



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Biociências
Câmpus do Litoral Paulista



ESTUDO ECOTOXICOLÓGICO E AVALIAÇÃO DO RISCO AMBIENTAL DA COCAÍNA EM ECOSISTEMAS MARINHOS

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São Vicente, SP.

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ESTUDO ECOTOXICOLÓGICO E AVALIAÇÃO DO RISCO
AMBIENTAL DA COCAÍNA EM ECOSISTEMAS MARINHOS

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Dedico esse trabalho a todos aqueles que acreditam na educação e na Ciência como principais ferramentas na busca de uma sociedade justa, desenvolvida e próspera.

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(Autor desconhecido)

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Lista de abreviações

- 6-ACM:** 6-monoacetyl morphine
- AChE:** acetylcholinesterase
- AMP:** amphetamines
- B:** bottom
- BAF:** bioaccumulation factor
- BE:** Benzoilecgonine
- CNS:** Central Nervous System
- COC:** Cocaine
- COD:** codeine
- COX:** Cicloxygenase
- DBF:** dibenzilfluoresceína
- DOPA:** dopamine
- EC:** Emerging contaminants
- EDDP:** 2-ethylidene-1,5-dimethyl-3,3-diphenylpy
- EME:** Ecgonine metil ester
- EPH:** Ephedrine
- EROD:** etoxiresorufin O-deetilase
- ETE:** estação de tratamento de esgoto
- GPx:** Glutathione Peroxidase
- GST:** Glutathione S- Transferase
- HER:** heroin
- ID:** illicit drugs
- LMS:** lysosomal membrane stability
- LOD:** limit of detection
- LOQ:** limits of quantification
- LPO:** lipoperoxidation
- LSD:** lysergic acid diethylamide
- MAMP:** Methamphetamines
- MAO:** Monoamine oxidase
- MDA:** 3,4-methylenedioxy amphetamine

MDMA: 3,4- methylenedioxy methamphetamine

MET: Mitochondrial electron transportation

MOR: morphine

MRM: multiple reaction monitoring

NOR-BZE: nor-benzoilecgonine

NOR-COC: norcocaine

NRRT: Neutral Red Retention Time

OCDE: Organização para a Cooperação e Desenvolvimento Econômico

PEPH: pseudoephedrine

PLANASA: Plano Nacional de Saneamento

S: surface

SSO: Sewage Submarine Outfall

STP: sewage treatment

THC: Δ^9 -tetrahydrocannabinol

THC-COO: 11- Nor- Δ^9 -carboxy-tetrahydrocannabinol

TLP: Total lipids

TRA: tramadol

UN: United Nations

UNODC: United Nations Office on Drugs and Crime

WWTPs: wastewater treatment plants

17 β -HSD: 17 β -hydroxysteroid dehydrogenase

3 β -HSD: 3 β -hydroxysteroid dehydrogenase

5-HT: serotonin

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Seasonal monitoring of cocaine and benzoylecgonine in a subtropical coastal zone (Santos Bay, Brazil)

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Cellular defenses and damages triggered by cocaine in marine mussels

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Resumo

Os esgotos urbanos representam uma importante fonte de poluição em ecossistemas marinhos, devido, principalmente, ao aumento populacional em regiões metropolitanas costeiras e à ineficiência dos processos de coleta, tratamento e disposição de efluentes domésticos. Entre as principais substâncias que podem ser encontradas nos efluentes estão os produtos farmacêuticos, de cuidados pessoais e drogas ilícitas, tais como a cocaína, que é considerada como um sério problema de saúde pública. Os emissários submarinos representam o principal mecanismo de liberação de contaminantes de preocupação emergente (como as drogas ilícitas) no ambiente marinho. Estudos anteriores identificaram a presença de cocaína (COC) em água superficial continental e costeira, bem como efeitos biológicos em concentrações ambientalmente relevantes. Dessa forma, o presente trabalho tem como objetivo a realização de uma avaliação do risco ambiental de cocaína em ambientes costeiros através de uma metodologia escalonada que contemple a quantificação em matrizes ambientais na Baía de Santos (água superficial, sedimento e mexilhões) e ensaios ecotoxicológicos empregando como modelos o mexilhão *Perna perna* e o peixe *Anguilla anguilla*. Os mexilhões foram expostos a duas diferentes concentrações de cocaína (200 ng.l⁻¹ e 2000 ng.l⁻¹), a um controle de água do mar e um controle de solvente, por 168h. Ao todo, foram 21 mexilhões divididos em 2 aquários por tratamento. Após os períodos de exposição (48h, 96h e 168h), 7 mexilhões eram aleatoriamente retirados dos aquários e submetidos à avaliação de citotoxicidade (pelo ensaio do Tempo de Retenção do Vermelho Neutro) e posteriormente eram excisados para retirada de tecidos (músculo adutor, glândula digestiva, brânquia e gônadas) para análise de biomarcadores (dopamina- DOPA; 5-hydroxytryptamine- 5-HT – serotonina; acetilcolinesterase- AChE; monoamina oxidase- MAO; ciclooxigenase- COX; transporte mitocondrial de elétrons- MET; lipídios totais- TLP; 7-ethoxiresorufin O-deetilase – EROD; dibenzilfluoresceína dealkilase – DBF; glutathione S-transferase – GST; glutathione peroxidase – GPX; DNA danos em DNA; e peroxidação lipídica – LPO). A capacidade da cocaína bioacumular nos mexilhões também foi verificada. Além disso, peixes (*Anguilla anguilla*) também foram expostos à cocaína (20 ng.l⁻¹) para investigar potenciais danos histopatológicos, imunohistoquímicos (3β-hidroxisteroide dehidrogenase- 3β-HSD; 17β- hidroxisteroid dehidrogenase- 17β-HSD type 3; e P450 aromatase) e endócrinos (hormônio folículo estimulante- FHS; hormônio luteinizante- LH and cortisol) da cocaína em vertebrados. Os resultados demonstram que a cocaína foi encontrada na Baía de Santos em concentrações variando de 1.91 – 203.6 ng.l⁻¹, sendo as maiores concentrações detectadas na

primavera/verão, período que coincide com o aumento populacional na Baixada Santista devido ao início da alta temporada. No sedimento, as concentrações quantificadas variaram de 0.94 – 46.85 ng.g⁻¹. A cocaína também foi detectada no tecido de mexilhões coletados na Baía em concentrações variando de 0.914-4.58 µg.kg⁻¹ (peso úmido). O fator de bioacumulação calculado variou de 163 – 1454 l.kg⁻¹. Quanto às vias metabólicas investigadas, foram observadas alterações nas atividades de EROD e DBF, além de GST e GPx, indicando comprometimento na metabolização e resposta antioxidante. A exposição à cocaína provocou citotoxicidade nos mexilhões, que foi observada pela reduzida estabilidade da membrana lisossômica dos hemócitos, além do aumento significativo de peroxidação lipídica e danos em DNA. A cocaína aumentou os níveis de neurotransmissores (dopamina e serotonina) nos mexilhões em todas as concentrações testadas. Já após 168h foi observada uma redução nos níveis de AChE e COX. Além disso, foi observada um aumento nos níveis de MET e TLP em ambas as concentrações. Os peixes expostos à cocaína apresentaram um atraso no desenvolvimento e maturação dos folículos ovarianos, fato que pode comprometer a reprodução desses organismos. Isso também foi demonstrado pela menor expressão de 3β-HSD, e P450 aromatase, bem como menores níveis de FHS e LH. Os resultados indicam que concentrações ambientalmente relevantes de cocaína, mesmo na ordem de ng.l⁻¹ são capazes de afetar negativamente a saúde dos animais marinhos a partir de perturbações no sistema reprodutivo e citogenotoxicidade. A avaliação de risco realizada apontou para quocientes situados entre os níveis moderado e alto para Baía de Santos. É preciso melhorar a estrutura de coleta e tratamento de esgoto em regiões costeiras a fim de minimizar o descarte de efluentes contaminados com drogas ilícitas em ambientes marinhos. Além disso, é importante que essas substâncias sejam incluídas em programas de monitoramento da qualidade de água, a fim de se estabelecerem parâmetros de segurança que permitam a preservação dos recursos naturais e da saúde humana.

Palavras-chave: Cocaína, ecossistemas marinhos, concentrações ambientais, mexilhões *Perna perna*, enguia europeia, biomarcadores.

Abstract

Urban sewage represents an important source of pollution to marine ecosystems, mainly due to the population growth on coastal metropolitan regions and the inefficiency of the processes of collection, treatment and disposal of domestic effluents. Pharmaceutical, personal care products and illicit drugs, such as cocaine, constitute some of the main substances found in domestic effluents representing serious problem to the public health. Submarine sewage outfalls represent the main source of contaminants of emerging concern (such as illicit drugs) into the marine environment. Previous studies have identified the presence of cocaine (COC) in inland and coastal surface water, as well as biological effects at environmentally relevant concentrations. The present work aimed to assess of the environmental risk of cocaine in coastal environments through a tiered approach that included the COC (and by products) quantification in environmental matrices of Santos Bay (surface water, sediment and mussels) and ecotoxicological tests using as models the mussel *Perna perna* and the fish *Anguilla anguilla*. Mussels were exposed to two different concentrations of cocaine (200 ng.l⁻¹ and 2000 ng.l⁻¹), a seawater control and a solvent control, for 168h. The mussels (21 specimens) were divided into 2 aquariums per treatment. After the exposure periods (48h, 96h and 168h), 7 mussels were randomly removed from the aquariums and submitted to cytotoxicity evaluation (by the Neutral Red Retention Time assay) and later they were excised for tissue removal (adductor muscle, digestive tract, gills and gonads) for the analysis of biomarkers (dopamine-DOPA; 5-hydroxytryptamine- 5-HT - serotonin; acetylcholinesterase-AChE; monoamine oxidase-MAO; cyclooxygenase-COX; mitochondrial electron transport-MET; total lipids- TLP; 7-ethoxyresorufin O-deethylase – EROD; dibenzylfluorescein dealkylase – DBF; glutathione S-transferase – GST; glutathione peroxidase – GPX; DNA damage to DNA; and lipid peroxidation – LPO). The bioaccumulation of COC in mussels soft tissues was also verified. In addition, fish (*Anguilla anguilla*) were also exposed to cocaine (20 ng.l⁻¹) to investigate potential histopathological, immunohistochemical (3β-hydroxysteroid dehydrogenase-3β-HSD; 17β-hydroxysteroid dehydrogenase-17β-HSD type 3; and P450 aromatase) and endocrine (follicle stimulating hormone-FHS); luteinizing hormone- LH and cortisol) damages caused by cocaine in marine vertebrates. Cocaine was found in Santos Bay at concentrations ranging from 1.9 – 203.6 ng.l⁻¹, with the highest concentrations detected in spring/summer, a period that coincides with the population increase in Baixada Santista due to the beginning of the warmer high season. In the sediment, the quantified concentrations ranged from 0.94 – 46.85 ng.g⁻¹.

Cocaine was also detected in the soft tissue of mussels collected in the Bay at concentrations ranging from 0.914-4.58 $\mu\text{g}\cdot\text{kg}^{-1}$ (wet weight). The calculated bioaccumulation factor ranged from 163 – 1454 $\text{l}\cdot\text{kg}^{-1}$. As for the metabolic pathways investigated, changes were observed in the activities of EROD and DBF, in addition to GST and GPx, indicating impairment in metabolism and antioxidant response. Cocaine exposure caused cytotoxicity in mussels, which was observed by the reduced stability of the lysosomal membrane of hemocytes, and the significant increase in lipid peroxidation and DNA damage. Cocaine increased the levels of neurotransmitters (dopamine and serotonin) in mussels at all concentrations tested. After 168h, a reduction in AChE and COX levels was observed. In addition, an increase in MET and TLP levels was observed at both concentrations. Fish exposed to cocaine showed a delay in the development and maturation of ovarian follicles, a fact that can compromise the reproduction of these organisms. This was also demonstrated by lower expression of 3 β -HSD, and P450 aromatase, as well as lower levels of FHS and LH. The results indicated that environmentally relevant concentrations of cocaine, even in the order of $\text{ng}\cdot\text{l}^{-1}$, are capable of negatively affecting the health of marine animals through disturbances in their reproductive system and cytogenotoxicity. The risk assessment carried out pointed to moderate to high risks in Santos Bay. It is necessary to improve the structure of sewage collection and treatment in coastal regions in order to minimize the disposal of effluents contaminated with illicit drugs in marine environments. Furthermore, it is important that these substances are included in water quality monitoring programs, in order to establish safety parameters that allow the preservation of natural resources and human health.

Keywords: Cocaine, marine ecosystems, environmental concentrations, *Perna perna*, European eels, biomarkers

General introduction, aims and structure of the thesis

1. Brazilian Sanitation

The basic sanitation can be defined as a set of services that aims to provide potable water supply, sewage collection and treatment, urban and cleaning services, solid waste management and urban rainwater drainage and management (Campos, 2017). The access to improved water and sanitation are recognized by the United Nations as human rights (UN, 2020) and are essentials to guarantee the environment quality, human health, and economic development (Kresh and Schneider, 2020). The supply of water in adequate quantity and quality is of extreme importance for the prevention of diseases, reduction of infant mortality and helps in the expansion of tourism and real estate appreciation mainly in areas with high population density (Alemu, 2017; Choumert et al. 2015; Yindong et al. 2017; Shidhar & Adejumo, 2020; Ali et al. 2018; O’Gorman, 2020; Ferreira et al. 2021). Furthermore, sanitation is also related to environmental protection, depollution of rivers and preservation of water resources (Trata Brasil, 2021).

In 1971 the “Banco Nacional da Habitação” launched the Water and Sewage National Plan (PLANASA) aiming to increase geographical coverage in urban centers, allocating resources for municipalities to create their own sanitation companies (Barbosa & Brusca, 2015). In 1992, with the dissolution of Planasa, the Federal Government made new investments through the creation of programs such as Sanitation for Urban Centers, Pro-Sanitation and the Social Action Program in Sanitation, defined the universalization of sanitation services (Ramos et al. 2020).

In 2007 the Basic Sanitation Law N. 11,445 was enacted and defined the principles of national operation and established the general bases and guidelines for a State policy to be developed such as universalization of access, as well as the prevention the abuse of economic power by the definition of tariffs to ensure the economic-financial stability of the contracts and general framework for each state to carry out its specific implementation strategy (UN, 2018). Newly, the Federal Senate approved the Basic Sanitation Legal Framework (Bill Law N. 4,162/2019) with the aim of universalizing of sanitation and water supply (“Services”) in Brazil until the end of 2033, changing the way such Services are provided in Brazil and its regulation, to open the sector to private initiative (Sampaio and Sampaio, 2020).

Brazil experienced an early urban transition compared to other developing countries of Asia and Africa, however it occurred unevenly between the different regions of the country (Martine and McGranahan, 2012). Due to its vast territory population, Brazil presents regional diversities which are reflected in the access to basic sanitation (von Sperling, 2016). The North and Northeast regions of the country had the lowest coverage (27.4% and 47.2%, respectively), while the highest coverage is observed in the southeastern region (88.9%); followed by the South and Midwest regions (68.7%) (Gomes, et al. 2020).

According to Brazilian Institute of Geography and Statistic, about 9 million households (12.6%) had a rudimentary cesspit, a ditch, in addition to other forms of waste disposal, being these conditions prevalent in the North region (29.6% of households- 1.6 million), exceeding the estimated 27.4% of households connected to the general network (IBGE, 2019). Regarding treatment, the Midwest has a treatment rate of 52.62% against only 18.3% in the North. In terms of national average, only 10 of the 100 largest Brazilian cities are able to treat over 80% of sewage and only 44.92% of all sewage in the country receives treatment (SNIS, 2021).

Coastal zonas are areas densely populated and exhibit higher rates of population growth and urbanization, that produced several economic benefits such as marine trade and transport, industrial and urban development, tourism and food production (Neumann et al. 2015). During holidays, the population in coastal areas may duplicate due to an increase in the number of tourists, overloading the water supply as well as sewage collection and treatment (Chili et al. 2017). However, the booming population growth on coastal areas occurs faster than the increase in supply of sanitation facilities, promoting the continuous disposal of contaminants that affect water quality, coastal biodiversity and consequently, human health (Feitosa et al. 2017).

2. Marine ecosystems

The oceans cover more than 70% of the Earth's surface and contain more than 90% of the living species, being deeply related to the evolution and development of humanity, because oceans provide food for billions of people worldwide, play an important role in the renewable energy (wind, wave, currents and tides) and have large oil and gas reserves (Sevilla & Le Bail, 2017). In addition to the ecological importance, the ocean asset value is estimated in US\$24,4 trillion/year and are responsible for two thirds of gross primary marine productivity, that would make the ocean the world's 7th largest economy (Hoegh-Guldberg *et al.* 2010). It is also estimated that the oceans produce about 50-80% of the oxygen production on Earth and captures

2.4 Gt carbon dioxide per year. Also, the ocean plays an important role in climate regulation (Rackley, 2009; NOAA, 2022a; 2022b).

Brazil has one of the largest coastal areas in the world (7, 491 km), with a total area covering about 3.5 million km² with coastal zones extraordinarily diverse, composed of subtropical to temperate waters (South and Southeast regions) and warm waters (North and Northeast regions), supporting a wide variety of marine ecosystems that include mangroves, coral reefs, dunes, sandbanks, sandy beaches, rocky shores, lagoons and estuaries (MMA, 2010; Obraczka *et al.* 2017). Seas and oceans stand out for hosting unique ecosystems and rich in biodiversity and have almost twice the number of animal phyla compared to terrestrial ecosystems, such as cnidarians, crustaceans, fish, birds, reptiles and mammals, many of which are endemic and endangered (Jefferson *et al.* 2021).

Marine ecosystems provide some goods (fish harvests, wild plant and animal resources, genetic material) and services (breeding and nursery habitats, shoreline stabilization and erosion control, carbon sequestration, biogeochemical cycling, flow energy and others) (Barbier *et al.* 2017). Despite their importance, marine ecosystems are threatened by several human activities directly related to population growth, urban and industrial, exploratory fish, climate change, habitat loss, nutrient enrichment, introduction of exotic species and pollution (Partelow *et al.* 2015; Santos & Schiavetti, 2014; Wang *et al.* 2021).

3. Marine pollution

The United Convention on the Law of the Sea defines pollution of the marine environment as the introduction by humans, directly or indirectly, of substances or energy into marine environment, resulting in deleterious effects to the living resources, quality water, marine activities, and hazards to human health (UN, 1982). Then, pollutants are defined as anthropogenically introduced substances that is present in concentrations that may harm several organisms or exceed an environment quality standard (OECD, 2005). Contaminants can be defined as substances (chemical elements or compounds) potentially toxic, persistent, and liable to bioaccumulate (Tornero & d'Alcalà, 2014).

According to Chapman (2007), contamination is the presence of a substance where it should not be or in concentrations above background and pollution is contamination that provokes or may provoke adverse biological effects. So, all pollutants are contaminants, but not all contaminants are pollutants. The distinction between pollutant and contaminant is not always clear, as the concentrations at which contaminants become pollutants cannot always be defined

and some negative effects are only observed in the long-term exposure (Stengel *et al.* 2006).

For a long time, the oceans were considered as a great reservoir for the safe elimination of contaminants, due to its high dilution and rapid dispersal capacity of contaminants (Feitosa *et al.* 2017). However, despite efforts to reduce the release of pollutants from anthropogenic sources into the environment, direct and indirect discharge of waste into coastal waters is a major environmental problem (Priya *et al.* 2021).

The marine environment is constantly threatened by various contaminants from various sources commonly found in the surrounding area (Kumar & Prasannamedha, 2021). Oil spills, nutrient enrichment, plastic waste, metals, pharmaceuticals, personal care products and even illicit drugs are recognized as the main contaminants that threaten the marine ecosystem (Vikas & Dwarakish, 2015; Pereira *et al.* 2016; Fontes *et al.* 2017; Thushari & Senevirathna, 2020).

Domestic sewage is a major contamination vector for the marine environment (Araújo *et al.* 2021). The dumping of sewage untreated or without primary treatment is a great concern because the effluents may carry several substances with potential contaminants (Roth *et al.* 2016). In Brazil, only 60.2% of the urban areas have sewage collection and 73.3% of these have adequate treatment (Brazil, 2019). In coastal areas the Sewage Submarine Outfalls (SSO) are proposed as an efficient alternative for the final destination of wastewater in densely populated coastal areas, mainly due to economic aspects (low cost) and high dispersal capacity compared with other forms of treatment (Ortiz *et al.* 2016; Birocchi *et al.* 2021).

The SSO is constituted from a pipeline or tunnel, or combination of the two, that collect the effluents produced in coastal cities for a final discharge in the ocean, considering the encompassing population, geographic, hydraulic, oceanographic and microbiologic aspects (Feitosa *et al.* 2017). However, the preliminary or primary treatments applied to the effluents before being sent to the outfall is insufficient to treat or remove some contaminants, such as drugs that are continuously discharged into the environment, representing a potential risk to the marine ecosystem (Pereira *et al.* 2016).

4. Emerging contaminants

According to the Environmental Protection Agency (EPA), an emerging contaminant (EC) is a chemical or material characterized by a perceived, potential, or real threat to human health or the environment or by a lack of published health standards (EPA, 2021). The ECs are natural or anthropogenic substances including pharmaceuticals, personal care products, endocrine disruption compounds, flame retardants and illicit drugs (Maranho *et al.* 2015; Pereira *et al.*

2016; Fontes *et al.* 2017; Kasonga *et al.* 2021). The term "emergent" does not necessarily refer to substances that have recently emerged, but to compounds that were synthesized or discovered a long time ago, but only recently recognized as potentially dangerous (Valbonesi *et al.* 2021). The ECs are currently not included in monitoring programs, but are candidates for future regulations depending on researching of potential health effects, ecotoxicity and data regarding monitoring and occurrence in the environment (Barcelò *et al.* 2005; Yadav *et al.* 2021).

The presence of ECs in the aquatic environment was first observed in 1970, but the studies have intensified since the 90s due to the development of new analytical methodologies that allowed the detection of these substances at very low concentrations (Vélez- *et al.* 2019). The ECs have a wide variety of chemical structure, mode of action and toxicity to the environment and human health (Raies and Bajic, 2016). Over the past 20 years, the global ECs presence in surface waters, wastewater effluents, groundwater and drinking water has been well documented (Luo *et al.* 2014).

ECs occur in several environments matrices in concentrations ranging from ng.l^{-1} to mg.l^{-1} and are continuously discharged into the environment due to inefficient treatment processes of WWTPs (Castiglioni *et al.* 2018). However, even in low concentrations ECs can affect the ecosystems and human health, causing bacterial resistance, reproductive abnormalities, oxidative stress and carcinogenic effects (Martini *et al.* 2021). Although there are several studies in the literature regarding the effects of pharmaceuticals and personal care products in freshwater environments, data on the occurrence and effects of illicit drugs in the aquatic environment are still scarce, especially in marine ecosystems.

According UNODC (2020), the number of drug users around the world was estimated at 269 million people in 2018: 192 million (cannabis), 58 million (opioids), and the use drugs is 27 million (amphetamines and prescription stimulants), 21 million (ecstasy) and 19 million (cocaine) users. The drug use is more widespread in developed countries than in developing countries (UNODC, 2020). Among the drugs mentioned, cocaine (COC) is considered one of the most potent and addictive illicit drug. In mammals, COC enhances monoamine neurotransmitter (dopamine, norepinephrine, and serotonin) activity in the central and peripheral nervous systems, blocking its reuptake into the nerve terminal (Volkow *et al.* 2000). Hence, cocaine addiction is recognized as the brain's dopamine reward system. Cocaine can also block sodium ion channels in the plasma membrane, which causes a local anesthetic effect and can contribute to cardiac arrhythmias (Cubo, 2014).

Illicit drugs are highly bioactive substances, able to interact with cellular receptors, even

at low concentrations, therefore, the occurrence of these substances in aquatic ecosystems is a concern as they can cause adverse effects on non-target organisms (Baker and Kaspzyk-Horder, 2013; Capaldo et al. 2019).

COC has been frequently detected in the aquatic environment (Sulej- Suchomska *et al.* 2020). Several studies have been reporting that COC and its metabolites (Benzoilecgonine-BE; Ecgonine metil ester- EME) affect freshwater organisms, causing oxidative stress, cytotoxicity, behavior changes, morphological damage (Binelli *et al.* 2013; Capaldo *et al.* 2018; De Felice and Parolini, 2019). However, ecotoxicological studies regarding effects of illicit drugs on marine organisms are still limited.

5 Aims and structure of the thesis

With the background presented by this introduction, we can hypothesize that COC and its metabolites are present in São Paulo coastal zone (Santos Bay) in concentrations able to bioaccumulate and affect marine organisms.

In this context, the main objective of the thesis is to assess the occurrence of cocaine in environmental matrices of Santos Bay as well as its biological effects leading to environmental risk. The specific objectives are:

1. To quantify the concentrations of COC and BE around the Santos submarine sewage outfall during the four seasons of the year;
2. To quantify the concentrations of COC and BE in surface water, sediment and mussels of the Santos Bay;
3. To determine the Field-measured Bioaccumulation Factor in *P. perna* mussels from Santos Bay;
4. To elucidate metabolic pathways involved in biotransformation, conjugation and excretion of cocaine in *Perna perna*
5. To assess sublethal effects related to oxidative stress and cytogenotoxicity in mussels exposed to COC (*in vivo*)
6. To assess disturbances on neurotransmitter and energy balance in mussels exposed to COC (*in vivo*)
7. To assess histopathological effects in fishes (*Anguilla anguilla*) exposed to COC (*in vivo*)
8. To perform a preliminary environmental risk of cocaine based on the adaptation of protocols already established for drugs;

9. To provide data for the inclusion of cocaine in water quality monitoring programs
To achieve what was proposed, this thesis was structured in five chapters:

Chapter 1 presents a review of the main groups of illicit drugs found in the aquatic ecosystem (opioids, cannabinoids, amphetamines and cocaine) and their effects on biota.

Chapter 2 presents the contamination of cocaine and benzoylecgonine in a subtropical coastal zone (Santos Bay), considering a seasonal monitoring (Article 1) and different environmental matrices (seawater, sediment and mussel *Perna perna*) collected from Santos Bay (Article 2).

Chapter 3 provides the assessment of cocaine's effects at environmentally realistic concentration on mussels *Perna perna* and fishes *Anguilla Anguilla*. Disturbances on neurotransmitters and energy balance in mussels exposed to COC (*in vivo*) are presented in Article 1. Metabolic pathways involved in biotransformation, conjugation and excretion of cocaine in *Perna perna*, as such as sublethal effects are assessed in mussels exposed to COC (Article 2), whereas histopathological effects in fishes (*Anguilla anguilla*) exposed to COC are shown in Article 3.

Chapter 4 presents an environmental risk assessment of COC in marine ecosystems.

Chapter 5 brings the conclusions and suggestions for future research.

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CHAPTER 1

Review on the occurrence and biological effects of illicit drugs in aquatic ecosystems

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Review on the occurrence and biological effects of illicit drugs in aquatic ecosystems

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Abstract

Illicit drugs (IDs) and their metabolites are recognized as contaminants of emerging concern. After consumption, illicit drugs are partially metabolized and excreted unchanged in urine and feces or as active metabolites reaching wastewater treatment plants (WWTPs). Furthermore, most WWTPs are insufficient in the treatment of effluents containing IDs, which may be released into aquatic ecosystems. Once in the water or sediment, these substances may interact and affect non-target organisms and some evidence suggest that illicit drugs may exhibit pseudo-persistence because of a continuous environmental input, resulting in long-term exposure to aquatic organisms that may be negatively affected by these biologically active compounds. We reviewed the literature on origin and consumption, human metabolism after consumption, aquatic occurrences, and toxicity of the major groups of illicit drugs (opioids, cannabis, synthetic drugs, and cocaine). As a result, it could be concluded that illicit drugs and their metabolites are widespread in diverse aquatic ecosystems in levels able to trigger sublethal effects to non-target organisms, besides to concentrate in seafood. This class of emerging contaminants represents a new environmental concern to academics, managers, and policymakers, whose would be able to assess risks and identify proper responses to reduce environmental impacts.

Keywords: Illicit drugs. Aquatic ecosystems. Non-target organisms. Emerging contaminants

1. Introduction

Illicit drugs and their metabolites have been described as a new class of emerging contaminants (Zuccato et al. 2008; Baker et al. 2012) which have been detected in influents and effluents from WWTPs, freshwater and drinking water (Campestrini and Jardim 2017), and seawater (Pereira et al. 2016). Contaminants of emerging concern are substances that are not explicitly contemplated in any environmental legislation but may be found in several environmental compartments (Feitosa et al. 2013). Recent studies have shown that illicit drugs may present risks to human health and the environment, since they can interact biologically with non-target organisms (Binelli et al. 2012, 2013). Furthermore, most of these chemicals occur at low concentrations in the environment (from ng L^{-1} to $\mu\text{g L}^{-1}$) and their threats to aquatic life, especially marine life, and public health of human population are still poorly understood (Zuccato and Castiglioni 2009; Parolini et al. 2015).

According to the United Nations Office on Drugs and Crime, the term “illicit drugs” determine substances whose possession, production, sale, or consumption is prohibited by law, considering the manner in which such substances are manufactured, distributed, and acquired, in addition to being used for non-medicinal purposes (UNODC 2014). Illicit drugs and their metabolites are a group of structurally diverse substances of chemical agents with extremely high potential for biological effects in humans and non-target organisms (Daughton 2011), and may be classified as hallucinogens (MDA: 3,4-methylenedioxy amphetamine; MDMA: 3,4-methylenedioxy methamphetamine; LSD: lysergic acid diethylamide), stimulants (cocaine, benzoylecgonine, norcocaine, norbenzoylecgonine), opioids (morphine derivatives, heroin, 6-acetyl-morphine, codeine, norcodeine, oxycodone) (Baker and Kasprzyk-Hordern 2013), and other psychoactive drugs as cannabinoids (THC-COO: 11-Nor- Δ^9 -carboxy-tetrahydrocannabinol; THC: Δ^9 -tetrahydrocannabinol) (Boix et al. 2014).

The global increased in production and consumption of illicit drugs have generated economic, social, and health problems to the populations and may affect the organisms (van Nuijs et al. 2011; Binelli et al. 2012, 2013). It is estimated that 250 million people have used drugs at least once in 2015 (UNODC 2017b). In this context, the presence of illicit drugs in the aquatic environment has attracted a broad scientific interest concerning their potential for environmental impacts (Evgenidou et al. 2015; Kasprzyk-Hordern et al. 2009a, b). Once consumed, illicit drugs are partially metabolized and excreted in urine and feces unchanged or as active metabolites and reach the WWTPs as a complex mixture of parent compounds and

metabolites (Zuccato and Castiglioni 2009; Campestrini and Jardim 2017). Due to insufficient treatments for removal, these substances are expected to reach aquatic ecosystems receiving WWTPs' effluents or untreated sewage (Baker and Kasprzyk-Hordern 2013; Bijlsma et al. 2014).

Evidence suggest that illicit drugs may exhibit pseudopersistence because of a continuous environment input (Rosi-Marshall et al. 2015), resulting in a long-term exposure to aquatic organisms that may be negatively affected since illicit drugs are biologically active compounds (Parolini et al. 2015, 2016a). There are four major drug groups that have been causing worldwide concern: opioids, cannabinoids, synthetic drugs (amphetamines and ecstasy), and cocaine (UNODC 2015, 2016, 2017a).

Due to a growing concern about the presence of illicit drugs and their metabolites in aquatic environment, this review is presented in order to assess information on the occurrence and potential biological effects in aquatic environments of the most used illicit drugs worldwide (opioids, amphetaminetype stimulants, cannabis, and cocaine), since they are suspected to promote negative effects even at low concentrations. Table 1 shows the main characteristics of illicit drugs contemplated in this review.

WWTPs play an important role in the life cycle and occurrence of illicit drugs and their metabolites in the aquatic environment. Once excreted in the sewage, these substances are transported to WWTPs, but most facilities are not designed to remove such compounds, resulting in continuous release of contaminated effluents into the water bodies (Terzic et al. 2010; Vazquez-Roig et al. 2013; Evgenidou et al. 2015).

Efficiency of removal and biotransformation of these chemicals may be affected by several operational conditions (retention time, hydraulic system, and biodegradation kinetic of the WWTPs) or environmental conditions (temperature, pH, and aerobic or anaerobic conditions) (Verlicchi et al. 2012; Evgenidou et al. 2015; Devault et al. 2017b). Similarly, physical-chemical properties such as molecular weight, solubility, log Kow, and acid dissociation constant (pKa) (Mastroianni et al. 2016) also may influence the ability of these compounds to remain in the aqueous phase (especially if the log Kow values are lower than 3.0) or bound to sediments and sludges (Behera et al. 2011; Vazquez-Roig et al. 2013).

Human excretion (urine, feces, saliva, and sweat) and intentional or accidental disposal from clandestine laboratories are mainly responsible for the presence of illicit drugs in STPs (Pal et al. 2013; Parolini et al. 2016a; UNODC 2017a). Illicit drugs may remain in untreated effluents, being transferred in the final effluent into aquatic environment (groundwaters, rivers,

ocean), affecting water quality and ecosystem health (Zuccato and Castiglioni 2009; Yadav et al. 2017). Moreover, the occurrence of illicit drugs and their metabolites in tap water represents relevant risk to human health (Campestrini and Jardim 2017), as summarized in Fig. 1.

The presence of ID metabolites in aquatic ecosystems may have seasonal characteristics, associated with social behavior and tourism. Several studies have shown an increase in the use of illicit drugs during holidays, weekends, and music festivals, evidencing a recreational pattern of consumption (HuertaFontela et al. 2008a; Bijlsma et al. 2014; Viana et al. 2011; Ort et al. 2014; Lopes et al. 2014; Krizman et al. 2016; Pereira et al. 2016; González-Alonso et al. 2017). Furthermore, illicit drugs also interact with the residues of other therapeutic substances, leading to unexpected pharmacological interactions that may cause toxic effects on aquatic organisms (Parolini et al. 2016a, b). However, available data on how different illicit drugs behave in different WWTP process as well as their interaction in aquatic ecosystems are still insufficient to understand the potential impacts of these compounds.

Our study reviewed chemical, epidemiological, and ecotoxicological studies concerning main illicit drugs to contribute to the understanding of occurrence, toxicity, and risks of introducing IDs into freshwater and coastal ecosystems, which could support further proposals for environmental monitoring and regulation. For this purpose, the occurrence of opioids, cannabis, synthetic drugs, and cocaine in aquatic ecosystems, such as biological effects to non-target organisms, were assessed in the scientific literature over the last decades. Recommendations and an outlook are also provided.

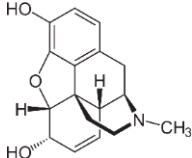
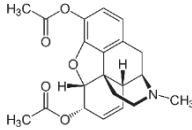
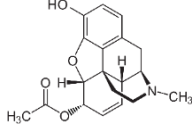
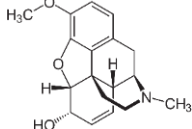
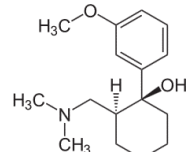
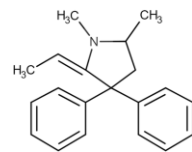
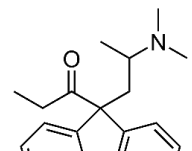
1.2 Environmental occurrence and biological effects

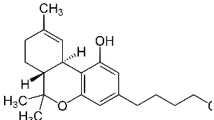
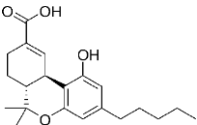
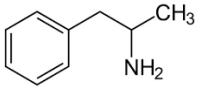
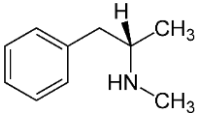
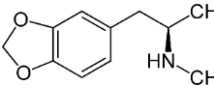
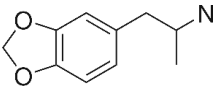
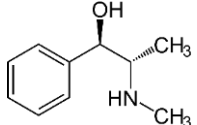
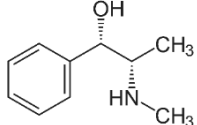
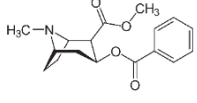
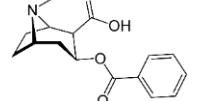
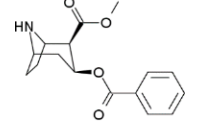
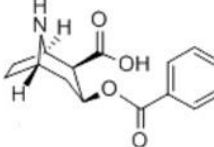
Data was presented according to the origin and consumption, human metabolism after consumption, environmental occurrences, and ecotoxicological assessment. Studies concerning drug concentrations on different environmental matrices are much more numerous than studies on biological effects on non-target biota. Chronic effects are observed in different species exposed to the drug or its metabolite under controlled conditions.

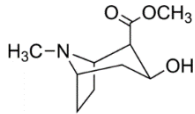
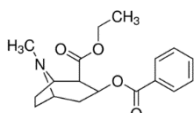
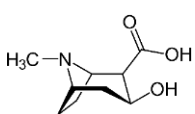
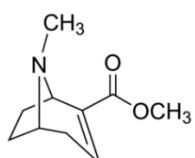
It is important to note that the order of magnitude of the concentrations found in the aquatic environment (ng L^{-1}) is potentially dangerous for aquatic organisms that inhabit affected areas. Developing countries still have considerable deficiencies regarding sewage treatment which directly compromises the performance of studies that accurately estimate the consumption of illicit drugs in these regions.

This fact combined with the lack of studies on the ecotoxicological effects of illicit drugs on aquatic organisms makes it difficult to perform studies on ecological risk. The lack of data regarding effect concentrations (ECs) on reproduction, growth inhibition, fertilization, and respiration rate, as well as other sublethal endpoints, represents an important field of study that needs to be explored and included in quality monitoring programs of water, especially in regions that do not yet have frameworks for the development of these protocols.

Table 1. Illicit drugs - basic properties.

Compound	Structure	Molecular weight (g/mol)	Log Kow	pKa	Water sol. (mg/L, 25°C)	Density
MOR C ₁₇ H ₁₉ NO ₃ CAS:57-27-2		285.34	0.89	8.21	149	1.32
HER C ₂₁ H ₂₃ NO ₅ CAS:561-27-3		369.41	1.58	7.95	600	1.56
6-ACM C ₁₉ H ₂₁ NO ₄ CAS: 2784-73-8		327.4	0.4	8.19	4093	1.38
COD C ₁₈ H ₂₁ NO ₃ CAS: 76-57-3		299.4	1.19	8.2	1	1.32
TRA C ₁₆ H ₂₅ NO ₂ CAS: 27203-92-5		263.4	3.01	9.41	1151	1.0±0.1
EDDP C ₂₀ H ₂₃ N CAS:30223-73-5		277.41	4.94	9.64	0.0012	1.051
METH C ₂₁ H ₂₇ NO CAS: 76-99-3		309.44	3.93	8.94	48.48	1.0 ± 1

THC C ₂₁ H ₃₀ O ₂ CAS:1972-08-3		314.45	6.97	10.6	2.8	NA
THC-COOH C ₂₁ H ₂₈ O ₄ CAS:64280-14-4		344.45	6.36	4.2	0.2335	NA
AMP C ₉ H ₁₃ N CAS:300-62-9		135.20	1.76	10.13	2.803 x 10 ⁴ (30°C)	0.9 ± 0.1
MAMP C ₁₀ H ₁₅ N CAS:537-46-2		149.23	2.07	9.09	1.329 x 10 ⁴	0.9 ± 0.1
MDMA C ₁₀ H ₁₅ NO ₂ CAS: 42542-10-9		193.25	2.28	10.14	7034	1.1 ± 0.1
MDA C ₁₀ H ₁₃ NO ₂ CAS:4764-17-4		179.22	1.64	9.67	2.83	1.2 ± 0.1
EPH C ₁₀ H ₁₅ NO CAS: 299-42-3		165.23	0.89	10.3	6.36 x 10 ⁴	1.0085
PEPH C ₁₀ H ₁₅ NO CAS:90-82-4		165.23	0.89	10.3	1.06 x 10 ⁵	1.0 ± 0.1
COC (C ₁₇ H ₂₁ NO ₄) CAS:50-36-2		303.35	2.3	8.61	1800 (22°C)	1.2 ± 0.1
BZE C ₁₆ H ₁₉ NO ₄ CAS:519-09-5		289.32	-1.32	2.15	1605	1.3 ± 0.1
NOR-COC C ₁₆ H ₁₉ NO ₄ CAS: 1871772-1		289.33	1.96	9.56	3067	1.2 ± 0.1
NOR-BZE C ₁₅ H ₁₇ NO ₄ CAS:60426-41-7		275.3	NA	NA	NA	NA

EME $C_{10}H_{17}NO_3$ CAS:7143-09-1		199.25	-0.29	9.04	7.968×10^5	1.2 ± 0.1
CE $C_{18}H_{23}NO_4$ CAS: 529-38-4		317.38	2.66	8.77	528.3	1.2 ± 0.1
ECG $C_9H_{15}NO_3$ CAS: 481-37-8		185.22	-3.78	9.69	7.432×10^5	1.3 ± 0.1
AME $C_{10}H_{15}NO_2$ CAS:43021-26-7		181.23	1.16		5.63×10^5	1.1 ± 0.1

Physical-chemical properties were obtained from EPI Suite™(Estimation Program Interface for Microsoft®Windows, v 4.0. United States Environmental Protection Agency (U.S. EPA), Washington, DC, USA. Download at <https://www.epa.gov/tsc-screening-tools/download-epi-suitetm-estimation-program-interface-v411>)

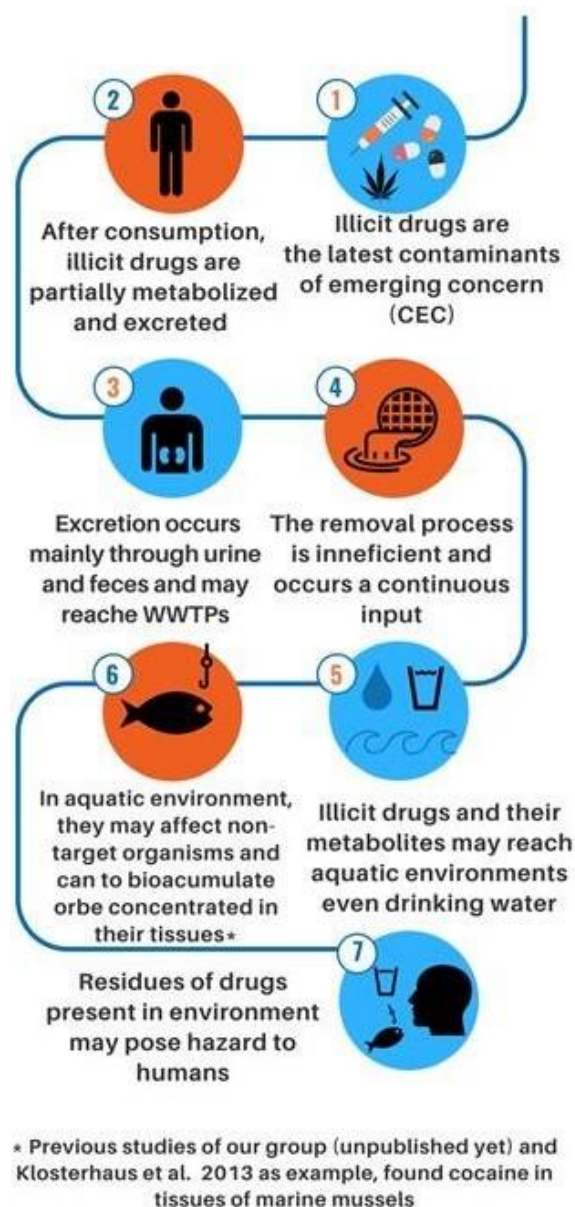


Fig. 1 Summary of sources, behavior, and effects of illicit drugs and metabolites in aquatic matrices

2. Opioids

The term “opioid” is used to denote all natural, synthetic, or semi-synthetic substances which react with opioid receptors as agonist or antagonist (Duarte 2005). Opium itself is derived from a plant known as the oriental poppy (*Papaver somniferum*) and the extraction takes place by the latex, which is withdrawn from the green leaves, producing a milky liquid that may contain up to 10% of medicinal alkaloids, such as morphine which may turn into heroin when heated (Martins et al. 2012). The number of opioid users is estimated in 35 million people worldwide, including opium, morphine, heroin, and synthetic derivatives (UNODC 2019).

Production is concentrated in Southwest Asia, and Afghanistan is the world's largest producer of opium accounting for almost two-thirds of the world's area of illicit poppy cultivation, with an area of cultivation of approximately 201,000 ha (UNODC 2019).

Opioids can be classified into three different groups: (a) natural opioids (morphine, codeine, thebaine, and some opium alkaloids); (b) semi-synthetic opioids (hydrocodone, oxycodone, and oxymorphone, come from morphine derivatives); and (c) synthetic opioids (buprenorphine, fentanyl, methadone, and tramadol); this last group being mainly used as a medicine after prescription (Campos-Mañas et al. 2018).

In the human body, these compounds can mimic the functioning of several other naturally produced substances such as endorphins and enkephalins (Garcia et al. 2012). Most opioids undergo extensive metabolism in the liver before entering the systemic circulation, which reduces the bioavailability of these drugs. Furthermore, they are metabolized through two major enzyme systems: CYP450 in phase-I metabolism and uridine diphosphate glucuronosyltransferases (UGT) in phase-II metabolism (Melis et al. 2011).

