Departamento de Química-Física
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ADVERSE EFFECTS OF CRACK/COCAINE TO MARINE ORGANISMS AFFECTED BY ACIDIFICATION CONDITIONS

Efectos adversos de crac-cocaína en organismos marinos en condiciones de acidificación

PhD. Thesis

TESIS DOCTORAL

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Esta Tesis Doctoral ha sido realizada dentro del Grupo de Investigación (Contaminación de Sistemas Acuáticos (-C.o.S.A.- P.A.I., nº RNM0375 del Plan Andaluz de Investigación, Desarrollo e Innovación), del Departamento de Química Física) en la Facultad de Ciencias del Mar y Ambientales de la Universidad de Cádiz.

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Lorena da Silva Souza

D. Camilo Dias Seabra Pereira, Profesor Asociado de la Universidad Federal de São Paulo y Profesor Titular del Departamento de Ecotoxicologia de la Universidad Santa Cecília como su director,

HACE CONSTAR:

Que esta Memoria, titulada "**Adverse Effects of Crack/Cocaine to Marine Organisms Affected by Acidification Conditions**" presentada por Dª. Lorena da Silva Souza, resume su trabajo de Tesis Doctoral y, considerando que reúne todos los requisitos legales, autorizan su presentación y defensa para optar al grado de Doctor en Gestión Marina y Costera/Marine and Coastal Management.

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"Know all the theories, master all the techniques,

but as you touch a human soul be just another human soul."

Carl G. Jung

ABSTRACT

After administration, illicit drugs and its metabolites are excreted and reaches the wastewater treatment plants where they may not be eliminated by conventional treatment technologies. Recent research demonstrates they can influence and alter physiology as well as behavior of aquatic organisms. Since 1970, cumulative carbon dioxide (CO_2) emissions from fossil fuel combustion, cement production and flaring have tripled, and cumulative CO_2 emissions from forestry and other sources have increased by about 40%. Rising atmospheric CO_2 concentration is causing global warming and ocean acidification. The inorganic carbon system is one of the most important chemical equilibria in the ocean and is largely responsible for controlling seawater's pH. Once pH values decreased, organic compound may also suffer some alterations in bioavailability. Based on that, the hypothesis of this work is that the acidification in the marine environment will modify toxicity of the illicit drug cocaine and its byproducts. Aiming to address the effects of the combination of the all different stressors on the organisms used, and besides, to distinguish the effect observed related to each of the stressors, it was developed an integrated, and more precise, interpretation of the risks associated with CO_2 enrichment in the marine environment: the multivariate analysis tools. Using Principal Component Analysis (PCA) it was possible to understand the correlation between the biological effects measured in laboratory associated with the concentration of protons and Crack-Cocaine. The experiments have shown negative effects mainly associated with high concentration of protons (lower pH values) and relatively high concentrations of crack-cocaine for all the organisms used in the different assays. This thesis demonstrate that acidification of coastal ecosystems will trigger enhanced adverse effects on marine organisms exposed to drugs.