Opioid method of administration is mainly intravenous injection. In addition, heroin is recognized as one of the most powerful drugs of abuse and is usually administered as nasal insufflation or inhalation and hydrochloride salt by intravenous or subcutaneous injection. In the human organism, heroin is quickly deacetylated to 6-acetyl-morphine in the liver (Baselt 2004). Some studies also observed that morphine and heroin metabolites may be found in wastewater samples (Castiglioni et al. 2006).

The most important reason why opioids reach the environment consists of both medical prescriptions and illicit drugs of abuse acquired through illegal sources (Campos-Mañas et al. 2018). The environmental occurrence of opioids is linked to WWTPs, but there are some studies showing their occurrence in surface receiving waters. Table 2 summarizes the main information on the occurrence of opioid residues in aquatic environments. The most frequently detected compounds are morphine (MOR), 6-monoacetyl morphine (6-ACM), codeine (COD), 2-ethylidene-1,5-dimethyl-3,3-diphenylpy (EDDP).

The concentrations of MOR ranged from $< 0.55 \text{ ng L}^{-1}$ in Po river and Thames river (Zuccato et al. 2008) to 2400 ng L^{-1} in WWTP from a Taipei hospital (Lin et al. 2014). Residues of 6-ACM ranged from $< 0.2 \text{ ng L}^{-1}$ in secondary effluent from Zagreb (Terzic et al. 2010) to 224 ng L^{-1} in influents from England (Baker and Kasprzyk-Hordern 2013). For EDDP, the concentrations ranged from 0.1 ng L^{-1} in Yellow River and Pearl River (Li et al. 2016) to 1150 ng L^{-1} in effluents from Catalonia (Boleda et al. 2009).

Table 2. Concentration of opioids in aquatic environment (ng/L).

Compound	Country	Local	Study site	Concentration (ng.L ⁻¹)	Reference
MOR	Spain	Spanish basin	Llobregat river	1.2-3.0	Mastroianni et al. (2016)
			Ebro river	3.6-21.9	
			Jucar river	<LOQ-0.8	
	Martinique	Fort de France	Guadalquivir river	1.5-2.0	Devault et al. (2017a)
			Influent WWTP1	119.0 ± 16.0	
			Influent WWTP2	154.0 ± 27.0	
			Effluent WWTP 2	58.7 ± 7.0	
			Influent	25.5-278.0	
	Spain	Catalonia	Effluent	12.0-81.1	Boleda et al. (2009)
			Llobregat river	2.8-7.0	
			Anoia river	10.7	
	Spain	Iberian Pensinsula	Rubí river	27.0-30.9	Postigo et al. (2010)
			Surface water	6.5-10.8	
			Influent	54.2-166.0	
	Spain	Catalonia	Effluent	5.4-80.5	Boleda et al. (2007)
			Influent	<LOQ- 96.7	
	Costa Rica	Liberia	Effluent	<LOQ- 81.1	Causanilles et al. (2017)
			Llobregat river	4.8-6.3	
			Influent	41.0-77.0	
	Switzerland	Zurich	Pooled WS	29.0-36.0	Berset et al. (2010)
			Influent	67.0-77.0	
	Croatia	Zagreb	Efluent	15.0-61.0	Terzic et al. (2010)
			Influent	<LOQ-1970	
	Canada	Quebec	Effluent	84.0-1270.0	Rodayan et al. (2014)
			River creeks	<LOQ-14.0	
	German		Raw wastewater	160.0- 476.0	Wick et al. (2009)
Secondary effluente			18.0-101.0		
USA	Various cities	WWTP grab	42.0 – 48.0	Gerrity et al. (2011)	
		WWTP POCIS	43.0 – 56.0		
USA	Various cities	WWTP 24h	46.0 – 76.0	Gerrity et al. (2011)	
		Influent	440		
USA	Various cities	Effluent	28	Gerrity et al. (2011)	
		Influent (Super Bowl weekend)	427.0-1540.0		
USA	Various cities	Effluent (Super Bowl weekend)	<50	Gerrity et al. (2011)	
		Influent (baseline weekend)	523.0-1060.0		
USA	Various cities	Effluent (baseline weekend)	<50	Gerrity et al. (2011)	
		WWTP1	90.0-206.0		
Martinique	Fort de France	WWTP2	56.0-212.0	Devault et al. (2014)	
		WWTP3	80.0-253.0		
		WWTP4	57.0-224.0		
Italy	Lombardia region	Olona river	38	Zuccato et al. (2008)	
		Lambro river	3.5		

		Po river	<0.55	
	Toscana region	Arno river	1.30-4.70	
England	London	Thames river	<0.55-42 ± 4.7	
		Hospital WWTP	36.0-2400.0	
	Taipei	Rivers	36	
Taiwan		Influent	38	Lin et al. (2014)
		Effluent	29	
	Pingtung	Hospital WWTP	130.0- 220.0	
England		Influent	65.6-985.5	Baker & Kasprzyk-Hordern (2013)
		Effluent	13.0-266.6	
Cyprus		IWW	15.0-36.0	Hapeshi et al. (2015)
		WWTP influent	819.2	
UK	Various locations	WWTP effluents	243.8	Baker & Kasprzyk-Hordern (2011a)
		River water	35.8	
		WWTP A	156.4- 335.0	
UK	Various locations	WWTP B	337.0-239.7	Baker & Kasprzyk-Hordern (2011b)
		WWTP C	777.9-274.3	
Spain	Barcelone	Influent	61.0-370.0	Mastroianni et al. (2017)
		Influent	90.0-275.0	
Spain	Southeast region	Effluent	60.0-155.0	Bueno et al. (2011)
		River	12.0-19.0	
Croatia	Various cities	STPs	178.0 ± 95.0	Krizman et al. (2016)
France	Paris	Seine River	< LOQ-1.83	Brieudes et al. (2017)
Italy	Nosedo	Influent	83.3 ± 11.8	
		Influent	204.4 ± 49.9	Castiglioni et al. (2006)
Switzerland	Logano	Effluent	55.4 ± 11.1	
		Influent	820	
German		Effluent	110	Hummel et al. (2006)
		Rivers	78	
Czech Republic		WW	81.7	Baker et al. (2012)
Ireland	Dublin	Swords effluent	874 ± 86.0	Bones et al. (2007)
		Navan effluent	452 ± 86.0	
Spain	Galicia	Rías Baixas area	15.7	Fernández-Rubio et al. (2019)
		Influent	<19.0-60.0	
Netherlands	Various cities	Effluent	<7.0-13.0	Bijlsma et al. (2012)
		Olona river	<0.93	
	Lombardia region	Lambro river	<0.93	
Italy		Po river	<0.93	Zuccato et al. (2008)
	Toscana region	Arno river	1.30-4.70	
6-ACM		Raw wastewater	3.30-28.0	
Croatia	Zagreb	Secondary effluent	<0.2-6.80	Terzic et al. (2010)
Croatia	Various cities	STPs	<0.12- 28.0	Krizman et al. (2016)
Spain	Catalonia	Llobregat river	3.4	Boleda et al. (2007)
Spain		Guadalquivir river	2.5	Mastroianni et al. (2016)
Spain	Barcelone	Influent	<4.90-27.0	Mastroianni et al. (2017)
Italy	Nosedo	Influent	11.80 ± 8.50	Castiglioni et al. (2006)

	Switzerland	Logano	Influent	10.40 ± 4.80	
	Spain	Iberian Pensinsula	Influent	1.30-4.0	Postigo et al. (2010)
			WWTP A	4.50-5.90	
	UK	Various locations	WWTP B	3.60-5.90	Baker & Kasprzyk-Hordern (2011b)
			WWTP C	7.30-21.60	
	UK	Various locations	WWTP influent	69.7	Baker & Kasprzyk-Hordern (2011a)
	England		Influent	3.0-224.0	Baker & Kasprzyk-Hordern (2013)
			Effluent	0.6-7.70	
			Influent	3.30-1029.0	
			Effluent	2.70-1150.0	
	Spain	Catalonia	Llobregat river	2.0- 16.60	Boleda et al. (2009)
			Cardener river	3.0- 7.0	
			Anoia river	2.50-54.0	
			Rubí river	53.60-54.0	
			Influent	4.50-41.30	
	Spain	Catalonia	Effluent	4.90-56.70	Boleda et al. (2007)
			Llobregat river	9.61-17.50	
EDDP	Croatia	Zagreb	Raw wastewater	71.0-156.0	Terzic et al. (2010)
			Secondary effluent	74.0-163.0	
	Spain	Barcelone	Influent	50.0-197.0	Mastroianni et al. (2017)
	Czech Republic	Various cities	Surface water	0.60-7.40	Fedorova et al. (2014)
			Influent	153.0-634.0	
			Effluent	151.0-442.0	
	Switzerland	Zurich	River creeks	0.60-12.20	Berset et al. (2010)
			Lakes	2.20-8.70	
			WWTP grab	93.0 – 144.0	
	Canada	Quebec	WWTP POCIS	103.0 – 130.0	Rodayan et al. (2014)
			WWTP 24h	87.0 – 103.0	
			WWTP influent	47.0 ± 6.0	
			WWTP mechanical step	25.0 ± 4.0	
MET	Slovakia	Petržalka	WWTP biological step	32.0 ± 3.0	Mackulak et al. (2015)
			WWTP sludge water	17.0 ± 2.0	
			WWTP effluent	44.0 ± 5.0	
	France	Corbeil	Influent	40	Hubert et al. (2017)
			Effluent	30	

	France	Paris	Seine River	0.50- 2.95	Brieudes et al. (2017)
			Olona river	18	
	Italy	Lombardia region	Lambro river	9.9	Zuccato et al. (2008)
		Toscana region	Po river	0.60-1.90	
			Arno river	1.60-6.60	
			WWTP influent	342.2	
	UK	Various locations	WWTP effluents	161.6	Baker & Kasprzyk-Hordern (2011a)
			River water	38.2	
			WWTP A	59.70-126.0	
	UK	Various locations	WWTP B	122.0-144.50	Baker & Kasprzyk-Hordern (2011b)
			WWTP C	134.10-145.90	
	England		Influent	3.70-342.20	Baker & Kasprzyk-Hordern (2013)
			Effluent	2.60-162.30	
			Llobregat river	13.90 – 49.50	
	Spain	Spanish basin	Ebro river	14.40- 44.80	Mastroianni et al. (2016)
			Jucar river	5.80- 6.20	
			Guadalquivir river	16.0- 33.80	
			Ringsed effluent	48.0 ± 1.0	
	Ireland	Dublin	Swords effluent	206.0 ± 10.0	Bones et al. (2007)
			Leixlip effluent	9.0 ± 1.0	
			Navan effluent	67 ± 10	
	Czech republic		WW	15.90-28.30	Baker et al. (2012)
	China		Various rivers	0.216- 0.317	Wang et al. (2016)
			Dianchi lake	1.9	
	China	Various cities	Yellow river	0.1-0.3	Li et al. (2016)
			Pearl river	0.1-0.4 ±0.2	
	Spain	Galicia	Rías Baixas areas	71.7	Fernández-Rubio et al. (2019)
	Italy	Nosedo	Influent	19.80 ± 3.10	
			Effluent	22.60 ± 0.6	
	Switzerland	Logano	Influent	91.30 ± 19.20	Castiglioni et al. (2006)
			Effluent	72.10 ± 8.70	
			Influent	3.40- 1531.0	
			Effluent	3.40-732.0	
	Spain	Catalonia	Llobregat river	0.5- 12.0	Boleda et al. (2009)
			Cardener river	1.0-4.80	
			Anoia river	2.0-18.10	
			Rubí river	15	
			Influent	4.0-23.90	
	Spain	Catalonia	Effluent	4.0-24.70	Boleda et al. (2007)
			Llobregat river	4.90-10.10	
	Croatia	Zagreb	Raw wastewater	25.0-94.0	Terzic et al. (2010)
COD			Secondary effluent	20.0-60.0	
	Spain	Barcelone	Influent	30.0-383.0	Mastroianni et al. (2017)

Spain	Galicia	Rías Baixas areas	22.7	Fernández-Rubio et al. (2019)
German		Influent	130	Wick et al. (2009)
		Effluent	120	
Switzerland	Zurich	Influent	42.0-202.0	Berset et al. (2010)
		Effluent	44.0-128.0	
		River creeks	<LOQ-4.60	
		Lakes	1.10-2.50	
Canada	Quebec	WWTP grab	34.0 – 69.0	Rodayan et al. (2014)
		WWTP POCIS	36.0 – 62.0	
		WWTP 24h	52.0 – 63.0	
		WWTP influent	25.0 ± 3.0	
Slovakia	Petržalka	WWTP mechanical step	6.2 ± 0.80	Mackuľak et al. (2015)
		WWTP biological step	16.0 ± 2.0	
		WWTP sludge water	31.0 ± 3.0	
		WWTP effluent	18.0 ± 3.0	
France	Corbeil	Influent	26	Hubert et al. (2017)
		Effluent	22	
Italy	Lombardia region	Olona river	8.6	Zuccato et al. (2008)
		Lambro river	3.4	
	Po river	0.2-0.8		
UK	Various locations	Toscana region	0.6-10.10	Baker & Kasprzyk-Hordern (2011a)
		Arno river	0.6-10.10	
		WWTP influent	171.1	
UK	Various locations	WWTP Effluent	68.8	Baker & Kasprzyk-Hordern (2011b)
		River water	38.2	
		WWTP A	69.10-69.40	
England		WWTP B	70.0- 80.10	Baker & Kasprzyk-Hordern (2013)
		WWTP C	99.60-104.80	
		Influent	2.60-171.10	
Spain	Spanish basin	Effluent	1.40-91.0	Mastroianni et al. (2016)
		Llobregat river	9.60-20.0	
		Ebro river	5.0-10.80	
Czech Republic		Jucar river	1.30 – 2.40	Baker et al. (2012)
		Guadalquivir river	12.50-14.0	
		Untreated WW	13.10-19.10	
China	Various cities	Dianchi lake	1.8	Li et al. (2016)
China	Bohai and Yellow sea	Various rivers	0.123 -0.313	Wang et al. (2016)
France	Paris	Seine River	0.54- 1.74	Brieudes et al. (2017)
Czech Republic	Various cities	Surface water	0.50-5.60	Fedorova et al. (2014)
Netherlands	Various cities	Influent	<45.0	Bijlsma et al. (2012)
		Effluent	8.70- 47.0	
Spain	Valencia	Turia river (2012)	2.02-39.30	Andrés-Costa et al. (2017)
		Turia river (2011)	2.29-40.10	
Italy	Nosedo	Influent	11.60 ± 1.70	Castiglioni et al. (2006)
		Efluent	9.10 ± 0.5	
Switzerland	Logano	Influent	49.70 ± 9.60	
		Effluent	36.20 ± 2.80	

Netherlands	Various cities	Influent	240.0-536.0	Bijlsma et al. (2012)
		Effluent	173.0-245.0	
Sweden	Stockholm	STP inlet	670	Wennmalm & Gunnarsson (2009)
		STP outlet	160	
German		Influent	25	Wick et al. (2009)
		Effluent	370	
German		Influent	540	Hummel et al. (2006)
		Effluent	260	
Canada	Quebec	Rivers	94	Rodayan et al. (2014)
		WWTP grab	405.0 – 508.0	
		WWTP POCIS	459.0 – 508.0	
		WWTP 24h	456.0 – 519.0	
Slovakia	Petržalka	WWTP influent	123.0 ± 11.0	Mackuřak et al. (2015)
		WWTP mechanical step	105.0 ± 11.0	
		WWTP biological step	25.0 ± 2.0	
		WWTP sludge water	35.0 ± 4.0	
		WWTP effluent	24.0 ± 2.0	
		Eskilstuna	290.0 – 410.0	
Sweden	Stockholm (Wetlands)	Nynäshamn	180.0 – 730.0	Breitholtz et al. (2012)
		Oxelösund	51.0 – 210.0	
		Trosa	39.0 – 73.0	
		Hospital WWTP	4.50-700.0	
Taiwan	Taipei	Rivers	1.30-15.0	Lin et al. (2014)
		Influent	41	
		Effluent	56	
		Hospital WWTP	58.0-180.0	
China	Bohai and Yellow sea	Various rivers	0.829-1.76	Wang et al. (2016)
France	Paris	Seine River	3.13 – 13.8	Brieudes et al. (2017)
Italy	Verone	Influent	275.0-335.0	Repice et al. (2013)
		Effluent	110.0-126.0	
Taiwan	Keeting	Effluent	3967	Jiang et al. (2015)
Cyprus		Influent	2316.0-6460.0	Hapeshi et al. (2015)
		Effluent	<LOD-3783.0	
Spain	Southeast region	Influent	234.0-1556.0	Bueno et al. (2011)
		Effluent	289.0-786.0	
		Rivers	32.0-174.0	
		Influent	2.64-25.30	
Korea		Olona river	51	Kim et al. (2017)
Italy	Lombardia region	Lambro river	12	Zuccato et al. (2008)
		Po river	1.60-2.70	
	Toscana region	Arno river	4.70-8.80	
		Influent	18.10-119.70	
Spain	Catalonia			Boleda et al. (2007)

		Effluent	3.10-397.0	
		Llobregat river	18.50-26.70	
Croatia	Zagreb	Raw wastewater	159.0-364.0	Terzic et al. (2010)
		Secondary effluent	88.0-206.0	
		Influent	5.70-120.0	
		Effluent	3.10-397.0	
Spain	Catalonia	Llobregat river	2.0-39.50	Boleda et al. (2009)
		Anoia river	1.60 -67.90	
		Rubí river	212.50 – 251.0	
		Cardener river	5.0-13.50	
UK	Various locations	WWTP influent	2703.5	Baker & Kasprzyk-Hordern (2011a)
		WWTP Effluent	1206.2	
		River water	341.7	
		WWTP A	667.0- 955.70	
UK	Various locations	WWTP B	949.0- 1101.90	Baker & Kasprzyk-Hordern (2011b)
		WWTP C	1075.20- 2041.50	
England river		Influent	236.40- 3972.80	Baker & Kasprzyk-Hordern (2013)
		Effluent	9.70-1502.10	
		Influent	143.0- 325.0	
	Liberia	Effluent	11.0 -29.0	
Costa Rica		Pooled WS	<LOQ- 252.0	Causanilles et al. (2017)
	El Roble	Influent	438.0-538.0	
		Effluent	503.0-665.0	
		Tárcoles River	10	
Czech Republic		WW	162.20 -216.90	Baker et al. (2012)
Czech Republic	Various cities	Surface water	11.0- 42.0	Fedorova et al. (2014)
Spain	Valencia	Turia river (2013)	81.50-101.0	Andrés-Costa et al. (2017)
		Influent	<LOQ-389.0	
Switzerland	Zurich	Effluent	94.0-274.0	Berset et al. (2010)
		River/creeks	<LOQ-18.0	
		Lakes	2.10-4.40	
Souht Africa	Guateng province	Upstream	11.3	Archer et al. (2017)
		Downstream	128.9	
		Surface water	<2.0 – 1780.0	
Nigeria	Lagos	Groundwater	<2.0 – 2440.0	Ebele et al. 2020
		Sachet water	<2.0 – 305.0	
UK	River Taff		<30.0 -5970.0	Kasprzyk-Hordern et al. (2008)

	River Ely		<30.0-7731.0	
		Upstream WWTP	<30.0-435.0	
	River Taff	Downstream WWTP	1302.0-5970.0	
		Influent	8505.0-89,026	
	Cilyfynydd	Effluent	24,132.0-97,616	Kasprzyk-Hordern et al. (2009c)
UK		Upstream WWTP Coslech	46.0- 3468.0	
	River Ely	Downstream WWTP Coslech	731.0 – 1898.0	
		Influent	23, 037-85,843	
	Coslech	Effluent	12,779 – 56,810	
Sweeden	Stockholm	STP inlet	320	Wennmalm & Gunnarsson (2009)
		STP outlet	460	
German		Influent	470	Wick et al. (2009)
		Effluent	370	
		WWTP influent	860.0 ± 120.0	
Slovakia	Petržalka	WWTP mechanical step	670.0 ± 80.0	Mackul'ak et al. (2015)
		WWTP biological step	570.0 ± 50.0	
		WWTP sludge water	680.0 ± 40.0	
		WWTP effluent	710.0 ± 50.0	
		Eskilstuna	660- 663	
Sweeden	Stockholm (Wetlands)	Nyn äshamn	660 - 730	Breitholtz et al. (2012)
		Oxel ösund	510 - 740	
		Trosa	410 - 460	
		Möckeln	340	
Sweeden	Various lakes	Hjälmaren	320	El Marghani et al. (2014)
		Mälaren	28	
Czech Republic	Various cities	Surface water	38.0- 663.0	Fedorova et al. (2014)
Greece	Saronikos Gulf and Elefsis Bay	Sewater	0.1 – 1.0	Alygizakis et al. (2016)
		WWTP grab	58.0 – 128.0	
Canada	Quebec	WWTP POCIS	41.0 – 73.0	Rodayan et al. (2014)
		WWTP 24h	44.0 – 77.0	
		Influent	1320.7 ± 59.30	Evans et al. (2015)
UK		Effluent	506.0 ± 46.60	
Souht Africa	Guateng province	Upstream	97.7	Archer et al. (2017)
		Downstream	299.9	
France	Paris	Seine River	12.4- 90.3	Brieudes et al. (2017)
Nigeria	Lagos	Surface water	<2.0 – 852.0	Ebele et al. 2020

Groundwater	<2.0- 883.0
Sachet water	<2.0 -6.0

POCIS: polar organic chemical integrative samplers

MET concentrations ranged from 0.123 ng L⁻¹ in various rivers from China (Wang et al. 2016) to 1531 ng L⁻¹ in effluent from Catalonia (Boleda et al. 2009). COD residues ranged from 0.829 ng L⁻¹ in Bohai Sea (Wang et al. 2016) to 6460 ng L⁻¹ in influents of Cyprus (Hapeshi et al. 2015). Concentrations of TRA ranged from < 2.0 ng L⁻¹ in samples of water from Lagos (Nigeria) (Ebele et al. 2020) to 97,616 ng L⁻¹ in effluent samples from Cilfynydd (UK) (Kasprzyk-Hordern et al. 2009c).

There are few studies regarding opioids' biological effects in aquatic organisms, although relevant environmental concentrations were toxic to different non-target species. Gagné et al. (2010) showed that *Elliptio complanata* mussels MOR exposed (30, 150, and 750 ng g⁻¹ wet weight) may exhibit physiological changes such as reduction in serotonin levels and increase in dopamine. Parolini et al. (2015) exposed zebra mussels in a mixture of illicit drugs containing MOR (100 ng L⁻¹) and observed damages in macromolecules such as proteins, lipids, and DNA, in addition to an increase in antioxidant defenses.

Specimen of crayfish (*Orconectes rusticus*) exposed to MOR (2000 ng g⁻¹ dose) exhibited an increase of mobility (Imeh-Nathaniel et al. (2017). Freshwater mussels (*Dreissena polymorpha*) exposed to a mixture of illicit drugs containing 100 ng L⁻¹ of MOR exhibited a significant increase of DNA fragmentation and triggered the apoptotic process and micronuclei formation (Parolini et al. 2016a). Environmental concentrations of 50 ng L⁻¹ and 5000 ng L⁻¹

of MOR induced a decrease of lysosome membrane stability of bivalves and an increase of lipid peroxidation (Magni et al. 2014).

A study performed by Buřič et al. (2018) demonstrated that crayfish (*Procambarus virginalis* Lyko 2017) exposed to TRA ($810 \pm 110 \mu\text{g L}^{-1}$) and citalopram ($890 \pm 90 \text{ ng L}^{-1}$) exhibited changes in behavior, especially a decrease in locomotion ability and lower velocity. Ložek et al. (2019) observed that crayfish (*Pacifastacus leniusculus*) exposed to tramadol (1000 ng L^{-1}) also presented a reduction in locomotion ability and lower velocity and a significant increase in heart rate. *Danio rerio* larvae showed behavioral changes (hypoactivity in dark period) when exposed in $434,000 \text{ ng L}^{-1}$ of TRA after 144 h of exposure (Bachour et al. 2020).

Fish embryo of *D. rerio* exposed to TRA concentrations of 10,000; 100,000; and 200,000 ng L^{-1} exhibited a delay in the hatching process, after 3 days of exposure. Similar results were observed in *Cyprinus carpio* exposed to 10,000; 50,000; and 100,000 ng L^{-1} of TRA. After 14 days of exposure, significantly retarded development was observed. Carp exposed to 100,000 and 200,000 ng L^{-1} exhibited morphological anomalies (elevated pigmentation) and a higher number of the skin mucous cells. Furthermore, carp also showed a decrease in body weight (50,000 and 100,000 ng L^{-1}) after 6 days and oxidative stress when exposed to TRA (100,000 and 200,000 ng L^{-1}) (Sehonova et al. 2016).

3. Cannabinoids

Cannabis is the drug most cultivated, produced, trafficked, and consumed worldwide, and the number of users is estimated in 183 million people. In 2015, the number of seizures reached 5781 tons of herb (marijuana) and 1533 tons of resin (hashish). The largest producers of hashish are in Morocco, followed by Afghanistan and Lebanon. Regarding marijuana, Mexico and the USA stand out as the largest producers in the North American region, while Albania, Colombia, Jamaica, Holland, and Paraguay are considered the main countries of origin of the herb that is destined for the international market (UNODC 2016, 2017a).

There are three most relevant species of cannabis: *Cannabis sativa* is the main variety, followed by *C. indica* and *C. ruderalis*. The Cannabis plant contains more than 460 chemicals, of which 60 are classified as cannabinoids, with the delta Δ^9 -tetrahydrocannabinol (THC) being the main hallucinogenic component of the plant, responsible for its psychotropic effects (Ben Amar 2006).

THC is a lipophilic substance that can bioaccumulate in fat tissue because of the amount

and frequency of smoking (Melis et al. 2011), being slowly released into the blood compartments, including the brain (Ashton 2001). In human body, Cannabis can induce euphoria, dysphoria, sedation, changes in the perception of time and sensorial functions, and impairment in motor control and short-term memory, as well as dry mouth, tachycardia, and postural hypotension (Crippa et al. 2009).

Table 3. Concentration of cannabinoids in aquatic environment (ng/L).

Compound	Country	Local	Study site	Concentration (ng.L ⁻¹)	Reference
THC	Spain	Catalonia	Influent	11.30-31.50	Boleda et al. (2007)
			Effluent	<LOQ	
			Llobregat river	<LOQ-13.60	
	Spain	Catalonia	Influent	11.30-127.0	Boleda et al. (2009)
			Effluent	20.50	
	Spain	Spanish basin	Llobregat river	1.0	Mastroianni et al. (2016)
			Ebro river	3.60	
	Spain	Iberian Pensinsula	Influent	48.40	Postigo et al. (2010)
			Influent (Super Bowl weekend)		
	USA	Various cities	Effluent (Super Bowl weekend)		Gerrity et al. (2011)
			Influent (baseline weekend)	<100	
				Effluent (baseline weekend)	
	Martinique	Fort de France	Influent WWTP1	722.0 ± 96.0	Devault et al. (2017a)
			Influent WWTP2	1267.0 ± 178.0	
Effluent WWTP 1			19.0- 61.0		
Effluent WWTP 2			169.0 ± 73.0		
Martinique	Fort de France	WWTP1	369.0- 925.0	Devault et al. (2014)	
		WWTP2	268.0-621.0		
		WWTP3	310.0-940.0		
		WWTP4	352.0-1158.0		
Spain	Catalonia	Influent	<LOQ- 96.20	Boleda et al. (2007)	
		Effluent	14.80-71.70		
			Llobregat river	16.40-34.10	
Croatia	Various cities	STPs	132.0 ± 27.0	Krisman et al. (2016)	
France	Corbeil	Influent	1040.0	Hubert et al. (2017)	
Spain	Barcelone	Influent	212.0-1231.0	Mastroianni et al. (2017)	
Spain	Valencia	Influent	918.0-1638.0	Bijlsma et al. (2014)	
THC-COOH	Netherlands	Various cities	Influent	<33.0- 375.0	Bijlsma et al. (2012)
			Effluent	<7.0-13.0	
	Colombia	Bogota	Influent	184.0-268.0	Bijlsma et al. (2016)
			Mendelín	264.0-344.0	
Czech Republic	Various cities	Surface water	1.10- 5.90	Fedorova et al. (2014)	
	Spain	Catalonia	Influent	23.50-402.0	Boleda et al. (2009)
			Effluent	14.80-71.70	

		Llobregat river	4.70-79.50	
		Cardener river	7.30-42.60	
		Anoia river	3.10-3.80	
		Rubí river	5.70	
		Olona river	<0.48	
Italy	Lombardia region	Lambro river	3.70	
		Po river	<0.48-0.5	Zuccato et al. (2008)
	Toscana region	Arno river	<0.48-1.0	
England	London	Thames river	<0.48- 1.0 ± 0.6	
	Liberia	Influent	169.0-502.0	
		Effluent	<LOQ-37.0	
Costa Rica		Pooled WS	<LOD- 127.0	Causanilles et al. (2017)
	El Roble	Influent	124.0-206.0	
		Effluent	10.0- 36.0	
	Petržalka	Sewage	140.0	
Slovakia	Bratislava	Sewage	105.0	Mackulak et al. (2014)
	Other cities	Sewage	42.0-74.0	
Croatia	Zagreb	Raw wastewater	21.0- 128.0	
		Secondary effluent	<53.0	Terzic et al. (2010)
		WWTP influent	124.0 ± 15.0	
		WWTP mechanical step	53.0 ± 7.0	
Slovakia	Petržalka	WWTP biological step	2.9 ± 0.5	Mackulak et al. (2015)
		WWTP sludge water	3.5 ± 0.5	
		WWTP effluent	<7.0	
Spain	Spanish basin	Guadalquivir river	17.0	Mastroianni et al. (2016)
China	Shangai	22 surface water	5.83- 10.3	Yao et al. (2016)
		Influent (Super Bowl weekend)		
		Effluent (Super Bowl weekend)		
USA	Various cities	Influent (baseline weekend)	<100.0	Gerrity et al. (2011)
		Effluent (baseline weekend)		
		Surface water	5.50	
Spain	Iberian Pensinsula	Influent	10.60- 21.70	Postigo et al. (2010)
		Effluent	5.40- 72.80	
		Influent Pinedo I	20.40- 652.40	
Spain	Valencia	Influent Pinedo II	22.10-396.90	Andrés-Costa et al. (2014)
		Influent Quart-Bernager	18.80-940.20	
Italy	Nosedo	Influent	62.70 ± 5.0	
Switzerland	Logano	Influent	91.20 ± 24.70	Castiglioni et al. (2006)
		Effluent	7.20 ± 3.70	

About 50% of THC present in marijuana is inhaled during smoking and almost everything is absorbed by the lungs, and it quickly enters the bloodstream reaching the brain within minutes. However, the oral bioavailability is much lower, with blood concentrations equivalent to 25–30% of that obtained through smoking, due to the first phase of metabolism that occurs in the liver, which causes a “delay” in the effects, but it allows a prolonged duration due to the slow absorption process that takes place in the intestine (Ashton 2001).

About 70% of a dose of THC is excreted within 72 h in feces and urine as metabolites. THC is rapidly metabolized by hepatic metabolism through CYP450 enzymes and is converted to 11-hydroxy-THC (11-OH-THC) (active metabolite) or 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THC-COOH) (inactive metabolite) (Melis et al. 2011).

The occurrence of THC and THC-COOH in the aquatic environment is reported in Table 3. Concentrations of THC ranged from 1 ng L⁻¹ in Llobregart river at Spain (Mastroiani et al. 2016) to 127 ng L⁻¹ in influents from Catalonia (Boleda et al. 2009). The THC-COOH concentrations ranged from < 0.48 ng L⁻¹ in Olona river, Arno river, Po river, and Thames river (Zuccato et al. 2008) to 1638 ng L⁻¹ in influents of Valencia (Bijlsma et al. 2014).

Environmental concentrations of THC should not cause acute toxicity effects. However, carps (*Cyprinus carpio*) exposed to extracts of *C. sativa* (1.88; 3.75; 7.50; 15; and 30 mg L⁻¹) showed a decrease in alkaline phosphatase activity in the gills and liver and high levels of transaminases and lactate dehydrogenase in the blood (Audu et al. 2015). Sublethal responses were observed in soft tissues from zebra mussels exposed to environmental concentration of THC (500 ng L⁻¹) which presented oxidative stress and DNA damage (Parolini and Binelli 2014).

Saoud et al. (2017) observed that tilapia (*Oreochromis niloticus*) fed with a diet containing 10.7 g kg⁻¹ of Cannabis extract exhibited lower growth rate, increase metabolism, and feed conversion. Furthermore, Bedini et al. (2016) showed that *C. sativa* essential oil induced high mortality rates in invasive species *Aedes albopictus* larvae (exposed to 400 μ L L⁻¹ and 500 μ L L⁻¹) and *Physella acuta* (since 100 μ L L⁻¹).

4.Synthetic drugs: amphetamines, derivatives, and ecstasy

Amphetamine-type stimulants are synthetic drugs that can be manufactured almost anywhere because they do not depend on the extraction of active compounds from plants. In 2015, the seizure rate was estimated at 132 tons of methamphetamine, 52 tons of amphetamines,

and 6 tons of ecstasy (UNODC 2017b).

Ephedrine (EPH) and pseudoephedrine (PEPH) are two of the main precursors used in the manufacture of methamphetamine. Both chemicals have widespread legitimate use in the pharmaceutical industry (UNODC 2017b). While EPH is widely used as an adrenergic stimulant, PEPH is commonly used as a nasal decongestant (Limberger et al. 2013).

Methamphetamines (MAMP) can be found in the form of tablets or crystals and are predominant in East and Southeast Asia and North America. Ecstasy is also found in the form of tablets, containing 3,4-methylenedioxymethamphetamine (MDMA), being predominant in Europe and Asia. The number of users is estimated at 37 million people for amphetamines and 22 million people for ecstasy (UNODC 2017b).

In the human body, MDMA presents stimulating and hallucinogenic activity and may interfere in several neurotransmitters causing releases of serotonin, dopamine, and norepinephrine, which are directly involved in mood control, sleep, thermoregulation, and appetite (Xavier et al. 2008). Other amphetamines may stimulate the central nervous system (CNS) and act as indirect monoamine agonists, interacting with membrane transporters involved in neurotransmitter reuptake (Melis et al. 2011).

The main acute effects caused using amphetamines (AMP) correspond to the increase of the heart beats and hypertension, inhibition of the appetite, dilation of the pupils, dry mouth, and intense euphoria. Chronic effects are represented by pictures of psychosis, irritability, and even cerebral cell degeneration (Moratalla et al. 2017).

AMP are oxidatively deaminated by monoamine oxidase, whose reaction is catalyzed by CYP450 and CYP2C, and the hydroxylation of the AMP and METH ring by CYP2D6 (Kraemer and Maurer 2002). However, most of the MDMA is demethylated to 3,4-dihydroxymethamphetamine (DHMA) by CYP2D6 and other enzymes such as CYP21A and CYP3A4 may contribute to a lesser extent (Oesterheld et al. 2004; Vizeli et al. 2017).

Table 4. Concentration of amphetamines, derivatives and ecstasy in aquatic environment

Compound	Country	Local	Study site	Concentration (ng.L ⁻¹)	Reference
EPH	Croatia	Zagreb	Raw wastewater	<MDL -108.0	Senta et al. (2015)
			Secondary effluent	< MDL- 43.0	
	UK		Influent	20.20 ± 28.60	Evans et al. (2015)
			Effluent	1.30 ± 0.2	
	Spain	Southeast	Influent	912.0-4817.0	Bueno et al. (2011)
			Effluent	217.0-2749.0	
		River	51.0- 90.0		

			Llogrebat river	18.80-88.60	
	Spain	Spanish basin	Ebro river	1.0-144.0	Mastroianni et al. (2016)
			Jucar river	10.70-23.90	
			Guadalquivir river	21.60-66.20	
	South Africa	Guateng province	Upstream	38.80	Archer et al. (2017)
			Dowstream	80.40	
			Surface water	0.7- 145.0	
	Spain	Iberic Peninsula	Influent	203.0- 660.0	Postigo et a. (2010)
			Effluent	2.60-276.0	
			WWTP Influent	1032.10	
	UK	Various locations	WWTP Effluent	126.90	Baker & Kasprzyk-Hordern (2011a)
			River water	16.50	
			WWTP A	500.0- 800.20	
	UK	Various locations	WWTP B	676.20-1079.50	Baker & Kasprzyk-Hordern (2011b)
			WWTP C	529.10-1071.0	
			WWTP grab	114.0 – 164.0	
	Canada	Quebec	WWTP POCIS	136.0 – 167.0	Rodayan et al. (2014)
			WWTP 24h	118.0 – 158.0	
	China	Beijing	Surface water	1.20-185.70	Zhang et al. (2016)
	Spain	Valencia	Turia river (2013)	5.28- 17.60	Andrés-Costa et al. (2017)
	Spain	Barcelone	Influent	611.0- 3257.0	Mastroianni et al. (2017)
			Raw wastewater	26.0-698.0	
	Croatia	Zagreb	Secondary effluent	18.0-551.0	Senta et al. (2015)
			River water	<MDL-30.0	
PEPH	Taiwan	Keeting	Effluent	44.667	Jiang et al. (2015)
	China	Shangai	22 surface water	6.05-33.50	Yao et al. (2016)
			WWTP	6.70-16.90	
			Influent	497.70 ± 28.50	
	UK		Effluent	30.0 ± 0.2	Evans et al. (2015)
			Raw wastewater	14.0-545.0	
	Croatia	Zagreb	Secondary effluent	<MDL-126.0	Senta et al. (2015)
			River water	<MDL	
			Untreated wastewater	< LOQ- 25.0	
AMP	Canada	Various cities	Treated wastewater	< LOD- 14.0	Metcalf et al. (2010)
	Italy	Nosedo	Influent	14.70 ± 10.60	Castiglioni et al. (2006)
			Olona river		
	Italy	Lombardia region	Lambro river		
			Po river	<0. 65	Zuccato et al. (2008)
		Toscana region	Arno river		
England	London		Thames river		
			Raw wastewater	<2.60-3.10	
Croatia	Zagreb		Secondary effluent	<0.9-9.40	Terzic et al. (2010)
			WWTP grab	17.0 – 68.0	
Canada	Quebec		WWTP POCIS	18.0 – 78.0	Rodayan et al. (2014)
			WWTP 24h	10.0 – 85.0	

Slovakia	Petržalka	Sewage	98.0-257.0	Mackulak et al. (2014)
		WWTP influent	100.0 ± 8.0	
		WWTP mechanical step	54.0 ± 6.0	
Slovakia	Petržalka	WWTP biological step	<2.80	Mackulak et al. (2015)
		WWTP sludge water	<3.40	
		WWTP effluent	<2.90	
Spain	Bracelone	Influent	3.0-688.0	Huerta-Fontela et al. (2008a)
		Effluent	4.0-210.0	
UK	River Taff		<1.0 -14.0	Kasprzyk-Hordern et al. (2008)
	River Ely		<1.0- 21.0	
England	Various cities	Influent	17.40- 3112.50	Baker & Kasprzyk-Hordern (2013)
		Effluent	4.30-145.20	
		Influent	212.0-1021.0	
Spain	Southeast	Effluent	215.0-325.0	Bueno et al. (2011)
		River water	309.0	
Switzerland	Zurich	Influent	<LOQ-82.0	Berset et al. (2010)
		River/ creeks	<LOQ- 1.2.0	
		Influent Pinedo I	23.60-98.50	
Spain	Valencia	Influent Pinedo II	13.10-71.60	Andrés-Costa et al. (2014)
		Influent Quart-Bernager	1.70-110.0	
Spain	Barcelone	Influent	27.0- 235.0	Mastroianni et al. (2017)
		Surface water	1.60-12.10	
Spain	Iberic Pensinsula	Influent	3.30-664.0	Postigo et al. (2010)
		Effluent	0.90-57.60	
		Llobregat river	0.4- 50.0 ±5.0	Huerta-Fontela et al. (2008b)
		DWTP intake	0.8-50.0	
Czech Republic	Various cities	Surface water	0.50-5.60	Fedorova et al. (2014)
		Influent	280.30 ± 4.10	
UK		Effluent	22.0	Evans et al. (2015)
		Digested sludge	7.90 ± 1.70	
	River Taff	Upstream WWTP	<1.0-11.0	
		Downstream WWTP	2.0-13.0	
	Cilyfynydd	Influent	292.0-12,020	
		Effluent	19-739	
UK		Upstream WWTP	<1.0	Kasprzyk-Hordern et al. (2009c)
	River Ely	Coslech		
		Downstream WWTP	<1.0- 3.0	
		Coslech		
		Influent	255.0- 3225.0	
		Effluent	<3.0 -11.0	
Czech Republic		Untreated WW	33.30 -118.90	Baker et al. (2012)
Spain	Spanish basin	Ebro ri<LOQ-39.3ver	< LOQ-6.50	Mastroianni et al. (2016)
Korea		Influent	1.81 - 5.64	Kim et al. (2017)
USA	California	San Francisco Bay	<RL- 9.70	Klosterhaus et al. (2013)

South Africa	Guateng province	Upstream	27.10	Archer et al. (2017)	
		Downstream	37.0		
China	Beijing	Surface water	5.0- 11.20	Zhang et al. (2016)	
		Influent	<LOQ- 39.3		
	Beijing	Effluent	< 4.0		
		Influent	18.3- 33.2		
	Shangai	Effluent	<4.0		
		Influent	<LOQ- 9.8		
	Nanjing	Effluent	<LOQ- 2.0		
		Influent	37.9- 39.9		
	Haerbin	Influent	10.0- 17.0		
	Shenyang	Influent	6.1- 158.0		
	Xi'an	Influent	6.6- 11.6		
	Lanzhou	Influent	6.1- 28.1		
	Shijiazhuang	Influent	11.8- 19.8		
	Luoyang	Influent	<LOQ- 6.0		Du et al. (2015)
	Jinan	Influent	9.8- 690.4		
	Xiamen	Effluent	3.3- 4.5		
		Influent	4.5- 28.4		
	Wuhan	Effluent	< 4.0		
Influent		6.4- 19.6			
Kunming	Effluent	<4.0			
	Influent	3.5- 49.5			
Yinchuan	Effluent	< 4.0			
	Influent	9.0- 52.6			
Hangzhou	Influent	<LOQ- 4.9			
Nanning	Influent	12.6-32.0			
Shenzen	Influent	<LOQ- 7.4			
Guiyang	Influent	255.0-355.0			
USA	Various cities	Influent (Super Bowl weekend)	<25.0	Gerrity et al. (2011)	
		Effluent (Super Bowl weekend)	261.0-361.0		
		Influent (baseline weekend)	<25.0		
China	Various rivers	Influent	1.41-3.26	Wang et al. (2016)	
Netherlands	Various cities	Influent	81.0- 682.0	Bijlsma et al. (2012)	
		Effluent	<4.0- 6.90		
UK	Various locations	WWTP Influent	2300.10	Baker & Kasprzyk-Hordern (2011a)	
		WWTP Effluent	24.40		
UK	Various locations	River water	4.30	Baker & Kasprzyk-Hordern (2011b)	
		WWTP A	46.0- 48.30		
		WWTP B	42.70- 116.40		
Various cities	Various cities	WWTP C	124.70- 255.50	Metcalf et al. (2010)	
		Untreated wastewater	< LOQ- 65.0		
		Treated wastewater	< LOQ- 95.0		
Netherlands	Various cities	Influent	<15.0-17.0	Bijlsma et al. (2012)	
		Effluent	<5.0		

MAMP	Czech Republic		WW	393.0-822.70	Baker et al. (2012)
			Olona river	1.70	
	Italy	Lombardia region	Lambro river	2.10	
			Po river		Zuccato et al. (2008)
		Toscana region	Arno river	<0.41	
	England	London	Thames river		
			WWTP grab	16.0 – 79.0	
	Canada	Quebec	WWTP POCIS	9.0 – 92.0	Rodayan et al. (2014)
			WWTP 24h	14.0 – 85.0	
		Petržalka	WW	658.0	
	Slovakia		Sewage	953.0	Mackulak et al. (2014)
		Prešov	WW	79.0	
		Other cities	WW	175.0-300.0	
			WWTP influent	763.0 ± 19.0	
			WWTP mechanical step	482.0 ± 18.0	
	Slovakia	Petržalka	WWTP biological step	26.0 ± 12.0	Mackulak et al. (2015)
			WWTP sludge water	54.0 ± 3.0	
			WWTP effluent	30.0 ± 2.0	
	Spain	Barcelone	Influent	3.0-277.0	Huerta-Fontela et al. (2008a)
			Effluent	3.90	
	England		Influent	0.60-70.30	Baker & Kasprzyk-Hordern (2013)
			Effluent	0.40-1.30	
	UK	Various locations	WWTP influent	3.80	Baker & Kasprzyk-Hordern (2011a)
			WWTP effluent	1.20	
	UK		WWTP C	0.60	Baker & Kasprzyk-Hordern (2011b)
	Spain	Southeast	Influent	475.0-700.0	Bueno et al. (2011)
			Influent Pinedo I	3.10-69.10	
	Spain	Valencia	Influent Pinedo II	2.90-58.10	Andrés-Costa et al. (2014)
			Influent Quart-Benager	1.10-51.70	
	Switzerland	Zurich	Influent	<LOQ-27.0	Berset et al. (2010)
		Effluent	<LOQ-11.0		
Spain		Llobregat river	<0.2- 2.0 ±1.0	Huerta-Fontela et al. (2008b)	
		DWTP intake	<0.2- 6.10		
		Surface water	0.3-0.7		
Spain	Iberic Pensinsule	Influent	0.8-8.40	Postigo et al. (2010)	
		Effluent	0.5-7.60		
		Influent (Super Bowl weekend)	1840.0-2670.0		
		Effluent (Super Bowl weekend)	<25.0-86.0		
USA	Various cities	Influent (baseline weekend)	1910.0-2640.0	Gerrity et al. (2011)	
		Effluent (baseline weekend)	<25.0-32.0		
		Influent	34.0 ± 1.41		
UK		Effluent	28	Evans et al. (2015)	
		Digestive sludge	3.18 ± 0.5		
Spain	Spanish basin	Llobregat river	0.4-1.90	Mastroianni et al.	

		Ebro river	2.50-2.90	(2016)
		Jucar river	0.4	
		Guadalquivir river	0.7	
Korea		Influent	18.30-53.0	Kim et al. (2017)
China		Various rivers	16.10- 23.80	Wang et al. (2016)
		Various lakes	0.2-95.90	
		Songhua	<LOQ- 3.90	
China	Various cities	Yellow	0.8- 1.70	Li et al. (2016)
		Yangtze	1.30 ± 0.5- 2.80 ± 0.8	
		Pearl	17.40 - 58.20	
	Beijing	Influent	42.20 – 447.20	
		Effluent	0.6 – 112.0	
	Shanghai	Influent	264.0 – 645.0	
		Effluent	3.50- 250.0	
	Nanjing	Influent	15.8- 160.8	
		Effluent	1.0- 23.2	
	Haerbin	Influent	485.6 – 510.8	
	Shenyang	Influent	146.0- 259.2	
	Xi'an	Influent	128.0- 217.6	
	Lanzhou	Influent	90.4- 140.8	
	Shijiazhuang	Influent	109.2- 181.2	
	Luoyang	Influent	201.2- 434.0	
China	Jinan	Influent	41.8- 59.8	Du et al. (2015)
		Influent	309.6- 364.0	
	Xiamen	Effluent	82.2- 243.6	
		Influent	59.6- 428.4	
	Wuhan	Effluent	28.5- 53.5	
		Influent	94.8- 276.4	
	Kunming	Effluent	22.2- 77.4	
		Influent	54.8- 781	
	Yinchuan	Effluent	31.0- 67.4	
	Hangzhou	Influent	156.0- 318.4	
	Nanning	Influent	7.6- 90.0	
	Shenzen	Influent	181.6- 642.0	
	Guiyang	Influent	34.1- 250.8	
China	Beijing	Surface water	3.30- 99.50	Zhang et al. (2016)
China		Various rivers	0.1-42.0	Wang et al. (2016)
Spain	Barcelone	Influent	23.0-225.0	Mastroianni et al. (2017)
Czech Republic	Various cities	Surface water	1.90- 277.0	Fedorova et al. (2014)
Italy	Nosedo	Influent	16.20 ± 7.10	Castiglioni et al. (2006)
		Effluent	3.50 ± 2.0	
		Raw wastewater	12.0 -160.0	
Croatia	Zabreg	Secondary effluent	8.80 -81.0	Senta et al. (2015)
		River water	2.20-8.60	
Canada	Various cities	Untreated WW	9.0- 35.0	Metcalf et al. (2010)

		Treated WW	<LOD- 32.0	
	Italy	Nosedo	Influent	14.21 ± 14.50
			Effluent	4.40 ± 3.70
	Switzerland	Logano	Influent	13.60 ± 12.60
			Effluent	5.10 ± 3.0
	France	Paris	Influent	<LOQ-28.0
			Effluent	<LOQ-10.20
			Olona river	1.70
	Italy	Lombardia region	Lambro river	1.10
			Po river	<0.35-0.4
		Toscana region	Arno river	<0.5-1.40
	England	London	Thames river	2.0 – 6.0 ± 0.3
	Netherlands	Various cities	Influent	<12.0- 140.0
			Effluent	30.0- 138.0
MDMA	Spain	Valencia	Influent	>27000
	Colombia	Bogota	Influent	12.0 -68.0
		Mendelin	Influent	10.0-14.0
			Surface water	0.2- 11.80
	Spain	Iberian Pensinsula	Influent	3.50-180.0
			Effluent	3.30-120.0
			WWTP influent	137.90
	UK	Various locations	WWTP effluent	155.70
			River water	24.80
			WWTP A	1.80 – 2.80
	UK	Various locations	WWTP B	1.80 -2.30
			WWTP C	10.60- 12.60
	France	Corbeil	Effluent	7.0
			Influent (Super Bowl weekend)	197.0 -402.0
	USA	Various cities	Effluent (Super Bowl weekend)	36.0 -118.0
			Influent (baseline weekend)	102.0 -476.0
			Effluent (baseline weekend)	28.0-83.0
	Czech Republic	Various cities	Surface water	0.80- 11.0
	Spain	Galicia	Rías Baixas areas	4.82
	Spain	Barcelone	Influent	23.0- 287.0
	France	Paris	Seine River	<LOQ- 1.57
	Croatia	Zagreb	Raw wastewater	2.20-33.0
			Secondary effluent	<0.3-8.40
			WWTP grab	140.0 – 192.0
	Canada	Quebec	WWTP POCIS	96.0 – 196.0
			WWTP 24h	99.0 – 216.0
		Petržalka	Sewage	73.0
	Slovakia	Bratislava	Sewage	31.0
		Trenčín	Sewage	239.0
	Slovakia	Petržalka	WWTP influent	<7.3

		WWTP mechanical step	<8.5	(2015)
		WWTP biological step	<3.8	
		WWTP sludge water	4.8 ± 0.5	
		WWTP effluent	<4.3	
		Influent Pinedo I	7.60- 101.40	
Spain	Valencia	Influent Pinedo II	5.70- 67.40	Andrés-Costa et al. (2014)
		Influent Quart-Benager	<LOQ- 159.10	
Spain	Valencia	Turia river (2012)	22.80	Andrés-Costa et al. (2017)
		Turia river (2013)	2.34- 7.21	
Spain	Barcelone	Influent	2.0-598.0	Huerta-Fontela et al. (2008a)
		Effluent	2.0-267.0	
England		Influent	0.7-455.4	Baker & Kasprzyk-Hordern (2013)
		Effluent	0.6-177.70	
Switzerland	Zurich	Influent	<LOQ- 108.0	
		Effluent	<LOQ- 29.0	Berset et al. (2010)
		River/ creeks	<LOQ- 1.40	
Spain		Llogrebat river	1±0.5 – 40.0 ± 5.0	Huerta-Fontela et al. (2008b)
		DWTP intake	0.3-55.0	
UK		Influent	34.3 ± 1.3	
		Effluent	45.3 ± 0.5	Evans et al. (2015)
Czech Republic		Digestive sludge	16.3 ± 0.7	
		Untreated WW	14.2 -101.6	Baker et al. (2012)
China			0.4 ± 0.2 – 2.1 ± 0.4	Li et al. (2016)
Taiwan	Keeting	Effluent	1267.0	Jiang et al. (2015)
Cyprus		Influent	< LOD-112.0	Hapeshi et al. (2015)
		Llogrebat river	7.60-56-80	
Spain	Spanish basin	Ebro river	3.10- 14.30	Mastroianni et al. (2016)
		Jucar river	0.3- 0.5	
		Guadalquivir river	3.0-23.0	
Italy	Nosedo	Influent	4.60 ± 7.30	Castiglioni et al. (2006)
		Effluent	1.10 ± 1.50	
		Effluent	0.9 ± 1.90	
Italy	Lombardia region	Olona river	<.1.18	
		Lambro river	<.1.18	
		Po river	<.1.18	Zuccato et al. (2008)
England	Toscana region	Arno river	<.1.18-1.50	
England	London	Thames river	<.1.18-4.0	
Spain	Barcelone	Influent	3.0-266.0	Huerta-Fontela et al. (2008a)
		Effluent	1.0-200	
UK	Various locations	WWTP Influent	15.20	Baker & Kasprzyk-Hordern (2011a)
		WWTP effluent	24.50	
England		Influent	7.20-32.40	Baker & Kasprzyk-Hordern (2013)
		Effluent	6.30-24.50	
Spain	Southeast	Influent	266.0	Bueno et al. (2011)
USA	Various cities	Influent (Super Bowl weekend)	34.0-54.0	Gerrity et al. (2011)

		Effluent (Super Bowl weekend)	<50.0	
		Influent (baseline weekend)	31.0 -70.0	
		Effluent (baseline weekend)	<50.0	
		WWTP influent	<5.3	
		WWTP mechanical step	<6.1	
Slovakia	Petržalka	WWTP biological step	<2.8	Mackuľak et al. (2015)
		WWTP sludge water	<3.4	
		WWTP effluent	<2.9	
China	Various cities	Various lakes	1.90 -2.70	Li et al. (2016)
Spain		Llobregat river	0.4- 20.0 ± 5.0	Huerta-Fontela et al. (2008b)
		DWTP intake	<0.2- 25.0	
		Influent	17.0 ± 1.40	
UK		Effluent	42.70 ± 1.30	Evans et al. (2015)
		Digestive sludge	2.20 ± 0.6	

MDMA elimination partially depends on the liver, and 65% of the dose is eliminated without metabolism by renal excretion (Xavier et al. 2008). A rate of 30 to 74% of the AMP dose is excreted unchanged. In the case of MAMP, 70 to 90% of the drug is excreted in the urine, 43% of which is excreted unchanged, and, for both drugs, urine pH may influence the rate of excretion (Baselt 2004). These substances have been detected in wastewater and used as target wastes to assess drug use in each region (Daughton 2011).

Regarding the occurrence of the AMP and amphetamine type stimulants, as well as synthetic drug group in aquatic environment, Table 4 presents a summary of main studies. The most frequently detected substances are EPH, PEPH, AMP, MAMP, MDMA, and MDA.

EPH concentrations ranged from 0.7 ng L⁻¹ in surface water samples from Iberic Peninsula (Postigo et al. 2010) to 4817 ng L⁻¹ in influents from Spain (Bueno et al. 2011). PEPH was detected in a smaller number of studies and their concentrations ranged from 6.05 ng L⁻¹ surface water samples from Shanghai (Yao et al. 2016) to 698 ng L⁻¹ in raw wastewater samples from Zagreb (Senta et al. 2015).

Residues of AMP ranged from < 0.65 river samples from Italy and England (Zuccato et al. 2008) to 3112.5 ng L⁻¹ in influent from England (Baker and Kasprzyk-Hordern 2013).

Concentrations of MAMP ranged from $< 0.2 \text{ ng L}^{-1}$ Llobregat river and samples from a DWTP intake (Hue) to 2670 ng L^{-1} in influent samples from the USA after Super Bowl weekend (Gerrity et al. 2011). The lowest concentration of MDMA was detected in secondary effluent ($< 0.3 \text{ ng L}^{-1}$) from Zagreb (Terzic et al. 2010) and the highest concentration was found in influent ($> 27,000 \text{ ng L}^{-1}$) from Valencia (Bijlsma et al. 2014). Huerta-Fontela et al. (2008b) detected low concentration of MDA ($< 0.2 \text{ ng L}^{-1}$) in samples of DWTP intake from Spain and Huerta-Fontela et al. (2008a). Bueno et al. (2011) found 266 ng L^{-1} of this compound in influent from Barcelona.

Environmental concentrations of AMP and their derivatives showed chronic effects on non-target organisms. Zebra mussels exposed to a mixture of illicit drugs containing AMP (300 ng L^{-1}) presented DNA, proteins, and lipid damages, and an increase in antioxidant defenses (Parolini et al. 2015). High concentrations of AMPs (5000 ng L^{-1}) induce oxidative and genotoxic damage in zebra mussels (Parolini et al. 2016a). Fish exposed to high concentrations of AMP ($5; 10 \text{ mg L}^{-1}$) presented alterations in levels of brain monoamines and behavior (Kyzar et al. 2013). Crayfish (*O. rusticus*) exposed to 2000 and $10,000 \text{ ng L}^{-1}$ of methamphetamine exhibited an increase of mobility (Imeh-Nathaniel et al. 2017). Stewart et al. (2011) observed that high doses of MDMA ($40\text{--}120 \text{ mg L}^{-1}$) reduced bottom swimming and immobility in zebrafish.

5. Cocaine

Cocaine (COC) is an alkaloid extracted from the *Erythroxylum coca* leaf that grows in the Andean Mountains, mainly in Peru, Bolivia, and Colombia (Melis et al. 2011; UNODC 2017b), and can be extracted from the leaf with the aid of organic solvents such as kerosene (Carrera et al. 2004). The land area used to coca cultivation is estimated at 245,000 ha, equivalent to 343,137 soccer fields. Global production of pure cocaine in 2017 reached 1545 tons and it is estimated that the number of users reaches 18 million people worldwide (UNODC 2019).

According to the World Drug Report (UNODC 2017a), South America is responsible for virtually all the world's COC production, whose use is mainly concentrated in that region, followed by Europe and Oceania. South America has been consolidated as a major consumer, as well as an important traffic route, mainly in Brazil, due to the factors of geographical location and high rate of urban population (UNODC 2014). Recent data point that 1.75% of the adult

population in the country uses cocaine, which in 2013 registered several seizures equivalent to 40 tons (UNODC 2015).

After opiates, COC is the most used drug, especially in the Americas, and is found in two forms: hydrochloride salt (used orally, intravenously, or intranasally) and “free base,” a mixture of cocaine with ammonia and sodium bicarbonate that is used for smoking and is better known as “crack” (Melis et al. 2011).

COC has the uncommon characteristic of being highly hydrophilic and also lipophilic. Usually, salts of acids (SO₄, NO₃, HCl) are added to cocaine, which turns into a polar, water-soluble compound. When mixed with sodium carbonate (Na₂CO₃), the boiling point decreases to 90 °C, making the compound practically insoluble in water and allowing crack pyrolysis. In the marine environment, both powdered cocaine and crack become free bases because of seawater pH on ionization of weak bases (Florence and Attwood 2006).

Different forms of consumption produce different patterns and levels of COC in the plasma: rapid absorption occurs through the intravenous route and through inhalation (smoking). Since COC has a high pKa (8.7), oral consumption promotes rapid ionization in the digestive system, reducing the rate of absorption (Carrera et al. 2004). In addition, cocaine acts by stimulating the CNS and enhances the action of dopamine, serotonin, and noradrenaline, producing feelings of euphoria, anxiety, and alertness (Carlini et al. 2001) as well as increased blood pressure, heart and respiratory rate, body temperature, pupil dilation, and increased motor activity (Amaral et al. 2010).

Table 5. Concentration of cocainics in aquatic environment (ng/L).

Compound	Country	Local	Study site	Concentration (ng.L ⁻¹)	Reference
		Milan		255.0	
	Italy	Como	Various STPs	98.0	Castiglioni et al. (2011)
		Sardinia		48.0-138.0	
	USA	Chicago	Stickney Water Reservation	868.0	
	Brazil	Santos	Santos Bay	12.60-537.0	Pereira et al. (2016)
			Influent	40.0 -820.0	
	Spain	Southeast region	Effluent	12.0 -496.0	Bueno et al. (2011)
			River	5.0 -87.0	
	Brazil	Federal District	Various WWTPs	174.0 -3690	Maldaner et al. (2012)
	Spain	Galicia	Rías Baixas areas	42.30	Fernández-Rubio et al. (2019)
	Italy	Pavia	River Po	1.20 ± 0.2	Zuccato et al. (2005)
		Other cities	Influent	42.0-120.0	
	Brazil	São Paulo	Surface water	6.0 – 62.0	Campestrini & Jardim (2017)
			Drinking water	6.0- 22.0	

COC	UK	River Taff		<0.3 – 7.0	Kasprzyk-Hordern et al. (2008)	
		River Ely		<0.3		
	River Taff	Upstream WWTP		0.3	Kasprzyk-Hordern et al. (2009c)	
		Downstream WWTP		<0.3-4.0		
	Cilyfynydd	Influent		21.0-1837.0		
		Effluent		48.0-324.0		
	UK	Upstream WWTP		<0.3		
		River Ely	Coslech	Downstream WWTP		<0.3
			Coslech	Influent		54.0- 471.0
				Effluent		<1.0
	Brazil	Manaus	Igarape do 40	5896		Thomas et al. (2014)
	Brazil	Santos	Santos Bay	<3.0- 203.60		Fontes et al. (2019)
			Ringsend influent	489.0 ± 117.0		
			Ringsend effluent	138.0 ± 20.0		
			River broadmeadow	25.0 ± 7.0		
	Ireland	Dublin	Shanganagh effluent	77.0 ± 25.0		Bones et al. (2007)
			Navan effluent	111.0 ± 15.0		
			Leixlip effluent	47.0 ± 10.0		
			River Liffey	33.0 ± 11.0		
	Frances	Paris	Influent	4.80- 282.0		Karolak et al. (2010)
Effluent			<LOQ- 20.70			
Spain	Barcelone	Influent	4.0 - 4.700	Huerta-Fontela et al. (2008a)		
		Effluent	1.0 -100			
Martinique	Fort de France	WWTP1	88.0 -473.0	Devault et al. (2014)		
		WWTP2	323.0 -1751.0			
		WWTP3	208.0 -554.0			
		WWTP4	64.0 -393.0			
Canada	Quebec	WWTP grab	70.0 – 869.0	Rodayan et al. (2014)		
		WWTP POCIS	333.0 –1841.0			
		WWTP 24h	72.0 – 903.0			
Slovakia	Petržalka	Sewage	62.0	Mackulak et al. (2014)		
	Bratislava	Sewage	96.0			
	Trenčín	Sewage	40.0			
Slovakia	Petržalka	WWTP influent	36.0 ± 7.0	Mackulak et al. (2015)		
		WWTP mechanical step	38.0 ± 6.0			
		WWTP biological step	6.7 ± 0.8			
		WWTP sludge water	<3.20			
Belgium	Flanders	WWTP effluent	6.30 ± 0.60	Gheorghe et al. (2008)		
		Influent	22.0- 678.0			
		Surface water	7.0 – 26.0			
		Olona river	44.0			
Italy	Lombardia region	Lambro river	15.0	Zuccato et al. (2008)		
		Po river	0.3-0.8			
	Toscana region	Arno river	0.3-2.90			
England	London	Thames river	<0.13-6.0			

Spain	Valencia	Influent	400.0 -450.0	Bijlsma et al. (2014)
Colombia	Bogota	Influent	64.0 -808.0	Bijlsma et al. (2016)
	Mendelín	Influent	60.0-1132.0	
France	Paris	Seine River	<LOQ - 8.26	Brieudes et al. (2017)
Croatia	Zagreb	Raw wastewater	30.0-114.0	Terzic et al. (2010)
		Secondary effluent	8.0 – 70.0	
Belgium	Various cities	WWTP	45.0- 2258.0	Van Nuijs et al. (2009)
		Surface water	<1.0 – 114.90	
Costa Rica	Liberia	Influent	763.0 -2710.0	Causanilles et al.(2017)
	El Roble	Influent	525.0 – 1050.0	
		Effluent	29.0 -62.0	
UK	Various locations	WWTP Influent	109.0	Baker & Kasprzyk-Hordern (2011a)
		WWTP effluent	65.20	
		River water	14.0	
UK	Various locations	WWTP A	37.50 -43.20	Baker & Kasprzyk-Hordern (2011b)
		WWTP B	43.90- 51.50	
		WWTP C	66.70- 81.90	
Czech Republic		WW	58.90- 155.30	Baker et al. (2012)
		Influent (Super Bowl weekend)	465.0-1030.0	
		Effluent (Super Bowl weekend)	<10.0	
USA	Various cities	Influent (baseline weekend)	580.0 -899.0	Gerrity et al. (2011)
		Effluent (baseline weekend)	<10.0	
Spain	Barcelone	Influent	308.0 – 2667.0	Mastroianni et al. (2017)
Spain		Llobregat river	0.1- 60.0 ± 10.0	Huerta-Fontela et al. (2008b)
		DWTP intake	2.10- 60.0	
Martinique	For de France	Influent WWTP2	778.0 ± 649.0	Devault et al. (2017a)
		Influent WWTP2	449.0 ± 127.0	
		Effluent WWTP2	51.0 ± 55.0	
Switzerland	Zurich	Influent	<LOQ-1920.0	Berset et al. (2010)
		Effluent	<LOQ-106.0	
		Rivers/ creeks	<LOQ-3.70	
		Llobregat river	7.10-23.80	
Spain	Spanish basin	Ebro river	25.40 -34.20	Mastroianni et al. (2016)
		Jucar river	4.50-8.10	
		Guadalquivir river	9.90 -15.30	
England		Influent	5.10- 208.90	Baker & Kasprzyk-Hordern (2013)
		Effluent	0.6 – 70.30	
France	Corbeil	Influent	194.0	Hubert et al. (2017)
		Effluent	8.0	
Spain	Valencia	Influent Pinedo I	195.10 -1871.70	Andrés-Costa et al. (2014)
		Influent Pinedo II	172.20- 3429.70	
		Influent Quart-Benager	110.30-3291.90	
USA	California	San Francisco Bay	<RL- 2.40	Klosterhaus et al. (2013)

	China	Various cities	Yantze	0.2 ± 0.2 – 0.7 ± 0.5	Li et al. (2016)
	China		Various rivers	0.829-1.76	Wang et al. (2016)
	Czech Republic	Various cities	Surface water	0.8-3.10	Fedorova et al. (2014)
			Hospital WWTP	6.30 -130.0	
	Taiwan	Taipei	Influent WWTP	1.20	Lin et al. (2014)
			Effluent WWTP	0.6	
	Canada	Various cities	Untreated WW	209.0- 823.0	Metcalf et al. (2010)
			Treated WW	< LOD- 530.0	
	Cyprus		Influent	<LOD-8.0	Hapeshi et al. (2015)
			Effluent	<LOD-1.0	
	Italy	Nosedo	Influent	421.40 ± 83.30	
	Switzerland	Logano	Influent	218.40 ± 58.40	Castiglioni et al. (2006)
			Effluent	10.70 ± 3.20	
	Netherlands	Various cities	Influent	118.0-559.0	Bijlsma et al. (2012)
			Effluent	<6.0- 35.0	
			Surface water	0.4-59.20	
	Spain	Iberian Pensinsula	Influent	195.0-961.0	Postigo et al. (2010)
			Effluent	1.90-31.10	
		Milan		712.0	
	Italy	Como	Vaious STP	380.0	
		Sardinia		144.0 -337.0	Castiglioni et al. (2011)
	USA	Chicago	Stickney Water Reservation	1553.0	
	Brazil	Santos	Santos Bay	4.60-20.80	Pereira et al. (2016)
	Brazil	Santos	Santos Bay	<1.20 -38.59	Fontes et al. (2019)
			WWTP influent	368.30	
	UK	Various locations	WWTP effluent	293.30	Baker & Kasprzyk-Hordern (2011a)
			River water	52.50	
			WWTP A	140.90 -192.80	
	UK	Various locations	WWTP B	172.60-242.20	Baker & Kasprzyk-Hordern (2011b)
			WWTP C	154.90- 205.10	
			Influent	851.0 -409.0	
	Spain	Southeast region	Effluent	487.0-2221.0	Bueno et al. (2011)
			River	10.0 -530.0	
BZE	Brazil	Federal District	Various WWTPs	663.0- 9717.0	Maldaner et al. (2012)
	Italy	Paiva	River Po	25.50 ± 5.0	Zuccato et al. (2005)
			Influent	390.0 -750.0	
	Brazil	São Paulo	Drinking water	10.0 -1019.0	Campestrini & Jardim (2017)
			Surface water	10.0 -652.0	
	France	Paris	Seine River	0.47- 4.82	Brieudes et al. (2017)
	Brazil	Amazonas	Igarapé do Mindu	366.0- 604.0	Thomas et al. (2014)
			Igarapé do 40	1639.0-3582.0	
	Italy	Verone	Influent	130.0 -715.0	Repice et al. (2013)
			Effluent	36.0 -58.0	
	Spain	Galicia	Rias Baixas area	142.0	Fernández-Rubio et al. (2019)

Ireland	Dublin	Ringsend influent	290.0 ± 11.0	Bones et al. (2007)
		Ringsend effluent	22.0 ± 4.0	
		Shanganagh effluent	31.0 ± 18.0	
USA	Various cities	Influent (Super Bowl weekend)	1620.0 -2330.0	Gerrity et al. (2011)
		Effluent (Super Bowl weekend)	<25.0	
		Influent (baseline weekend)	944.0 -1580.0	
France	Paris	Effluent (baseline weekend)	<25.0	Karolak et al. (2010)
		Influent	64.0 -849.20	
Spain	Barcelone	Effluent	7.90- 149.0	Huerta-Fontela et al. (2008a)
		Influent	9.0- 7500	
Martinique	Fort de France	WWTP1	440.0-1024.0	Devault et al. (2014)
		WWTP2	1151.0-2494.0	
		WWTP3	692.0-1652.0	
		WWTP4	233.0-1669.0	
UK	River Taff		<1.0 -123.0	Kasprzyk-Hordern et al. (2008)
	River Ely		<1.0 - 84.0	
UK	River Taff	Upstream WWTP Cilyfynydd	<1.0	Kasprzyk-Hordern et al. (2009c)
		Downstream WWTP Cilyfynydd	3.0-92.0	
	Cilyfynydd	Influent	126.0- 2114.0	
		Effluent	202.0-3275.0	
	River Ely	Upstream WWTP Coslech	<1.0	
		Downstream WWTP Coslech	<1.0- 54.0	
Canada	Quebec	Influent	187.0- 3715.0	Rodayan et al. (2014)
		Effluent	<1.0 -29.0	
		WWTP grab	356.0 – 1594.0	
Slovakia	Petržalka	WWTP POCIS	18.0 – 78.0	Mackulak et al. (2014)
		WWTP 24h	298.0 – 1855.0	
		Sewage	219.0	
		Sewage	202.0	
Slovakia	Petržalka	Sewage	88.0	Mackulak et al. (2015)
		Sewage	24.0	
		WWTP influent	65.0 ± 9.0	
		WWTP mechanical step	60.0 ± 10.0	
Colombia	Bogota	WWTP biological step	9.20 ± 1.0	Bijlsma et al. (2016)
		WWTP sludge water	5.60 ± 0.6	
UK	Mendelín	WWTP effluent	18.0 ± 3.0	Jones et al. (2014)
		Influent	1148.0 -1716.0	
Belgium	Flanders	Influent	4748.0 -6160.0	Gheorghe et al. (2008)
		Surface water	907.0-1544.0	
Italy	Lombardia region	Olona river	82.0 – 1898.0	Zuccato et al.
			53.0 -191.0	
			183.0	

		Lambro river	50.0	(2008)
		Po river	2.20 -5.10	
	Toscana region	Arno river	8.10 -37.20	
England	London	Thames river	4.0- 17.0 ±0.8	
Belgium	Various cities	WWTP	46.0 – 2258.0	Van Nuijs et al. (2009)
		Surface water	<0.5- 520.20	
Spain	Valencia	Influent	1000.0-1400.0	Bijlsma et al. (2014)
Croatia	Zagreb	Raw wastewater	89.0 -325.0	Terzic et al. (2010)
		Secondary effluent	47.0-174.0	
	Liberia	Influent	2100.0 -4500.0	
Costa Rica		Effluent	1260.0 -1450.0	
	El Roble	Influent	2280.0 -3520.0	Causanilles et al.(2017)
		Effluent	340.0 -792.0	
		Tárcoles River	72.0	
Czech Republic		WW	115.80- 309.20	Baker et al. (2012)
Spain	Barcelone	Influent	729.0- 3642.0	Mastroianni et al. (2017)
Spain		Llobregat river	15.0 -150	Huerta-Fontela et al. (2008a)
		DTWP intake	20.0- 770.0	
Croatia	Various cities	STPs	190.0 ± 89.0	Krizman et al. (2016)
		Influent WWTP1	2260.0 ± 697.0	
Martinique	Fort de France	Influent WWTP2	1781.0 ± 312.0	Devault et al. (2017a)
		Effluent WWTP 2	169.0 ± 73.0	
		Influent	<LOQ-1860.0	
Switzerland	Zurich	Effluent	37.0- 425.0	Berset et al. (2010)
		River/ creeks	<LOQ-11.0	
		Lakes	0.3-2.40	
		Llobregat river	25.20 -44.0	
Spain	Spanish basin	Ebro river	94.60 -129.0	Mastroianni et al. (2016)
		Jucar river	6.60 -21.40	
		Guadalquivir river	30.30 -62.90	
England		Influent WWTP	15.80- 566.60	Baker & Kasprzyk-Hordern (2013)
		Effluent WWTP	0.80- 293.30	
France	Corbeil	Influent	592.0	Hubert et al. (2017)
		Effluent	44.0	
		Influent Pinedo I	870.90 -3525.10	
Spain	Valencia	Influent Pinedo II	879.20- 2972.30	Andrés-Costa et al. (2014)
		Influent Quart-Benager	1011.0- 7752.50	
Spain	Valencia	Turia river (2013)	81.50 -101.0	Andrés-Costa et al. (2017)
USA	California	San Francisco Bay	2.80 - 7.20	Klosterhaus et al. (2013)
Czech Republic	Various cities	Surface water	0.5-8.40	Fedorova et al. (2014)
China	Various cities	Yangtze	0.8– 1.4	Li et al. (2016)
		Pearl river	0.50-0.60	
China		Various rivers	0.070-16.20	Wang et al. (2016)
Antartica		Stream	91.67	Gonzalez-Alonso

					et al. 2017)
	Canada	Various cities	Untreated wastewater	287.0 -2624.0	Metcalf et al. (2010)
			Treated wastewater	62.0 -775.0	
	Cyprus		Influent	13.0 -53.0	Hapeshi et al. (2015)
			Effluent	1.0 -7.0	
		Nosedo	Influent	1132.10 ± 197.20	
	Italy	Lugano	Influent	547.40 ± 169.40	Castiglioni et al. (2006)
			Effluent	100.30 ± 28.60	
	Netherlands	Various cities	Influent	409.0-2306.0	Bijlsma et al. (2012)
			Effluent	<2.0- 196.0	
			Surface water	1.40 -346.0	
	Spain	Iberian Pensinsula	Influent	545.0 -3790.0	Postigo et al. (2010)
			Effluent	4.10 -510.0	
		Milan		20.0	
	Italy	Como	Various STP	12.0	
		Sardinia		5.30 -11.0	Castiglioni et al. (2011)
	USA	Chicago	Stickney Water Reservation	51.0	
	UK		Influent	27.40-53.95	Jones et al. (2014)
	Italy	Nosedo	Influent	36.60 ± 7.80	
			Influent	18.80 ± 5.60	Castiglioni et al. (2006)
	Switzerland	Logano	Effluent	7.50 ± 2.90	
NOR-BZE			WWTP influent	15.20	
	UK	Various locations	WWTP effluent	12.0	Baker & Kasprzyk-Hordern (2011a)
			River water	2.80	
			WWTP A	4.60- 5.40	
	UK	Various locations	WWTP B	5.0- 7.60	Baker & Kasprzyk-Hordern (2011b)
			WWTP C	4.30 – 6.30	
	Czech Republic		WW	2.30 -8.0	Baker et al. (2012)
	England		Influent WWTP	1.80 - 19.40	Baker & Kasprzyk-Hordern (2013)
			Effluent WWTP	0.5- 14.60	
		Milan		4.0	
	Italy	Como	Various STP	3.20	
		Sardinia		0.30 -3.50	Castiglioni et al. (2011)
	USA	Chicado	Stickney Water Reservation	28.0	
	Italy	Nosedo	Influent	13.70 ± 5.30	
			Influent	4.30 ± 0.90	Castiglioni et al. (2006)
	Switzerland	Logano	Effluent	0.70 ± 0.90	
NOR-COC			WWTP influent	1.0	
	UK	Variou locations	River water	0.10	Baker & Kasprzyk-Hordern (2011a)
			Influent (Super Bowl weekend)	13.0 -36.0	
			Effluent (Super Bowl weekend)	<10.0	
	USA	Various cities	Influent (baseline weekend)	11.0 -32.0	Gerrity et al. (2011)
			Effluent (baseline weekend)	<10.0	
	Italy	Milan	Various STP	6.40	Castiglioni et al.

		Como		2.70	(2011)
		Sardinia		0.40 -5.10	
	USA	Chicago	Stickney Water Reservation	23.0	
			WWTP influent	5.40	
	UK	Various locations	WWTP effluent	5.40	Baker & Kasprzyk-Hordern (2011a)
			River water	1.40	
			WWTP A	1.30 – 1.40	
CE	UK	Various locations	WWTP B	1.10 – 1.80	Baker & Kasprzyk-Hordern (2011a)
			WWTP C	3.50- 5.60	
	Czech Republic		WW	1.80 -10.70	Baker et al. (2012)
	England		Influent WWTP	0.90 -15.0	Baker & Kasprzyk-Hordern (2013)
			Effluent WWTP	0.50 -7.90	
	Spain	Galicia	Rías Baixas area	5.20	Fernández-Rubio et al. (2019)
		Lombardia region	Olona river	1.30	
	Italy		Lambro river	0.20	Zuccato et al. (2008)
			Po river	<0.70	
		Toscana region	Arno river	<0.70- 1.0	
	Italy	Nosedo	Influent	11.50 ± 5.10	
	Switzerland	Logano	Influent	5.90 ± 2.60	Castiglioni et al. (2006)
			Effluent	0.20 ± 0.50	
				176.0	
	Italy	Various cities	Various STP	84.0	Castiglioni et al. (2011)
				96.0	
	USA	Chicago	Stickney Water Reservation	346.0	
			WWTP1	63.0 -121.0	
			WWTP2	120.0 -302.0	Devault et al. (2014)
	Martinique	Fort de France	WWTP3	101.0 -223.0	
			WWTP4	46.0 -233.0	
EME			Influent (Super Bowl weekend)	347.0 -492.0	
			Effluent (Super Bowl weekend)	<25.0	
	USA	Various cities	Influent (baseline weekend)	258.0 – 440.0	Gerrity et al. (2011)
			Effluent (baseline weekend)	<25.0	
	Spain	Valencia	Turia river (2013)	15.03	Andrés-Costa et al. (2017)
		Milan		97.0	
	Italy	Como	Various STP	58.0	Castiglioni et al. (2011)
		Sardinia		<LOQ	
	USA	Chicago	Stickney Water Reservation	199.0	
ECG			Influent (Super Bowl weekend)	590.0 -798.0	
			Effluent (Super Bowl weekend)	<50.0	
	USA	Various cities	Influent (baseline weekend)	570.0 -856.0	Gerrity et al. (2011)
			Effluent (baseline weekend)	<50.0	

AME	Italy	Milan, Como and Sardina	Various STP	<LOQ	Castiglioni et al. (2011)
	USA	Chicago	Stickney Water Reservation		

After consumption, cocaine is metabolized by three different esterases called pseudocholinesterase-2, human carboxylesterase-1 (hCE-1), and carboxylesterase-2 (hCE-2). In the liver, cocaine is mainly hydrolyzed by hCE-1 in benzoylecgonine (the main metabolite excreted in the urine) or by hCE-2 in ecgonine methyl ester (EME) (Maurer et al.2006). In urine, benzoylecgonine (BZE) accounts for 54% of the administered dose and only a 1 to 9% rate of the parent compound is excreted unchanged (Binelli et al. 2013).

In addition, COC may undergo oxidative metabolism, where the production of a pharmacologically active metabolite called norcocaine occurs. This process occurs when cocaine is directly transformed in *N*-demethylated by the enzymes CYP450 and CYP3A4. Then, a first oxidation to *N*-oxide occurs by a flavin containing monooxygenase, followed by *N*-demethylation catalyzed by several CYP subfamilies including 1A, 2A, 3A, and even 2B (Maurer et al. 2006).

COC is usually eliminated within 12 h and 72 h after ingestion (depending on the dose and the administered route), whereas a BZE may take up to 5 days to be eliminated (Gonzalez-Alonso et al. 2017). Being excreted mainly by urine, they can access WWTPs, soils, and a wide variety of ecosystems. Several recent studies are concerned about the potential danger of the cocaine presence and its metabolites in the aquatic environment. These studies (Table 5) are focused on the determination of environmental concentrations of the drug in both marine and freshwater environments (Castiglioni et al. 2006; Metcalfe et al. 2010; Hernández et al. 2014; Kasprzyk-Hordern et al. 2008; Borova et al. 2014; Dodder et al. 2014).

COC is the illicit drug most often detected in aquatic ecosystems. In freshwater environment, Zuccato et al. (2008) detected low concentrations of COC in Thames river ($< 0.13 \text{ ng L}^{-1}$), while Thomas et al. (2014) detected the highest concentration of COC (5896 ng L^{-1}) in Igarapé of 40 river, Brazil. At coastal zone, the concentrations of COC ranged from 2.4 ng L^{-1} (Klosterhaus et al. 2013) in San Francisco Bay, California, to 537 ng L^{-1} in Santos Bay, Brazil (Pereira et al. 2016).

Residues of BZE were also detected in both freshwater and seawater environment. In freshwater, the concentrations ranged from $< 0.5 \text{ ng L}^{-1}$ in surface water from Belgium (van Nuijs et al. 2009) to 9717 ng L^{-1} in influent from Paranao, Federal District, Brazil (Maldaner et al. 2012). The low concentration of BZE detected in coastal zone was 2.8 ng L^{-1} in San Francisco Bay (Klosterhaus et al. 2013) and the highest (38.59 ng L^{-1}) concentration was found in Santos Bay (Fontes et al. 2019).

Other metabolites of COC have been also detected in aquatic environment. Concentrations of cocaethylene (CE) ranged from 0.2 ng L^{-1} in effluent from Losano (Castiglioni et al. 2006) to 15 ng L^{-1} in influent from Engalnd (Baker and Kasprzyk-Hordern 2013). Residues of norbenzoylecgonine (NOR-BZE) ranged from 0.5 ng L^{-1} in effluent of England (Baker and Kasprzyk-Hordern 2013) to 53.95 ng L^{-1} in influent from UK (Jones et al. 2014). Norcocaine (NOR-COC) presented concentrations that ranged from 0.1 ng L^{-1} in river water samples from UK (Baker and Kasprzyk-Hordern 2013) to 36.0 ng L^{-1} influent samples from USA after Super Bowl weekend (Gerrity et al. 2011). These authors also found the lowest concentration ($< 25 \text{ ng L}^{-1}$) and the highest concentration (492.0) of EME in the same conditions. For the ecgonine (ECG), the concentrations ranged from < 50 to 856 ng L^{-1} , both in the USA. Concentrations of anhydroecgonine methyl ester (AME) ranged from 4.3 to 35 ng L^{-1} in Italy and US STPs, respectively (Castiglioni et al. 2011).

Some studies have demonstrated the ability of environmental concentrations of COC and BZE to interact with non-target organisms, causing chronic effects. Binelli et al. (2012) demonstrated that COC (40 ng L^{-1} ; 200 ng L^{-1} ; $10,000 \text{ ng L}^{-1}$) can interact with freshwater mussels, inducing cytotoxicity, oxidative stress, DNA damage, and apoptosis in *D. polymorpha* mussels. The metabolite BZE (500 and 1000 ng L^{-1}) induced alterations in important proteins that act in the metabolism, compromising the survival of these organisms. Crustaceans (*Orconectes rusticus*) exposed to COC (2000 and $10,000 \text{ ng L}^{-1}$) showed increased mobility and behavioral changes (Imeh-Nathaniel et al. 2017). Freshwater mussels exposed to BZE (500 and 1000 ng L^{-1}) exhibited a significant increase of protein carbonylation and oxidative modifications in proteins of cytoskeleton, energetic metabolism, and stress response (Pedriali et al. 2013). Parolini and Binelli (2013) observed that COC metabolites (BZE and EME) (150 and 500 ng L^{-1}) induced sublethal effects to different levels of biological organization as cytotoxicity in hemocytes and oxidative stress in mussels *D. polymorpha*.

Souza et al. (2019) assessed the toxicity of crack cocaine combined with different scenarios of ocean acidification. The data showed that a decrease in the pH value (7.5 and 7.0)

associated with high concentrations of crack cocaine (6.25; 12.5; and 25 mg L⁻¹) had a negative effect in embryo larval development of *Echinometra lucunter* sea urchin.

Mersereau et al. (2015) observed that zebrafish embryos exposed to COC present tachycardia. Mussels exposed to BE (500 and 1000 ng L⁻¹) exhibited changes in important proteins involved in metabolism and homeostasis (Binelli et al. 2013). Decreased cell viability, increased DNA fragmentation, and apoptosis were observed in zebrafish embryos exposed to COC and its metabolite (BE and EME) (Parolini et al. 2018b).

Gay et al. (2013, 2016) have found that COC (20 ng L⁻¹) induces an increase of brain dopamine and plasma catecholamines, affecting endocrine system of eel (*Anguilla anguilla*), and might decrease the number and size of mucous cells, alter the thickness of the epidermis, and increase prolactin, cortisol, and dopamine levels in eels after 50 days of exposure. In these same conditions, Capaldo et al. (2012, 2018, 2019) observed that COC is able to accumulate into the eel tissues, mainly the brain, muscle, and liver, suggesting potential risks for eels, since COC could affect their physiology and contribute to their decline, and for humans consuming contaminated fish. These organisms have also exhibited severe damage in morphology and physiology of the skeletal muscle, as well as histological damage in gills.

Marine mussels (*Perna perna*) exposed to crack cocaine (0.5; 50; and 500 µg L⁻¹) exhibited cytotoxicity (decreased of lysosomal membrane stability) and genotoxicity (500,000 ng L⁻¹) after 48 h of exposure (Maranho et al. 2017). Ortega et al. (2018) evaluated the sublethal effects of crack cocaine in mussels and observed changes in biochemical biomarkers (EROD, DBF, GST, GPx enzymatic activities) after 48 h of exposure to 5 and 50 µg L⁻¹ and cytotoxicity after 96 h and 168 h of exposure to all concentrations, including environmental concentration of 500 ng L⁻¹.

A study performed by De Felice et al. (2019) showed that *Daphnia magna* exposed to COC (50 ng L⁻¹ and 500 ng L⁻¹) presented an overproduction of reactive oxygen species and a modulation of the activity of defense enzymes. Moreover, COC affected the swimming behavior and altered the reproductive success of treated specimens.

An environmental mixture of illicit drugs containing COC (50 ng L⁻¹) and BZE (300 ng L⁻¹) also induced genotoxicity and apoptotic cell in zebra mussels (Parolini et al. 2015). Furthermore, Parolini et al. (2017) observed that COC and both metabolites EME and BZE (0.4, 4, and 40 nM) significantly reduced cell viability, increased DNA fragmentation, and promoted the onset of apoptotic cells and micronuclei in zebrafish embryos. Recently, a study performed by Parolini et al. (2018a) reported that BZE (500 and 1000 ng L⁻¹) has also induced

oxidative stress and inhibited AChE activity affecting swimming behavior and reproduction of *Daphnia magna*.

As summarized by Fig. 2, illicit drugs and metabolites are aquatic contaminants dispersed throughout the world in environmental concentrations able to trigger chronic toxicity and sublethal effects to non-target aquatic organisms, representing a new concern to academics, environmental managers, and policymakers.

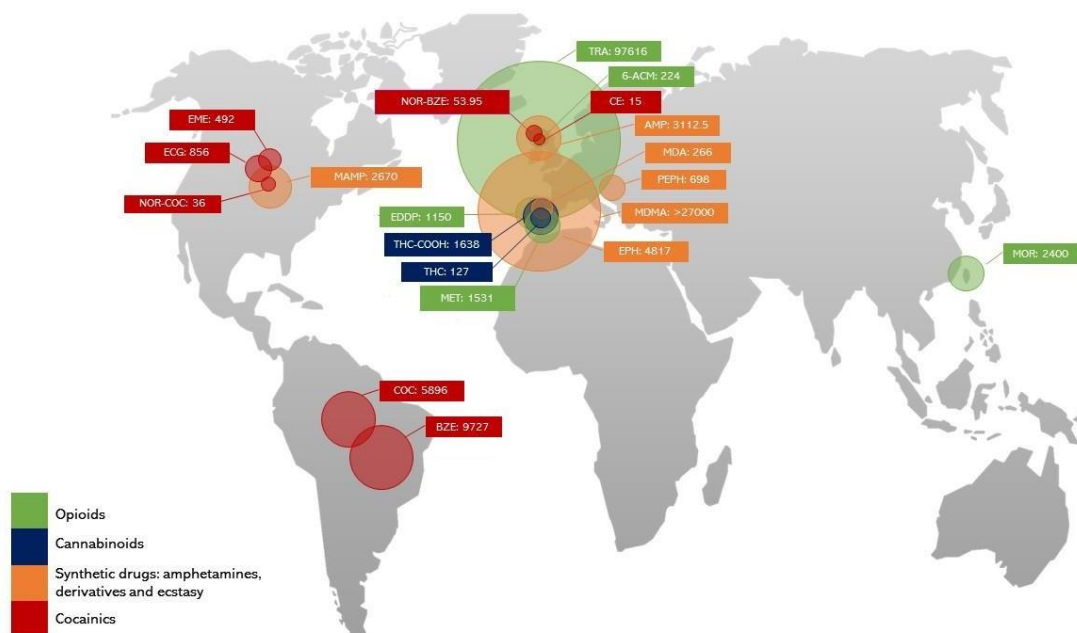


Fig. 2 Illicit drugs and metabolites as aquatic contaminants dispersed throughout the world. COC cocaine, BZE benzoylecgonine, NOR-COC norcocaine, AME anhydroecgonine methyl ester, NOR-BZE norbenzoylecgonine, 6ACM 6-acetylmorphine, THC Δ^9 -tetrahydrocannabinol, ECG ecgonine, CE cocathylene, MDA 3,4-metilenodioxianfetamina, MDMA 3,4-methylenedioxyamphetamine, EME ecgonine methyl ester, MET methadone, MAMP methamphetamine, EDDP 2-ethylidene-1,5- dimethyl-3,3-diphenylpyrrol, TCH-COOH Δ^1 -nor-9-carboxy-9- tetrahydrocannabinol, MOR morphine, AMP amphetamines, PEPH pseudoephedrine, EPH ephedrine, COD codeine.