RESUMEN

Las drogas ilícitas y sus metabolitos son excretadas tras su administración y consumo humano. Tras esto, sus residuos acaban llegando a las plantas de tratamiento de aguas residuales, donde las tecnologías de tratamiento convencionales no permiten su total eliminación. El desarrollo de las investigaciones más recientes demuestra que las mismas pueden influir y alterar la fisiología y el comportamiento de los organismos acuáticos. Desde 1970, las emisiones acumuladas de dióxido de carbono (CO_2) proveniente de la combustión de combustibles fósiles, producción y quema de cemento se han triplicado, y las emisiones acumuladas de CO_2 de la repoblación forestal y otras fuentes han aumentado en aproximadamente un 40%. El aumento de la concentración atmosférica de CO_2 está causando el calentamiento global y la acidificación de los océanos. El sistema de carbono inorgánico es uno de los equilibrios químicos más importantes en el océano y es en gran parte el responsable del control del pH del agua de mar. Si los valores de pH disminuyen, aumentando la concentración de los protones, el compuesto orgánico, en este caso crack-cocaína también puede sufrir algunas alteraciones en su biodisponibilidad. La hipótesis de este trabajo determina que la acidificación producida por el aumento de CO_2 en el medio marino modificará la toxicidad asociada a las concentraciones de esta droga ilícita crack-cocaína y sus subproductos. Con el objetivo de abordar los efectos de la combinación de todos los factores estresantes se han utilizado diferentes organismos que han sido expuestos a la combinación de ambos contaminantes (concentración de protones y de crack-cocaína). Además, se han diseñado y utilizado para distinguir el efecto observado relacionado con cada uno de estos factores mediante el desarrollo de un método integrado como aquella más precisa para determinar y cuantificar los riesgos asociados con el enriquecimiento por CO_2 en el medio marino, entre ellas, el uso del análisis multivariante. Utilizando el análisis de componentes principales (PCA) fue posible comprender la correlación entre los efectos biológicos medidos en laboratorio asociados con la concentración de protones y la crack-cocaína. Los experimentos han mostrado resultados relacionados con efectos negativos principalmente asociados con una alta concentración de protones (valores de pH más bajos) y concentraciones relativamente altas de crack-cocaína para todos los organismos utilizados en los diferentes ensayos. Esta tesis demuestra que la acidificación de los ecosistemas costeros provocará efectos adversos que pueden aumentar aquellos asociados con los contaminantes existentes en el medio, como se ha demostrado con las concentraciones de crack-cocaína probadas en esta Tesis sobre organismos marinos expuestos a las drogas.

RESUMO

Após a administração, drogas ilícitas e seus metabólitos são excretados e chegam às estações de tratamento de águas residuais, onde não são totalmente eliminados pelas tecnologias convencionais de tratamento. Pesquisas recentes demonstram que drogas ilícitas podem influenciar e alterar a fisiologia, bem como o comportamento de organismos aquáticos. Desde 1970, as emissões cumulativas de dióxido de carbono (CO_2) provenientes da combustão de combustíveis fósseis e produção e queima de cimento triplicaram, e as emissões cumulativas de CO_2 do desmatamento e outras fontes aumentaram cerca de 40%. O aumento da concentração atmosférica de CO_2 está causando aquecimento global e acidificação oceânica. O sistema de carbono inorgânico é um dos equilíbrios químicos mais importantes no oceano e é o principal responsável pelo controle do pH da água do mar. Uma vez que os valores de pH diminuem, o composto orgânico também pode sofrer algumas alterações em sua biodisponibilidade. Com base nisso, a hipótese deste trabalho é que a acidificação no ambiente marinho possa modificar a toxicidade da droga ilícita cocaína e de seus derivados. Com o objetivo de abordar os efeitos da combinação de estressores sobre os organismos utilizados, além de distinguir o efeito observado em relação a cada um desses fatores, foi desenvolvida uma interpretação integrada e mais precisa dos riscos associados ao enriquecimento de CO_2 no meio marinho: as ferramentas de análise multivariadas. Utilizando a Análise de Componentes Principais, foi possível compreender a correlação entre os efeitos biológicos medidos em laboratório, associados à concentração de prótons e/ou as concentrações de crack-cocaína. Os experimentos mostraram efeitos negativos principalmente associados a alta concentração de prótons (valores mais baixos de pH) e concentrações relativamente altas de crack-cocaína para todos os organismos utilizados nos diferentes ensaios. Esta tese demonstra que a acidificação de ecossistemas costeiros provocará efeitos adversos em organismos marinhos expostos a drogas**.**

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Chapter I. Introduction and Objectives

1. Introduction

1.1 Emerging Contaminants

Pharmaceuticals, Personal Care Products and illicit drugs comprise a diverse group of chemicals recognized as contaminants of emerging concern. This class of contaminants have received growing global attentions due its potential for threatening drinking water safety and aquatic organisms (Sui et al., 2011). Considering the pathways by which they enter into the water environment (Figure 1), effluent from wastewater treatment plants (WWTPs) has been identified as an important source (Quinn et al., 2005). Although nowadays it is mandatory in developed countries to perform sewage treatment to minimize the pollution of the receiving waters, there are many substances (e.g., antibiotics, hormones, cocaine) that are not efficiently removed in wastewater treatment plants (WWTPs) (Heberer, 2002).

The environmental fate and ecosystem consequences represent a crosscutting frontier in aquatic ecology (Rosi-Marshall and Royer, 2012) and environmental chemistry (Ankley et al., 2007). Recent research demonstrates that pharmaceuticals can influence and alter the structure of aquatic communities (Drury et al., 2013; Muñoz et al., 2009) as well as the behavior of aquatic organisms (Brodin et al., 2013; Jonsson et al., 2014). In addition, they have the potential to influence ecosystem functions such as primary production and microbial respiration (Rosi-Marshall et al., 2013) and invertebrate secondary production (Muñoz et al., 2009).

Figure 1: Conceptual overview of the illicit drug transfer pathway, from production and consumption to the receiving environments by Yadav et al. (2017)

Recently, some investigations have shown that occurrence, fate and effects of illicit drugs in aquatic ecosystems are also of environmental concern (Binelli et al., 2013, 2012; Parolini et al., 2013). In fact, the amounts of illicit drugs consumed worldwide are comparable with those of therapeutic drugs, as millions of individuals are current users of cocaine, heroin, amphetamine like stimulants, marijuana and other drugs (Zuccato and Castiglioni 2009). In analogy with that observed for therapeutic drugs, the residues of drugs of abuse persisting in consumers urine and

entering sewage networks with wastewater are also only partially removed by some sewage treatment plants (STPs) (Baker and Kasprzyk-Hordern 2013; Borova et al. 2014; Pal et al. 2013)

Cocaine and cannabis are the two most consumed illicit drugs around the world. Brazil has been identified by the United Nations Office on Drug and Crime as one of the emerging nations where the use of stimulants such as cocaine – used either intranasally ("powder") or smoked (crack) – is increasing (UNODC, 2014). There are many reasons for the suggested elevated consumption rate: (i) Brazil's geographic position, neighbouring the world's largest cocaine producers — Peru, Colombia and Bolivia, (ii) its young population (Brazil has nearly 35% of its population is 15 to 34 years of age (IBGE, 2015), (iii) the socio-economic raise seen in the last decade in Brazil,which represents higher purchasing power and (iv) the cheap price of cocaine in the country (UNODC, 2012).

After its consumption by humans, most cocaine is bio transformed in the liver, being eliminated in the form of metabolites such as benzoylecgonine (BE, 45%) and ecgonine methyl ester (40%), while only a small proportion (1–9%) is eliminated unaltered (García-Cambero et al., 2015). Once cocaine and its metabolites are excreted, they reach inland waters directly by sewage outfalls. At best, cocaine and its metabolites reach a treatment plant (Castiglioni et al., 2011), however, it appears that these substances are only partially removed with conventional treatments (Zuccato et al. 2008; Domènech, Peral, and Muñoz 2009), so most of cocaine and its metabolites will reach surface waters, contributing to an increased pollutant loading (Pereira et al., 2016).

The free base form of cocaine is crack, an alkaloid compound, with unusual characteristic of being either highly hydrophilic or lipophilic (Figure 2). Typically, many drugs are reacted with acids (H_2SO_4, HNO_3, HCl) as part of the production process offering advantages over storing the

pure drug, resulting a salt compound, where the cocaine is more stable, more soluble in water and more easily absorbed by the cells. However, in the case of cocaine, the salt is not practical to consume due to is higher boiling point than the freebase, and if heated too much it tends to decompose. In order to turn possible the use, a base such as baking soda (Na_2CO_3) is added, and the equilibrium reaction is reversed, turning the salt back into freebase cocaine (crack-cocaine) that can be consumed by smokers. (Florence and Attwood, 2006).