6. Conclusion and future outlook

The worldwide increase in consumption of illicit drugs represent not only a social problem but it has also been recognized as a relevant environmental concern, due to the occurrence of large amounts of compounds and metabolites into different water matrices such as wastewater, freshwater, drinking water, groundwater, and seawater. The occurrence of illicit drugs and their metabolites in aquatic ecosystems has shown the fragility of sewage treatment systems in the removal and treatment of contaminated effluents, especially in developing

countries. The presence of these substances in WWTPs and environment has been used as a tool to estimate the consumption of the local community. However, reliable information on trafficking remains subjective as it is an illegal activity.

Furthermore, ecotoxicological studies have identified that single IDs (cocaine and amphetamine) and metabolites (benzoylecgonine) showed pseudo-persistence, bioavailability, and toxicity after exposures at environmentally relevant concentrations (ng L^{-1}). Long-term or chronic exposure scenarios should be assessed in order to investigate environmental hazards and risks of IDs, their metabolites, and mixtures already detected in aquatic ecosystems.

The future inclusion of these compounds in environmental legislation as priority contaminants may promote ID monitoring considering standardized sampling methodologies, as well as improvements in WWTPs to completely remove this class of bioactive compounds.

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CHAPTER 2

Occurrence of cocaine in marine environments: Santos Bay case study

Seasonal monitoring of cocaine and benzoylecgonine in a subtropical coastal zone (Santos Bay, Brazil)

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Mussels get higher: A study on the occurrence of cocaine and benzoylecgonine in seawater, sediment and mussels from a subtropical ecosystem (Santos, Bay, Brazil)

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Seasonal monitoring of cocaine and benzoylecgonine in a subtropical coastal zone (Santos Bay, Brazil)

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Abstract

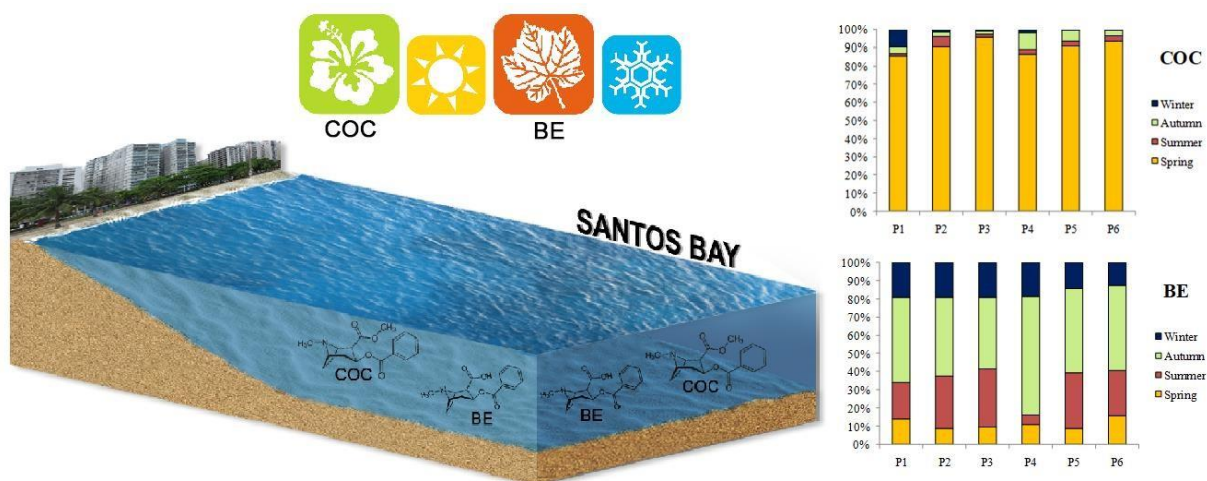
Illicit drugs and their metabolites represent a new class of emerging contaminants. These substances are continuously discharged into wastewater which have been detected in the aquatic environment in concentrations ranging from ng.L^{-1} to $\mu\text{g.L}^{-1}$. Our study detected the occurrence of cocaine (COC) and benzoylecgonine (BE) in a subtropical coastal zone (Santos Bay, SP, Brazil) within one year. Water samples (surface and bottom) were collected from the Santos Submarine Sewage Outfall (SSOS) area. COC and BE were measured in the samples using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–ESI-MS/MS). Concentrations ranged from 12.18 to 203.6 ng.L^{-1} (COC) and 8.20 to 38.59 ng.L^{-1} (BE). Higher concentrations of COC were observed during the end of spring, following the population increase at summer season. COC and its metabolite occurrence in this coastal zone represent a threat to coastal organisms.

Keywords: Cocaine . Benzoylecgonine . Marine Pollution . Submarine sewage outfall . Contaminants of emerging concern

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Graphical Abstract



1. Introduction

The increase in illicit drugs consumption has been caused not only social and public health problems, but also environment impacts around the world (Binelli et al., 2012). Illicit drugs represent a group of emerging contaminants in the aquatic environment (Berset et al., 2010), and have been detected in municipal sewage treatment plants (STPs), surface and drinking waters (Campestrini and Jardim, 2017), and seawater (Pereira et al., 2016). Illicit drugs are continuously discharged unaltered into wastewater due to improper disposal, or as metabolites due to consumption and excretion (Bijlsma et al., 2014; Pal et al., 2012). In this context, the occurrence of illicit drugs in the aquatic environment is a concern due to high biological activity, psychoactive properties, and unknown effects on biota (Baker and Kasprzyk-Hordern, 2013).

Cocaine (COC) use has increased over the past decade (Bijlsma et al., 2013). Several studies have shown increased use of COC on holidays, weekends and during music festivals, demonstrating a recreational pattern of consumption (Huerta-Fontela et al., 2008; Viana et al., 2011; Ort et al., 2014; Lopes et al., 2014; Krizman et al., 2016; Pereira et al., 2016). According to the World Drug Report (UNODC, 2017), South America is responsible for virtually all of the world's COC production. Consumption and trafficking of COC in South America have become more prominent, mainly in Brazil, due to geographical location. Recent data indicates that an estimated 3.35 million Brazilians used COC in 2012 (UNODC, 2014), and in 2015, 40 tons of the drug were seized in the country (UNODC, 2015).

After consumption, COC is rapidly metabolized; 35–54% of the parent compound is hydrolyzed to benzoylecgonine (BE), 32–49% to ecgonine methyl ester, and 5% to norcocaine (Pal et al., 2012). These compounds are continually released into the environment via wastewater treatment plants (WWTPs), recognized as the main source of surface water contamination as a result of inefficient removal and treatment of effluents (Terzic et al., 2010; Vazquez-Roig et al., 2013; Borova et al., 2014; Evgenidou et al., 2015). This problem becomes more evident in coastal zones such as Santos Bay (São Paulo, Brazil), where the submarine sewage disposal system is known to cause different types of disturbances that may alter the water quality (Ortiz et al., 2011). Disposal of domestic effluents via submarine sewage outfalls is considered a significant source of pollution, since the low level of treatment combined with adverse release conditions in the region (shallow waters) have resulted in high concentrations of contaminants in seawater (Subtil et al., 2012). This in turn makes the water unsuitable for producing and maintaining fish stocks, and also degrades aesthetic and landscape aspects while undermining the ecological balance (Abessa et al., 2012). Furthermore, dumping raw sewage contaminated with illicit drugs represents an important health concern, and although the reported concentrations are negligible for humans (ranging from ng.L^{-1} to $\mu\text{g.L}^{-1}$), these compounds might present potential risks for aquatic biota (Parolini et al., 2015).

Once in the environment, COC and BE could interact with non-target organisms, causing negative effects. COC induced cytotoxicity, oxidative stress, and changes to the metabolism of *Dreissena polymorpha* exposed to 40 ng.L^{-1} , 220 ng.L^{-1} , and $10 \mu\text{g.L}^{-1}$ (Binelli et al., 2012, 2013). Crustaceans (*Orconectes rusticus*) exposed to a dose of $2.0 \mu\text{g.g}^{-1}$ of COC showed increased mobility and behavioral changes (Imeh-Nathaniel et al., 2017). The genotoxic action of crack cocaine was observed in a significant increase in DNA strand breaks observed in the digestive glands from *Perna perna* mussels exposed to a dose of $500 \mu\text{g.L}^{-1}$ (Maranho et al., 2017). DNA fragmentation was measured in *Danio rerio* embryos exposed to 40 nM of BE (Parolini et al., 2017).

The frequency and levels at which illicit drugs are detected in the environment depend on local prevalence efficiency and coverage of sanitation infrastructure (Campestrini and Jardim, 2017). Seasonal and spatial variations have also been found on illicit drugs concentrations in the aquatic environments (Jiang et al., 2015; Lai et al., 2016; Kim et al., 2017). Some studies have monitored COC and its metabolites in surface water around the world (Huerta-Fontela et al., 2008; Zuccato et al., 2008; VanNuijs et al., 2009; Metcalfe et al., 2010; Castiglioni et al., 2011; Baker et al., 2012), but wastewater concentrations and ecotoxicological impacts have

addressed aquatic biota in temperate countries, which differ from tropical ecosystems (Devault et al., 2014). Data on the occurrence of these substances in tropical coastal zones are still lacking. In fact, this is the first study on the occurrence of COC and BE in seawater samples from a subtropical coastal zone (Santos Bay, São Paulo, Brazil) comparing four seasons.

2. Material and methods

2.1. Sample sites

Santos is the most populated city on the southeast coast of São Paulo (Brazil), which comprises estimated 434,742 inhabitants. However, the WWTP in Santos also receives discharge from the São Vicente municipality, serving an estimated 795,122 inhabitants (IBGE, 2017). The bay receives $7.363 \text{ m}^3 \cdot \text{s}^{-1}$ of sewage through the Santos submarine sewage outfall system (SSOS) (Abessa et al., 2005). During the summer and over holidays, the population may increase 15.56% in São Vicente and 19.62% in Santos to reach 897,000 inhabitants (SPDR, 2011; São Paulo, 2014).

Despite the large amount of sewage discharged, WWTP only employs railing and sifting process to remove solids, and disinfection is performed using chlorination (Abessa et al., 2012). The preconditioned sewage is then sent through pipes into Santos Bay and released in a 10-meter-deep area located 4.5 km from the beach (Pereira et al., 2016). Previous studies denoted water/sediment contamination (Cesar et al., 2007) and ecological impacts in Santos Bay (Sánchez-Jérez et al. 2001).

The present study investigated the presence of COC and BE throughout the year (spring, summer, autumn, and winter) at Santos Bay. Water column sampling was conducted at five stations surrounding the SSOS, considering all possibilities for effluent plume dispersion (P1–P5) indicated by monitoring conducted by the São Paulo Environmental Agency (CETESB, 2014) and at P6, a not directly influenced area (Fig. 1).

Three liters of surface water (S, collected at 1 m) and bottom water (B, collected at 8 m) were collected at each station using a Van Dorn bottle, always at the end of each season (spring: 2016, December; summer: 2017, March; autumn: 2017, June; winter: 2017, September). Water samples were placed into amber glass bottles previously cleaned with HNO₃, methanol and distilled water. Samples were transported to the laboratory into insulated cooler with ice (< 6 °C) and placed in a freezer at –20 °C until the sample preparation.

2.2. Sample preparation

The extraction technique used was the same described by Pereira et al. (2016). Before extraction, the pH of each seawater sample was adjusted to 7 ± 0.5 using HCl solution (1 M). The samples were filtered through Whatman filter paper (GF/C diameter 47 mm, particle retention 1.2 μm ; Merck, Darmstadt, Germany). The filters were washed with 2 mL of methanol (Sigma-Aldrich, St. Louis, USA) to prevent the loss of relevant compounds. The extracted methanol was collected and added to the filtered sample. Subsequently, solid-phase extraction (SPE) was performed using Chromabond HR-X cartridges (3 mL, 200 mg; Marcherey-Nagel, Duren, Germany) as described by Wille et al. (2010) and Ghoshdastidar et al. (2015). The cartridges were preconditioned with 5 mL of methanol and 5 mL Milli-Q water (Merck, Darmstadt, Germany). After preconditioning, the filters were loaded with 1 L of the filtered sample combined with the methanol from filter washings. The cartridges were dried under vacuum for 30 min. Elution was performed using 5 mL of acetone and 2×5 mL of methanol. After the SPE procedure, the samples were dried under nitrogen flow (at 50 °C) and eluted with water/acetonitrile (95: 5 v/v) prior to mass spectrometry analysis.

2.3. LC-MS/MS analysis

The method employed was described and validated by Shihomatsu et al. (2017). An aliquot (10 μL) of each sample was analyzed by an HPLC Agilent 1260 device (Agilent Technologies, CA, USA) combined with a 3200 QTRAP hybrid triple quadrupole/LIT (linear ion trap) mass spectrometer ABSciex, Ontario (Canada). The COC and BE standards used were acquired from Cerilliant®. Samples were analyzed by an Agilent Eclipse XDB-C18 4.6×50 mm, 1.8 μm columns at 25 °C. The eluent flow rate was $0.7 \text{ mL} \cdot \text{min}^{-1}$, and the mobile phase for positive mode analysis was 0.1% formic acid (Sigma-Aldrich LC-MS Grade) in water (solvent A) and acetonitrile (MS grade; J.T. Baker LC) (solvent B).

A linear gradient of $0.7 \text{ mL} \cdot \text{min}^{-1}$ was used for this mode of ionization (positive), starting with a mixture of 95% solvent A and 5% solvent B. The percentage of solvent A was decreased linearly from 95% to 5% over the course of 5 min, and maintained at 5% for 1 min. The mixture was then returned to the initial conditions over the course of 2 min. Analytes were 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 each compound. Data were recorded and processed using Analyst 1.5.2 software (ABSciex, Ontario, Canada). MRM parameters for the positive mode for COC and BE, limit of detection (LOD), and limit of

quantification (LOQ) are shown in the Table 1. Solvent calibration curve was employed, as described by Shihomatsu et al. (2017). Individual standard solutions at 200 $\mu\text{g}\cdot\text{mL}^{-1}$ were prepared in acetonitrile-ACN/HPLC grade water (1:1, v/v). Linearity, limit of detection (MDL), limit of quantification (MQL), precision, accuracy and recovery were the parameters to evaluate the performance of the SPE-LC-MS/MS methodology. Calibration curves of the compounds showed satisfactory determination coefficients ($0.993 \leq r^2 \leq 1$). The SPE extraction recoveries were obtained in the range from 49% to 109%. The uncertainty for COC and BE analyses were $\pm 3\%$ and $\pm 5\%$, respectively.

Table 1. Multiple reaction parameters for positive mode, limit of detection, limit of quantification, and retention time.

Compound	Q1	Q3	DP (V)	CE (V)	CXP (V)	LOD ($\text{ng}\cdot\text{L}^{-1}$)	LOQ ($\text{ng}\cdot\text{L}^{-1}$)	RT (min.)
COC	304.2	182.2	36	27	4	3.0	12.0	3.90
		105.1	36	39	4			
BE	290.2	168.2	31	25	4	1.2	7.7	3.63
		105.3	31	37	4			

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); MIM (multiple ion monitoring). In Q3, the quantifier ion is in the upper cell and the qualifier ion is in the lower cell.

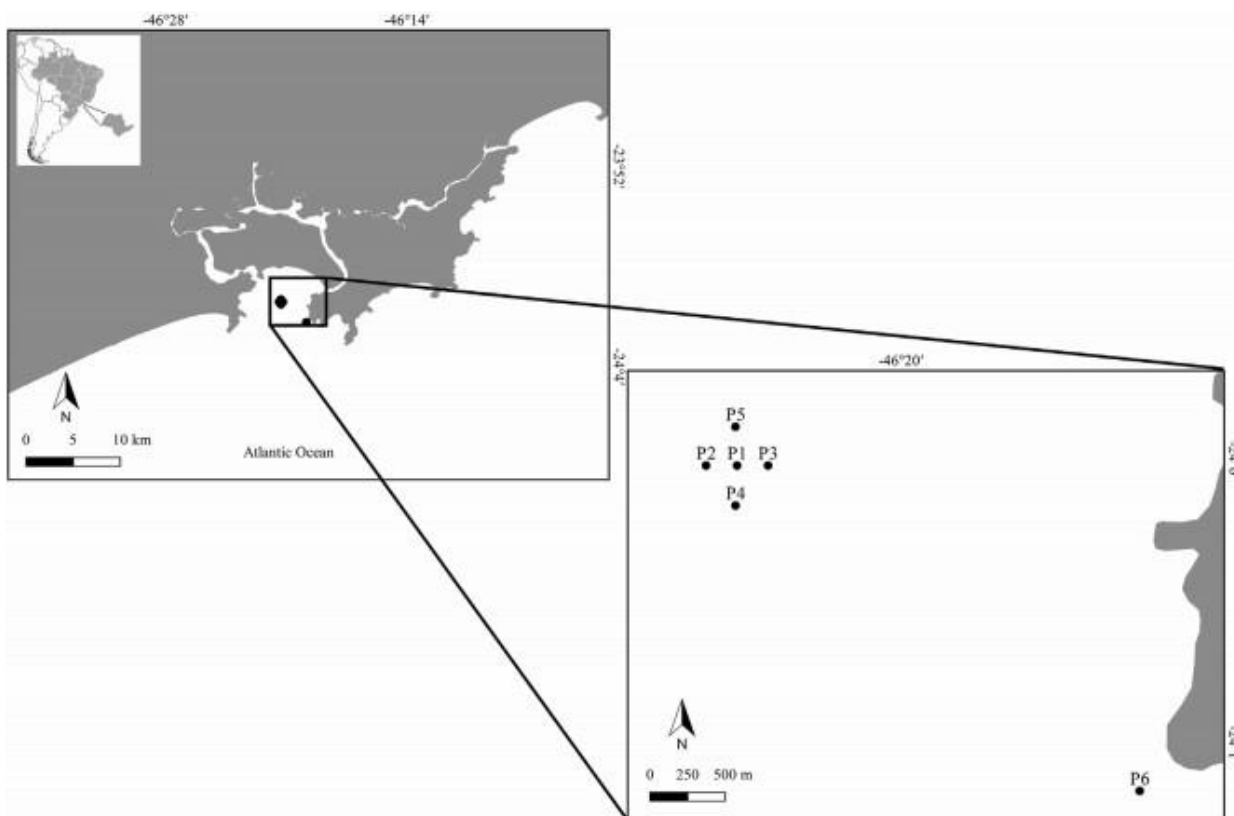


Fig. 1. Sampling stations located in Santos Bay (São Paulo, Brazil).

2.4. Statistical analyses

Considering that the replacement of the non-detected data (concentrations below the LOD or LOQ) with some substitute value such as 0, half the detection time or the detection limit would lead to significant distortions in the statistics, we applied the Kaplan-Meier non-parametric method, which makes use of those censored data to identify significantly difference for the sampling locations (surface and bottom) and seasons analyzed. Statistical differences were considered significant when $p \leq 0.05$. R package NADA (Helsel, 2005) were used to perform such analyses.

3. Results

The physical and chemical parameters are shown in the Table 2. Higher temperatures were observed in spring ($26.13 \text{ }^{\circ}\text{C} \pm 0.63$) and summer ($26.73 \text{ }^{\circ}\text{C} \pm 0.65$) in the surface water samples, whereas pH values did not vary significantly. The highest values were observed in bottom water samples in spring (7.99 ± 0.06), while the lowest values were observed in bottom water samples in autumn (7.8 ± 0.23). The lowest dissolved oxygen (DO) values were found in

bottom water samples in summer ($3.35 \text{ mg.L}^{-1} \pm 0.30$), while samples taken in winter presented the highest values ($7.77 \text{ mg.L}^{-1} \pm 0.73$) in surface water. Salinity was also highest during winter ($31.67 \pm 0.08 \text{ PSU}$) in bottom water samples.

Table 2. Values of the physical-chemical parameters obtained in Santos Bay water samples in different seasons. T°C: temperature, degrees Celsius; DO: dissolved oxygen, mg.L^{-1} ; Sal: salinity, PSU.

Physical and chemical parameters																
Stat	Spring				Summer				Autumn				Winter			
	T°C	pH	DO	Sal	T°C	pH	DO	Sal	T°C	pH	DO	Sal	T°C	pH	DO	Sal
1S	25.1	7.8	6.4	27.7	26.6	7.2	5.4	30.4	21.8	7.6	6.3	29.0	22.5	7.0	6.6	31.1
2S	26.1	7.9	7.2	27.6	26.4	7.9	5.7	30.8	21.8	7.6	6.3	29.1	23.4	7.9	8.2	30.6
3S	26.2	8.0	7.4	27.6	26.7	8.0	5.8	30.9	21.8	7.9	6.3	29.1	23.5	8.0	8.2	30.5
4S	26.2	7.9	7.6	27.7	26.6	7.9	5.7	31.2	21.8	7.9	6.4	29.2	23.1	8.1	7.5	31.1
5S	26.1	8.0	7.2	27.7	26.1	7.9	5.8	30.2	21.8	8.0	6.4	29.1	23.3	8.0	8.6	30.7
6S	27.1	8.0	9.6	27.2	28.0	8.0	6.2	31.3	21.8	7.9	6.5	29.2	23.0	8.1	7.5	31.6
1B	24.8	8.0	6.2	28.1	27.0	7.7	5.8	31.0	21.7	7.2	6.0	29.5	22.4	7.5	6.4	31.7
2B	24.2	8.0	6.3	29.2	22.4	7.8	1.9	32.4	21.8	7.7	6.2	29.4	22.6	7.9	6.6	31.7
3B	24.2	7.9	6.4	28.8	22.6	7.9	1.0	31.8	21.7	7.9	6.4	30.0	22.5	8.0	6.6	31.7
4B	24.3	8.0	6.4	29.4	23.5	7.9	3.8	31.5	21.8	7.9	6.5	30.0	22.4	8.1	6.3	31.5
5B	24.3	8.0	6.3	29.0	23.1	7.9	3.4	32.2	21.8	8.0	6.2	29.6	22.5	8.1	6.7	31.7
6B	24.2	8.0	6.4	29.4	23.5	8.0	4.3	32.2	21.8	8.0	6.7	29.2	22.7	8.1	7.1	31.7

COC were quantified in all samples from spring. In autumn, COC was quantified at only station 4 (surface and bottom) and at only station 1 (surface) in winter. Meanwhile, the BE was detected in all samples from autumn; quantified in stations 1, 2, 3, and 5 (surface) from summer, and 1, 2, and 4 (surface) from winter. We did not detect concentrations of BE during spring season. The environmental concentrations are shown in the Table 3. Concentrations of COC ranged from 12.18 to 203.6 ng.L^{-1} and BE ranged from 8.20 to 38.59 ng.L^{-1} . Only for BE were observed significant statistical differences between concentrations found in surface and bottom water sample ($p = 0.03$). Concentrations of both compounds differed significantly between

seasons ($p < 0.0001$), with the highest concentrations of COC occurring in the spring and the highest concentrations of BE was found in autumn (Figs. 2 and 3).

Table 3. Environmental concentrations of cocaine (COC) and benzoylecgonine (BE) in samples of surface (1S–6S) and bottom (1B–6B) water from Santos Bay.

Station	Concentration (ng.L ⁻¹)							
	Spring		Summer		Autumn		Winter	
	COC	BE	COC	BE	COC	BE	COC	BE
1S	169.22	< 7.7	< 12.0	19.82	< 12.0	27.93	38.44	16.07
2S	95.50	< 7.7	< 12.0	11.11	< 3.0	11.17	< 3.0	8.99
3S	91.29	< 7.7	< 12.0	8.77	< 3.0	10.53	< 3.0	< 7.7
4S	94.74	< 7.7	n.a*	n.a*	12.57	16.22	< 3.0	9.31
5S	168.92	< 7.7	< 12.0	13.08	< 12.0	13.29	< 3.0	< 7.7
6S	135.59	< 7.7	< 3.0	< 7.7	< 12.0	8.33	< 3.0	< 7.7
1B	203.60	< 7.7	< 3.0	< 1.2	< 12.0	19.22	< 12.0	< 7.7
2B	120.57	< 7.7	< 12.0	< 7.7	< 12.0	11.34	< 3.0	< 1.2
3B	100.90	< 7.7	< 3.0	< 7.7	< 3.0	8.20	< 3.0	< 7.7
4B	145.05	< 7.7	< 12.0	< 7.7	12.18	38.59	< 3.0	< 7.7
5B	68.62	< 7.7	< 12.0	< 7.7	< 12.0	12.55	< 3.0	< 7.7
6B	114.41	< 7.7	< 12.0	< 7.7	< 12.0	< 7.7	< 3.0	< 1.2

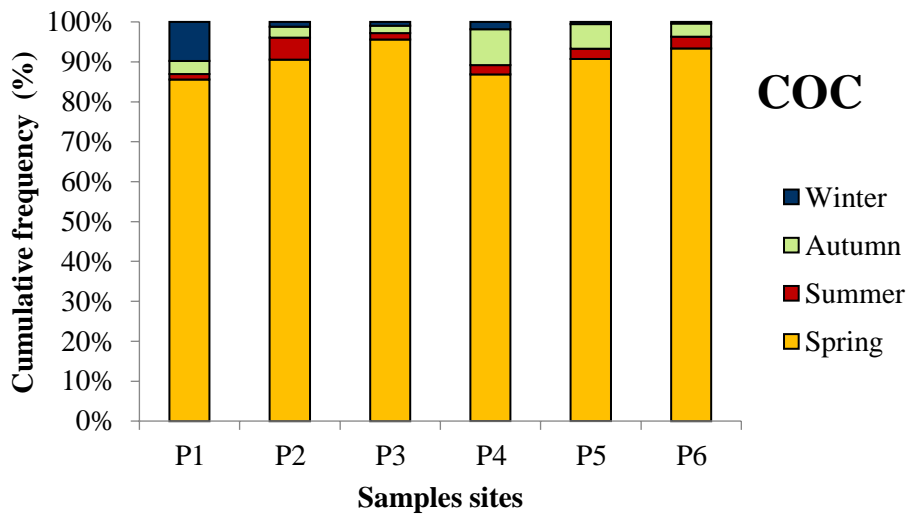


Fig. 2. Concentrations of cocaine found during different seasons in Santos Bay.

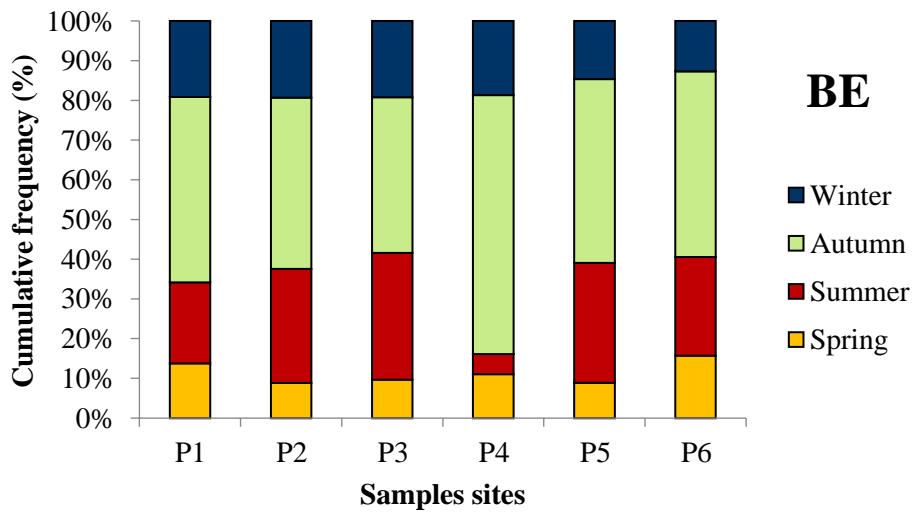


Fig. 3. Concentrations of benzoylecgonine found during different seasons in Santos Bay.

4. Discussion

Illicit drug use is a global issue affecting millions of people and leading to serious health and social costs (Castiglioni et al., 2014). We found concentrations of COC and BE in all samples of surface and bottom waters. COC concentrations range from 12.18 to 203.6 ng.L⁻¹. BE concentrations ranged from 8.33 to 38.59 ng.L⁻¹. Pereira et al. (2016) recorded COC higher levels (12.6–537 ng.L⁻¹) than BE (4.6–20.8 ng.L⁻¹) in samples taken from Santos Bay after Carnival, in March 2014. Klosterhaus et al. (2013) found COC and BE concentrations in San Francisco Bay of 0.6 ng.L⁻¹ and 5.2 ng.L⁻¹ respectively. Borova et al. (2014) reported 2.6–7.8 ng.L⁻¹ of COC and 2.2–6.6 ng.L⁻¹ of BE in wastewater samples from five WWTPs at Santorini. Furthermore, the concentrations found in the present study corroborate with previous studies reported on freshwater environments (Karolak et al., 2010; Baker et al., 2012; Castiglioni et al., 2011; Lopes et al., 2014; Thomas et al., 2014; Bijlsma et al., 2016).

In Brazil, 59% of sewage reaches water bodies without primary treatment, introducing substances with high contaminant potential directly into aquatic ecosystems (Campestrini and Jardim, 2017). Several factors affect removal of illicit drugs from effluent, such as type of treatment technology, temperature, local industrial output, sunlight, dilution of wastewater and physical and chemical properties of analytes (Baker and Kasprzyk-Hordern, 2013). Due to the COC and BE low $\log K_{ow}$ and high pKa, such substances are predominantly present in the dissolved aqueous phase (Gheorge et al., 2008; Domènech et al., 2009; Binelli et al., 2013). This may explain the occurrence of both compounds in samples taken from surface water, since most illicit drugs have polar characteristics which provide high solubility and facilitate ionization in the aquatic environment (Vazquez-Roig et al., 2013).

Removal of compounds with $\log K_{ow}$ values < 3.0 is less efficient in primary treatment (Evgenidou et al., 2015), which might contribute to the presence of these substances in Santos Bay, since urban effluent is only preconditioned and chlorinated before disposal. This process is not sufficient to remove drugs from effluents, releasing untreated wastewater into a low-energy coastal zone (Hortellani et al., 2005; Subtil et al. 2012).

There is also a concern about chlorination, since a variety of halogenated organic compounds such as halogenated acetic acids (HAAs) and trihalomethanes (THMs) might be formed during this process which might cause contamination in coastal environments, since

chlorinated wastewater is discharged into the ocean through outfall pipes (Yang et al., 2000). Furthermore, as reported by Bijlsma et al. (2013), chlorination might generate unwanted transformation products (TPs) derived from COC and BE, representing another threat to the aquatic environment.

Temperature and pH affects the stability of illicit drugs in aquatic environments. McCall et al. (2016) observed that COC is stable at low temperature and pH conditions (≈ 2). Bisceglia and Lippa (2014) noted that COC is readily hydrolyzed at pH 7.5 and 20 °C, but BE hydrolyzes slowly enough to be considered stable under the same conditions. Devault et al. (2014) analyzed potential degradation of COC in a tropical context and observed that at average temperatures of 25 °C, COC rapidly degraded into BE. These researchers also noted that COC is known to be transformed into BE as a function of temperature and BE increased concentration is proportional to decreased of COC concentration (Devault et al., 2016). However, in our study we did not observe a clear relation between high spring and summer temperatures with the decreased concentrations of COC at Santos Bay, since the highest concentrations of COC were detected during these seasons. Furthermore, as reported by Yang et al. (2000), temperature changes in seawater are insignificant in coastal areas, where most of wastewater from land is discharged, indicating that this parameter is not as significant during the observed seasonal variation.

Values for pH near 7.0 and 7.6 significantly increase the half-life of COC as well as concentrations of BE (Devault et al., 2017), which may explain the concomitant occurrence of the parent drug and metabolite at the SSOS. In our study, pH ranged from 7.78 to 7.99. The alkalinity detected in coastal zones may influence the bioavailability of compounds such as COC with high pKa values, since they become partially ionized. This increased log Kow values for COC from 0.10 (for the ionic form) to 2.30 (for the non-ionic form), which can make COC more hydrophobic, promoting bioaccumulation in the biota (EPISuite, 2012).

Seasonal variations were observed, with the highest concentrations in spring and summer, coinciding with the beginning and peak of the tourist season, respectively. We therefore believe that these variations are more associated with the population's social behavior than environmental factors. In fact, Santos is an important center for tourism, and the COC levels observed during these seasons may have been affected by the influx of tourists. This suggests that consumption of COC is recreational in this area. Similar patterns were observed in several other studies (Gheorge et al., 2008; VanNuijs et al., 2012; Jiang et al., 2015; Kankaanpää et al., 2014; Been et al., 2016; Kim et al., 2017). As reported by Lai et al. (2012), drug availability is

likely to be driven by consumer demand at specific times such as holidays and weekends and during music festivals and other cultural events, when population increase and illicit drug use is likely to be more prevalent. Furthermore, COC is more easily available in urban areas with population enough to support a regular market (Lai et al., 2012).

Previous studies have already reported adverse effects on biota exposed to environmentally significant concentrations of COC and BE in fresh water, but aquatic ecotoxicological data for marine organisms are still scarce. A recent study by Maranhão et al. (2017) indicated that marine mussels (*Perna perna*) exposed to crack cocaine exhibited DNA damage and induced cytotoxicity in concentrations ranging from 5 to 500 $\mu\text{g.L}^{-1}$. Ortega et al. (2018) reported that crack cocaine also induced alterations in lysosome stability membrane in mussels exposed to concentrations ranging from 0.5 to 50 $\mu\text{g.L}^{-1}$. Studies performed by Capaldo et al. (2012, 2018, 2019) observed that COC is able to accumulate into eel (*Anguilla anguilla*) tissues, generate serious injury in skeletal muscle and affecting the gill epithelium and increasing plasma levels of cortisol and prolactin when the eel were exposed to environmental concentrations of COC (20 ng.L^{-1}). Moreover, these compounds may interact with other therapeutic substances, leading to unexpected pharmacological interactions (Parolini et al., 2016).

Conclusion

This is the first study involving seasonal monitoring of COC and BE in a tropical coastal zone. High concentrations of COC were quantified during the spring, while higher BE concentrations were found during the summer/autumn, indicating a recreational pattern of consumption. Our results suggested the submarine sewage outfall as a source of these compounds, but COC was also found in a not directly influenced area (P6), indicating multiple sources. A long-term monitoring program would be needed for a more comprehensive understanding of the sources and environmental fate of illicit drugs on this coastal zone in order to predict and prevent their occurrence and biological effects.

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Mussels get higher: A study on the occurrence of cocaine and benzoylecgonine in seawater, sediment and mussels from a subtropical ecosystem (Santos Bay, Brazil)

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Highlights

- Santos Bay showed widespread contamination by cocaine and benzoylecgonine.
- Surface water was contaminated by both compounds.
- Sediment and mussels were contaminated only by cocaine.
- Cocaine had preferential bioaccumulation over its demethylated metabolite.
- A field-measured BAF was determined for cocaine.

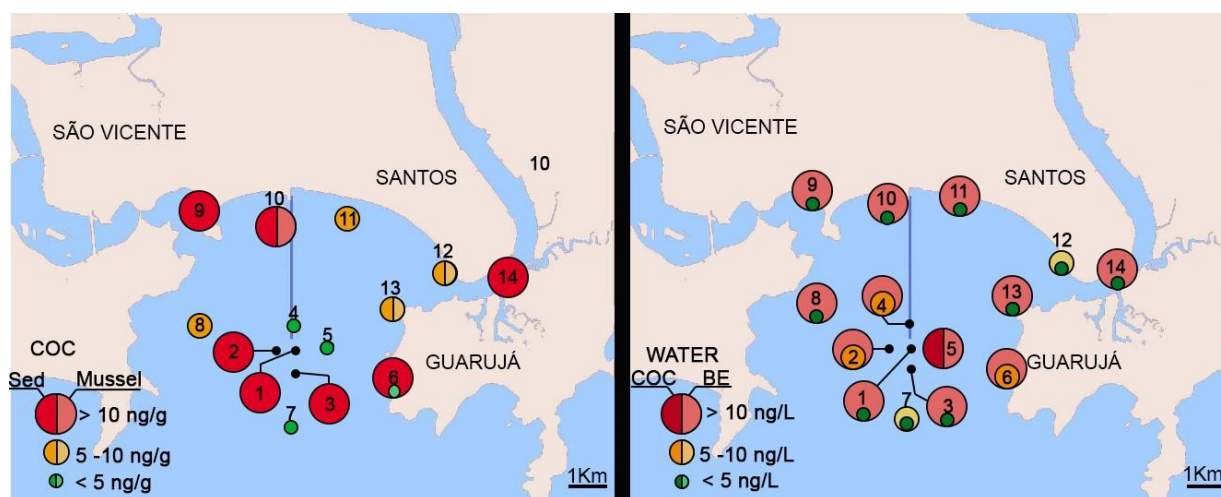
Abstract

Data on the occurrence of cocaine (COC) and benzoylecgonine (BE) in marine environmental compartments are still limited, with few studies reporting superficial water contamination, mainly in tropical zones. In this sense, environmental data of these substances are essential to identify potential polluting sources, as well as their impact in coastal ecosystems. The aim of this study was to evaluate the occurrence of COC and BE in seawater, sediment and mussels from a subtropical coastal zone (Santos Bay, São Paulo, Brazil), as well as to determine a field measured Bioaccumulation Factor (BAF). COC and BE were detected in all water samples in concentrations ranging from 1.91 ng·L⁻¹ to 12.52 ng·L⁻¹ and 9.88 ng·L⁻¹ to 28.53 ng·L⁻¹, respectively. In sediments, only COC was quantified in concentrations ranging from 0.94 ng·g⁻¹ to 46.85 ng·g⁻¹. Similarly, only COC was detected in tissues of mussels 0.914 µg·kg⁻¹

to $4.58 \mu\text{g}\cdot\text{kg}^{-1}$ (ww). The field-measured BAF ranged from 163 to 1454 ($\text{L}\cdot\text{kg}^{-1}$). Our results pointed out a widespread contamination by cocaine and its main human metabolite benzoylecgonine in Santos Bay. Mussels were able to accumulate COC in areas used by residents and tourists for bathing, fishing, and harvest, denoting concern to human health. Therefore, our data can be considered a preliminary assessment, which indicates the need to evaluate drugs (including illicit as COC) in environmental and seafood monitoring programs, in order to understand their risks on the ecosystem and human health.

Keywords: Illicit drugs . Bioaccumulation . *Perna perna* . BAF . Marine pollution

Graphical abstract



1. Introduction

According to the last World Drug Report of the United Nations, drug use around the world has been on the rise, in terms of both overall numbers and the proportion of the world's population that uses drugs. In 2009, the estimated 210 million users represented 4.8% of global population aged 15–64, compared with the estimated 269 million users in 2018, or 5.3% of the population (UNODC, 2020).

The use of illicit drugs causes effects on health, social life and economy (Campestrini and Jardim, 2017), and represents a threat to the environment. Illicit drugs and their metabolites may enter the sewage collection system after consumption and excretion (Bijlsma et al., 2012),

and are continuously released into aquatic environment due to the inefficiency of the wastewater treatment plants (WWTP) (Evgenidou et al., 2015; Yadav et al., 2017).

Cocaine (COC) is the third most widely used drug in North America, Western and Central Europe, and the second in Latin American and Caribbean (Capaldo et al., 2019). After consumption, COC is rapidly metabolized by the liver and excreted through the urine as two main metabolites: benzoylecgonine (BE, 45%) and ecgonine methyl ester (EME, 40%) (Baselt, 2004). Furthermore, a limited amount (1–9%) is eliminated unchanged (Binelli et al., 2012; Parolini et al., 2018).

Occurrence and effects of COC and BE in aquatic environment have been reported in several studies. In Santos Bay, Pereira et al. (2016) found concentrations of COC ranging from 12.6 to 537 ng·L⁻¹ and BE ranging from 4.6 to 20.8 ng·L⁻¹ in Santos Bay. A recent study performed by Fontes et al. (2019) in the same region identified concentrations from 12.18 to 203.6 ng·L⁻¹ (COC) and from 8.20 to 38.59 ng·L⁻¹ (BE). Residues of COC (42.3 ng·L⁻¹) and BE (142 ng·L⁻¹) were also identified in Galicia, Spain (Fernández-Rubio et al., 2019). Fedorova et al. (2014) found residues of COC (0.8–3.10 ng·L⁻¹) and BE (0.5–8.4 ng·L⁻¹) in samples from surface water from Czech Republic. Regarding negative effects, Binelli et al. (2012) demonstrated that COC (40 ng·L⁻¹, 200 ng·L⁻¹, 10 µg·L⁻¹) causes oxidative stress, cytotoxicity and DNA damage in freshwater mussels *Dreissena polymorpha*. Apoptosis and DNA fragmentation were observed in freshwater mussels exposed to COC (0.3 and 10 µg·L⁻¹) (Parolini et al., 2018). *Daphnia magna* exposed to COC (50 ng·L⁻¹ and 500 ng·L⁻¹) presented an overproduction of reactive oxygen species and change of swimming behavior and reproduction. Despite the availability of data on the presence and effects of COC and BE in aquatic organisms and ecosystems, this knowledge is still limited for coastal environments.

Mussels are filter-feeding and can accumulate pathogenic bacteria, organic and inorganic pollutants dissolved in the surrounding water column or attached to sediments (López-García et al., 2019). Because of these features, they have been used as sentinel organisms for coastal pollution monitoring (Beyer et al., 2017). *Perna perna* is an intertidal mussel widely distributed in warm-temperate regions of the Mediterranean Sea, Indian and Atlantic Ocean (Cunha et al., 2014), being widely cultivated and consumed in South Africa, Venezuela and Brazil (Santos et al., 2018).

In 2017, FAO estimated the worldwide production of marine mussels (Mytilidae) at 2.2 million tons with an economic value of about US\$ 4.2 billion (FAO, 2019). Brazilian production of mussels was about 18,000 tons in 2016 and 95% of which was farmed in Santa

Catarina state (Alves et al., 2020). *P. perna* is one of the most cultivated bivalves, representing 19% of the total produced by the entire national mariculture (Galvao et al., 2015). A study performed by Renó (2009) identified that in Santos Bay the *P. perna* extraction is an important socioeconomic activity, since mussel fisherman from traditional communities used the extraction as their most mean of subsistence. The annual extraction of *P. perna* was estimated at 60 tons. In addition, *P. perna* is also recognized as an important bioindicator of marine pollution, due its ability to concentrate a large variety of inorganic and organic contaminants (Pereira et al., 2012; Ferreira et al., 2013).

The harvest of *P. perna* from rock shores of Santos Bay for human consumption is a common activity, regardless of the history of contamination in the region (Casarini and Henriques, 2011). Metals, PAHs and microplastics (Catharino et al., 2008; Santana et al., 2016; Torres et al., 2012) have often been detected in bivalves from Santos Bay. More recent studies have also reported the occurrence of pharmaceuticals, COC and BE in superficial water near the submarine sewage outfalls (Pereira et al., 2016; Fontes et al., 2019), raising concerns about seafood contamination by drugs.

Therefore, considering the lack of scientific data on COC and BE contamination in tropical coastal zones, and the importance of *P. perna* within these socioecological systems, the aim of this study was to evaluate the occurrence of these compounds in superficial water, sediment and mussels from Santos Bay and adjacent estuarine mouths.

2. Material and method

2.1. Study area

The Baixada Santista Metropolitan Region (BSMR) is considered one of the most economically important areas in South America (Moreira et al., 2017). The BSMR is relevant mainly due to the largest port in South America (Port of Santos). This area is located in the southeast of Brazil, in the central region of Sao Paulo State coast and has an estimated population of 433,311 inhabitants (IBGE, 2017). Furthermore, Santos Bay is adjacent to a highly urbanized region and has been affected by different sources of contaminants from the urban activities, port, as well as the industrial pole (Cesar et al., 2007; Martins et al., 2011).

The Wastewater Preconditioning Plant (WWPP) of Santos is integrated with the neighboring municipality of São Vicente. Their effluents are conducted together to a Pre-Conditioning Station. The system is comprised of 503 km of collection network (São Paulo,

2010). The preconditioned sewage is sent through a pipeline into Santos Bay and released by diffusors in a 10 m-deep area located 4.5 km out coastline (Fontes et al., 2019).

The Santos Bay receives 7.363 m³ of sewage through the Submarine Sewage Outfall (SSO) (Abessa et al., 2005). During holidays, the population size increases and can reach 897,000 inhabitants (SPDR, 2011). This is a concern because WWPP only employs railing and sifting processes to remove large solids, without primary or secondary treatments before disposal.