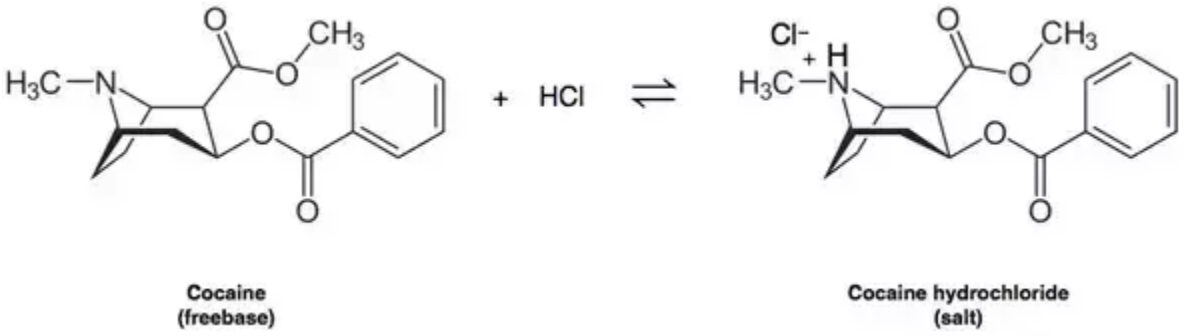

Figure 2: Process where cocaine (freebase) receives a proton in the basic amino group by the addition of a hydrochloric acid resulting in a salt (cocaine hydrochloride). Source: https://www.quora.com

Recently studies were focused mainly on the determination of environmental concentrations of illicit drugs in freshwater (Baker et al., 2012; Baker and Kasprzyk-Hordern, 2013; Castiglioni et al., 2006; Hernández et al., 2015; Metcalfe et al., 2010) (Table 1) and marine environments (Klosterhaus et al., 2013, Borova et al. 2014; Pereira et al. 2016). In England, cocaine and the major metabolite (benzoylecgonine) were quantified in all Sewage Treatment Plants (STPs) influential samples analyzed, at concentrations ranging from 5,1 to 208.9 ng L^{-1} (average load: 2,8 g day^{-1}) and 15,8 to 566,6 ng L^{-1} (average load: 6,7 g d^{-1}) (Baker and Kasprzyk-Hordern, 2013).

Table 1: A review of environmental concentration of cocaine (COC) in ng/L in surface freshwater around the world. Adapted from Yadav et al. (2017)

COC Concentration (ng/L)	River Location	Reference
1.2	Po, Italy	(Zuccato et al., 2005)
0.5	Po, Italy	(Zuccato et al., 2008a)
44	Olona, Italy	
15	Lambro, Italy	
1.7	Arno, Italy	
4.7	Thame, UK	
25	Broad Meadow, Ireland	(Bones et al., 2007)
33	Liffey, Ireland	
6	Llobregat, Spain	(Huerta-Fontela et al., 2008a)
28.3		
1.4 – 3	Ebro River basin, Spain	(Huerta-Fontela et al., 2007)
0.3 - 4	Taff, UK	(Kasprzyk-Hordern et al., 2009, 2008, 2007)
0.3	Ely, UK	
26	Grote Molembeek, Belgium	(Gheorghe et al., 2008)
13 – 14.3	DEmer, Belgium	
7	Senete, Belgium	
0.1	Minnesota lakes, USA	(Ferrey et al., 2015)

Considering the alkaline pH of marine waters and the pKa of the pharmaceuticals and drugs detected in coastal zones, some compounds could be more bioavailable to the marine biota when compared to freshwater environments (Pereira et al., 2016). Taking cocaine as an example in Santos Bay, this illicit drug with a pKa = 8.5 tends to be partially found in its non-ionic form in the pH of the sampling area (ranging from 7.9–8.1), which increases cocaine octanol–water partition coefficient (log Kow) values from 0.10 (for the ionic form) to 2.30 (for the non-ionic

form) (EPISuite, 2012). Higher Kow values favor absorption and bioaccumulation processes in organisms exposed to these compounds, and an increased toxicity may therefore be expected (Pereira et al., 2016).

1.2 Ocean Acidification:

Studies have demonstrated a substantial amplification of the annual oceanic CO_2 cycle over the twenty-first century (Global CCS Institute, 2017; IEA, 2010). In particular, for regions within the Southern, Pacific and North Atlantic oceans, our data-based projections show a five to eightfold amplification of CO_2 concentration in the annual CO_2 cycle over the twenty-first century under increasing atmospheric CO_2 concentration (McNeil and Sasse, 2016). Since 1970, cumulative CO_2 emissions from fossil fuel combustion, cement production and flaring have tripled, and cumulative CO_2 emissions from forestry and other land have increased by about 40%. In 2011, annual CO_2 emissions from fossil fuel combustion, cement production and flaring were 34.8 ± 2.9 $GtCO_2$/yr. For 2002–2011, average annual emissions from Forestry and Other Land Use were 3.3 ± 2.9 $GtCO_2$/yr (IPCC, 2014a).

Due to the strong dependence of global economies on carbon fuel for electricity generation, CO_2 has been pointed as the most important greenhouse gas (GHG) (Pires et al., 2011). This rate of increase, driven by human fossil fuel combustion and deforestation, is at least an order of magnitude faster than has occurred for millions of years (Doney and Schimel, 2007), and the current concentration is higher than experienced on Earth for at least the past 800,000 years (Lüthi et al., 2008). Rising atmospheric carbon dioxide (CO_2) concentration is causing global warming and ocean acidification (Schmittner et al. 2008; Caldeira and Wickett 2005; Feely et al. 2004),

which increasingly are recognized as important drivers of change in biological systems (Del Valls et al. 2004; Basallote et al. 2012; De Orte et al. 2014; Riba et al. 2004).

Ocean acidification is a predictable consequence of rising atmospheric CO_2 and does not suffer from uncertainties associated with climate change forecasts. Absorption of anthropogenic CO_2, reduced pH, and lower calcium carbonate ($CaCO_3$) saturation in surface waters, where the bulk of oceanic production occurs, are well verified from models, hydrographic surveys, and time series data (Caldeira and Wickett, 2005; Feely et al., 2004; Schmittner et al., 2008).

The inorganic carbon system is one of the most important chemical equilibria in the ocean and is largely responsible for controlling the pH of seawater (Figure 3). Dissolved inorganic carbon (DIC) exists in seawater in three major forms: bicarbonate ion (HCO_3^-), carbonate ion (CO_3^{2-}), and aqueous carbon dioxide ($CO_{2(aq)}$), which here also includes carbonic acid (H_2CO_3) (Fabry et al., 2008). When CO_2 dissolves in seawater, H_2CO_3 is formed. Most of the H_2CO_3 quickly dissociates into a hydrogen ion (H^+) and HCO_3^-. A hydrogen ion can then react with a CO_3^{2-} to form bicarbonate. Therefore, the net effect of adding CO_2 to sea water is to increase the concentrations of H_2CO_3, HCO_3^-, and H^+, decreasing CO_3^{2-} concentration and lowering pH ($pH = - \log[H^+]$). These reactions are fully reversible, and the basic thermodynamics of these reactions in seawater are well known (Millero et al., 2002). The atmospheric CO_2 value today is ~100 ppmv greater than the pre-industrial value (280 ppmv), and the average surface ocean pH has dropped by 0.1 unit, which is about a 30% increase in $[H^+]$. Under the IPCC emission scenarios (IPCC, 2014a), average surface ocean pH could decrease by 0.3–0.4 pH units from the pre-industrial values by the end of this century.

In order to reduce atmospheric CO_2 levels, many mitigation strategies have been developed and proposed. One such strategy is large-scale carbon dioxide capture and storage (CCS) in geological formations. According to the International Energy Agency (IEA 2010), it could contribute to a reduction of 19% of CO_2 emissions by 2050. The CCS methodologies comprise three steps: CO_2 capture, CO_2 transportation and CO_2 storage (Pires et al., 2011).