Another problem that deserves attention is the large number of people living in irregular settlements, where it is not possible to install basic sanitation infrastructure. Therefore, even if sewage collection covers a significant fraction of the population, sewage generated in irregular areas continues to compromise water quality (CETESB, 2019).

2.2. *Samples sites*

Surface waters and sediments were collected from fourteen nearshore stations at Santos Bay, SP, Brazil during September of 2018, covering areas close to the municipalities of São Vicente, Guarujá and Praia Grande. Sampling stations are distributed as follows: S1-S5 around the SSO diffusors, considering all possible directions for the dispersion of the effluent plume, indicated by a monitoring conducted by the São Paulo Environmental Agency (CETESB, 2017). The other sampling sites (S6-S14) were chosen in order to comprise the entire bay, including beaches and estuarine mouths. Also, mussels were collected in 4 sites (M1-M4) as reported in Fig. 1.

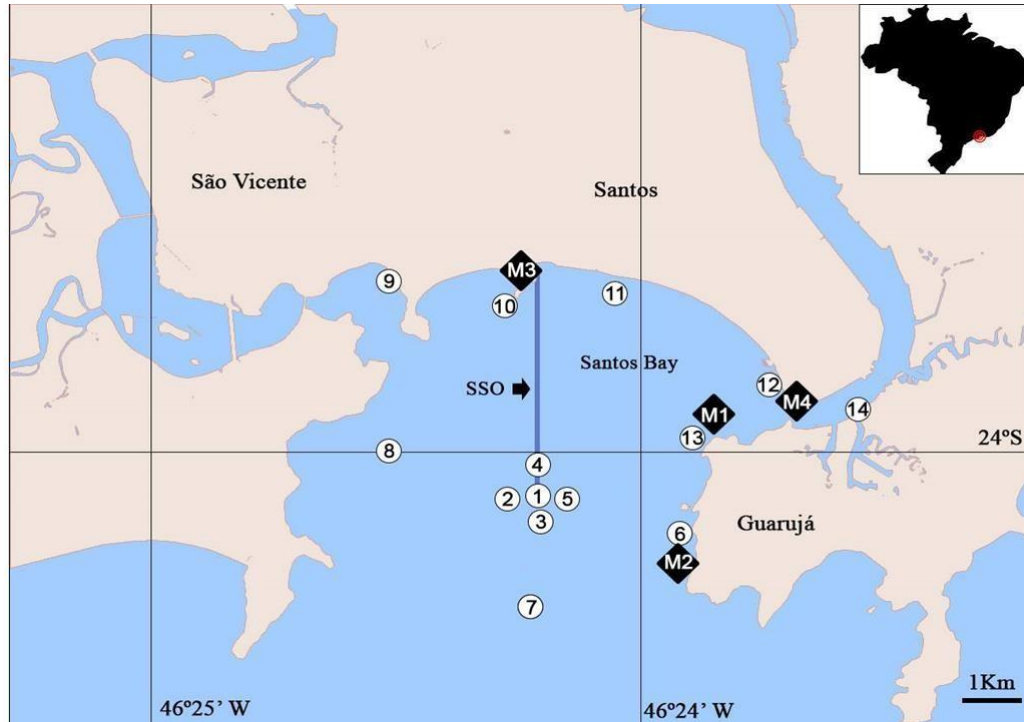


Fig. 1. Water, sediment (1–14) and mussels (M1–M4) sampling sites. Sites M1, M2, M3 and M4 correspond to sites 13, 6, 10 and 12, respectively.

2.2.1. Water samples

Water samples were collected at each station using a Van Dorn bottle. The samples were placed into amber glass bottles previously cleaned with HNO₃, methanol and distilled water. Samples were transported to the laboratory into an insulated cooler with ice (<6 °C) and placed in a freezer at –20 °C until sample preparation.

2.2.2. Sediment samples and properties

Sediment samples were collected using a stainless steel “Van Veen” grab sampler (0.026 m²) and kept on ice in Styrofoam boxes. Aliquots of sediment were stored at –20 °C for geochemical analyses. For texture analyses, they were dried at 60 °C for three days before use.

We analyzed the sediment grain size distribution according to Mudroch and Macknight (1994). A two-step sieving process was performed: in the first step, the sediment (100 g) was previously dried and wet-sieved through a 0.062 mm mesh to separate the sludge and clay (mud) fractions. The difference between the initial and final weights represented the mud fraction. In the second step, the material retained in the 0.062 mm mesh was dried and then dry-sieved on a set of sieves (scale Φ) to separate different sand classes, according to Wentworth (1992).

Calcium carbonate was determined according to the protocol described by Grant-Groos (1971). The sediment was digested with 30 volumes hydrochloric acid (HCl), followed by a new weighing and weight difference calculation. Organic matter (OM) in decarbonated sediment samples was estimated by the ignition method (Luczak et al., 1997). Dry sediment samples (5 g) were separated and incinerated in a muffle furnace (500 °C) for 4 h. Organic matter quantities were determined by calculating the difference between initial and final weights. The textural classes, OM, and CaCO₃ quantities were expressed as percentages.

An aliquot of 10 g of sediment was separated to analyze cocaine (COC) and benzoylecgonine (BE) content through a solid phase extraction (SPE) process described in Section 2.3.

2.2.3. *Mussels samples*

The mussel *P. perna* is very abundant along the coast of São Paulo, Brazil (Catharino et al., 2012). Mussels were collected in 4 sites (M1, M2, M3, M4). At each site, 9 mussels were obtained and divided into pools of 3 organisms. Mussels were transported to the laboratory in Styrofoam boxes. The organisms were measured, weighed, sex identified and then fully excised from their shells. Tissue samples were frozen at -80 °C until procedures for COC and BE quantification.

2.3. **Sample extraction and analysis**

2.3.1. *Water samples*

The extraction technique used was the same described by Pereira et al. (2016). Before extraction, the pH of each seawater sample was adjusted to 7 ± 0.5 using HCl solution (1 M). The samples were filtered through Whatman filter paper (GF/C diameter 47 mm, particle retention 1.2 µm; Merck, Darmstadt, Germany). The filters were washed with 2 mL of methanol (Sigma-Aldrich, St. Louis, USA) to prevent the loss of relevant compounds. The extracted methanol was collected and added to the filtered sample. Subsequently, solid-phase extraction (SPE) was performed using Chromabond HR-X cartridges (3 mL, 200 mg; Marcherey-Nagel, Duren, Germany) as described by Wille et al. (2010) and Ghoshdastidar et al. (2015). The cartridges were preconditioned with 5 mL of methanol and 5 mL Milli-Q water (Merck, Darmstadt, Germany). After preconditioning, the filters were loaded with 1 L of the filtered sample combined with the methanol from filter washings. The cartridges were dried under

vacuum for 30 min. Elution was performed using 5 mL of acetone and 2×5 mL of methanol. After the SPE procedure, the samples were dried under nitrogen flow (at 50 °C) and eluted with water/acetonitrile (95:5 v/v) prior to mass spectrometry analysis.

2.3.2. *Preparation of sediment and mussel samples*

Sediment (10 g) and mussel (whole soft tissues) samples were prepared according to Klosterhaus et al. (2013) and EPA-1694 extraction processes with adaptations. Mussel tissues were analyzed in triplicate, each replicate being a pool of 3 mussels from each sampling site (M1-M4). Sediment and tissue samples were lyophilized for two days before extraction. After this period, sediment and tissues (1 g dry weight) were first extracted with an aqueous phosphate buffer (pH 2.0). In order to compare our results with previous studies and to determine a field measured BAF, results of COC concentration in mussels were converted from dry weight (dw) to wet weight (ww). For this purpose, it was assumed a Conversion Factor (CF) of 5, which is supported by our previous studies with this species, with the percentage of humidity in wet tissues ranging around 80% (Pereira et al., 2007).

Each sample received 10 mL of acetonitrile and placed in an ultrasonic bath for 30 min. Afterwards the supernatant was collected and centrifuged at 2500 rpm for 5 min. The supernatant formed was stored. This procedure was repeated with phosphate buffer and once again with acetonitrile. At the end, all accumulated supernatant was centrifuged again. A volume of 25 mL of the final supernatant was collected and added to a bottle with 250 mL of Milli-Q water. The same extraction process described for water samples was performed with this solution.

2.3.3. *LC- MS/MS analysis*

The method employed was described and validated by Shihomatsu et al. (2017). An aliquot of 10 μ L of sample that was subjected to analysis by HPLC Agilent 1260 (Agilent Technologies, CA, USA) combined with a triple mass spectrometer quadrupole triple QTR/LIT (linear ion trap) ABSciex, Ontario (Canada). The COC and BE standards were obtained from Cerilliant® (FE07271503). The samples were analyzed using a 4.6×50 mm Agilent Eclipse XDB-C18 column, 1.8 μ m at 25 °C. The eluent flow was 0.7 mL min⁻¹ and the mobile phase

for positive analysis was 0.1% formic acid (Sigma-Aldrich LC-MS Grade) in water (solvent A) and acetonitrile (MS grade; JT Baker LC) (solvent B).

A linear gradient of 0.7 mL min⁻¹ was used for this (positive) ionization mode, starting with a mixture of 95% solvent A and 5% solvent B. The percentage of solvent A was decreased linearly from 95% to 5% over 5 min, and kept at 5% for 1 min. This mixture was then returned to initial conditions over 2 min and the analytes were detected and quantified using the ESI ionization and multiple reaction monitoring (MRM), with the selection of a precursor ion and two ion products to quantify and qualify each compound. The data were recorded and processed using the Analyst 1.5.2 software (ABSciex, Ontario, Canada).

A solvent calibration curve was employed as described by Shihomatsu et al. (2017). Individual standard stock solutions at 100 µg·mL⁻¹ were prepared in water/acetonitrile (1:1, v/v). Linearity, limit of detection, limit of quantification, precision, accuracy and recovery were the parameters to evaluate the performance of the SPE-LC-MS/MS methodology.

The linearity of the method was evaluated constructing a six-point calibration curve. The calibration curves of the compounds showed satisfactory coefficients of determination ($0.993 \leq r^2 \leq 1$). The recoveries of SPE extraction for water matrix were obtained in the range of 49% to 109%, whereas uncertainties for the COC and BE analyses were ±3% and ±5%, respectively. The accuracy for sediment and mussels samples was reduced, ranging from 35 to 40%. Two transitions were selected for the identification and confirmation of each analyte with the most intense fragment ion selected for analyte quantification, being 304–182 (m/z) and 290–168 (m/z) to COC and BE, respectively. The MRM parameters for positive mode, detection limit (LOD) and quantification limit (LOQ) are shown in Table 1.

Table 1. Parameters of Multiple Reactions Monitoring (MRM) for the positive and negative ion mode, limit of detection (LOD), limit of quantification (LOQ) and retention time (RT).

Compound	Q1	Q3	DP (V)	CE (V)	CXP(V)	LOD (ng.L ⁻¹)	LOQ (ng.L ⁻¹)	RT (min)
COC	304.2	182.2	36	27	4	0.16a	0.52 ^a	3.90
		105.1	36	39	4	0.50b	1.70 ^b	
						0.53c	1.80 ^c	
BE	290.2	168.2	31	25	4	0.30a	1.00 ^a	3.63
		105.3	31	37	4	0.43b	1.44 ^b	
						1.15c	3.84 ^c	

Q1(first quadrupole); Q3 (last quadrupole); DP (Declustering Potential); CE (Collision Energy); CXP (Collision Exit Potential). ^a Water (ng L⁻¹); ^b Sediment (ng g⁻¹); ^c Mussel (ng g⁻¹).

2.4. Field- measured bioaccumulation factor (BAF)

According to USEPA (2003), a field-measured BAF is determined from measured chemical concentrations in an aquatic organism and the ambient water collected from the same field location. Because the data are collected from a natural aquatic ecosystem, a field-measured BAF reflects an organism's exposure to a chemical through all relevant exposure routes. The bioaccumulation factor (BAF) was determined employing the average COC concentration in tissues for each site (M1-M4), as described in Section 2.3.2. According to USEPA (2003) and Arnot and Gobas (2006), BAF ($L \cdot kg^{-1}$) was calculated by the following equation:

$$BAF = C_{org} / C_{water}$$

C_{org} = concentration of the contaminant in the organism expressed in $\mu g \cdot kg^{-1}$ (ww); and C_{water} = concentration of the contaminant in water expressed in $\mu g \cdot L^{-1}$.

3. Results and discussion

3.1. Concentration of COC and BE in water and sediment

Physico-chemical parameters of water measured at the sampling sites were within the normal range for the region and season. Salinity ranged from 32.8 to 35.5 (mean = 34.64 ± 0.85); OD ranged from 5.3 to 9.6 (mean = $8.34 \pm 1.4 \text{ mg} \cdot L^{-1}$); pH ranged from 7.27 to 7.91 (mean = 7.74 ± 0.21) and temperature ranged from 22.4 to 23.6 (mean = $22.91 \pm 0.34 \text{ } ^\circ\text{C}$).

Both compounds (COC and BE) were detected in all water samples from Santos Bay (Fig. 2). The COC concentrations (mean = $4.53 \pm 2.78 \text{ ng} \cdot L^{-1}$) ranged from $1.91 \text{ ng} \cdot L^{-1}$ (S14) to $12.52 \text{ ng} \cdot L^{-1}$ (S5). BE concentrations (mean = $15.18 \pm 5.91 \text{ ng} \cdot L^{-1}$) ranged from $9.88 \text{ ng} \cdot L^{-1}$ (S7) to $28.53 \text{ ng} \cdot L^{-1}$ (S4).

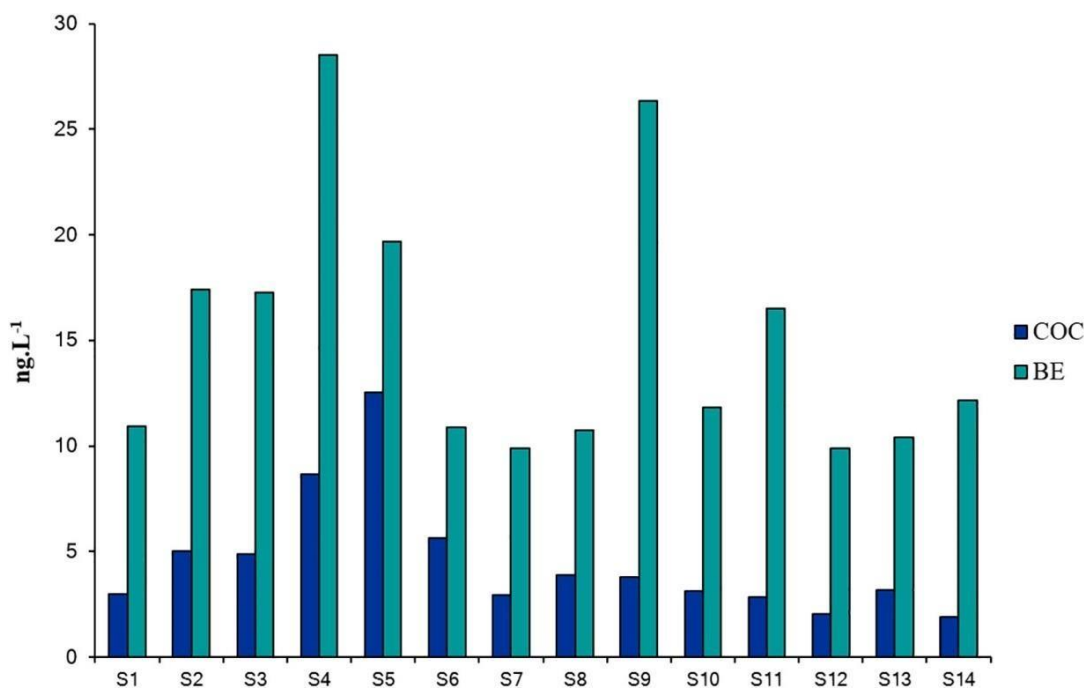


Fig. 2. Concentrations of COC and BE in water samples from Santos Bay.

A previous study performed by Pereira et al. (2016) in Santos Bay also showed occurrence of COC and BE in concentrations ranging from 12.6 to 537 ng·L⁻¹ and 4.6 to 19.5 ng·L⁻¹ respectively. In a recent study, Fontes et al. (2019) carried out a seasonal monitoring in the same region and reported concentrations ranging from 12.18 to 203.60 ng·L⁻¹ for COC and 8.20 to 38.59 ng·L⁻¹ for BE. Klosterhaus et al. (2013) detected COC and BE (2.4 and 7.8 ng·L⁻¹, respectively) in water samples from San Francisco Bay, California. Our results are also corroborated by Nodler et al. (2014) who found BE in water samples from Aegean Sea (16 ng·L⁻¹) and Venice (13 ng·L⁻¹).

It may be suggested that the low levels of drug residues detected in superficial water to date are so low that they pose no environmental risk or toxicological threat. However, illicit drugs as COC are bioactive compounds able to cause specific effects even at low concentrations. Freshwater mussels *Dreissena polymorpha* exposed to BE (0.5 and 1.0 µg·L⁻¹) presented oxidative stress and oxidative modification in different proteins from cytoskeleton, energetic metabolism and stress response (Pedriali et al., 2013). Binelli et al. (2012) found that mussels exposed to COC (40 ng·L⁻¹; 220 ng·L⁻¹; and 10 µg·L⁻¹) exhibited DNA damage (increase in micronucleated cells), apoptosis, and decreased stability of lysosomal membranes. Ortega et al. (2019) reported cytotoxicity induced by crack cocaine (500 ng·L⁻¹) in *P. perna* exposed during 96 h. COC and BE has been continuously detected in Santos Bays surface

waters at average concentrations in the mid to high $\text{ng}\cdot\text{L}^{-1}$ range, denoting chronic exposure with potentially sublethal responses, such as increased oxidative stress leading to cytogenotoxic effects.

Marine sediments act as final sinks of several contaminants produced by anthropogenic activities in coastal areas (Sciarrillo et al., 2020). The evaluation of contamination and toxicity of marine sediments is a major challenge in preventing hazard to marine biota and, consequently, human health (Oral et al., 2012). Although the current knowledge in the occurrence of these compounds in surface and wastewater is well documented (Pal et al., 2013; Li et al., 2016), data regarding illicit drugs in marine sediments are still limited.

In our study, COC was detected and quantified in all sediments samples, in concentrations ranging from $0.94 \text{ ng}\cdot\text{g}^{-1}$ (S5) to $46.85 \text{ ng}\cdot\text{g}^{-1}$ (S2) (mean = 12.27 ± 10.39) However, BE was below the limit of detection (LOD) in all samples (Fig. 3).

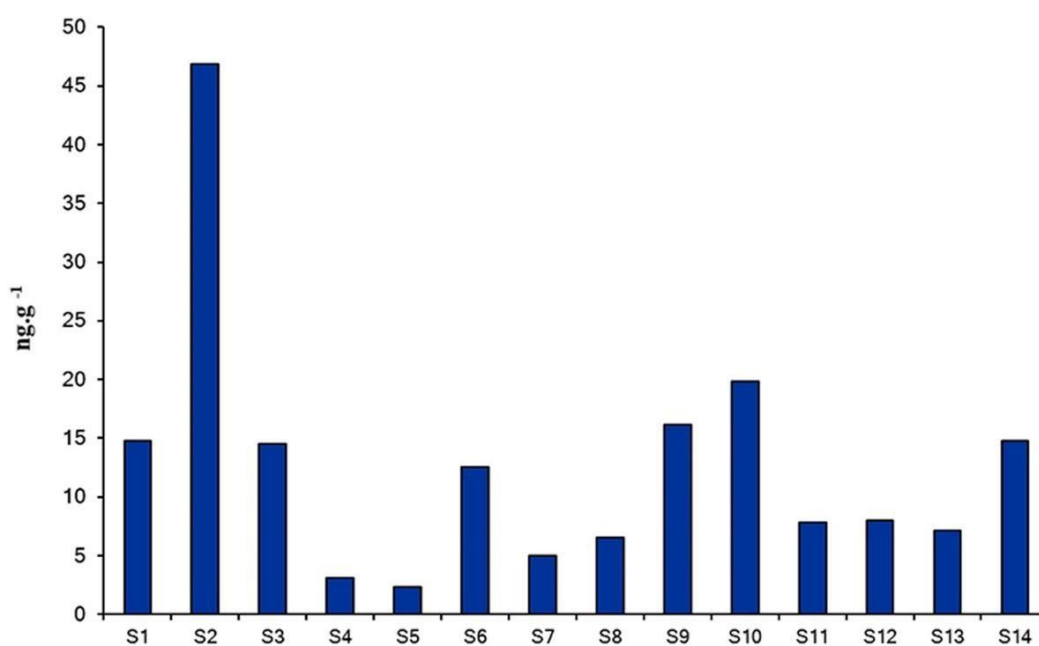


Fig. 3. Concentrations of COC in sediment samples from Santos Bay.

Similarly, Álvarez-Ruiz et al. (2015) observed the presence of COC and BE in samples of particulate matter ($14\text{--}127 \text{ ng}\cdot\text{L}^{-1}$; $3\text{--}258 \text{ ng}\cdot\text{g}^{-1}$) and sewage sludge ($4\text{--}58 \text{ ng}\cdot\text{L}^{-1}$; $1\text{--}4 \text{ ng}\cdot\text{g}^{-1}$) from a WWTP, and in sediments ($1 \text{ ng}\cdot\text{L}^{-1}$) from Turia river in Valencia, Spain. Furthermore, as observed in the present study, lower concentrations of metabolites were

reported probably because they are more easily degraded than unaltered drugs (Álvarez-Ruiz et al., 2015).

After carrying out granulometric analysis, sediments were classified predominantly as muddy at sites S3, S4, S5, S7, S13 and S14; and predominantly as sandy at sites S1, S2, S6, S8, S9, S10, S11, and S12, with a high concentration of very fine sand, followed by fine sand, medium sand and coarse. The highest OM values were found in sediments from S4 (14.99%), S5 (12.22%), S7 (17.34%) and S14 (17.42%). Highest values of CaCO₃ were found in S9 (59.69%), S6 (48.09%) and S13 (32.18%). It should be pointed out that, independently of sediment characteristics, COC was found predominantly in sampling stations surrounding the SSO (S1-S3) and the estuarine mouths (S9, S10 and S14).

This fact is due the disposal of untreated sewage from illegal housing in estuarine areas, as such as through the Submarine Sewage Outfall (SSO), which effluents presenting only preconditioning treatments (Abessa et al., 2005). Preconditioning has been considered inefficient in removing solids, nutrients and emerging contaminants as illicit drugs and their metabolites (Roth et al., 2016; Yadav et al., 2017). In addition to the inefficiency of sewage treatment, it is worth to note that in 2019 Brazil registered a record number of COC seizures (47.1 tons), 85% of which seized in ports. The Port of Santos is responsible for 40% of Brazil's maritime shipping, making it an attractive target for traffickers (Robbins, 2019). Therefore, we hypothesize that the occurrence of COC in this coastal zone could be also related to losses during handling, crack-cocaine manufacturing, or deliberate disposal to avoid seizures.

3.2. COC concentration in mussels and BAF

Body parameters of mussels (size, weight, sex) were determined. Mussels from M1 showed an average size of 50.60 ± 4.03 mm and weight of 6.29 ± 1.81 g. Mussels from M2 showed an average size of 56.0 ± 6.67 mm and weight of 7.46 ± 2.67 g; organisms from M3 showed an average size of 37.0 ± 4.94 mm and weight of 2.51 ± 0.92 g, whereas from M4 showed an average size of 58.48 ± 3.75 mm and weight of 10.7 ± 2.05 g. Forty mussels were collected, twenty-four females and sixteen undetermined.

COC was detected in all mussels samples (Fig. 4). The average concentration of COC ranged from $0.914 \mu\text{g}\cdot\text{kg}^{-1}$ to $4.58 \mu\text{g}\cdot\text{kg}^{-1}$ (ww). The BE was not detected in mussels, therefore, it was not possible to determine the BAF for this metabolite.

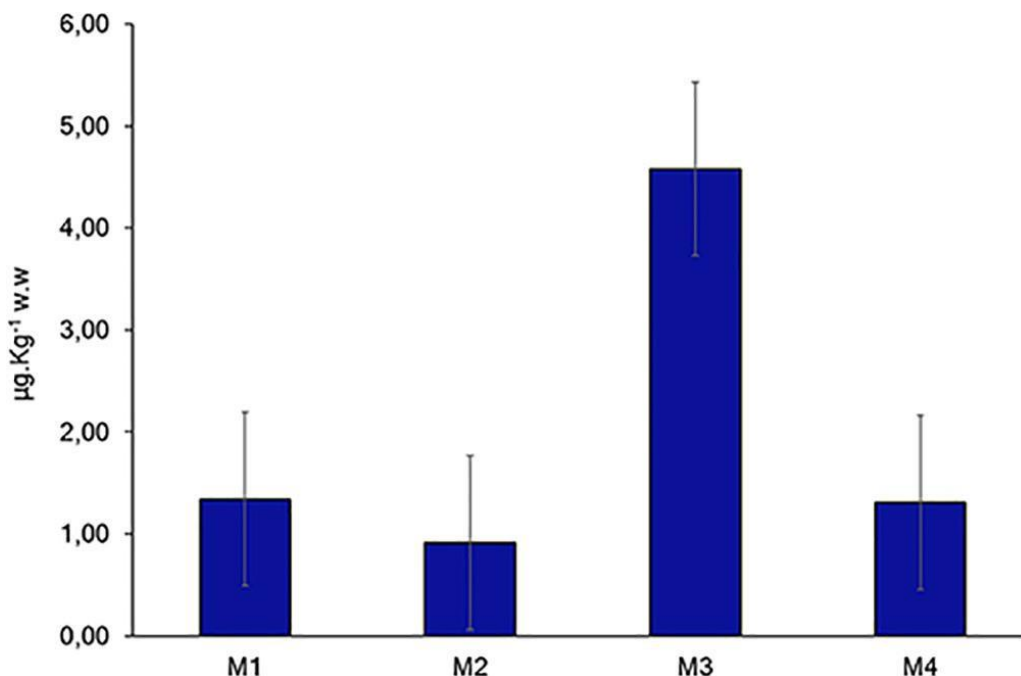


Fig. 4. Concentrations of COC in mussel samples from Santos Bay.

Our results are similar to those observed in a study performed by Miller et al. (2019), which detected an average of COC concentration of $5.9 \pm 4.3 \text{ ng}\cdot\text{g}^{-1}$ dw (max. $30.8 \text{ ng}\cdot\text{g}^{-1}$) in *Gammarus pulex* and showed that cocaine had preferential accumulation over its demethylated metabolite BE. Klosterhaus et al. (2013) identified COC ($0.3 \text{ ng}\cdot\text{g}^{-1}$ d.w.) in tissues of mussels *Geukensia demissa* from San Francisco Bay, California. Similarly, Dodder et al. (2014) determined COC at an average concentration of $0.28 \text{ ng}\cdot\text{g}^{-1}$ dw in *Mytilus* spp. from California coast. Capaldo et al. (2011) verified that European eel (*Anguilla anguilla*) can to bioaccumulate COC in concentrations ranging from 0.47 to $30.5 \text{ pg}\cdot\text{g}^{-1}$ ww depending on tissue type at an exposure concentration of $20 \text{ ng}\cdot\text{L}^{-1}$.

Bioaccumulation factor is an important tool for the assessment of contaminants toxicity to non-target organisms, relating the concentration of a chemical in biota with its corresponding concentration in water (Serra-Compte et al., 2018). In our study, the field-measured BAF was

determined in mussels collected at M1 (S13), M2 (S6), M3 (S10) and M4 (S12) (Table 2). The highest BAF values were determined in mussels sampled close to the Santos beaches (M3 and M4), denoting contamination by untreated sewage and concern to the consumption of these organisms easily accessible to collectors.

Table 2. Field- measured BCF in mussels from Santos Bay

Sampling sites	COC average concentration in tissues	COC concentrations in	BCF
	($\mu\text{g}\cdot\text{kg}^{-1}$ ww)	water ($\mu\text{g}\cdot\text{L}^{-1}$)	($\text{L}\cdot\text{kg}^{-1}$)
M1 (S13)	1.342	6.71	2.10
M2 (S6)	0.914	4.57	0.81
M3 (S10)	4.580	22.90	7.27
M4 (S12)	1.306	6.53	3.22

The physico-chemical properties of contaminants can influence their ability to accumulate in organisms. The log Kow (octanol-water partition coefficient) is one of the most used parameter to estimate the bioaccumulation potential of a contaminant, that is, the higher the Kow, the greater the capacity to bioaccumulate (EU, 2011). Furthermore, considering the alkaline pH of marine waters and pKa of COC (pKa = 8.61), it tends to be partially found in its non- ionic form, considering the pH of our sample area (ranging from 7.60 to 7.91) which increases the log know for COC from 0.10 (ionic form) to 2.30 (non-ionic form) (Pereira et al., 2016). Thus, COC would have a greater ability to bioaccumulate in mussels compared to BE (log Kow = -1.32 ; pKa = 2.15), which may explain the non-detection of BE in mussel samples from Santos Bay. Despite evidence that illicit drugs may accumulate in aquatic organisms, these compounds are rarely monitored in the aquatic biota (Miller et al., 2019).

To the best of our knowledge, this is the first study reporting BAF values for cocaine in marine mussels. However, according to Huerta et al. (2016) BAFs should be considered as tendency of bioaccumulation. Nevertheless, COC water concentrations are within the range of levels found in other campaigns at the same sites and/or seasons (Fontes et al., 2019).

Mussel meat is a high-quality marine protein, and its consumption is estimated between 200 g and 4 kg per capita (Monfort, 2014). Consumers of seafood are concerned about potential health risks associated with the presence of chemical contaminants, including inorganic compounds such as methylmercury and other metals, as well as persistent organic pollutants (Nesheim and Yaktine, 2007). Because of concerns about global circulation through the atmosphere, oceans, and other pathways, since 2003 the UNEP has established a list known as

“dirty dozen” which includes some pesticides, biphenyls, dioxins for representing the POPs to which seafood consumers are most likely exposed. Furthermore, the presence of veterinary and human drugs in marine organisms may pose a threat to human health, if residues persist in the edible portions of fish, shellfish, mussels, etc. Tolerance levels have already been determined for pharmaceuticals by some control agencies around the world (such as Food and Drugs Administration at USA or European Union) (Ahmed, 1991; FAO, 2002).

According to the recently published “Guidelines for rapid risk analysis following instances of detection of contaminants in food where there is no regulatory level” (FAO, 2019), the detection of chemical contaminants in foods where there is no regulatory level is increasing due to both the diversity of the food supply and the continuing advancement of analytical capabilities. This guideline suggests the application of a cut-off value of $1 \mu\text{g}\cdot\text{kg}^{-1}$, above which relevant stakeholders should be informed of such measurements and all available information should be shared for risk assessments. In this context, our results should be considered as a preliminary assessment of seafood contamination by drugs in this coastal zone, and information on toxicological data and dietary intake should be provided for further risk assessment approaches.

Conclusion

The Santos Bay showed a widespread contamination by cocaine and its main human metabolite benzoylecgonine. The three matrices (water, sediment and mussels) demonstrated the extent of contamination, especially in the São Vicente and Santos estuarine mouths, indicating the contribution of untreated sewage from irregular disposal. Mussels were able to accumulate the unaltered drug cocaine, pointing out the presence of untreated sewage in areas used by residents and tourists for bathing and harvesting. The occurrence of illicit drugs in different environmental matrices represents a concern to this coastal zone and its ecosystem services. The inclusion of drugs in environmental monitoring programs would be an important tool to assess risks for aquatic biota and human health.

CRedit authorship contribution statement

Mayana Karoline Fontes performed field work, samples preparations and analyses, as such as manuscript writing and revision.

Bruno Galvão de Campos performed sediment and mussels samples preparations and analyses, as such as manuscript writing and revision.

Fernando Sanzi Cortez performed field work, water samples preparations and analyses, as such as manuscript writing.

Fabio Hermes Pusceddu performed field work, water samples preparations and analyses, as such as manuscript writing.

Caio Rodrigues Nobre performed water, sediment and mussel samples preparations and analyses, as such as manuscript writing.

Beatriz Barbosa Moreno performed water, sediment and mussel samples preparations and analyses, as such as manuscript writing.

Daniel Temponi Lebre performed water, sediment and mussel samples preparations and analyses, as such as manuscript writing.

Luciane Alves Maranhão performed sediment and mussels samples preparations and analyses, as such as manuscript writing and revision.

Camilo Dias Seabra Pereira has coordinated and performed field work, samples preparations and analyses, as such as manuscript writing and revision.

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.

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CHAPTER 3

Effects of cocaine at environmentally realistic concentration in marine organisms

Environmentally realistic concentrations of cocaine in seawater disturbed neuroendocrine parameters and energy status in the marine mussel *Perna perna*

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Cellular defenses and damages triggered by cocaine in marine mussels

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Morphological and hormonal responses in ovaries of *Anguilla anguilla* exposed to environmental cocaine concentrations

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Environmentally realistic concentrations of cocaine in seawater disturbed neuroendocrine parameters and energy status in the marine mussel *Perna perna*

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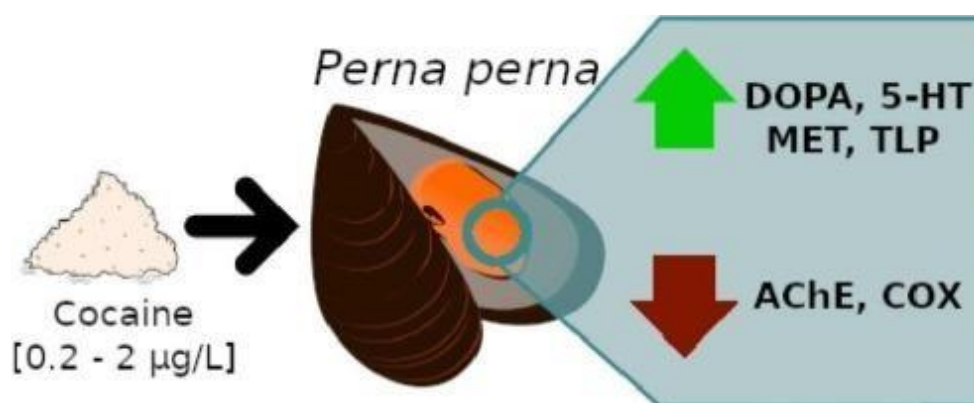
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Graphical abstract



Abstract

Cocaine (COC) is a powerful drug with anesthetic and stimulant properties that is consumed around the world and frequently detected in the aquatic environment. COC acts by inhibiting the reuptake of dopamine (DOPA) and 5-hydroxytryptamine (5-HT – serotonin), and causes endocrine disturbances in mammals. This study investigated similar effects from cocaine exposure in the marine mussel *Perna perna*, as well as neurotoxicity and energy imbalances. Mussels were exposed to COC (0.2 $\mu\text{g.L}^{-1}$ and 2 $\mu\text{g.L}^{-1}$) for periods of 48, 96, and 168 h. Acetylcholinesterase (AChE) was measured in adductor muscle tissue to determine neurotoxicity, and neurotransmitter levels (DOPA and 5-HT), monoamine oxidase (MAO) and cyclooxygenase (COX) activity, and energy status (mitochondrial electron transport, MET, and total lipids, TLP) were evaluated in the mussels' gonads. COC decreased AChE activity in the mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ and 2 $\mu\text{g.L}^{-1}$ after 168 h, and all concentrations of COC increased neurotransmitter levels. Increases in MET (0.2 $\mu\text{g.L}^{-1}$, for all exposure periods) and TLP (0.2 $\mu\text{g.L}^{-1}$ after 48h, and 2 $\mu\text{g.L}^{-1}$ after 96 h and 168 h) were also observed. No significant change was detected in MAO activity. COC also decreased COX activity in the mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ (48 h and 96 h) and 2 $\mu\text{g.L}^{-1}$ (96 h). These results suggest that COC may compromise gonad maturation in mussels as well as their spawning process, since these events are affected by neurotransmitter balance and COX activity. Furthermore, the changes in MET and LPT suggest that COC affects the energy balance of the mussels, and could negatively affect physiological processes such as metabolism, hormone production, and embryonic development. As of this writing, this is the first investigation of the neurotoxicological and endocrine effects of environmentally relevant concentrations of cocaine in marine mussels.

Keywords: illicit drugs, neurotoxicity, energy balance, bivalves, marine pollution.

1. Introduction

Cocaine (COC) is a powerful psychostimulant that affects the central nervous system, specifically the mesolimbic system. It acts as a monoamine transporter blocker, with affinity for dopamine (DOPA), serotonin (5- hydroxytryptamine), and norepinephrine transporters (Peña et al. 2015). Cocaine also acts as local anesthetic agent in cell membranes and may block sodium channel activity, precluding nerve impulse generation and conduction (Knuepfer, 2003). The physiological effects of COC include pupil dilation, hypertension, tachycardia,

psychomotor stimulation, anorexia, and euphoria (Dunwiddie, 1988). This substance is widely used by an estimated 19 million people around the world (UNODC, 2020). Beyond the socio-economic problems caused by consuming COC, it has also been recognized as an environmental contaminant (Binelli et al. 2012).

Once consumed, COC is rapidly metabolized and excreted through the urine. Approximately 40% is metabolized by hepatic esterases and plasma pseudocholinesterase (Shindler & Goldberg, 2012), but a small fraction (1–9%) of the parent compound may be excreted in its original form (Baselt, 2004; Maurer et al. 2006). Because sewage treatment plants cannot effectively remove these substances, COC and its metabolites are constantly introduced into the aquatic environment (Campestrini et al. 2017). Residues of illicit drugs have been detected in these environments, in concentrations ranging from ng.L⁻¹ to µg.L⁻¹ (Zuccato et al. 2008; Kasprzyk-Hordern et al. 2009; Gerrity et al. 2011; Thomas et al. 2014; Fontes et al. 2020).

Chemicals discharged into the ocean may alter the environmental structure, with negative impacts on biotic communities and the marine ecosystem as a whole (Bellas et al. 2020). Previous studies have reported the presence of COC and its metabolites in coastal zones (Klosterhaus et al. 2013; Pereira et al. 2016; Fontes et al. 2019), which was mainly attributed to discharge from submarine sewage outfalls.

Marine bivalves live in the intertidal zones, and are continuously subjected to various stress factors such as pH, dissolved oxygen, salinity, and temperature (Dong et al. 2017). Bivalves also have a long life cycle, are sessile, and filter large volumes of suspended matter and contaminants dissolved in the surrounding water (Gagné & Blaise, 2003). Mussels and other bivalves are especially threatened by compounds contained in municipal effluents, and have been frequently used as sentinel organisms to assess contamination in coastal zones (Pereira et al. 2014; Beyer et al. 2017; López-García et al. 2019). Bioaccumulation of cocaine in marine mussels was reported by Klosterhaus et al. (2013) in *Geukensia demissa* in the San Francisco Bay (US), Dodder et al. (2014) in *Mytilus* spp. from the California coast (US), and Fontes et al. (2021) in *Perna perna* from Santos Bay (Brazil).

Perna perna is a bivalve of great ecological and economic importance, particularly in South Africa and South America (Venezuela and Brazil), and is widely distributed in warm temperate regions of the Mediterranean Sea and the Atlantic and Indian Oceans (Santos et al. 2018). In addition to its importance as a source of protein for human nutrition, *P. perna* has

been also used in ecotoxicological studies to monitor the quality of the aquatic environment (Pereira et al. 2011; 2012; 2014).

These bivalves have a relatively simple and symmetrical nervous system, with pedal, cerebral, and visceral ganglia involved in the control of reproductive behavior such as releasing gametes into the water, fertilization, and larvae development (Siniscalchi et al. 2004). There is also a branchial nerve that supplies the gills and muscle, and nerves connecting the cerebrum-visceral network with the gonads, influencing gametogenesis. Dorsally, bivalves feature another nerve cord (the siphonal nerve) that allows sperm to enter the female cavity of the mantle, permitting egg fertilization (Meechonkit et al. 2012). Many of the nerve activities in mussels are controlled by neurotransmitters such as the indolamine serotonin (5-HT) and the catecholamine dopamine (DOPA), which regulate key physiological functions such as reproduction, sexual differentiation, spawning, gamete development, relaxation, and opening the mussel siphon and adductor muscle (Almeida et al. 2003; Gagné & Blaise, 2003; Garnero et al. 2006; Cubero-Leon et al. 2010).

There is scarce data on the effects of COC at environmental concentrations in marine invertebrates such as bivalves, especially considering impacts on the nervous system, considering that COC is known to alter neurotransmitter levels (Krause & Hocker, 2020). Within this context, this study investigated sublethal responses of the marine brown mussel *P. perna* after exposure to environmental concentrations of COC by assessing a battery of biomarkers related to neuroactivity (monoamine oxidase, acetylcholinesterase, serotonin, and dopamine). Furthermore, because COC affects the neuroendocrine system and in turn can alter the organism's energy demand (Maranho et al. 2015), mitochondrial electron transport chain activity and total lipid level were also measured to determine potential consequences for reproduction. Cyclooxygenase activity was also assessed, since this enzyme plays a key role in the immune system and spawning in bivalves (Matsutani & Nomura, 1987).

2. Material and methods

2.1 Mussel acclimation

Adult *P. perna* specimens (70.9 ± 4.88 g) were obtained from an aquaculture farm in Caraguatatuba, SP, Brazil and transported to the laboratory in thermal boxes filled with sea water. In the laboratory, the mussels were acclimatized for 72 h in a 300 L tank and fed with

microalgae. The tanks were kept below $20\pm 2^\circ$ C, with a 12:12h photoperiod and constant aeration.

2.2 Exposure of mussels to COC

After acclimation, the mussels were randomly distributed in 8 aquariums filled with sterile and filtered seawater, comprising 4 experimental groups of 2 replicates in which the mussels were exposed to 0 (control with acetonitrile), 0.2, and 2 $\mu\text{g}\cdot\text{L}^{-1}$ of COC. These concentrations correspond to the environmental concentration of COC previously reported by Fontes et al. (2019) in Santos Bay, Brazil. One of the replicate aquariums received 10 mussels (in 10 L of water) and the other 11 mussels (in 11 L of water) ($n = 21$ mussels per treatment), maintaining the proportion of 1 animal per liter in each aquarium. Standard COC solution (1 $\text{mg}\cdot\text{ml}^{-1}$ in acetonitrile, purity >99%) was obtained from Cerilliant Corporation (Round Rock, TX, USA) and added to the aquariums at the respective concentrations from this stock solution, and the mussels were exposed for 48, 96, and 168h.

The mussels exposed to COC and the controls were collected and processed at the same time after the respective exposure. Considering that the level of cocaine was found to drop 33.17% after 24 h in seawater (Ortega et al. 2019), the seawater and test solutions were changed every 24 hours. The mussels were fed daily with an algae solution (*Tetrasselmis* sp), and kept in controlled conditions at $20\pm 2^\circ$ C, 12:12h photoperiod, dissolved oxygen of 8.0 $\text{mg}\cdot\text{L}^{-1}$ (constant aeration), salinity 35 ppt, and pH 8.5. After exposure (48, 96, and 168 h), 7 mussels were collected randomly from each treatment tank; their gonads and adductor muscle were dissected and frozen at -80°C for subsequent biochemical analysis. After each collection, the volume of water was adjusted to maintain the same proportion of 1 animal per liter. The volume of workingsolution was also adjusted to maintain the concentrations of COC.

2.3 Neurotoxicity

Neurotoxicity of COC was measured by determining acetylcholinesterase (AChE) activity in the adductor muscle tissue, according to Ellman et al. (1961). This method detects 2-nitrobenzoate-5-mercaptothiocholine and 5-thionitrobenzoate (DTNB), derivatives formed by the reaction between thiocoline (resulting from hydrolysis of acetylthiocholine by acetylcholinesterase) and DTNB (0.75 mM). AChE activity was measured by absorbance (405 nm for 7 min), and results were expressed as $\text{DTNB min}^{-1}\cdot\text{mg}^{-1}\text{proteins}$.

2.4 Detection of dopamine and serotonin levels

L-dopamine (DOPA) and serotonin (5-hydroxytryptamine, 5-HT) were obtained from Sigma-Aldrich (St. Louis, MO, USA); levels of these substances were quantified using high performance liquid chromatography coupled to a fluorescence detector (HPLC-FD), according to De Benedetto et al. (2014). Gonads (20 mg) were homogenized in a buffer prepared with perchloric acid (0.2 μ M) and cysteine (3 mM). The homogenate was centrifuged for 12 min at 4 °C and 14,000 rpm. The mobile phase was composed of acetic acid (12 mM), Na₂-EDTA (0.26 mM), and methanol (10%) diluted in ultrapure water. An aliquot from supernatant (10 μ l) was filtered and injected directly into the HPLC-FD system (HPLC Shimadzu Series 20A, with Fluorescence RS10AXL detector). DOPA and 5HT were separated using an ACE C18 column (250 \times 4.6 mm, 5 μ m) at 35°C. The mobile phase was isocratically pumped at a flow of rate of 1 ml.min. A fluorescence detector (Shimadzu, model RF-20 A) was used to monitor the monoamines via a wavelength of 279 nm excitation and 320 nm emission. A calibration curve was prepared according to the 5HT and DOPA concentration levels in the samples to evaluate neurotransmitter levels, and the results were expressed in pg.g⁻¹.