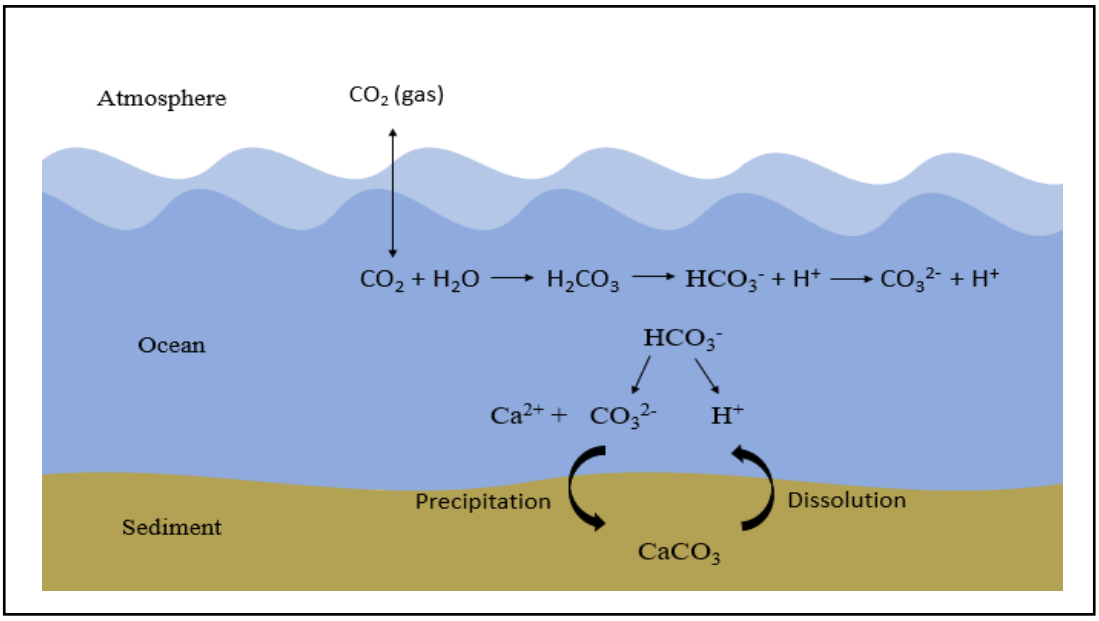

Figure 3: Diagram of the Physicochemical system for the Carbonic Acid in sea water. It shows the chemical reactions that lead to ocean acidification by the input of atmospheric carbon dioxide.

The sequestration of CO_2 is proposed as mitigation to reduce the amount of carbon dioxide available in the atmosphere. This technology consists of trapping CO_2 from industrial and energy related sources, transporting it to a storage site, injecting and storing it for a long time instead of releasing this gas into the atmosphere (Kirchsteiger, 2008). Considering that oceans have the

largest potential capacity for CO_2 storage, sub-seabed geological formations, such as depleted oil and gas reservoirs and saline aquifers, have been designated as potential storage locations for CO_2 sequestration. According to Lackner and Brennan 2009 some principles must be addressed: (i) storage must be safe; (ii) the environmental impact should be minimal; and (iii) storage must be verifiable.

The underground storage of CO_2 remains a lack of knowledge of the behavior of this gas under leaks. The complexity in foresee the location and magnitude of possible seepages turns difficult the evaluation of the potential effects on aquatics ecosystems. Two main sources of CO_2 escape are from transport facilities and storage areas (Leung et al., 2014). The effects of CO_2 leakage will depend on the amount and/or rate of leakage, the transport and dispersion processes and the chemical buffering capacity of the sedimentary or water system, contributing with the imbalance of seawater's chemistry. In this context, many studies have been performed in order to analyze the impacts from changes in the marine carbonate system as well as pH reduction by CO_2 enrichment to the organisms (Basallote et al., 2012; de Orte et al., 2014a; Passarelli et al., 2017b, 2017a; Szalaj et al., 2017).