2.5 Energy status

Mitochondrial electron transport activity was evaluated to determine mitochondrial energy consumption, based on the p-iodonitrotetrazolium reduction method (Smolders et al. 2004; King & Packard, 1975). Gonad samples were centrifuged (3000 g at 4°C for 20 min). The supernatant was mixed with Tris-HCl (0.1 M, pH 8.5), MgSO₄ (0.1 mM), Triton X-100 (0.1%), and polyvinylpyrrolidone (5%) for 1 min. NADH (1 mM) and NAPDH (0.2 mM) were added, and the reaction was initiated by adding p-iodonitrotetrazolium (1 mM). Mitochondrial electron transport activity was measured by absorbance at 520 nm for 5 min intervals, and results were expressed as Abs 520 nm.min⁻¹.mg⁻¹proteins.

Total lipid content (TLP) was determined by the phosphovaniline method (Frings et al. 1972). Gonad samples were incubated in phosphovaniline and H₂SO₄ for 10 min at 80 °C; TLP was measured by absorbance at 540 nm and results were expressed as μ g total lipids.mg⁻¹proteins.

2.6 Monoamine oxidase and cyclooxygenase activity

Monoamine oxidase (MAO) activity was measured using tryptamine serotonin analog as a substrate (Gagné et al. 2007). The homogenized gonad fraction was incubated with aminotriazole (1 mM), dichlorofluorescein (100 mM), HEPES-NaOH (pH 7.4), tryptamine (10 mM), NaCl (140 mM), and $0.1\mu\text{g}\cdot\text{ml}^{-1}$ of horseradish peroxidase. The reaction was analyzed at 30°C at 0, 15, 30, and 60 min. Fluorescein standard solution was used for calibration; fluorescence was determined at 485 nm (excitation) and 535 nm (emission), and results were expressed as $\text{nmol RFU}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins.

Cyclooxygenase (COX) activity was measured according to Fujimoto et al. (2002). Samples of gonad tissue were incubated in a buffer solution containing Tris-HCl (50 mM), 0.05% Tween 20 (50 mM), arachidonate (50 μM), dichlorofluorescein (2 μM), and horseradish peroxidase ($0.1\mu\text{g}\cdot\text{ml}^{-1}$). The activity was measured by fluorescence (484 nm emission and 530 nm excitation), and results were expressed as $\text{RFU}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ proteins.

2.7 Statistical analyses

Normality was tested with the Shapiro-Wilk test, and homogeneity was determined using Bartlett's test. Student's t-test was used to test distribution between controls (water and solvent). To identify differences between concentrations as well as time of exposure, the data was analyzed using two-way ANOVA followed by Dunnett's test, with significance of $p < 0.05$. The statistical ANOVA and post hoc analyses were conducted with Prism v.7a software (GraphPad Software, San Diego, CA, USA). The biomarker response profile of the gonad tissue was evaluated using the integrated biomarker response (IBR) calculated according to Beliaeff & Burgeot (2002) for all concentrations of COC.

3. Results and Discussion

3.1 Neurotoxicity: adductor muscle

Mussels are key components of the benthic community and have major ecological and economic importance in coastal zones. They are especially susceptible to contamination due to filtering behavior (Gagné et al. 2011). As far as we know, this is the first study to investigate the neurochemical effects of COC in marine mussels.

Hydrolysis of the neurotransmitter acetylcholine to choline and acetate is carried out by AChE, which plays an important role in the cholinergic system (Ferreira-Vieira et al. 2016). In mammals, COC metabolism occurs mainly via hydrolysis in the plasma and liver by esterases such as serum cholinesterases, pseudo-cholinesterases, and carboxylesterases, and the resulting metabolites are eliminated through the kidneys (Zentner, 1985; Loper, 1989; Martin & Faranoff, 2014). In aquatic organisms such as mussels, AChE is considered one of the most effective biomarkers in assessing neurological changes caused by xenobiotics (Rickwood & Galloway, 2004).

We found decreased AChE activity in mussels exposed to $0.2 \mu\text{g.L}^{-1}$ (54.36%) and $2.0 \mu\text{g.L}^{-1}$ (50.44%) of COC after 168 h of exposure ($p < 0.05$) (Fig 1). Decreased enzyme activity is a typical reaction in mussels after exposure to environmental contaminants (Ricciardi et al. 2006). Reduced plasma levels of AChE activity is also associated with enhanced COC toxicity (Hoffman et al. 2008). In mussels, a decrease in AChE activity also suggests a state of relaxation (Gagné et al. 2010). In fact, during the experiment we observed less rigidity in closing the valves of mussels exposed to COC. In this sense, the decreased AChE activity observed may indicate difficulty metabolizing COC. Furthermore, considering the alkalinity of the marine environment, COC can become more hydrophobic and bioaccumulate in the biota (Fontes et al. 2021).

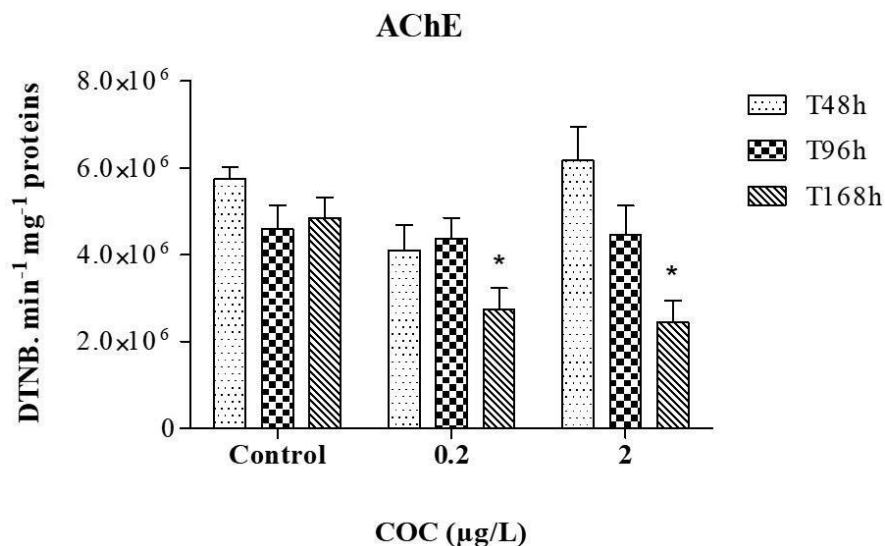


Fig 1. Mean (\pm standard derivation) AChE activity in the adductor muscle tissues of *Perna perna*: controls and mussels exposed to cocaine ($0.2 \mu\text{g.L}^{-1}$ and $2.0 \mu\text{g.L}^{-1}$). *represents significant differences (ANOVA, Dunnett's test, $p < 0.05$).

3.2 Dopamine and serotonin levels

DOPA levels were significantly higher in mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ COC after 48 h and 96 h, and to 2 $\mu\text{g.L}^{-1}$ after 48 h ($p < 0.05$), than in the controls. Similarly, 5-HT levels increased in mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ after 48 h and 96 h and to 2 $\mu\text{g.L}^{-1}$ after 48 h and 168 h of exposure ($p < 0.05$) (Fig. 2).

According to Klouche et al. (2015), *P. perna* display temporal cycles of reproduction; these are directly influenced by 5-HT and DOPA levels, which govern spawning, gametogenesis, and later stages of gamete maturation. The serotonergic and dopaminergic systems are extremely important to regulate stress response in both vertebrates and invertebrates (Liu et al. 2018; Bacqué- Cazenave et al. 2020). In aquatic organisms, these neurotransmitters play important roles in physiology and reproduction (Capaldo et al. 2012). Furthermore, in mollusks DOPA and 5-HT are responsible for regulating the processes of filtration, relaxation of the adductor muscle, and gonadal maturation (Ono et al. 1992; Deguchi & Osanai, 1995; Murakami et al. 1998; Almeida et al. 2003).

Beiras & Widdows (1995) observed that 5-HT and DOPA influence the ciliary beating of mollusk larvae, and affect their food absorption capacity. In mollusk gonads, 5-HT acts as a neurohormone and performs an important function in the regulation of responses to adverse external and internal stress, modulating several physiological processes such as metabolism, behavior, immune response, and homeostasis (Dong et al. 2017). Interactions between ganglion neurosecretions control the reproductive cycle in bivalves (Garnero et al. 2006), and gamete maturation and spawning are directly related to increases in 5-HT levels in these animals (Matsutani & Nomura, 1987).

Considering the altered neurotransmitter levels found in *P. perna*, we predict that chronic exposure to COC can lead to significant changes in the physiological and reproductive processes essential for these organisms to survive. Reproduction could also be significantly impacted, since sexual differentiation and the release of larvae in mollusks also appears to be determined by the activity of the neurotransmitters investigated in this study (Joyce & Vogeler, 2018).

3.3 Energy and endocrine status

Energy status in the gonads was measured by analyzing MET and TLP (Fig. 2). Levels of MET were higher in mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ of COC compared to the controls ($p < 0.05$)

for all exposure periods; TLP levels also increased significantly in the mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ after 48 h and 2 $\mu\text{g.L}^{-1}$ of COC after 168 h ($p < 0.05$).

Because COC is a weak base, it can accumulate within cell compartments and reach high concentrations in the mitochondria, affecting the electrical potential of the transmembrane and causing it to rupture (Cunha-Oliveira et al. 2013a). We noted increased MET only in those mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ of COC. Studies on mice exposed to COC (0.2 mM, 0.4 mM, and 1 mM) found that this compound induced mitochondrial depolarization and production of H^+ ions, changing cellular oxygen consumption and affecting hydrolysis of ATP and the activity of ATPases (Cunha-Oliveira et al. 2013b). Devi & Chan (1997) observed that mice exposed to injections of COC (25 mg.Kg over a 3-day period) exhibited a decrease in the electron transport rate in complexes I and IV, with consequent production of malondialdehyde and oxidative stress in hepatic tissue. Similarly, neuronal mouse cells *in vitro* exposed to COC (600 μM) exhibited mitochondrial dysfunction. These data suggest that COC may cause important changes in mitochondrial transmembrane potential, compromising the cellular energy balance.

Organisms can store energy as glycerol and/or lipids when energy intake exceeds growth, maintenance, and reproduction requirements (Maranho et al. 2015). In aquatic organisms, lipids also provide an excellent source of energy and are an important component of oocytes (Martins et al. 2020).

According to Fokina et al. (2015), lipids are very important for mussels to adapt to various environmental factors, which involves modifying cell membrane fluidity (by changing lipid composition). Lipid intake also influences the production of bioactive compounds such as eicosapentaenoic acid and arachidonic acid, which act as precursors of short-lived hormone-like substances (including leukotrienes, thromboxanes, and prostaglandins) that impact various physiological processes such as immunity, inflammatory response, neural function, and reproduction (Copeman et al. 2003; Gizem et al. 2017). In this present study, increased TLP levels were seen in the mussels exposed to 0.2 $\mu\text{g.L}^{-1}$ and 2 $\mu\text{g.L}^{-1}$ of COC, suggesting that this substance may induce lipid accumulation, in turn potentially affecting intermediate metabolism, energy balance, hormone production, and also embryonic and larval development (Baek et al. 2014).

Contrary to our predictions, COC was not seen to affect MAO activity ($p > 0.05$). MAO is an intracellular enzyme found mainly in the mitochondria that degrades monoamines such as 5-HT, norepinephrine, epinephrine, and DOPA (Fišar, 2016). For this reason, when COC is

present MAO activity is often inhibited, preventing reuptake of the neurotransmitters that accumulate in the synaptic cleft and increasing the feelings of pleasure and euphoria that are characteristic in COC users (Knuepfer, 2003). However, our results do not indicate any significant change in MAO activity, suggesting that the tested concentrations may not have been sufficient to affect this enzyme.

Increased COX activity was seen in the mussels exposed to $2 \mu\text{g.L}^{-1}$ of COC, indicating an inflammatory response in the gonads. Similar results were found by Gagné et al. (2003) in *Ellipio complanata* mussels exposed to municipal effluents with emerging contaminants. COX is an enzyme involved in the regulation of immune function and inflammation in mammals (Rouzer & Marnett, 2009), and produces important biological mediators such as prostacyclin, prostaglandins, and thromboxane (McPhee et al. 2007). In marine invertebrates, prostaglandins are involved in reproduction (gamete maturation and spawning agent), osmoregulation, ion transport, and defense (Osada & Nomura, 1992; Costanzo et al. 2019). Furthermore, prostaglandins play an important role in sodium regulation and may be associated with cyclic AMP and serotonin (Singh et al. 2017) and are consequently important in reproduction.

3.4 Integrated biomarker response- IBR index

The responses which were most strongly affected by COC concentrations were identified with an integrated biomarker index (IBR) first described by Beliaeff & Burgeot (2002) which has been used in several monitoring studies (Serafim et al. 2012; Maranhão et al. 2015; Ortega et al. 2018) to integrate multiple biomarker responses into a single index (Serafim et al. 2012). These IBR indexes are shown in Fig. 3 for the two different concentrations ($0.2 \mu\text{g.L}^{-1}$ in green and $2 \mu\text{g.L}^{-1}$ in yellow). IBR values ranged from -40 to 20 for all concentrations after 48 h and 96 h of exposure, and from -40 to -20 after 168 h of exposure. The IBR index indicated that COC affected energy status (MET and TLP) and neurotransmitter levels (5-HT and DOPA) in the mussels exposed to $0.2 \mu\text{g.L}^{-1}$; in the mussels exposed to $2 \mu\text{g.L}^{-1}$, COC exposure affected MET, COX, 5-HT, DOPA, and TLP.

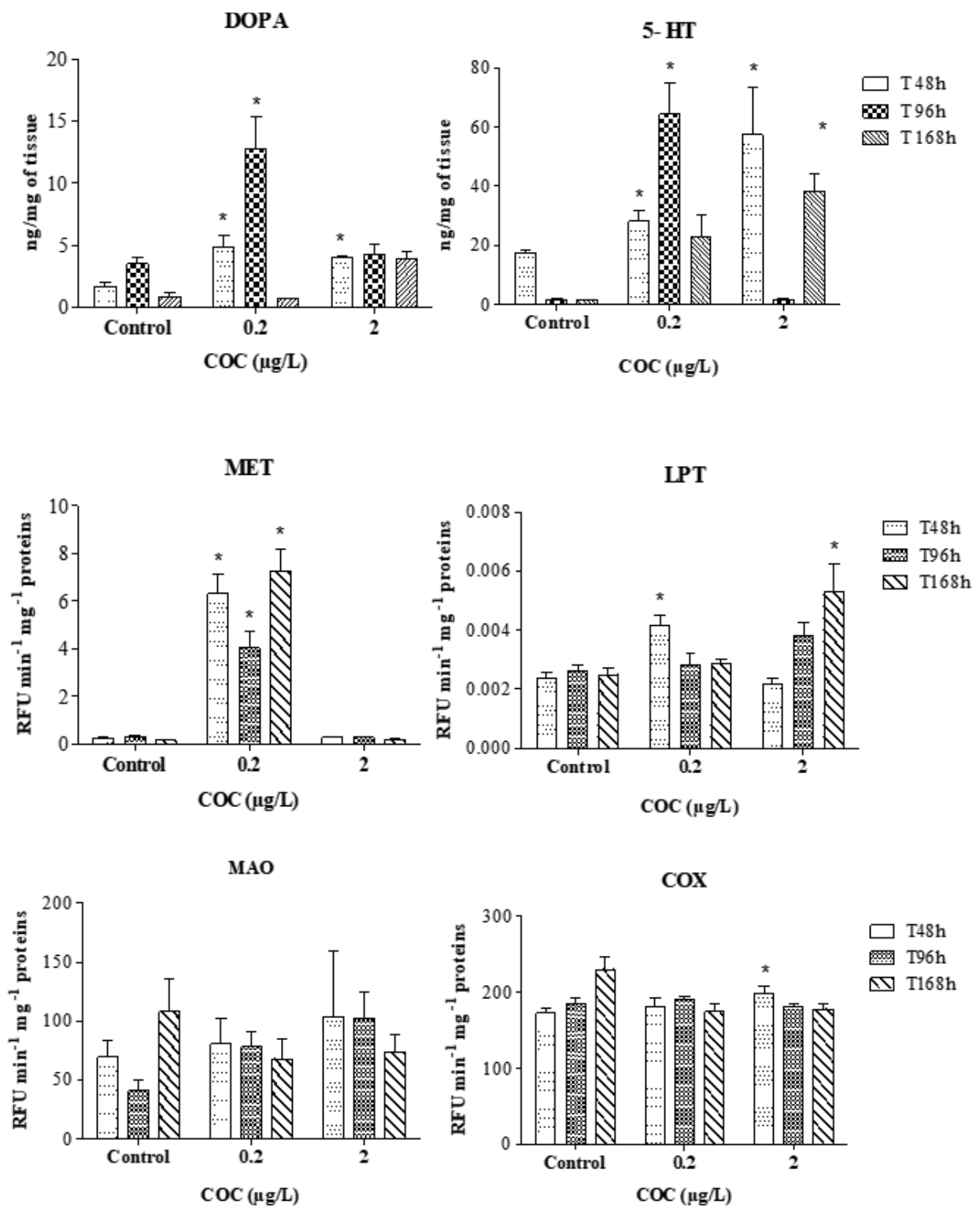


Fig 2. Mean (\pm standard derivation) biomarker activity (DOPA, 5-HT, MET, TLP, MAO, and COX) in the gonads of *Perna perna* exposed to COC (0.2 $\mu\text{g}\cdot\text{L}^{-1}$ and 2.0 $\mu\text{g}\cdot\text{L}^{-1}$) and controls; *represents significant differences (ANOVA, Dunnett's test, $p < 0.05$) between the controls and COC concentrations.

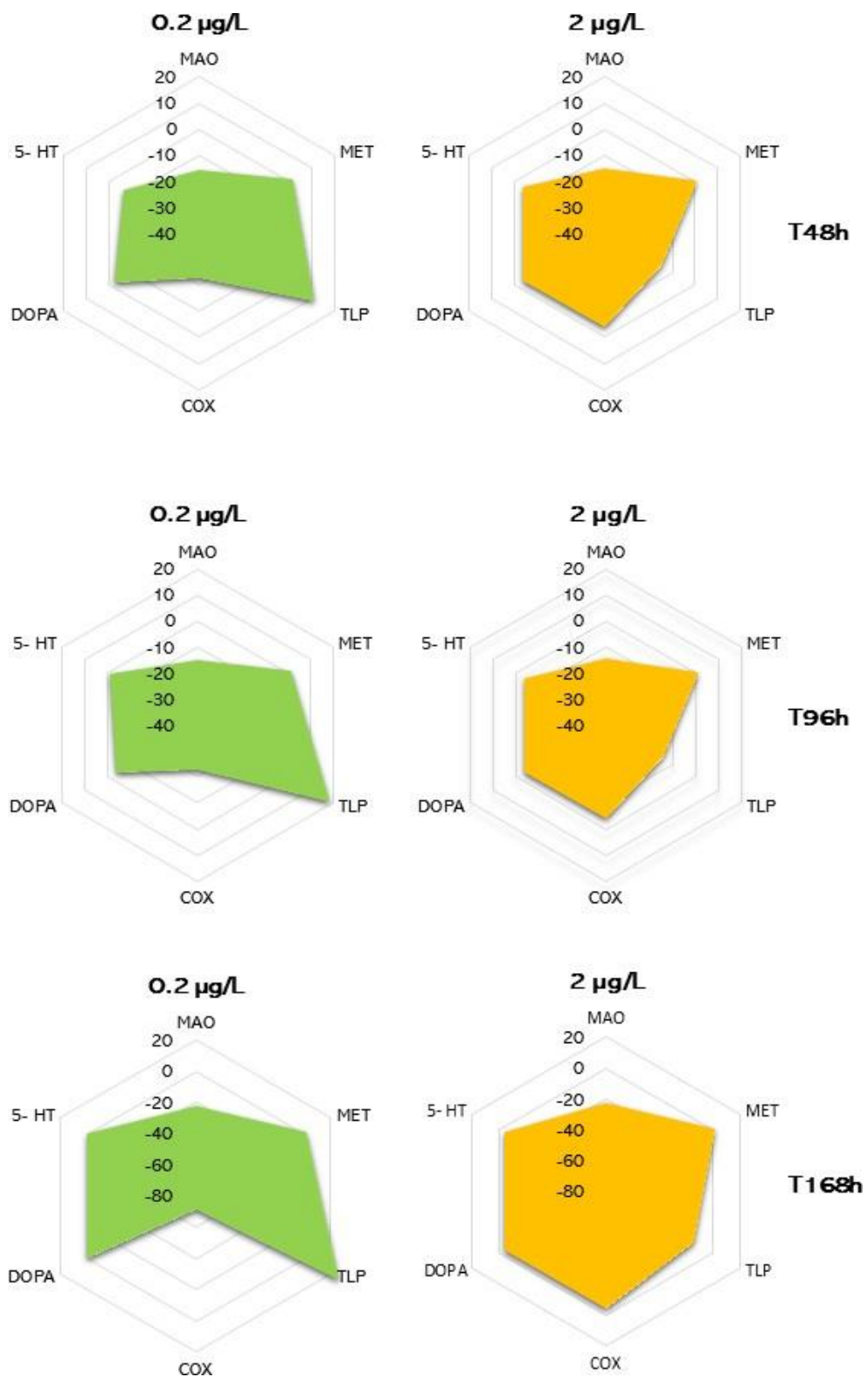


Fig 3. Integrated biomarker response (IBR) and COC star plots. IBR index for biomarker response includes MAO (monoamine oxidase activity), MET (mitochondrial electron transport activity), TLP (total lipids), COX (cyclooxygenase activity), DOPA (dopamine), and 5-HT (serotonin).

Conclusion

Cocaine triggered a complex pattern of neuroendocrine responses evidenced by reduced AChE and COX activity, as well as increased levels of 5-HT, DOPA, MET, and TLP. Our results indicate that environmentally relevant concentrations of cocaine can alter the energy balance and affect reproductive processes such as gonadal maturation and spawning in marine mussels.

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Cellular defenses and damages triggered by cocaine in marine mussels

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Abstract

Cocaine (COC) is one of the most used drug worldwide. Residues of COC have been frequently measured in aquatic environment, in concentrations ranging from $\mu\text{g.l}^{-1}$ and ng.l^{-1} . The presence of COC in aquatic ecosystem represents a potential risk for aquatic organisms, but little is known about the ecological effects, mainly on marine organisms. The aim of this study was to evaluate biochemical and cellular responses linked to biotransformation, oxidative stress and cytogenotoxicity of COC on marine mussel *Perna perna*. Organisms were exposed to two different concentrations of COC (200 ng.l^{-1} and 2000 ng.l^{-1}) for 168 h. Biological effects were determined by biomarkers of exposure (enzymatic activities of 7-ethoxyresorufin O-deethylase - EROD, dibenzylfluorescein dealkylase - DBF, glutathione S-transferase - GST, glutathione peroxidase – GPX), effects (lysosomal membrane stability - LMS, DNA damage - strand breaks and lipid peroxidation - LPO), All concentrations tested decreased the LMS, demonstrating the cytotoxic potential of COC. EROD and DBF activities indicate that the organisms had difficulties in metabolizing COC. Similarly, GST and GPx activities were compromised, suggesting that cocaine hinders an antioxidant response on mussels. In fact, DNA damage and increased LPO have been observed since the lowest concentration. Our results show that even low concentrations of COC harmed mussels, suggesting damages to their functions and survival.

Key-words: Cocaine, biomarkers, *Perna perna*, marine environment

1. Introduction

Coastal zones are ecological and economically important areas, subjected to several forms of disturbance as chemical and industrial pollution, associated with high urbanization and others anthropogenic activities (Vidal-Liñan et al. 2010). Emerging contaminants such as pharmaceutical and personal care products, estrogens hormones, pesticides, illicit drugs and their metabolites have been detected in different coastal ecosystem at trace concentrations (Klosterhaus et al. 2013; Pereira et al. 2016) and have been cause of concern because of their potential human and environmental risk (Omar et al. 2018). The major sources of ECs to the environment are via sewage treatment plants (STPs) and WWTP (Ebele et al. 2017).

Globally, it was estimated that the number of drug users is about 271 million people, or 5.5 per cent of the global population aged 15–64 (UNODC, 2019). Abuse of illicit drugs is a significant problem in the society and cause not only health, social life and economy problems, but also environmental impacts (Baker & Kasprzyk-Hordern et al. 2011; Binelli et al. 2012). After consumption, drugs are mainly excreted by urine continuously released into aquatic environment, unaltered or as metabolites, due to their incomplete removal in the WWTPs (Bijlsma et al. 2012; Campestrini et al. 2017).

Cocaine (COC) is the most widely used illicit drug in North America, Europe (Western, Central, Eastern and South-Eastern regions) and Latin America. The total number of users is estimated at 18.1 million (UNODC, 2019). In Brazil, the COC and crack cocaine market is the second highest in the world (Campestrini et al. 2017).

Residues of COC and their metabolites have been frequently detected in surface water, drinking water (Mendoza et al. 2014; Campestrini et al. 2017), seawater (Klosterhaus et al. 2013; Pereira et al. 2016; Devault et al. 2017; Fontes et al. 2019) and sediment, in concentrations ranging from ng/L to µg/L (Castiglioni et al. 2006; 2011; Zucatto et al. 2008; Berset et al. 2010; Gerrity et al. 2011; Li et al. 2016; Fontes et al., 2021).

The oceans' capability of diluting microcontaminants do not prevent the bioaccumulation in aquatic organisms (Mazzelani et al. 2018), because the release of these chemicals is usually punctual and concentrated in shallow parts, where the local circulation does not allow their expected (infinite) dilution (Weber, 1982). Capaldo et al. (2012) showed the presence of COC in several tissues of *Anguilla anguilla* in concentrations ranging from 0.47 to 30.50 pg g⁻¹

¹. In a recent study, Fontes et al. (2021) showed that COC can bioaccumulate in marine mussels from a coastal zone contaminated by untreated sewage.

Even at low concentrations, long term exposures to low doses of COC showed adverse effects in the aquatic organisms (Binelli et al. 2012; 2013; Parolini et al. 2015; 2016; 2017). However, data regarding effects of COC environmental concentrations on marine organism are still limited, because they are not routinely monitored in any National environmental legislation.

Marine mussels are abundant organisms and play important ecological role such as nutrient cycling, creation and modification of habitats, besides affecting food webs directly and indirectly (Vaughn & Hoellein, 2018). They are sedentary and filter feeders, which allow them to accumulate contaminants from the surrounding water (Vosloo et al. 2012). Furthermore, bivalves are used in the human diet as source of protein (Casarini & Henriques, 2011). Mussels of the family Mytilidae, such as of the genera *Perna* have been widely used in monitoring programmes around the world in order to assess chemical pollution of coastal waters (Francioni et al. 2007; Pereira et al. 2012; Cortez et al. 2018). *Perna perna* is an intertidal mussel widely distributed in Atlantic, Mediterranean Sea and Indian Ocean, sessile, easy to collect and tolerant to salinity variations, considered suitable bioindicator of the aquatic environment quality (Santos et al. 2018; Birnstiel et al. 2019).

Thus, due to the ecological and economic importance of the bivalves and the scarcity of data regarding the effects of COC on marine organisms, this present study investigated the sublethal effects of environmental concentration of COC using the brown mussel *P. perna* as a model. To establish the organisms' health status, a suite of biomarkers of exposure and effects was evaluated after different exposure times and environmental realistic concentrations.

2. Material and Methods

2.1 Mussel acclimation

P. perna adult specimens (70.9 ± 4.88 mm) were acquired from an aquaculture farm in Caraguatatuba, SP, Brazil, which has good bathing conditions (CETESB, 2019; 2020). In the lab, the organisms were acclimatized for 72h (300 L tank), receiving food supply (microalgae), kept in tanks under controlled conditions: temperature ($20 \pm 2^\circ$ C), photoperiod 12:12h and constant aeration.

2.2 *Mussels exposure to COC*

COC standard (1 mg/ml in acetonitrile, purity > 99%) was purchased from Cerilliant Corporation (Round Rock, TX, USA). The mussels were distributed in 4 experimental groups: natural seawater (filtered) control, acetonitrile control, 200 ng.l⁻¹ and 2000 ng.l⁻¹ of COC, based on the concentrations previously reported by Fontes et al. (2019) in the Santos Bay. For each group, 2 aquariums of 10 liters were used, totaling 8 aquariums: 4 aquariums were filled with 10 mussels each and the other 4 aquariums were filled with 11 mussels, (n = 21 organisms per treatment), respecting the proportion of 1 animal per liter in each aquarium

The total exposure time was 168h. The seawater and test solutions were renewed at each 48h to ensure the constant concentration of COC. The COC was added to the aquariums at respective concentrations from the stock solution. The mussels were feed daily with an algae solution (*Tetrasselmis* sp). The laboratory conditions were daily controlled: temperature (20 ± 2° C), photoperiod (12:12h), salinity (35 ppt), dissolved oxygen (8.0 mg.l⁻¹) and pH (8.5). After 48h, 96h, and 168h of exposure, 7 organisms from each treatment were chosen randomly and the hemolymph was extracted to analyze cytotoxicity through Neutral Red Retention Time (NRTT) assay. Then, the mussels were dissected and the tissues (gills and digestive gland) were frozen at -80°C until biomarkers analysis.

2.3 *Tissue preparation*

Gills and digestive gland tissues were excised and homogenized in a buffer solution containing Tris-HCl (50 mM), EDTA (1 mM), sucrose (50 mM), KCl (150 mM), dithiothreitol (DDT) (1 mM) and phenylmethylsulfonyl fluoride (PMSF) (100 mM). A fraction of the gills and digestivegland homogenate was reserved to assess DNA damage (strand breaks) and lipid peroxidation(LPO). The homogenized samples of all tissues were centrifuged at 4°C, 15.000 g for 20 min. Gills and digestive gland were employed to determine ethoxyresorufin O- deethylase (EROD), dibenzylfluorescein dealkylase activity (DBF), glutathione- S- transferase (GST), glutathione peroxidase (GPx) activities.

All the biomarker responses (homogenized and centrifuged) were normalized by the total protein content of each tissue following Bradford (1976) methodology.

2.4 Biochemical responses

2.4.1 Biotransformation

Ethoxyresorufin O-deethylase (EROD) activity was evaluated according Gagné and Blaise (1993). Samples of gills and digestive gland (S_{15}) were added in a dark microplate with 160 μM of 7-ethoxyresorufin, 10 μM of reduced nicotinamide adenine dinucleotide phosphate (NADPH), 100 μM KH_2PO_4 buffer (pH 7.4). A standard calibration curve of resorufin (5 μM) was used and the fluorescence was measured at 485 nm (excitation) and 580 nm (emission). The results were expressed as $\text{pmol min}^{-1} \text{mg}^{-1}$ proteins.

Dibenzylfluorescein dealkylase activity (DBF) activity was determined according Gagné et al. (2007) using dibenzylfluorescein as substrate. Similarly to EROD, samples of gills and digestive gland (S_{15}) were added in a dark microplate with dibenzylfluorescein (50 μM), being incubated with NADPH (10 μM). Fluorescence was measured at 485 nm (excitation) and 516 nm (emission). Results were expressed as $\text{pmol min}^{-1} \text{mg}^{-1}$ proteins.

2.4.2 Conjugation

Glutathione-S-transferase activity (GST) was analyzed using the method proposed by McFarland et al. (1999). Samples of gills and digestive gland (S_{15}) were added in a transparent microplate with 1-chloro-2,4-dinitrobenzene (CDNB 1 mM), buffer of 10 mM (HEPES- NaOH, pH 6.5) and NaCl (125 mM). The activity was measured by absorbance at 340 nm at every 50 seconds for 5 min. Results were expressed as $\text{OD GST min}^{-1} \text{mg}^{-1}$ protein.

2.4.3 Antioxidant enzyme

Glutathione peroxidase (GPX) activity was performed according McFarland et al. (1999). Gills and digestive gland (S_{15}) were incubated with assay solution (3 mM GSH and 1mM NADPH), buffer (200 mM KH_2PO_4 at pH 7) in transparent microplates and a substrate of 1 mM cumene hydroperoxide was used. Absorbance was measured at 340 nm for 5 minutes every 50 seconds. Results were expressed as $\text{nmol min}^{-1} \text{mg}^{-1}$ proteins.

2.5. Cytogenotoxicity

To evaluate the cytotoxicity of COC, we applied the NRRT assay proposed by Lowe and Pipe (2015). It is an indicator of general cellular stress in bivalves, through the analysis of the

lysosomal membrane stability (LMS). The hemolymph was withdrawn from posterior adductor muscle of living bivalves and it was mixed with physiological saline solution with pH 7.3 and containing HEPES (4.77 g l⁻¹); NaCl (25.48 g l⁻¹); MgSO₄ (13.06 g l⁻¹); KCl (0.75 g l⁻¹); CaCl₂ (1.47 g l⁻¹). The hemocytes (40 µl) were stained with neutral red dye (40 µl) and incubated in the dark for 15 min. After this time, cells were examined systematically thereafter by optical microscopy (400 x) at 15 min intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. Tests endpoint was the time when dye loss was evident in at least 50% of the hemocytes. The retention time was calculated in relation to the last time the dye remained retained in the lysosomes.

DNA damage was evaluated by alkaline precipitation assay proposed by Olive (1998). The method is based on K-SDS precipitation of DNA–protein cross links using fluorescence (360 nm: excitation/ 450 nm: emission) to detect DNA strand breaks (Gangé et al. 1995), after staining with Hoescht dye. Results were expressed as µg DNA strand break mg⁻¹protein.

Lipid Peroxidation (LPO) was determined by the thiobarbituric acid reactive substances (TBARS) according Wills (1987). Trichloroacetic acid (TCA 10%), TBAR and 1mM FeSO₄ solution were added to samples homogeneized (gills and digestive gland) and incubated in water bath at 70°C for 10 min. A standard curve with tetramethoxypropane (TMP) (0, 0.6, 1.5, 3, 4, 6, 10 mM) was prepared. Fluorescence (530 nm: excitation/ 590 nm: emission) were measured and results were expressed as TBARS mg⁻¹ min⁻¹ proteins.

2.6 Statistical analyses

Normality and homogeneity of biomarker data were tested using Shapiro Wilk's and Bartlett's tests respectively. A Student ® t- test was performed between controls (water and solvent). Two-way ANOVA followed by Dunnett's test were used to identify differences between concentrations as well as differences between times of exposure. Significance level was set at p<0.05. Prism v.7a software was employed for ANOVA and post hoc analysis.

Principal Component Analyses (PCA) on biomarkers according to biochemical response patterns. The PCA was conducted grouped the results of gills and digestive glands after 168h exposure. Only variables whose coefficient was > 0.45 were considered components of the factors. The analyzes were performed in Statistica software ® version 10.

3. Results

3.1 Organisms

All organisms were measured, weighed and sexed. The average size recorded was 70.99 ± 4.88 mm and the average of weight was 25.22 ± 7.53 mm. The proportion of males and females remained at 1:1. Only 2 organisms died during the experiments.

3.2 Physical-chemical parameters

Temperature (T°C), salinity (sal), dissolved oxygen (DO) and pH were measured every day (Table 1). The average values obtained indicate that the parameters remained within the values considered adequate for a marine environment.

Table 1. Physical-chemical parameters of aquariums during experiment of exposure to COC

Physical-chemical parameters				
	pH	T°C	Sal	DO
Water control	7.67 ± 0.09	19.70 ± 0.65	34.67 ± 0.75	6.33 ± 0.16
Solvent control	7.72 ± 0.12	19.60 ± 0.61	34.67 ± 0.75	6.95 ± 0.81
200 ng.l⁻¹	7.69 ± 0.15	19.53 ± 0.62	34.67 ± 0.75	6.00 ± 0.32
2000 ng.l⁻¹	7.74 ± 0.12	19.58 ± 0.56	34.67 ± 0.75	6.92 ± 1.03

3.3 Biomarker responses

There was no statistically significant difference between water control and solvent control ($p > 0.05$), therefore further description of solvent control will be omitted.

3.3.1 Biochemical responses- gills

Biochemical responses in gills were presented in Fig. 1. EROD activity decreased significantly after 168h of exposure at 200 ng.l^{-1} and $2000 \text{ } \mu\text{g.l}^{-1}$ of COC ($p < 0.05$). Regarding DBFactivity, we observed a significantly decreased at 200 ng.l^{-1} after 96h and 168h of exposure. However, activity increased after 48h of exposure in organisms exposed to 2000 ng.l^{-1} of COC ($p < 0.05$).

GST activity was affected for all concentrations of COC. After 96h of exposure, GST significantly decreased compared to control in mussels exposed to 200 ng.l^{-1} and $2000 \text{ } \mu\text{g.l}^{-1}$ ($p < 0.05$). GPX activity decreased significantly in mussels exposed to 200 ng.l^{-1} of COC in all times of exposure ($p < 0.05$). After 96h, an increased activity was observed in organisms

exposed to 2000 ng.l⁻¹ of COC (p < 0.05).

Oxidative effects were evaluated through DNA strand break and LPO. Significant increase in DNA damage was observed in mussels exposed to 200 ng.l⁻¹ and 2000 ng.l⁻¹ of COC after 48h of exposure (p < 0.05). Regarding LPO, higher levels were observed after 168h in mussels exposed 200 ng.l⁻¹ of COC (p < 0.05).

3.3.2 Biochemical resposed- digestive gland

Mussels exposed to 200 ng.l⁻¹ of COC showed a significantly increased of EROD activity after 48h of exposure (p < 0.05). However, after 168h of exposure, EROD activity decreased significantly in all concentrations tested (p < 0.05). Regarding DBF activity, we observed a significantly decreased at 200 ng.l⁻¹ of COC in all times of exposure (p < 0.05).

GST activity was significantly affected only at 200 ng.l⁻¹ of COC when compared to control (p < 0.05). GPX activity increased significantly in mussels exposed to 200 ng.l⁻¹ of COC after 48h (p < 0.05).

DNA damage was significantly increased in mussels exposed to 200 ng.l⁻¹ of COC after 48h and 96h of exposure (p < 0.05). LPO levels were higher in mussels exposed to 200 ng.l⁻¹ of COC after 48h and 96h when compared to control (p < 0.05) (Fig.2).

3.3.3 Cytotoxicity (LMS)

Data of cytotoxicity are presented in Fig. 3. In this study, mussels were considered healthy if NRRT was ≥ 60 min; stressed but compensated if NRRT was between 60 min and 30 min and severely stressed if NRRT ≤ 30 min as described by Ortega et al. (2018). The LMS of *P. perna* was significantly affected by COC at all concentrations tested in all times of exposure regarding control (p < 0.05).

After 48h of exposure, organisms exposed to 200 ng.l⁻¹ of COC showed a decreased of retention time by 53.2 % (47.14 min) and at 2000 ng.l⁻¹. At 96h, mussels exposed to 200 ng.l⁻¹ showed a NRRT reduced by 51.5 % (34.29 min); at 2000 ng.l⁻¹ by 66.7% (23.57 min). Finally, after 168h of exposure, at 200 ng.l⁻¹ the retention time was reduced by 61.1% (27.50 min) and 2000 ng.l⁻¹ by 48.5% (36.43 min).

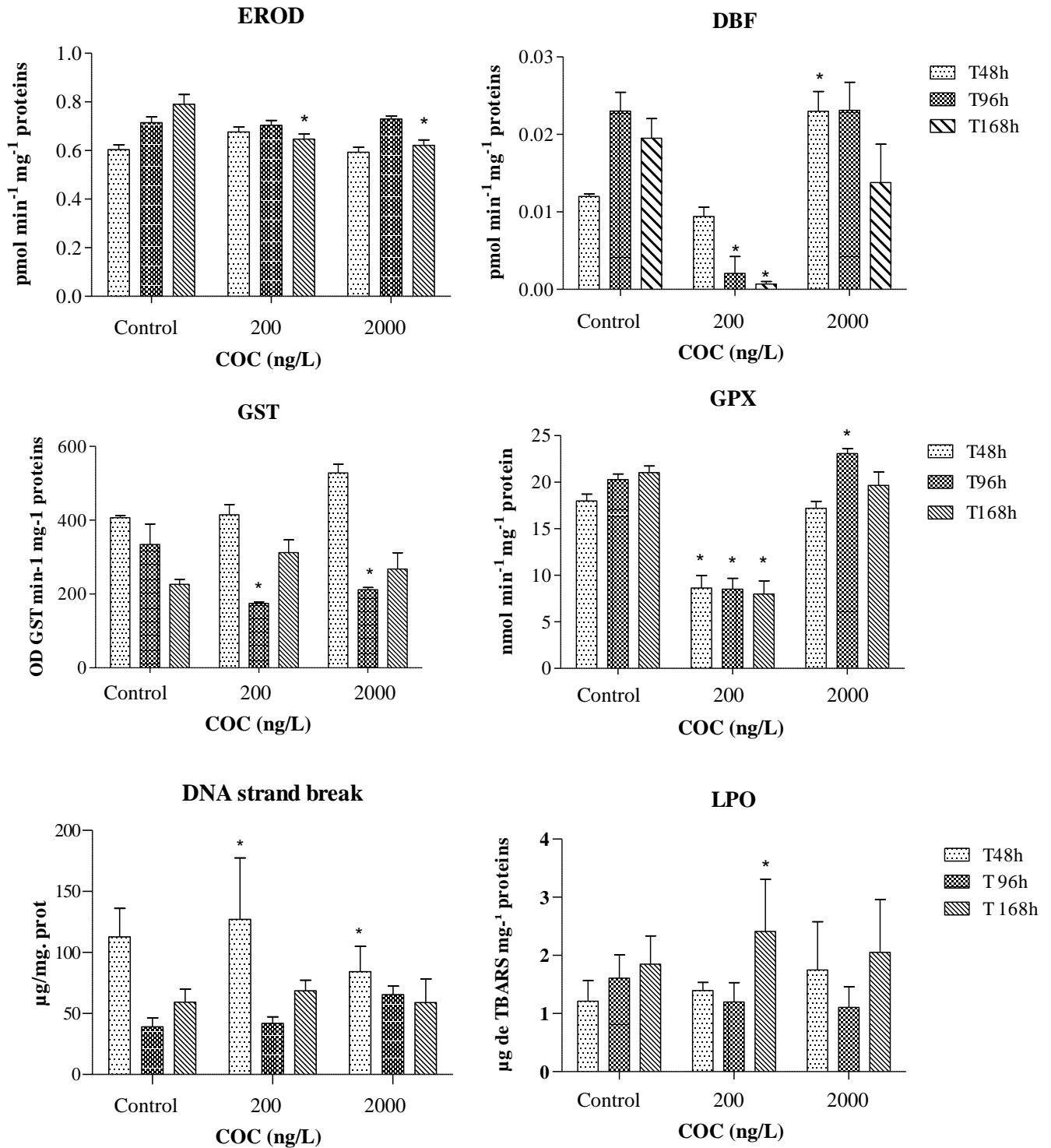


Fig 1. Mean (standard deviation) biomarker activity EROD, DBF, GST, GPX, DNA strand break and LPO in gills tissue of *Perna perna* exposed to COC (200 ng.l⁻¹ and 2000 ng.l⁻¹) and controls; *represents significant differences (ANOVA, Dunnett's test, p<0.05) between the controls and COC concentrations.

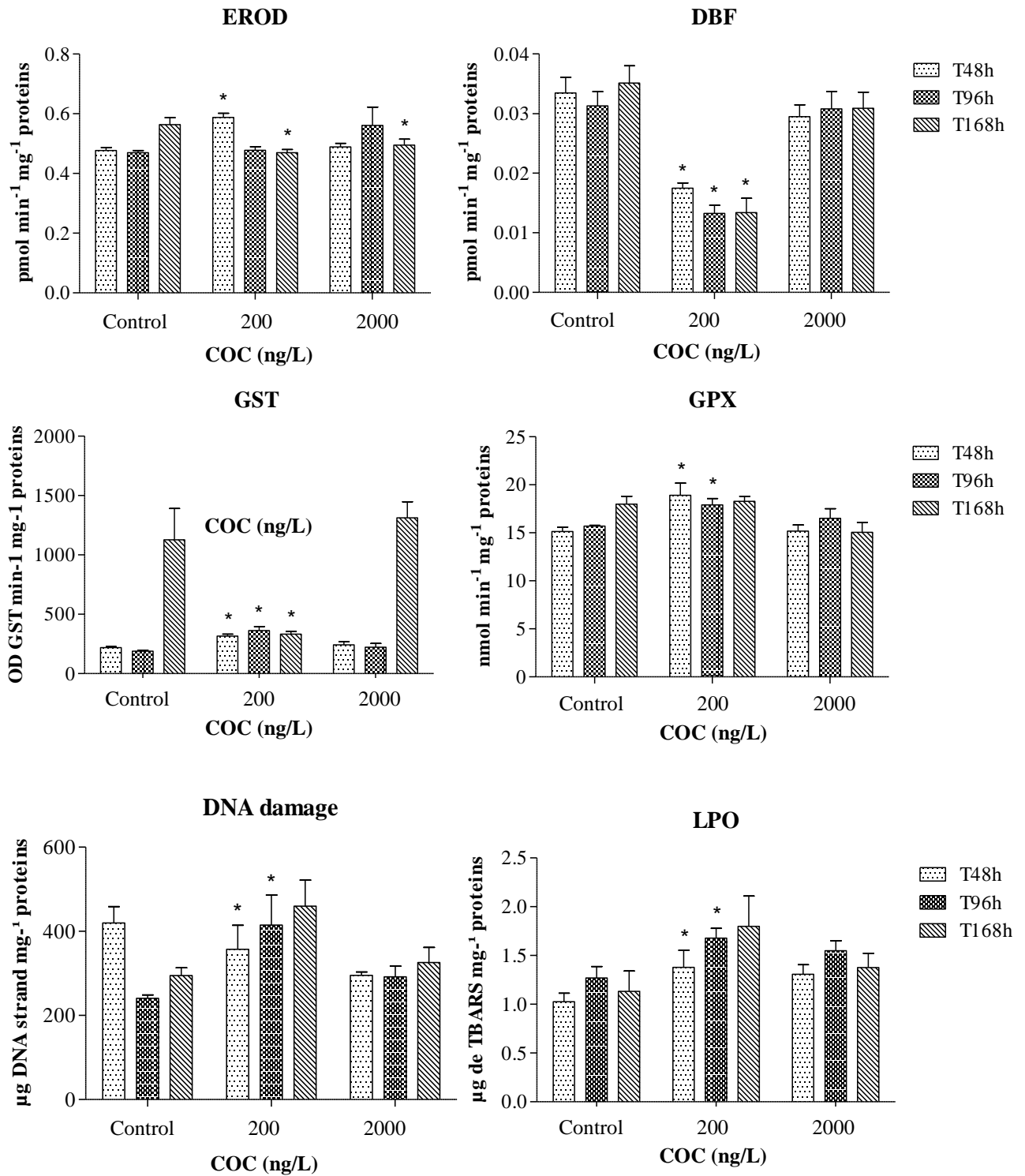


Fig 2. Mean (standard deviation) biomarker activity EROD, DBF, GST, GPX, DNA strand break and LPO) in digestive gland tissue of *Perna perna* exposed to COC (200 ng.l⁻¹ and 2000 ng. l⁻¹) and controls; *represents significant differences (ANOVA, Dunnett's test, p<0.05) between the controls and COC concentrations.

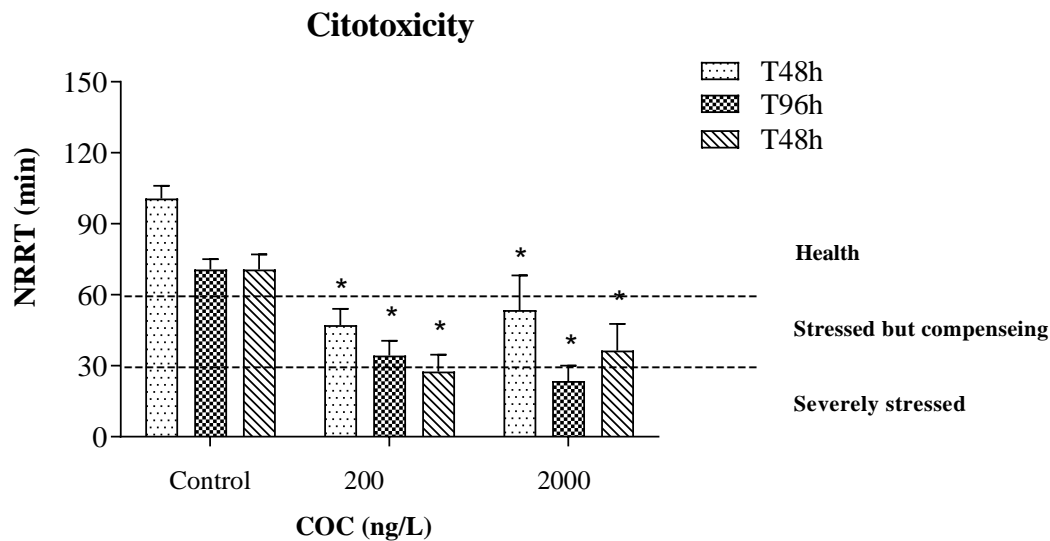


Fig 3. NRRT in hemocytes of *Perna perna* exposed to COC (ANOVA- Dunnet's test, $p < 0.05$). Asterisk indicate significance compared to control. Error bars indicate the standard errors.

We organized the different responses of biomarkers into an integrated biomarker response index (IBR) developed by Beliaeff and Burgeout (2002) and adapted by Guerlet et al. (2010). This index was also used by Serafim et al. (2012) and Maranhão et al. (2015) to assess the susceptibility of aquatic organisms to contaminants. The IBR was calculated to both tissues (gills and digestive gland) from mussels exposed to all concentrations of COC at the different exposure times. The results observed in the gills tissue suggest that after 48h, mussels exposed to 200 ng.l⁻¹ and 2000 ng.l⁻¹ presented important changes at EROD and GPx, followed by cellular responses represented by LMS. Similar situation was observed after 96h in both concentrations. At 168h, mussels exposed to 200 ng.l⁻¹ showed EROD and GPx activity; mussels exposed to 200 ng.l⁻¹ presented effects on LMS and GPx (Fig. 4). In the digestive gland (Fig. 5), we observed a relevant response to EROD and DBF activity, but also LPO and DNA damage to 200 ng.l⁻¹ and 2000 ng.l⁻¹. After 96h and 168h, in both concentrations, mussels presented EROD activity, followed by LPO and increase of damage on LMS (Fig. 5).

In order to get an overview of how COC affect the mussels, the biochemical responses in both tissues after 168h were grouped in a PCA (Fig. 6). The variables were described by two factors and the criteria selected to interpret variables associated with factor > 0.45. The factor 1 explained 80.4 % of the variance and the factor 2 explained 19.60 % of the variance (Table 2).

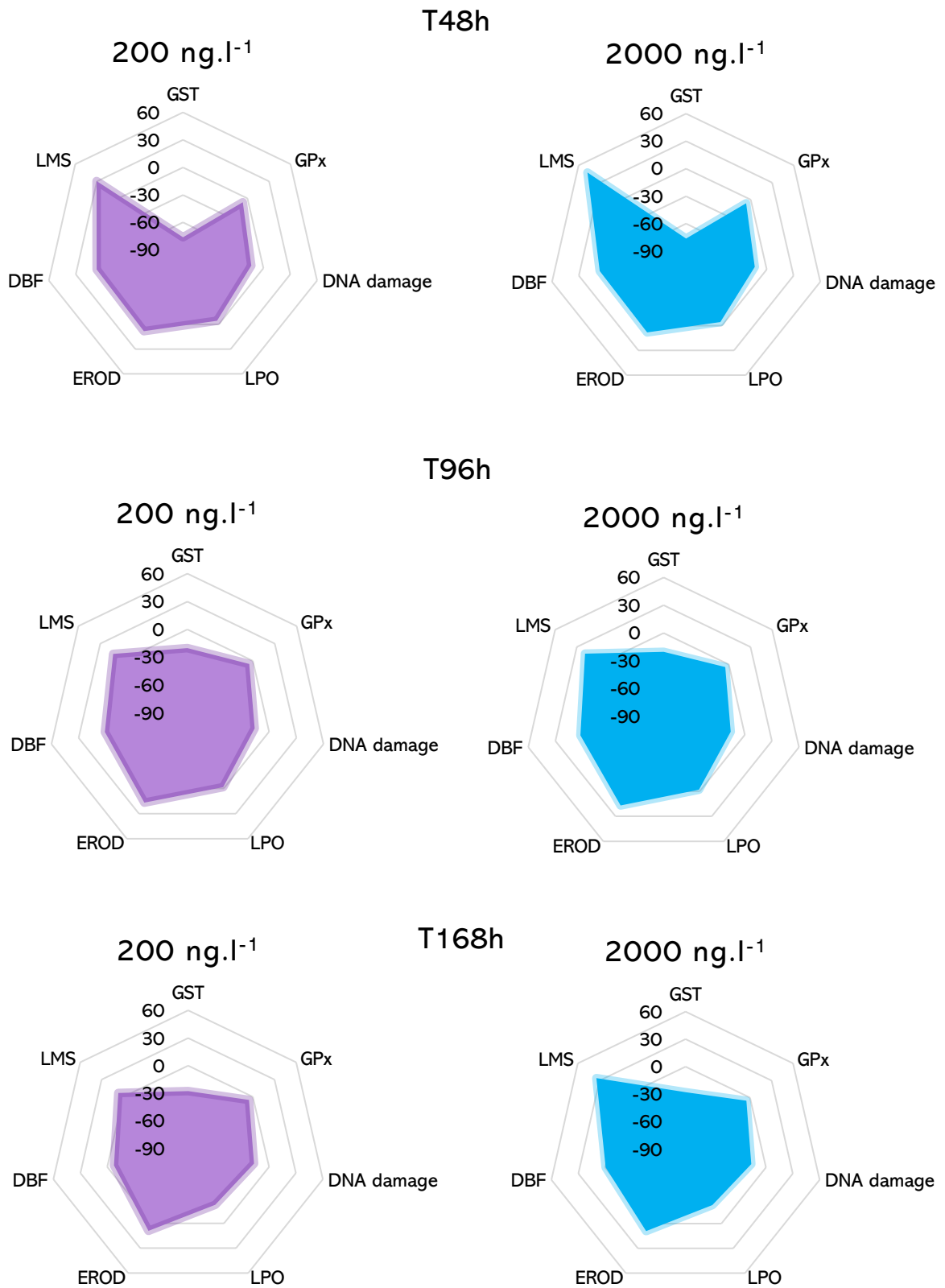


Fig 4. . Integrated biomarker response (IBR) and COC star plots in gills tissue. IBR index for biomarker response includes LMS, EROD, DBF, GST, GPx, DNA strand break and LPO.

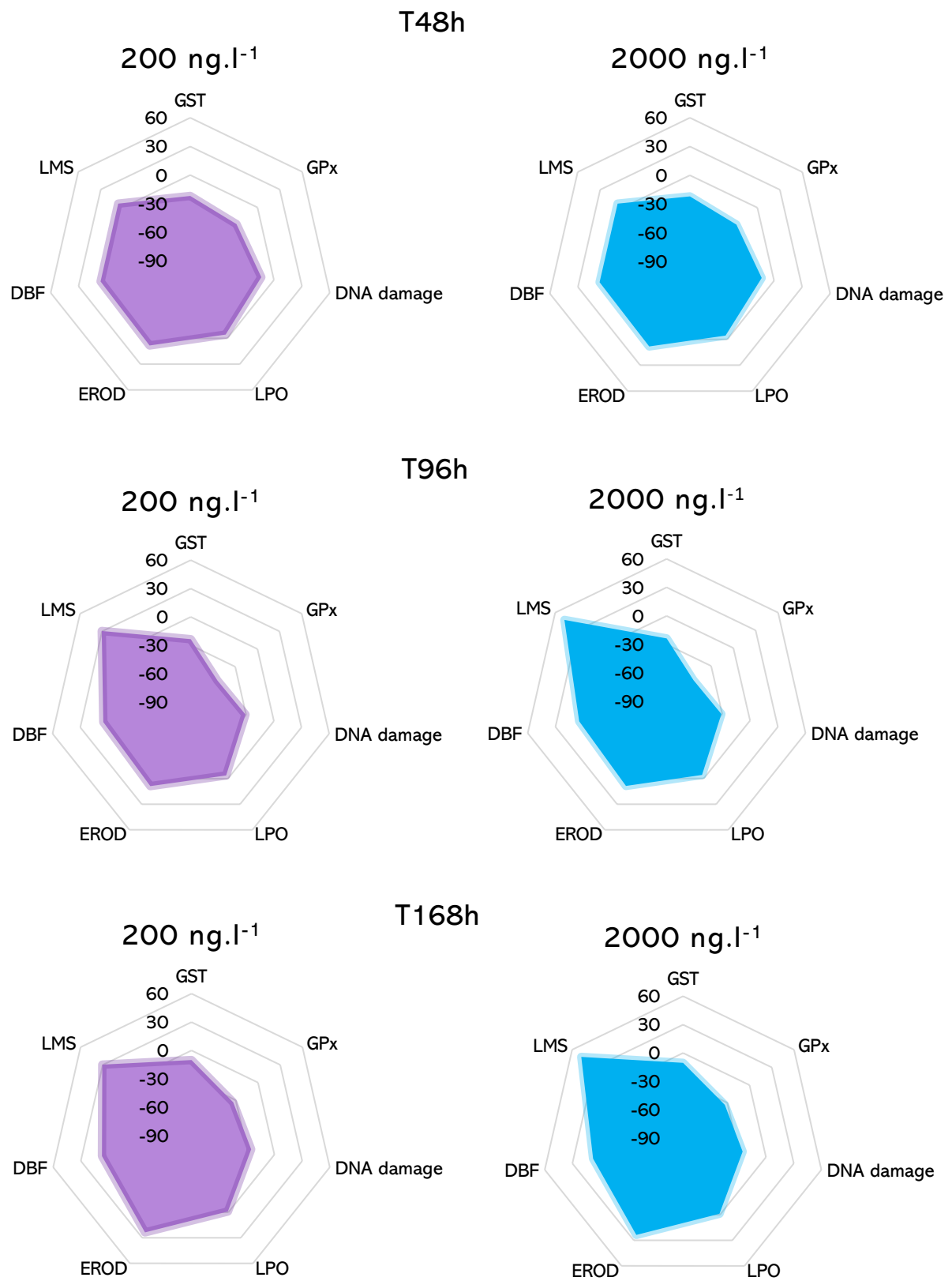


Fig 5 . Integrated biomarker response (IBR) and COC star plots in digestive gland tissue. IBR index for biomarker response includes LMS, EROD, DBF, GST, GPx, DNA strand break and LPO.

Table 2. Multivariable analysis of results obtained from biochemical and LMS responses on *Perna perna* mussel exposed to COC.

	Factor 1	Factor 2
GST	-0,954047	-0,299659
GPx	0,962434	0,271516
DNA damage	0,981027	0,193870
LPO	-0,409991	-0,912089
EROD	0,102181	0,994766
DBF	0,892525	0,450998
LMS	0,516194	0,856472
Expl; Var	4,040490	2,959510
Prp. Totl	0,577213	0,422787

Factor 1: combined the increase of oxidative effects (DNA damage), antioxidant system (GPx), induction of phase I (DBF) and lysosomal membrane stability (LMS). These data indicated activation of the biotransformation and antioxidant systems and genotoxicity. The decreased of LMS represent a decrease of health status.

Factor 2: associated the phase I (EROD and DBF) and LMS in the positive part of the axis. Oxidative stress biomarker (LPO) appeared on the negative part of the axis. The results also indicated activation of the biotransformation and a decreased of LMS, probably related to the increase in LPO.

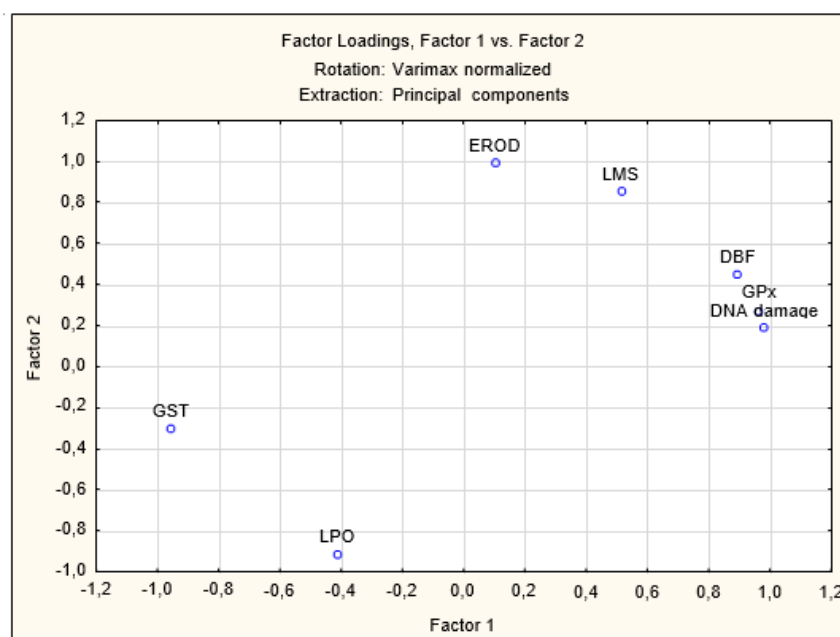


Fig. 6 Principal components analysis (PCA) based on biochemical and cellular endpoints for mussels exposed after 168h exposure. LPO, lipid peroxidation; GPx, glutathione peroxidase; GST, glutathione-S-transferases; EROD, 7-ethoxyresorufin O-deethylase; DBF, dibenzylfluorescein dealkylase; GST, glutathione S-transferase DBF, LMS, lysosomal membrane stability; DNA damage.

4. Discussion

Emerging contaminants such as illicit drugs are recognized as important threat to aquatic ecosystems worldwide (Parolini et al. 2016; Capaldo et al. 2019; 2021). Some ecotoxicological studies have been carried out, but little is known about ecological effects of COC in marine organisms from tropical ecosystems. In our study, mussels exposed to COC shown significantly changes in biochemical and cellular parameters.

Early disruption of organisms homeostasis due to contaminants can be detected by biochemical, cellular, molecular and physiological responses. When integrated, the biomarkers can clarify issues of contaminant bioavailability, bioaccumulation, and ecological effects (Hook et al. 2014).

In our study we observed that even low concentrations of COC can affect the LMS, causing a decrease in NRRT. Lysosomes are cytoplasmatic organelles with optimal activities at an acidic pH (4.5 – 5.0), maintained by active proton pumping due to the activity of a H⁺-ATPase (Martínez-Gómez et al. 2015). When exposed to a xenobiotic, the efficiency of the pump can be reduced, causing an increase in the pH inside the lysosomes, which become unable to retain the neutral red dye (Francioni, 2007). In this sense, lysosomal alterations have been used to identify environmental impacts on marine organisms (Moore et al. 2008). In our study, mussels exposed to COC (200 ng l⁻¹ and 2000 ng.l⁻¹) shown damage in LMS in all times of exposure, indicating that COC causes cytotoxicity in mussels exposed to environmentally relevant concentrations.

Our results corroborate with Binelli et al. (2012) that also observed a decrease of the stability of lysosomal membrane in mussels *Dreissena polymorpha* exposed to 40 ng.l⁻¹, 220 ng.l⁻¹ and 10 ng.l⁻¹ of COC, since 24h of exposure. Specimens of *P. perna* exhibited damage on lysosomal membrane when exposed to crack cocaine (5 ng.l⁻¹; 50 ng.l⁻¹; 500 ng.l⁻¹), a by-product of COC after 48h of exposure (Maranho et al. 2017). Similarly, Ortega et al. (2018) also recorded cytotoxicity induced by crack cocaine in *P. perna* exposed to crack cocaine (0.5 ng.l⁻¹; 5 ng.l⁻¹; 50 ng.l⁻¹) since 96h of exposure. When mussels are exposed to xenobiotics, one

of the characteristic pathological alterations is decreased integrity of the lysosomal membrane (Svendsen et al. 2004). The ability of COC to cause cytotoxicity in non-target organisms is a concern, because it implies the generation of oxidative stress in mussels. Reactive oxygen species (ROS) cause negative effects on lysosomal membranes and the intra-lysosomal environment is already an oxiradical production site (Livingston et al. 2001; Binelli et al. 2012)

Human drug metabolism is a biochemical process and involves different phases: oxidation, reduction and hydrolysis that are enzymatically mediated by cytochrome P450 (Plósz et al. 2013). In mussels, the biotransformation of COC is associated with EROD and DBF activity (1A like-proteins and 3A like proteins, respectively) (Gagné et al. 2007) and in our study, the activity of these enzymes was measured in the gills and digestive gland tissues. Gills and digestive gland are the mainly biotransformation sites of xenobiotics in mussels, since these tissues have important enzymes involved in the detoxification process (Aguirre-Martinez et al. 2013). In both of tissues, EROD decreased since environment concentration of COC (200 ng.l⁻¹), but DBF shown a different patter. While in the gills, DBF activity was strongly suppressed in environmental concentration, (but increased in the highest concentrations), in the digestive gland the activity was affected only by the environmental concentration, decreasing in relation to the control. Then, mussels exposed to COC can trigger a series of reactions that aim to protect the organism from the toxic effects of this substance. Our results corroborated with Ortega et al. (2018) who recorded changes in EROD and DBF activities gills of *P. perna* exposed to crack-cocaine, exhibiting increase of DBF activity (5 ng.l⁻¹; 50 ng.l⁻¹). Studies performed in mammal also demonstrated that COC may to activate the CYP450 (Kovacic 2005; Valente *et al.*, 2012).

Glutathione plays an important role in several cellular processes such as catalysis, metabolism, protein synthesis transport and metabolism of peroxides and free radicals (Forman et al. 2009; Morris et al. 2014). In our study, GST levels decreased in gills (all concentrations tested, since 96h of exposure) and digestive gland (200 ng.l⁻¹), but GPx was affected only in gills tissue (200 ng.l⁻¹ in all times of exposure). Ortega et al. (2018) demonstrated that mussels exposed to crack cocaine (5 µg. l⁻¹ after 48h of exposure) also shown changes in GST and GPx levels. Zebrafish embryos exposed to a mixture of illicit drugs containing 50 ng.l⁻¹ of COC exhibited an increase of GPx (Parolini et al. (2015). An increased of GST and GPx also were observed in embryos exposed to 0.4 nM, 4 nM and 40 nM of COC after 96h of exposure.

Studies performed in mammals shown that COC increased hydrogen peroxide and decreased endogenous antioxidant glutathione (GSH and GPx) due to production of ROS by acute and chronic exposure (Lipton et al. 2003; Dietrich et al. 2005; Muriach et al. 2010; Baiser

& Yaka, 2019). Also, COC is known to provoke an increase in hydrogen peroxides apoptosis, cell death and lipid peroxidation. In bivalves, GST represents an important conjugation enzyme, playing an important role in phase II of xenobiotic metabolism, while GPx acts in antioxidant defense, reducing organic and inorganic peroxides (Gagné et al. 2007; Manduzio et al. 2004). Our results suggested that COC changes the antioxidant defenses of mussels, making it difficult to perform phase II detoxification in metabolism due to impaired glutathione. Furthermore, the generation of ROS may also be responsible for the damage observed in the lysosomal membrane.

In addition to the damage of the antioxidant system, COC induced DNA damage and lipid peroxidation in both gills and digestive gland tissues. Data on the environmental hazards associated with these compounds are emerging, but are still scarce (Maranho and Pereira, 2017). Our results corroborated with Maranho et al. (2017) that observed DNA damage in mussels exposed to 500 ng.l⁻¹ of crack cocaine after 48h of exposure. Binelli et al. (2012) observed genotoxicity induced by COC (10 ng.l⁻¹) through micronuclei formation in mussels *Dreissena polymorpha*, indicating that exposure to COC caused chromosomal aberrations. Damage genetic in embryos from zebra mussel exposed to 0.4 nM, 4 nM and 40 nM of COC significant presented an increase in DNA fragmentation, confirming the capability to induce primary genetic lesions (Parolini et al. 2017).

The occurrence of damage is a concern, since changes in DNA may be accompanied by mutations, chromosomal changes and changes in the expression of certain proteins that are important in the processes of cellular respiration and metabolism (Boess et al. 2000; Parolini et al. 2017). Regarding LPO, our results corroborated with Parolini et al. (2013), where the cocaine metabolite (benzoylecgonine) induced LPO in mussels exposed to 1 ng.l⁻¹. In mammal, Öztezcan et al. (2000) observed that COC induced an increase in LPO of the liver of rats exposed to concentrations of 25 mg.kg leading to a picture of hepatotoxicity. LPO is also associated with the production of ROS and its occurrence is worrying, as it can lead to the direct cell injury, damaging proteins present in cell membranes (Kovacic, 2005).

Conclusion

Our study showed that cocaine (COC), at environmental concentrations, induced biochemical and cellular disturbs on marine mussel *Perna perna*. Such effects are associated to oxidative stress and cytogenotoxicity, which could culminate in long-term changes in growth, reproduction and survival of coastal population. The suite of biomarkers selected proved

adequate for monitoring exposure and effects of COC to marine organisms. These findings also show the importance of including illicit drugs in coastal water monitoring programs.

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Morphological and hormonal responses in ovaries of *Anguilla anguilla* exposed to environmental cocaine concentrations

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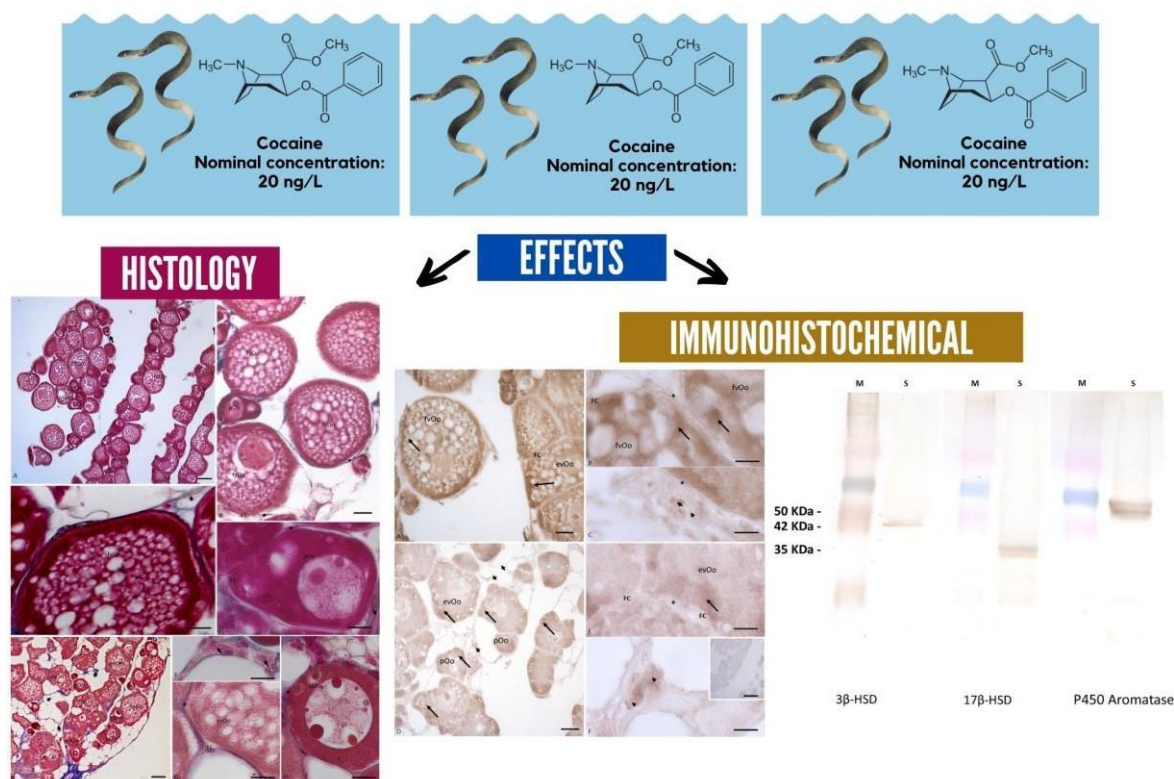
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Abstract

Cocaine is an illicit drug widely found in surface waters in concentrations ranging from $\mu\text{g.L}^{-1}$ to ng.L^{-1} . Cocaine in the environment presents a potential risk to aquatic organisms, but its ecological effects are still poorly understood. This study evaluated the influence of an environmentally relevant concentration of cocaine (20 ng.L^{-1}) on the morphology of European eel ovaries (*Anguilla anguilla*), the presence and distribution of enzymes involved in oogenesis, and cortisol, FSH, and LH levels. Animals exposed to cocaine had a smaller follicle area and higher percentage of connective tissue than controls ($p < 0.05$). Histological analysis found numerous fully vitellogenic and early vitellogenic oocytes in the control animals, while previtellogenic oocytes were frequently seen in the eels exposed to cocaine; moreover, the presence and location of 3β -hydroxysteroid dehydrogenase, 17β -hydroxysteroid dehydrogenase, and P450 aromatase differed in the two groups. Finally, cocaine exposure decreased FSH and LH levels while increasing cortisol levels. These findings show that even a low environmental concentration of cocaine affects the ovarian morphology and activity of *A. anguilla*, suggesting an impact on reproduction in this species.

Keywords: Cocaine, eel, ovary morphology, histopathology, gonadotropins, pollution effects.

Graphical abstract



1. Introduction

Illicit drugs are a class of emerging contaminants frequently detected in aquatic environments worldwide. The presence of these drugs and their metabolites in surface waters is considered a hazard for the aquatic environment, but their effects on aquatic organisms are poorly known (Capaldo et al. 2011). These substances are continuously discharged into aquatic environments and have been detected in municipal sewage treatment plants, surface and drinking water, seawater, marine sediment, and mussels (Berset et al. 2010; Pereira et al. 2016; Campestrini and Jardim et al. 2017; Fontes et al. 2019; 2021). The presence and occurrence of illicit drugs and their metabolites in aquatic environments are concerning due to their high biological activity, psychoactive properties, and possible effects on aquatic biota (Baker and Kasprzyk-Hordern et al. 2013; Capaldo et al. 2019).

Cocaine is the most widely used illicit stimulant drug in Africa, North America, Latin America, the Caribbean, and Europe, with an estimated 19 million users (UNODC, 2020). After consumption, cocaine is rapidly metabolized; 35–54% of the parent compound is hydrolyzed to benzoylecgonine (BE), 32–49% to ecgonine methyl ester (EME), 5% to norcocaine, and only 1–9% of the parent compound is excreted intact (Pal et al. 2013; Binelli et al. 2013).

Cocaine has been observed in aquatic ecosystems around the world in concentrations ranging from ng.l^{-1} to $\mu\text{g.l}^{-1}$ (Terzic et al., 2010; Castiglioni et al. 2011; Evgenidou et al. 2015; Pereira et al. 2016; Fontes et al. 2019; 2021). Once in the environment, cocaine may interact with non-target organisms and cause negative effects. Some *in vivo* studies conducted by Maranhão et al. (2017) and Ortega et al. (2018) reported that marine mussels (*Perna perna*) exposed to crack cocaine in concentrations ranging from 5 to 500 $\mu\text{g.l}^{-1}$ exhibited DNA damage and cytotoxicity after 48 h and 96 h of exposure, respectively. Crustaceans (*Orconectes rusticus*) injected with cocaine for 3 days (2 and 10 $\mu\text{g.l}^{-1}$) showed increased mobility and behavioral changes (Imeh-Nathaniel et al., 2017).

Fish play an important role as bioindicators of water pollution and demonstrate great sensitivity to changes in their normal behavioral and physiological functions such as cellular, molecular, biochemical, and hormonal responses (Plessi et al. 2017). Marine fish are cultivated extensively as important food sources with high economic value, and can be used as sentinel organisms to monitor marine pollutants such as illicit drugs (Saravanan et al., 2019).

The European eel (*Anguilla anguilla*) is a catadromous fish distributed throughout Europe and North Africa, and tolerates a wide range of environmental conditions (Bevacqua et al. 2015). After hatching in the Sargasso Sea, *A. anguilla* performs one of the longest migrations in the animal kingdom, traveling approximately 6000 km to the European continent. Several years later, mature eels migrate from fresh water back to the Sargasso Sea to complete their life cycle and die after spawning (Egg et al. 2017). European eels are highly valued for aquaculture, but the International Union for Conservation of Nature (IUCN) has classified them as critically endangered due to anthropogenic disturbances such as habitat loss and/or degradation, overfishing, environmental changes, and pollution (Guarniero et al. 2020), parasitic diseases, and overexploitation (Sjöberg et al. 2009; Geeraerts et al. 2010; Quadroni et al. 2012; Bevacqua et al. 2015; Belpaire et al. 2019). They are benthic and opportunistic predators that build up significant amounts of body fat, thus accumulating and biomagnifying lipophilic and persistent organic pollutants and other chemicals (Belpaire et al. 2007; Michel et al. 2016). This is concerning because cocaine can be accumulated in adipose tissue and muscles (Capaldo et al.

2012; 2018; 2019) but the fat stores are catabolized during fish migration releasing the cocaine and its metabolites into the bloodstream, contaminating and affecting the reproductive development of the gonads and reproduction itself (Belpaire, et al. 2019).

Biaccumulation of COC in soft tissues of *A. anguilla*, lead to serious injury in skeletal muscle, affecting the gill epithelium, and increasing plasma levels of cortisol and prolactin when eels were exposed to environmental concentrations of cocaine (20 ng.l^{-1}) for 30 days. But since the effects of this substance on reproduction are still unknown, the present study uses morphological, immunohistochemical, and biochemical analyses to investigate how an environmentally relevant concentration of cocaine (20 ng.l^{-1}) affects ovarian function in European eels. The following parameters were evaluated: general morphology of the ovary, including percentage of connective tissue and size and number of follicles; presence of the enzymes 3β -hydroxysteroid dehydrogenase (3β -HSD), 17β -hydroxysteroid dehydrogenase (17β -HSD) type 3, and P450 aromatase; and serum levels of cortisol, FSH, and LH.

2. Materials and methods

2.1. Animals

Forty-five female specimens of the European eel (*A. Anguilla*) in silver stage 52.0 ± 3.93 cm and 290.70 ± 36.40 g (mean \pm s.d.) were purchased from a fish dealer. Once in the laboratory, the eels were acclimatized for one month in 300 L glass aquariums in natural photoperiod. The water in the aquariums was dechlorinated and well-aerated, with the following characteristics: dissolved oxygen $8.0 \pm 0.7 \text{ mg L}^{-1}$, ammonia $< 0.1 \text{ mg L}^{-1}$, salinity 0, pH 7.4 ± 0.8 , and temperature $15^\circ\text{C} \pm 1^\circ\text{C}$. The water was refreshed every 24 h; because the eels were at silver stage and do not eat, they were not fed. This study was carried out in accordance with EU Directive 2010/63/EU for animal experimentation and institutional guidelines for care and use of laboratory animals, and was authorized by the Italian Ministry of Health's General Directorate of Animal Health and Veterinary Drugs.

2.2. Experimental design

For this fifty-day experiment, a stock solution of 0.006 mg mL^{-1} free base cocaine ($\geq 97\%$ purity, Sigma-Aldrich Inc., St. Louis, MO, USA) in ethanol was prepared. The eels were

randomly divided into three groups (untreated controls, vehicle, and cocaine) of 15 animals each. The experiment was carried out in triplicate, therefore the specimens in each experimental group were divided into three aquaria containing 5 specimens each. The eels in the cocaine treatment group were exposed to a nominal dose of 20 ng l⁻¹ cocaine (1 mL of stock solution, added to the aquariums after water changes every 24 h). Because nearly 90% of cocaine is degraded in 24 h at room temperature in water (Gheorghe et al., 2008), the water removed from the aquariums containing the experimental groups was stored in special containers for three days before being discharged as wastewater. At the same time, three control groups of five eels were exposed to tap water only, and three vehicle groups of five eels were exposed to ethanol at the same concentration as the experimental groups. All groups were kept in 300 L glass aquariums under the conditions described above, and water was changed every 24 h.

The nominal dose of cocaine (20 ng l⁻¹) was selected since this is the average concentration reported for surface water in Italy, China, and Brazil (Zuccato et al. 2008; Li et al; Fontes et al. 2019). After the exposure period, the eels were anesthetized using MS-222 (ethyl 3-aminobenzoate, methanesulfonic acid salt 98%, Aldrich Chemical Corporation Inc., Milwaukee, WI, USA) at a concentration of 100 mg L⁻¹, weighed, measured, and killed by decapitation. Blood was taken from the posterior cardinal vein with a 5 ml syringe to assess hormone levels; after coagulation in Eppendorf tubes for 2–4 h, the blood was centrifuged for 15 min at 2000 g and the serum collected and stored at –22 °C until the hormone assay was performed. Ovaries were removed from each animal and processed for light microscopy or immunoblot.

2.3. *Histological analysis*

The general morphology of the ovary was evaluated by fixing the ovaries in Bouin's solution, dehydrating in graded alcohols, clearing in Histolemon, and embedding in Paraplast. Serial 6- μ m sections were processed for routine histological analysis and stained with Mallory trichrome stain. Histomorphometric analysis of the ovaries was also performed. Five slides were selected for each ovary; three sections were randomly chosen from each slide and the area occupied by the connective tissue and number and area of the ovarian follicles were evaluated. All morphological observations and measurements were performed using a Zeiss Axioskop microscope (Carl Zeiss MicroImaging s.p.a., Milan, Italy). The images were captured using a camera connected to an IBM computer, with a Kontron Elektronik KS 300 image analysis system

(Carl Zeiss MicroImaging s.p.a., Milan, Italy) and Adobe Photoshop (Di Lorenzo et al., 2021) software.

2.4. Immunohistochemical analysis

2.4.1 Immunoblot

First, antibody specificity was tested via immunoblot analysis. The *Anguilla anguilla* ovaries were homogenized and lysed for 30 min on ice using RIPA lysis buffer containing a mixture of phosphatase and protease inhibitors (Santa Cruz Biotechnology, Milan-Italy). The homogenate was centrifuged at 14,000 g for 10 min at 4° C, and total proteins were measured by Bradford assay (Bio-Rad, Melville, NY) (Rosati et al., 2019a). Briefly, 30 mg of proteins were boiled for 5 min in SDS buffer (50 mM Tris-HCl [pH 6.8], 2 g 100 ml 1 SDS, 10% [v/v] glycerol, 0.1 g 100 ml 1 Bromophenol blue], separated on 12% SDS-PAGE and transferred to a PVDF membrane for blotting (Trans-Blot1 Semi-Dry Transfer Cell, Bio-Rad), as previously reported (Forte et al., 2019; Zizza et al., 2018). The membranes were then incubated for 1 h at room temperature with a blocking buffer (TBS, 0.05% Tween-20 and 5% BSA). Next, the membranes were incubated overnight in the presence of the primary antibody diluted in TBS-T containing 3% BSA at 4° C: 1) rabbit anti-mouse 3 β -HSD (Abcam) diluted 1:1000; 2) rabbit anti-human 17 β -HSD (type 3, isoform directly involved in the testosterone synthesis) (Abcam) diluted 1:500; 3) and rabbit anti-P450 aromatase (Elabscience Biotechnology Inc.) diluted 1:2000.

The membranes were washed four times for 10 min in TBS, 0.05% Tween-20 before a 1 h incubation with goat anti-rabbit IgG (HRP) (1:2000; Abcam ab-6721) secondary antibody diluted in TBS-T containing 2% BSA. The membranes were washed another four times in TBS and specific protein bands were detected using diaminobenzidine (DAB, Sigma-Aldrich, Milano, Italy) as a chromogen. Negative controls were performed by omitting the primary antibodies.

2.4.2 Immunohistochemistry

For immunohistochemical analysis, 5- μ m thick sections placed on poly-L-lysine slides (Menzel-Glaser, Braunschweig, Germany) were dewaxed, rehydrated in a graded series of alcohol, and heat-treated (microwave) for 20 min in 10 mM citrate (pH 6.0) antigen-retrieval buffer. The slides were then washed in PBS 1X, treated with 2.5% H₂O₂ for 40 min to reduce endogenous peroxidase activity, and blocked for 1 h at room temperature with normal goat serum

(Pierce, Rockford, IL, USA) to reduce non-specific background, as described elsewhere (Rosati et al., 2017, 2020). Sections were incubated overnight at 4°C with the following three primary antibodies: rabbit anti-mouse 3 β -HSD (1:750, Abcam); rabbit anti-human 17 β -HSD (type 3 isoform, directly involved in testosterone synthesis; 1:350, Abcam); rabbit anti-P450 aromatase (1:150, Elabscience Biotechnology Inc.). The following day, sections were washed in PBS and incubated with HRP conjugated goat anti-rabbit/mouse secondary antibody diluted 1:2000 in normal goat serum for 1 h at room temperature. Finally, sections were stained using diaminobenzidine (DAB) as a chromogen, and counterstained with Meyer's hematoxylin. For the negative controls, the primary antibody was omitted from incubation.

2.5. Hormone determination

This study determined the levels of cortisol, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) with an enzyme-linked immunosorbent assay (ELISA, DIAMETRA), as described previously (Manzo et al., 1994; Rosati et al., 2019a). The detection limit for cortisol sensitivity was 2.44 ng/mL, with an analytical range of 10–500 ng/mL and incubation time of 60 \pm 15 minutes. The detection limit for FSH sensitivity was 0.17 IU/mL, with an analytical range of 5–100 IU/mL and incubation time of 60 \pm 15 minutes. The detection limit for LH sensitivity was 0.22 IU/mL, with an analytical range of 5.0–200 IU/mL and incubation time of 60 \pm 15 minutes.

2.6. Statistical analysis

The quantitative data were subjected to statistical analysis, and values were expressed as means \pm SD. Normality was confirmed for all data, along with homogeneity of variance using the Bartlett test. The data were compared using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test. Commercial software (Sigma Stat Version 4.0; SPSS) was used to perform all statistical analyses, and $p < 0.05$ was considered significant.

3. Results

3.1 General morphology

No differences were observed between the vehicle and control specimens, but the ovaries were organized differently in the animals exposed to cocaine. *A. anguilla* is characterized by asynchronous oocyte development; the ovaries contain oocytes in the first growth phase characterized by absent or single yolk vesicles (previtellogenic oocytes, pvOo), oocytes in which vitellogenesis has begun with yolk vesicle accumulation in one part of the cytoplasm and a nucleus visible at the center (early vitellogenic oocytes, evOo), and large oocytes in which the yolk vesicles have accumulated throughout the cytoplasm covering the nucleus (fully vitellogenic oocytes, fvOo). All oocytes are present in a follicular structure composed of follicle cells and surrounded by connective cells such as theca cells, which have an endocrine function (Fig. 1). The control specimens contained several fvOo and evOo, and little connective tissue between the cells (Figs. 1 A-D). The ovaries from the animals exposed to cocaine contained more pvOo, with few fvOo and evOo (Figs. 1 E-H).

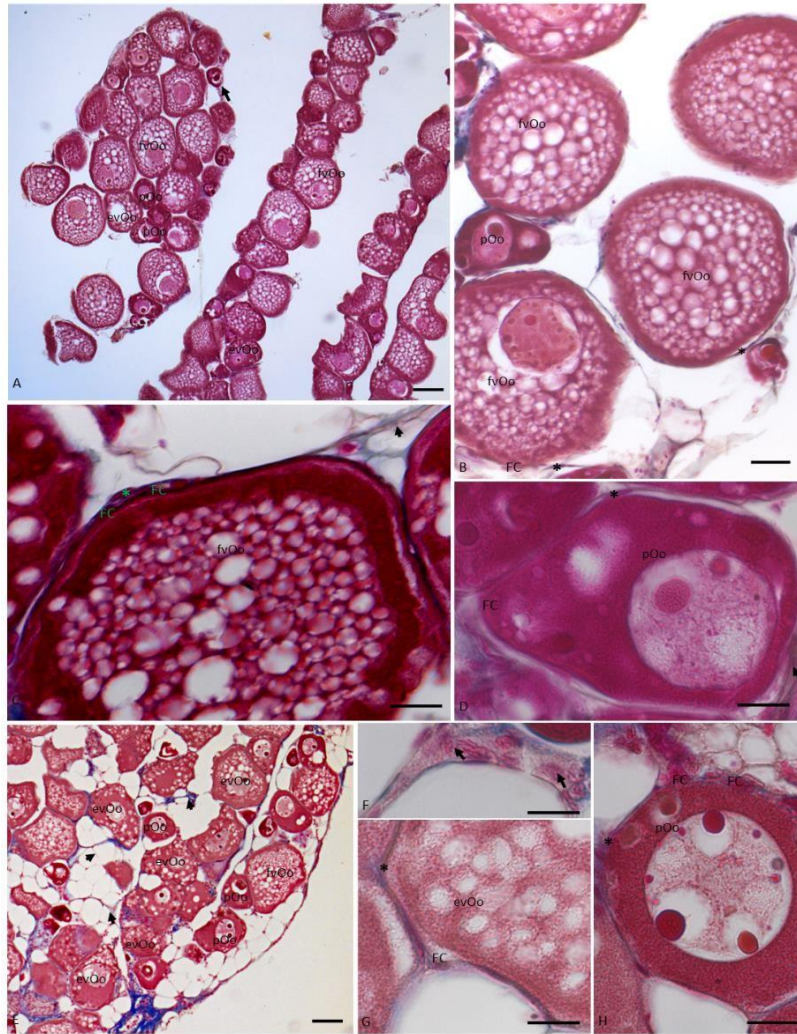


Fig. 1. Light micrographs of *Anguilla anguilla* ovaries with Mallory staining. (A-D): control specimens. The ovary shows numerous fully vitellogenic oocytes (fvOo) and early vitellogenic oocytes (evOo), along with few previtellogenic oocytes (pvOo) and connective interfollicular cells (arrowheads). (E-H): Ovary tissue from animals exposed to cocaine, containing more connective tissue (arrows) and previtellogenic oocytes (pvOo) than the control specimens. (FC): follicle cells; (asterisk): theca cells. Scale bars: A, E=100 μ m; B=50 μ m; C, D, F, G, H=5 μ m.

The morphological observations agreed with the results of the histomorphometric analysis; connective tissue occupied less area in the control animals than in the animals exposed to cocaine ($p < 0.05$) (Fig. 2A). Moreover, the follicles were smaller in the exposed animals than the control specimens ($p < 0.05$) (Fig. 2B), while the number of follicles was nearly identical in both the control and exposure groups ($p > 0.05$), although the eels exposed to cocaine had slightly more (Fig. 2C).

3.2. Immunoblot

Western blot assay performed on protein extracts from *A. anguilla* showed that the antibodies against 3 β -HSD, 17 β -HSD, and P450 aromatase reacted with the eels' ovarian proteins. One band of 42 kDa was observed to be positive to rabbit anti-3 β -HSD antibody, one band of 35 kDa positive to rabbit anti-17 β -HSD antibody, and one band of 50 kDa positive to rabbit anti-P450 aromatase antibody (Fig. 3). The bands corresponded to the molecular weight of these enzymes, demonstrating the validity of using these antibodies in the ovarian follicles of *A. anguilla* (Fig. 3).

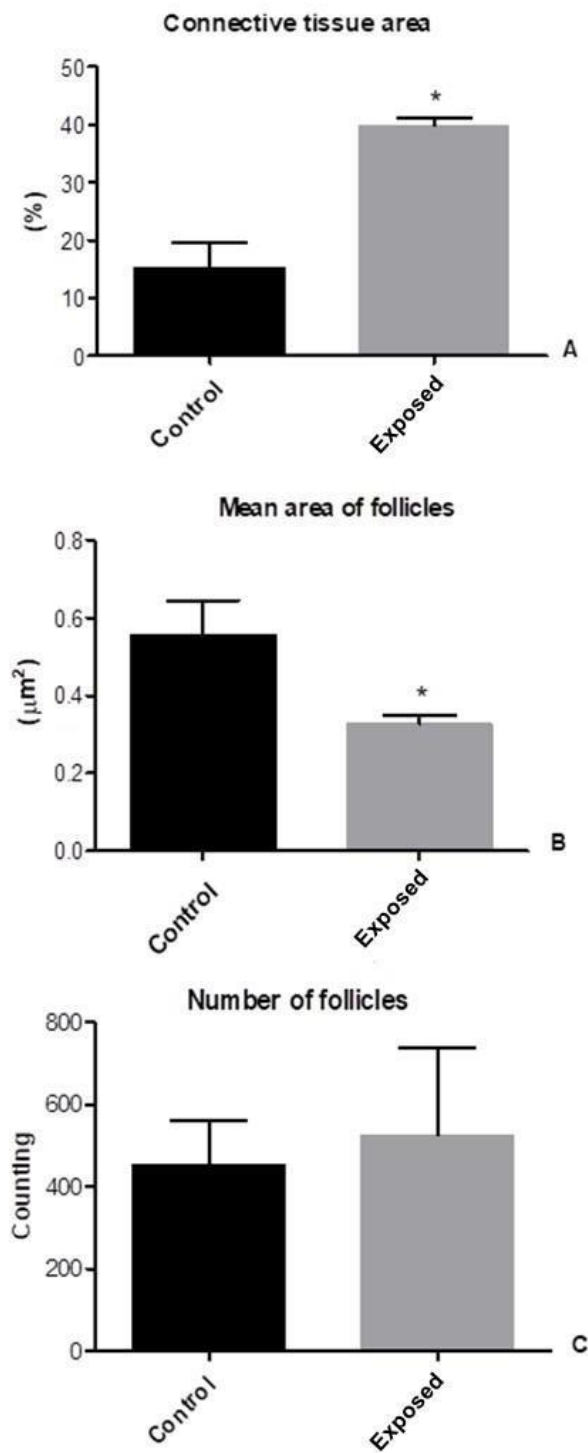


Fig. 2. Histomorphometric analysis of *Anguilla anguilla* ovaries. (A) Percentage of connective tissue relative to gonad area; connective tissue occupied more area in the gonads from the eels exposed to cocaine compared to the control animals. (B) Mean area of follicles; follicles were smaller in the gonads from the exposed group than in the controls. (C) Number of follicles present within the gonads; no difference in the number of follicles was found between the exposed and control specimens. (*) Significantly different ($p < 0.05$) from control values.

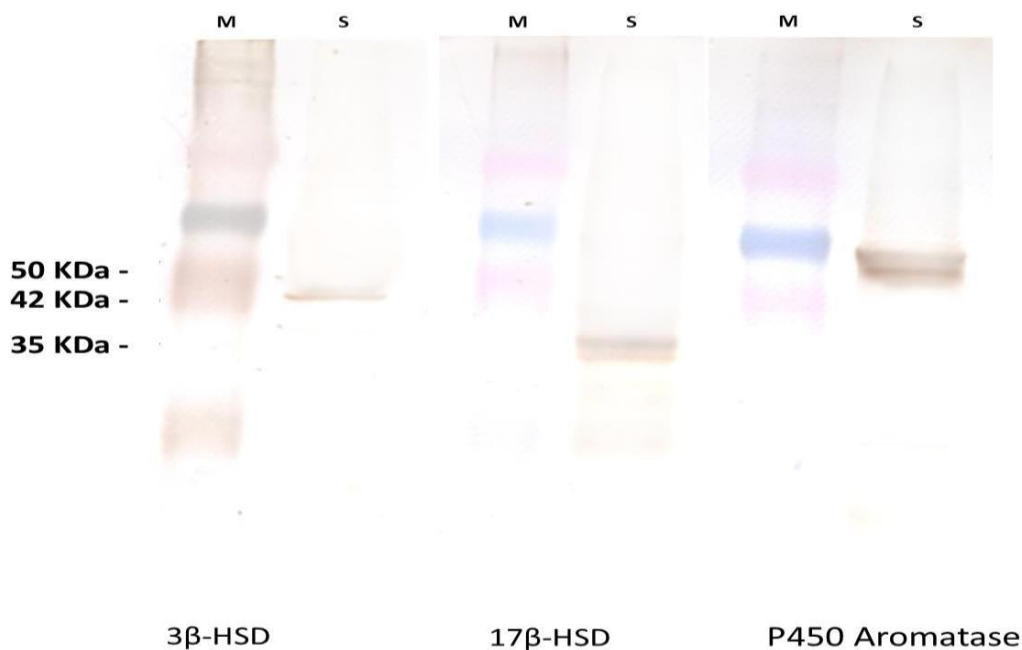


Fig. 3. Immunoblots on *Anguilla anguilla* proteins. The 17 β -HSD antibody reacted with a protein band of ~35 kDa, the 3 β -HSD antibody with a protein band of ~42 kDa, and the P450 aromatase with a band of ~50 kDa. S: sample; M: protein marker ladder.

3.3. 3 β -HSD localization

Distribution of the immunohistochemistry signal for 3 β -HSD, aromatase, and 17 β -HSD was similar in the ovarian follicles of the control specimens; a strong and diffuse signal was observed within evOo and fvOo, in the cytoplasm as well as at the edge of the cells. A weak signal was also observed in the follicular and theca cells and in the connective tissue (Figs. 4 A-C). Expression of 3 β -HSD in the ovarian follicles of the exposed specimens was less evident compared to the other enzymes, and was also seen on the edge of pvOo and evOo; a weak signal was observed in the connective tissue (Figs. 4 D-F). No signal was evident in the follicular and theca cells (Figs 4 D-F), and no positive reaction was detected in the control section (Fig. 4 F inset).

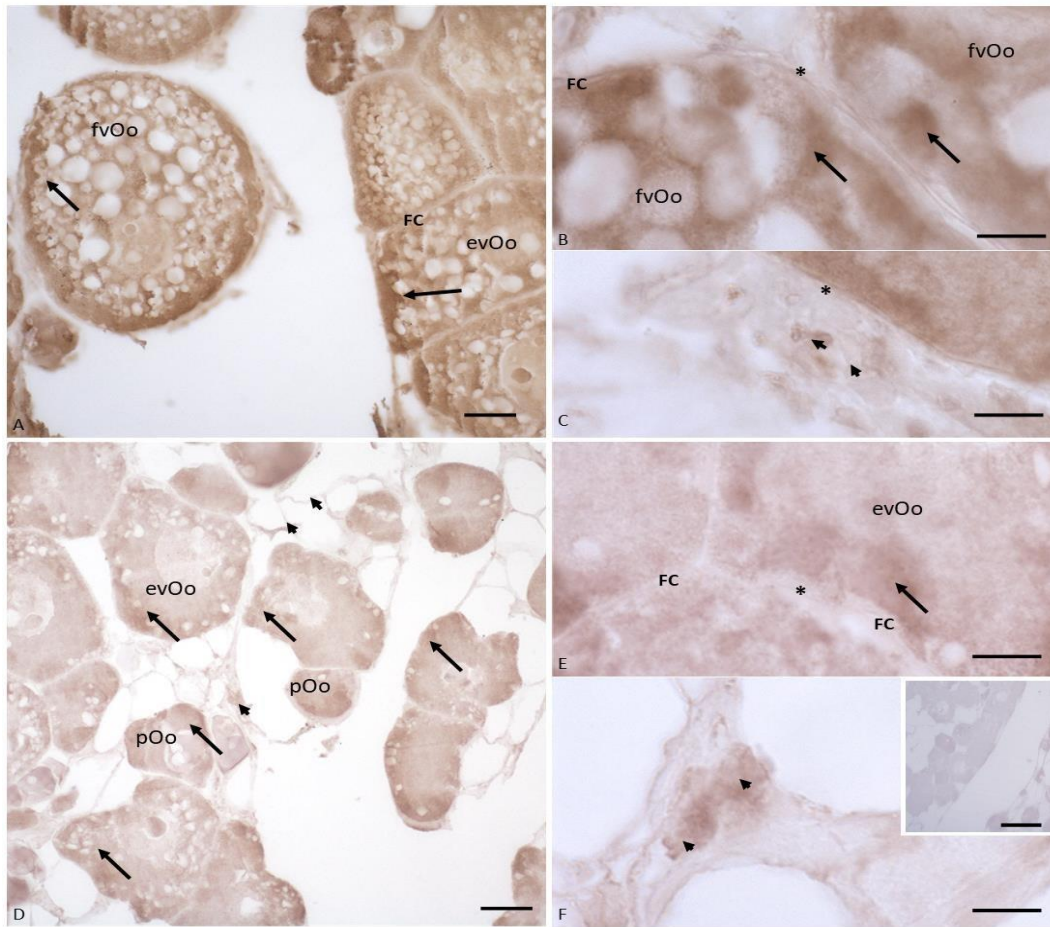


Fig. 4: Light micrographs of *Anguilla anguilla* ovary specimens from control animals (A, B, C) and eels exposed to cocaine (D, E, F) immunolabeled with anti-3 β -HSD antibody. (A, B, C) A wide and strong positive reaction (arrow) was visible at the level of the fully vitellogenic oocytes (fvOo) and early vitellogenic oocytes (evOo); a weak signal was evident in the follicular (FC) and theca cells (asterisk) as well as in the connective tissue (arrowheads). (D, E, F) Previtellogenic oocytes (pvOo) and early vitellogenic oocytes (evOo) were immunolabeled. In these cells, the immunohistochemical signal was located in the cytoplasm as spots (arrow). A weak signal was evident in the connective cells (arrowheads). No positivity was found in the follicular (FC) or theca (asterisk) cells. (F) (insert) Negative control: no labeling was evident in the ovary. Bars: A=20 μ m; B, C, E, F=5 μ m, D=50 μ m, F inset=100 μ m.

3.4. 17 β -HSD localization

Immunohistochemistry also revealed a strong signal for 17 β -HSD in the control specimens: oocytes were positive to anti-17 β -HSD antibody, with a strong signal in the fvOo and evOo, whereas a faint signal was registered in the follicular and theca cells as well as in the connective tissue (Figs. 5 A-C). In contrast, the oocytes from the exposed animals exhibited a less diffuse signal, mainly in the pvOo, evOo, follicular cells, and connective tissue. No signal

was found in the theca cells (Figs 5 D-G), nor was any positive reaction detected in the control section (Fig.5E inset).

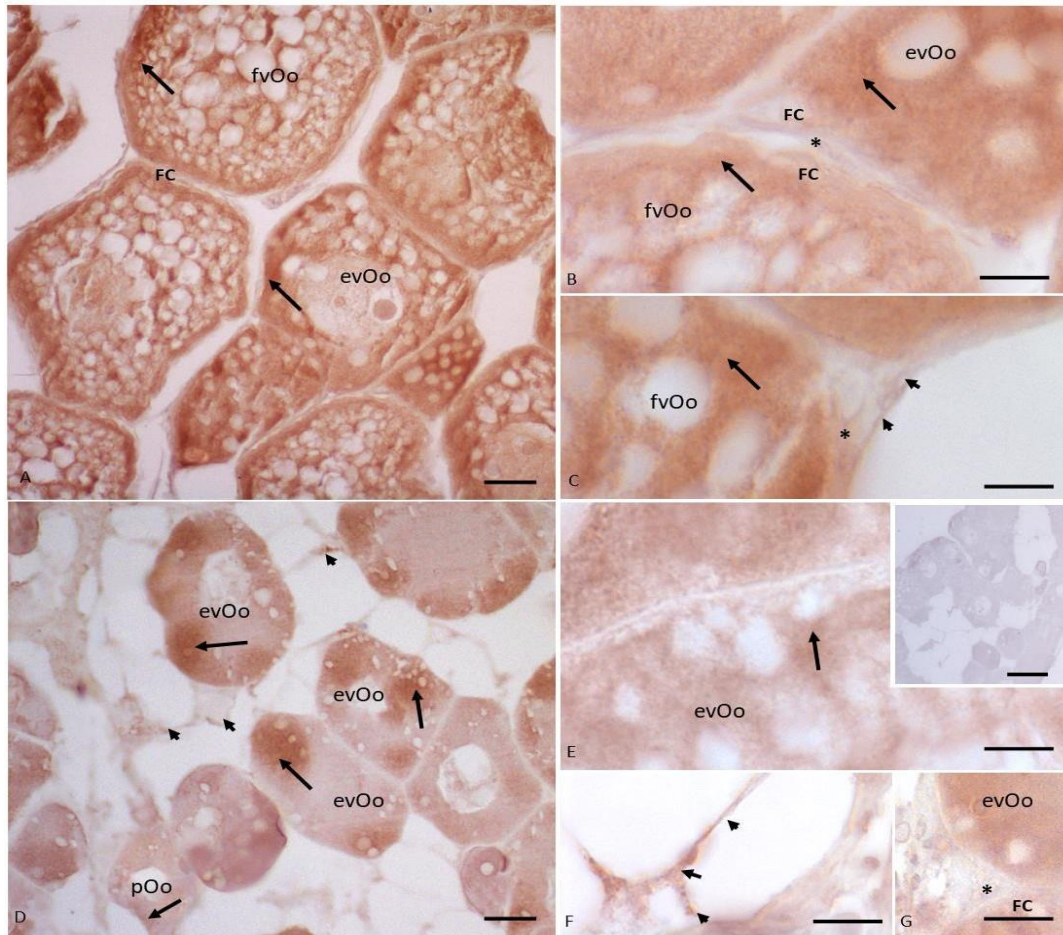


Fig. 5: Light micrographs of *Anguilla anguilla* ovaries. Control (A, B, C) and exposed specimens (D, E, F) immunolabeled with anti-17 β -HSD antibody (A, B, C). A wide and strong positive reaction was present in the fully vitellogenic oocytes (fvOo) and early vitellogenic oocytes (evOo); a weak signal was evident in the follicular (FC) and theca (asterisk) cells as well as in the connective cells (arrowheads). (D, E, F) Early vitellogenic oocytes (evOo) were immunolabeled and the signal was located in some places of the cytoplasm as spots (arrow); meanwhile, weak positivity (arrow) was localized within previtellogenic oocytes (pvOo), follicular (FC), and connective cells (arrowheads). No signal was evident in the theca cells (asterisk). (E) (inset) Negative control: no labeling was evident in the ovary. Bars: A=20 μ m; B, C, E, F, G=5 μ m, D=50 μ m, E inset=100 μ m.

3.5. P450 aromatase localization

Immunohistochemistry analysis showed the presence of the enzyme P450 aromatase on the ovarian follicles from *A. anguilla*. The enzyme showed a strong and widely distributed

signal in the control specimens, specifically in the cytoplasm of both fvOo and evOo and in the follicular, theca, and connective cells (Figs 6 A-C). Similarly, in the exposed animals the signal of P450 aromatase was visible in the pvOo and evOo as well as the theca and connective cells, but not in the follicular cells (Fig. 6 E-G). Figure 8F (inset) demonstrates the lack of signal for P450 aromatase in the negative control section.

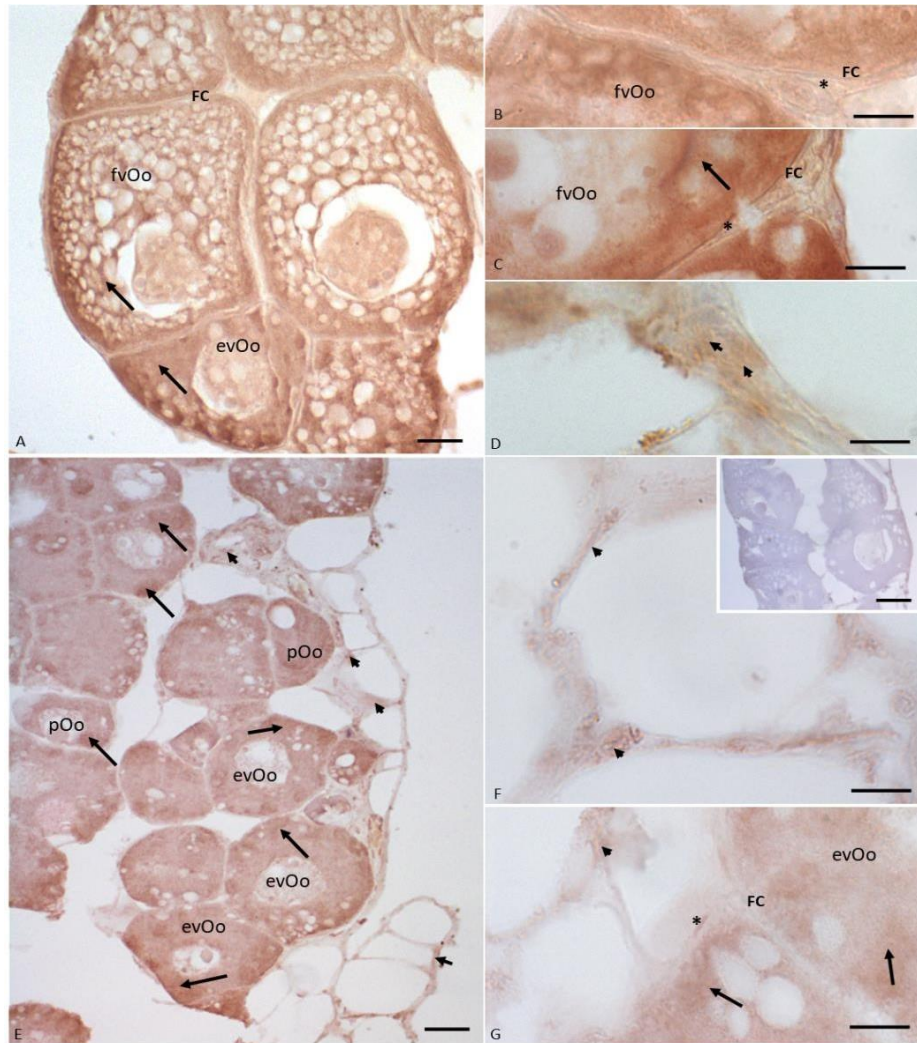


Fig.6. Light micrographs of *Anguilla anguilla* ovaries from control (A, B, C) and exposed specimens (D, E, F), immunolabeled with anti-P450 aromatase antibody. (A, B, C) A wide and strong positive reaction was present in the fully vitellogenic oocytes (fvOo), early vitellogenic oocytes (evOo), as well as in the follicular (FC), theca (asterisk), and connective cells (arrowheads). (D, E, F) Previtellogenic (pvOo) and early vitellogenic oocytes (evOo) were immunolabeled and the signal located in some areas of the cytoplasm as spots (arrow); positivity was also found in the theca (asterisk) and connective cells (arrowheads), while no signal was seen in the follicular cells (FC). (F) (inset) Negative control: no labeling was evident in the ovary. Bars: A=20 μ m; B, C, D, F, G=5 μ m, E=50 μ m, F inset=100 μ m

3.6. Serum cortisol, FSH and LH concentrations

Figure 7 presents the serum levels of cortisol, FSH, and LH. Higher cortisol concentrations and lower FSH and LH concentrations were found in the animals exposed to cocaine compared to the control specimens.

4. Discussion

Teleost fish are one of the main groups of animals used to monitor pollution in marine environments. The negative effects of cocaine on European eels have already been reported (Capaldo et al. 2011; 2018; 2021; Gay et al. 2013; 2016), but this present study provides the first evidence that an environmentally relevant concentration of cocaine induces histological and immunohistochemical changes in the gonads of the European eel, supporting our hypothesis that cocaine may impair reproduction in this species through changes in oocyte morphology, expression of crucial enzymes, and altered serum levels of cortisol and gonadotropins.

Our histological analyses showed that oocytes from the eels exposed to cocaine exhibited lower maturation than those taken from control specimens. In fact, greater mean follicle area, lower percentage of connective tissue, and more frequent presence of evOo (oocytes with small yolk vesicles restricted to the cell cortex) and fvOo (oocytes with enlarged and more abundant yolk vesicles) were observed in the control animals, while more pvOo (few lipid droplets without yolk vesicles) and connective tissue were found in the exposed animals.

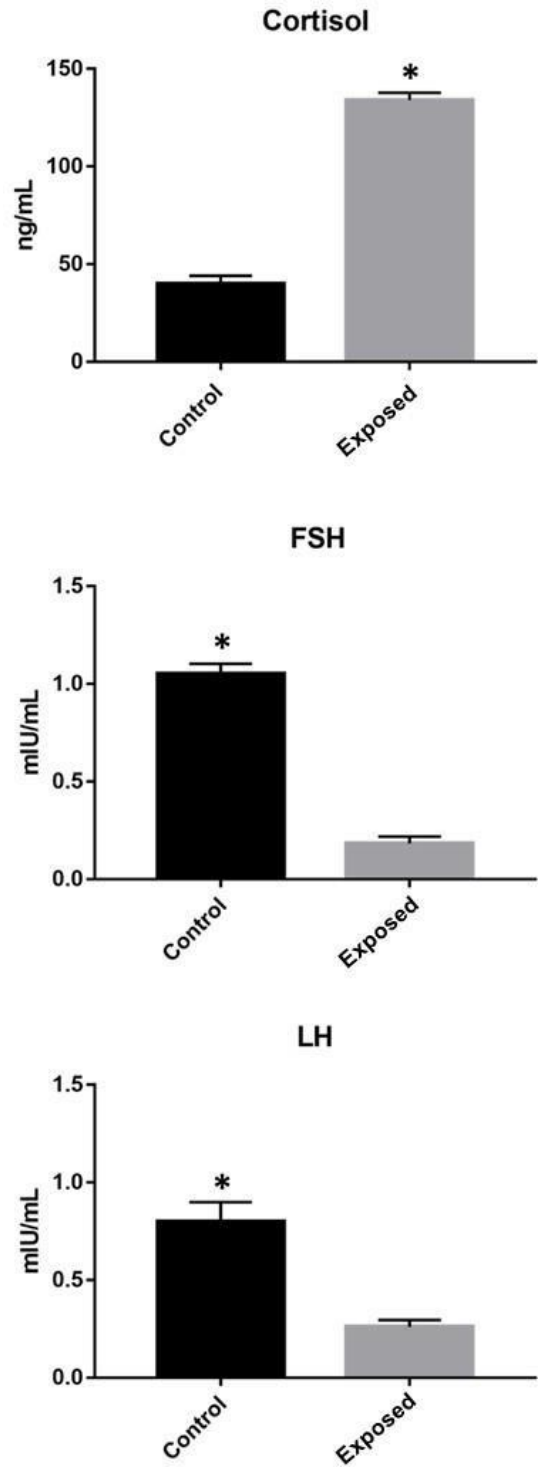


Fig.7. Values presented as mean±SD for serum cortisol, FSH, and LH levels in *Anguilla anguilla* specimens exposed to cocaine and control animals. Note that cortisol levels were lower in the exposed eels than in the controls. In contrast, FSH and LH concentrations were higher in the control specimens. (*) Significantly different ($p < 0.05$) than the control values.

Other authors have also reported negative effects on the reproduction of organisms exposed to cocaine. For example, Kaufmann et al. (1990) reported that oocyte development and follicular fluid in rats were affected by cocaine (10, 20, 40, or 80 mg/kg), decreasing periovulatory serum progesterone. Potter et al. (1998) observed that cocaine (4 mg/kg) disrupted folliculogenesis in female rhesus monkeys due to decreased estradiol levels.

In *Drosophila melanogaster*, developmental defects were induced during oogenesis (including aberrant follicle morphogenesis and vitellogenic follicle degeneration) after two weeks of cocaine exposure (0.75 mg/ml; 1.5 mg/ml; 2.0 mg/ml) (Willard et al., 2006). To our knowledge, no other studies have investigated the effects of cocaine on fish. Our results suggest that cocaine could directly affect the eel ovary and/or be mediated via decreased gonadotropin levels; further study is required to clearly identify the mechanisms involved.

Development and maturation of vertebrate gonads are positively controlled by the activity of several enzymes such as P450 aromatase, 17 β -HSD, and 3 β -HSD. We found that European eel ovaries contain these enzymes, concurring with other studies in teleost fish: Kobayashi et al. (1996) detected 3 β -HSD in the ovary, testis, and interrenal gonads of rainbow trout (*Oncorhynchus mykiss*). In gonads of the Japanese eel, P450 aromatase was immunolocalized in the innermost follicle layer (Ijiri et al. 2004), while Priyadarshini (2018) showed that 3 β -HSD and 17 β -HSD are expressed in the ovary of *Clarias batrachus*.

All the enzymes investigated in this study play a key role in oogenesis. P450 aromatase catalyzes the conversion of androgens into estrogen hormones, thus regulating sexual differentiation (Blakemore et al. 2016), while 17 β -HSD controls the last step in the formation of all estrogens and plays a key role in sex steroid biology (including 17 β -estradiol, E₂) and gametogenesis (Aranyakonont et al. 2020); moreover, 3 β -HSD regulates the production of progesterone (Kostic et al. 2011). Expression of enzymes in the gonads has been suggested as a key step in steroidogenesis synthesis, which is crucial for ovarian maturation and female reproduction in teleosts (Cheshenko et al. 2000; Tenugu et al. 2020). Our findings that European eels exposed to cocaine exhibited a weaker antibody-labeled signal than controls as well as a different localization of these enzymes suggest that chronic exposure to cocaine may negatively affect oogenesis and steroidogenesis in these animals. Indeed, P450 aromatase levels rise during maturation, and expression of this enzyme is a main limiting factor for synthesis of E₂ in the eel ovary because the 17 β -HSD-I gene is highly transcribed at the vitellogenic stage (Kazeto et al. 2000). Furthermore, decreased 3 β -HSD affects the conversion of pregnenolone, 17 α -hydroxypregnenolone, and dihydroepiandrosterone to progesterone, 17 α -hydroxyprogesterone,

and androstenedione, respectively, which are crucial steps in the biosynthesis of sex steroids (Bhat et al. 2018). Therefore, by considering the physiological role of the enzymes and their involvement in oogenesis, our results suggest that cocaine may affect this process and, in turn, reproductive success in eels, even if more detailed studies are required to confirm this hypothesis.

Furthermore, development and maturation of eel gonads are positively controlled by two gonadotropins (LH and FSH) and cortisol (Dufour et al., 2003; Song et al. 2006; Rojo-Bartolomè et al. 2017). In teleost fishes, FSH controls androgen and estrogen synthesis as well as spermatogenesis and oogenesis, while LH is responsible for production of a progesterone-like hormone, final gamete maturation, and ovulation or sperm release (Norris, 2007).

In *A. australis*, FSH promotes 17 β -estradiol (E2) biosynthesis in ovarian follicle cells, while LH regulates final oocyte maturation by stimulating the production of maturation-inducing hormone (Nguyen et al. 2020). Furthermore, cortisol stimulates LH synthesis in the European eel (Dufour et al., 2003). We found that cocaine exposure decreased both FSH and LH levels while increasing cortisol levels, as previously observed in eels (Gay et al. 2013). Our findings contrast with those of human studies, however, where both FSH and LH level increased after acute cocaine administration (Heesch et al., 1996), as well as in female rhesus monkeys, where cocaine increased LH levels in the presence of low basal estradiol levels (Mello et al., 2004). This discrepancy could result from differences in type of exposure (chronic vs acute), animal species, and/or doses administered, but dopamine may also have played a role. Cocaine is known to affect the dopamine system, blocking reuptake of catecholamines (dopamine, norepinephrine) and serotonin, leading to increased synaptic concentration of these neurotransmitters. Dopamine plays a critical role in the sexual maturation of eels by inhibiting the synthesis and release of gonadotropins (Dufour et al. 2010), resulting in inhibited gonadal development (Sébert et al. 2008).

Previous studies on European eels (Gay et al., 2013) showed that cocaine increased brain and plasma dopamine levels, so the inhibition of gonadotropin synthesis/release by dopamine may have caused the decreased release of FSH and LH. Although cocaine exposure increased cortisol levels, which should stimulate LH release, the dopamine-induced inhibition presumably prevailed over increased cortisol. We can hypothesize that chronic exposure to cocaine impairs the fertility and reproductive success of eels, potentially causing significant population and ecological damage to the species.

Conclusion

This study provides the first evidence that even low environmentally relevant concentrations of cocaine induce histological changes in eel gonad morphology, mainly in relation to the maturation of ovarian follicles. Furthermore, cocaine negatively influenced the expression and distribution of important enzymes and hormones involved in oogenesis and steroidogenesis, suggesting a potential risk for reproduction of *A. anguilla*.

CRedit author statement

Mayana Karoline Fontes: validation, investigation, formal analysis, resources, writing - original draft, review and editing. **Luigi Rosati:** validation, investigation, methodology, writing - original draft, review & editing. **Mariana Di Lorenzo:** validation, investigation, methodology, writing - original draft, review & editing. **Camilo Dias Seabra Pereira:** conceptualization, validation, methodology, supervision, writing - review & editing. **Luciane Alves Maranhão:** conceptualization, validation, supervision, writing - review & editing. **Vincenza Laforgia:** conceptualization, writing - review & editing. **Anna Capaldo:** conceptualization, validation, methodology, supervision, writing - original draft, review & editing, project administration.

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.

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Preliminary environmental risk assessment of COC in marine ecosystems

1. Introduction

The occurrence of illicit drugs in aquatic environment has raised a concern about the effects on aquatic fauna (Capaldo et al. 2018). After the consumption, illicit drugs are mainly excreted by urine, reaching wastewater treatment plants (WWTPs) and consequently aquatic environments (Fontes et al. 2021). The removal and treatment of effluent contaminated with illicit drugs depends on applied technologies, but they are less efficient in developing countries (Pal et al. 2013). Among illicit drugs, Cocaine (COC) is a psychostimulant and affects human behavior and brain physiology, changing the levels of neurotransmitters, mainly dopamine and serotonin (Ellefsen et al. 2016). The global number of users is estimated in 20 million people (UNODC, 2021).

COC is an illicit drug widespread in surface water, and continuously enters the sewage, whereby it has been quantified in concentrations that ranged from between 0.13 ng L⁻¹ (Zucatto et al. 2008) and 5896 ng L⁻¹ (Thomas et al. 2014). In marine environment, COC was detected in concentrations ranging from 2.4 ng L⁻¹ (Klosterhaus et al. 2013) and 537 ng L⁻¹ (Pereira et al. 2016). Despite its occurrence in aquatic environment, the risk has proven hard to determine, in part due to limited availability of data on their effects in aquatic organisms, communities, and ecosystems (Fernández-Rubio et al. 2020).

Some studies have been reported the effects of COC in aquatic organisms: freshwater mussel *Dreissena polymorpha* (Binelli et al. 2012; Parolini et al. 2017) exhibited genotoxicity and cytotoxicity. Studies performed by Kirla et al. (2016) showed the ability COC to reduce activity and locomotion of zebrafish. Eels exposed to environmental concentration of COC showed an increase of brain dopamine and plasma catecholamines (Gay et al 2013; 2016). Capaldo et al. (2012; 2018; 2019) observed that COC can accumulate into the eel tissues, mainly the brain, muscle, and liver, suggesting potential risks for fish. They have also exhibited severe damage in morphology and physiology of the skeletal muscle and histological damage in gills.

Fontes et al. (2021) also reported COC in tissues from *P. perna* mussel, collected in Santos Bay, indicating that the drug can bioaccumulate. As COC has been found in aquatic environments and can cause adverse effects on biota, it is necessary to calculate the risk. In this context, the main objective of this study was assessing the potential environmental risk posed by the COC in a tropical coastal area. We compared the results obtained to previously published data for other coastal/marine regions around the world.

2. Material and methods

2.1 Study and samples collection

Baixada Santista is a coastal metropolitan area from Brazil, densely urbanized and subject to multiple human activities such as industrial activities, fishing and tourism (Lima et al. 2021). Santos is the largest city of Baixada Santista with an estimated urban population of 433, 991 inhabitants, being 1,497/km² inhabitants (IBGE, 2021). It hosts the largest industrial complex along the coast of Brazil and a major commercial port in Latin America (Begliomini et al.2017). This area is economically important for the country, and it is the most impacted zone of São Paulostate (Albergaria-Barbosa et al. 2017).

Wastewaters from urban, port and industrial activities are released in Santos Bay several hazardous chemicals, such as trace metals (Kim et al., 2017), polycyclic aromatic hydrocarbons (Albergaria- Barbosa et al. 2017), pharmaceuticals (Pereira et al. 2016) and illicit drugs (Fontes et al. 2019). The WWTP local only employs railing and sifting process to remove solids, and disinfection is performed using chlorination, not involving treatments that removed the pollutant load (Abessa et al., 2012). Therefore, the contaminants present in the effluent are considered pseudo-persistent (Pereira et al. 2016).

The sample collection, preparation and analysis of water samples were performed according Fontes et al. (2019; 2020). The measured environmental concentration (MEC) obtained in these studies was used to calculate the risk quotient (RQ).

2.2 Environmental risk assessment

The environmental risk assessment was performed calculating the risk quotient (RQ) for three different aquatic organisms (algae, crustaceans, fishes and mussel) following the equation $RQ = MEC / PNEC$, in which MEC is the maximum Measured Environmental Concentration, and PNEC the Predicted No Effect Concentration, both expressed in $ng.l^{-1}$. The PNEC values for the acute and chronic were estimated using the Ecological Structure Activity Relationships Programme (ECOSAR, v 2.0), since ecotoxicological data on cocaine in marine environments are very limited. Considering the effects of COC on the lysosomal membrane stability observed by Fontes et al. (2022), we employ the concentration of cytotoxic effects, represented by neutral red retention time (NRRT) to calculate the PNEC for mussels. When the data were not available for marine organisms, data from freshwater communities were used.

The PNEC values for the acute, sub-chronic and chronic toxicity data were calculated by dividing each toxicological endpoint by an assessment factor (AF). For marine environments, an AF of 10,000 and 100 should be considered in short- and long-term data sets, respectively. The toxicological endpoints selected for the calculation of the PNECs are shown in **Table 1**. Finally, RQ was categorised into four levels: no ($RQ < 0.01$); low ($0.01 \leq RQ < 0.1$); moderate ($0.1 \leq RQ < 1.0$); high ecological risk ($RQ \geq 1.0$) to aquatic organisms (Hernando et al. 2006).

Table 1. Toxicological endpoints selected for the calculation of the PNECs

Organism	Duration	End point	Concentration ($mg.L^{-1}$)	Reference
Fish	96h	LC50	45.1	ECOSAR
Daphnid	48h	LC50	5.48	ECOSAR
Green algae	96h	EC50	4.35	ECOSAR
Mussel	168h	NRRT	0.0002	Fontes et al. 2022*
Fish		ChV	2.47	ECOSAR
Daphnid		ChV	0.459	ECOSAR
Green algae		ChV	1.46	ECOSAR

* data not yet published

3. Results and Discussion

Coastal areas are important for the development of several socioeconomics activities, such as shipping, agriculture, fisheries and tourism and that's why they are under constant

anthropogenic pressure (Dias et al. 2013). Discharge of domestic effluents is one of the principal anthropogenic impacts on estuarine waters and compromises the ecosystem quality, health of local human populations, their socioeconomic activities, local aesthetics, landscape, and the ecological preservation of the coastal areas (Costa et al. 2018; Pereira et al. 2019). In addition, coastal zones have been threatened by the occurrence of illegal activities related to the transport of illicit drugs (Wrathall et al. 2020; Devine et al. 2021).

South America is almost responsible for all the world's COC production and Brazil is an important trafficking route, mainly for due to the factors of geographical location and high rate of urban population (Fontes et al. 2020). In addition to being a threat to public health and the economy, COC has also been considered an emerging contaminant in aquatic environment (Pereira et al. 2016) and the potential risk to aquatic fauna cannot be ignored.

The hazard quotient risk (HQ) or risk quotient (RQ) or risk characterization ratio (RCR) are used by regulatory authorities to describe the risk category of a chemical substance. The United States Environmental Protection Agency (US EPA) defined HQ as the ratio between potential exposure to a substance and the concentration thereof below which no adverse effects are expected (US EPA, 1997). This is usually calculated as the ratio between two values: the predicted environmental concentration (PEC) or measured environmental concentration (MEC) and the predicted no-effect concentration (PNEC) (ECHA, 2012).

Using the PNEC from ECOSARr programme, the RQ in Santos Bay was calculated using the maximum MEC reported by Fontes et al. (2019). The acute, sub-chronic and chronic RQ for COC are showed in table 2.

Table 2. Risk quotient (RQ) of COC in Santos Bay.

MEC (ng.l ⁻¹)	Exposure	Trophic level	Organism/ specie	Endpoint (ng.L ⁻¹)	AF	PNEC (ng.l ⁻¹)	Reference	RQ
203.60	Acute	Algae	Green algae	EC50 (4.35E + 06)		435	ECOSAR	0.46
		Crustacea	Daphnid	LC50 (5.48E + 06)	10 ⁴	548	ECOSAR	0.37
		Fish	Fish	LC50 (45.1E + 06)		4510	ECOSAR	0.045
	Sub-chronic	Mussel	<i>Perna perna</i>	NRRT (2.0 E + 02)	10 ⁴	0.02	Fontes et al. 2022	10.180
	Chronic	Algae	Green algae	ChV (1.46E + 06)		14600	ECOSAR	0.01
		Crustacea	Mysid	ChV (4.59E + 08)	10 ²	45900	ECOSAR	0.004
Fish		Fish	ChV(2.47E + 08)		24700	ECOSAR	0.008	

The table presents the: MEC: maximum measured environmental concentration in the Santos Bay (ng.l⁻¹) reported by Fontes et al. 2019; acute and chronic toxicity data: [(trophic level; organism's test, toxicological endpoint and

concentration (ng.l^{-1}), assessment factor (AF), predicted no-effect concentration (PNEC, ng/L) and risk quotients (RQ, signalled in white, green, yellow, orange and red for no, low, moderate and high risk, respectively). Data from the toxicological endpoints were estimated from the ECOSAR program. Note: 1 freshwater; 2 seawater; EC50: 50% effective concentration; LC50: 50% lethal concentration; NRRT: Neutral Red Retention Time; ChV: chronic value; MEC reported by Fontes et al. 2019.

The presence of COC in marine environment represents not only an ecological concern, but also human health, especially if we consider that organisms exposed to the contaminant are consumed as food. In environmental risk assessment, identifying changes in ecological systems in relation to the contamination level is a crucial step (Chaumot et al. 2014). In our study we applied a PNEC for mussels to calculate the RQ in marine environment. We found a high RQ ($\text{RQ} > 1$), even considering low concentrations of COC (ng.l^{-1}). Our data corroborated with studies carried out by Fernández- Rubio et al. (2019) that estimated a $\text{HQ} = 1.17$ in the Santos Bay. Rovieri et al. (2020) detected COC ($0.2 - 30.3 \text{ ng.l}^{-1}$) and BE ($0.9 - 278 \text{ ng.l}^{-1}$) in a beach area of Guarujá, but the calculated low risk ($\text{RQ} < 1$) for all trophic levels analyzed.

The municipality of Santos is characterized by its rainwater drainage channels that cut through the entire city (Gandra et al. 2020). The urban drainage channels are potential anthropogenic sources of contaminants such as pharmaceuticals and illicit drugs (Roveri et al. 2020). Furthermore, in Santos Bay, the sewage disposal occurs through submarine outfall, being recognised as vehicles for the transport illicit drugs to the marine environment (Abessa et al. 2012).

Chlorine is one of the water disinfectants most used all over the world. It is applied during a pre-treatment (primary disinfection) and as a post-treatment to avoid microbial regrowth (González-Mariño et al. 2012). However, Several studies have been shown that chlorine can react with organic substances present in the aquatic environment such as pharmaceutical and personal care products, forming disinfection by products (DBP) that are toxic and/or have suspected carcinogenic or mutagenic activity (Richardson et al. 2010; Wang et al. 2013; Teo et al. 2016). Fantuzzi et al. (2018) showed that chlorine in water can react with COC producing the norcocaine, a pharmacological activity metabolite. González-Mariño et al. (2012) observed that in the presence of chlorine, COC undergoes an oxidative *N*-dealkylation reaction producing norcocaine, while benzoylecgonine produces norbenzoylecgonine. In mammals, norcocaine is hepatotoxic and causes oxidative stress (Larrey, 2013). Therefore, considering that it can be produced by the reaction of chlorine with cocaine present in water, future studies are needed to evaluate the effects of these substances on marine biota.

Legislation and regulation of domestic and industrial chemicals for the protection of the environment have been implemented in Europe and North America for decades (Jørgensen and Fath, 2011), but in Brazil, the inclusion of pharmaceuticals and illicit drugs in a water quality monitoring program is still unregulated. Our study reinforces the need to carry out more ecotoxicological studies, especially with tropical marine organisms to assess the acute and chronic toxicity of illicit drugs.

The evidences presented here suggest that COC is potentially dangerous in the Santos Bay and the ecological risk assessment performed indicates that this drug can potentially exert deleterious effects on the marine biota. In order to maintain the quality of Santos Bay and, therefore to prevent environmental and public risks, it is necessary to invest in the improvement of structures for: a) collection and treatment of effluents; b) establish a program for the control and elimination of sewage sources; c) expansion and adaptation of the collection and treatment network; d) treatment of effluents from ships; e) land tenure regularization of housing; to f) updating of environmental legislation for the development of quality guides that allow the establishment of the safety limits of these substances in the marine environment.

Conclusion

The present study reported that the maximum measured environmental concentration of, COC even in the order of ng.l^{-1} may pose risk to marine biota. Despite the efforts of the scientific community to carry out studies on the occurrence and effects of illicit drugs in aquatic environments, data on the effects of these substances in the marine environment are still limited. Given the ecological and economic importance of Santos Bay further research into the possible ecotoxicological effects of COC in local biota (mainly fish and mussels that are consumed as food), further investment should also be provided to improve the treatment processes of effluents that are discharge directly into the ocean. Establish guidelines for oceanic sewage disposal along the Brazilian coastal zone, implement environmental monitoring programmes of illicit drugs in marine environment; and performed further ecotoxicological studies on the about occurrence and the potential ecological risk of illicit drugs are essential tools to maintain the quality and preservation of resources from marine ecosystems.

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Conclusion and suggestions for future research

Illicit drug use is now recognized not only as a socio-economic but also an environmental problem, especially in aquatic environment. The occurrence of illicit drugs in aquatic ecosystems is a consequence of the frequent use of these substances, but it also highlights the inability of sewage treatment systems to remove these substances from effluents, especially in developing countries. Coastal zones are even more vulnerable to contamination by illicit drugs due to the use of sewage submarine outfalls (SSO) that often dispose of untreated effluent directly into the open ocean. In this context, it is necessary to carry out more ecotoxicological studies on the effects of cocaine (and other illicit drugs) in the marine environment, given the importance that this ecosystem has on world economic development.

The present study showed that environmentally concentration of COC, even in ng.L^{-1} , cause cytotoxic and genotoxic effects, affected neuroendocrine and biochemical parameters and bioaccumulated in mussels. Furthermore, COC also caused histopathological damage to fish ovary tissue and affected the levels of FSH, LH and increased cortisol., suggesting a potential risk for reproduction of *A. anguilla*. The use of the *Perna perna* mussel and the *Anguilla anguilla* fish as biological models proved to be adequate for carrying out ecotoxicological studies with COC.

The data reported several correlations between COC exposure and biomarker response. The use of biomarkers in ecological risk assessment is already applied in studies with other contaminants. The present study showed that the inclusion of these *endpoints* in ecological risk assessment for COC appears to be promising. The development of an adverse outcome pathway (AOP) for COC is also suggested to minimize potential limitations that biomarkers may present to adverse outcomes (fluctuations in community structure, size or decreases in population for example).

Based on the presented findings, COC can be considered hazardous and pose risk to the marine ecosystems, especially when these ecosystems are found in places where sewage treatment is inefficient. Minimizing the impacts of illicit drugs on the marine environment requires the inclusion of these substances in water quality monitoring programs in coastal areas, making data publicly available to better manage and protect aquatic ecosystems.

The results demonstrate the need to improve the processes of removal and treatment of effluents contaminated with illicit drugs to protect aquatic communities. Furthermore, it is necessary to intensify the performance of ecotoxicological studies in marine environments, focused on the mechanism of action of illicit drugs. The generation of data will allow the elaboration of specific guidelines for the establishment of emission standards, monitoring and environmental risk assessments for illicit drugs. The presented findings can be useful in a regulatory context, and to better characterize the risk associated with the use of COC (and other illicit drugs) in marine environment.