1.3 Technology simulating CO_2 enrichment

In order to understanding how ocean acidification can affect marine environment and organisms, experiments in laboratory have been performed worldwide to assess the possible effects of H^+ ions (Beardall et al., 2009; Kapsenberg et al., 2017; Nogueira et al., 2017; Taylor et al., 2015; Zhan et al., 2017).

A CO_2 injection system (Figure 4) was developed to simulate in laboratory the acidification process in marine environment caused by CO_2 enrichment for instance by means of leaks during the carbon capture and storage process in stable underwater structures. The design of this equipment allows the development of toxicity assays under laboratory condition. In this sense, organisms are exposed to several pH values, so the potential adverse effects of acidification on marine ecosystem can be determined. The CO_2 injection system used for this experiment is an adaptation of the experimental set up described by De Orte et al. (2014), patent process n^o: P201200753, Cadiz University, Faculty of Marine and Environmental Sciences, Physical Chemistry Department (RNM 375).

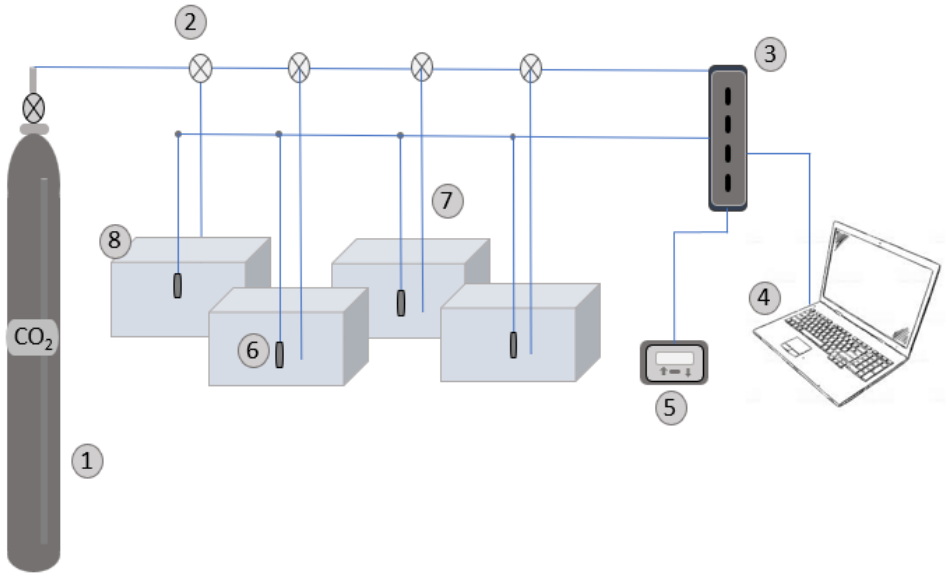

Figure 4: CO_2 injection system (1. CO_2 gas bottle. 2. Solenoid valves for the electronic regulation of the CO_2 injection 3. Power strips and USB connectors. 4. Laptop with software (Aquamedic 8.0). 5. AT-Control system. 6. pH interface to connect the pH sensor to the AT- Control System. 7. CO_2 injection hose. 8. Aquariums). Adapted from De Orte et al. (2014).

The CO_2 injection system includes twelve solenoid valves that control the CO_2 released in the system, injecting it at atmospheric pressure and can be controlled individually, according to each condition, through the AT-control system installed in a computer. Each one of the aquariums, containing seawater, has the pH continuously measured through a pH electrode, whose connection to a computer via an interface, allowed controlling the gas released according to the pH reduction, proportionally to the increase in CO_2 concentration in the system. (De Orte et al. 2014).

The same approach was used for all experiments, changing the system characteristic such as chambers test, the tests-organisms and procedure of each bioassay that are described in detail in the following chapters of this Thesis. The Figure 5 shows in detail the schematic design used for each experiment included in this Thesis.

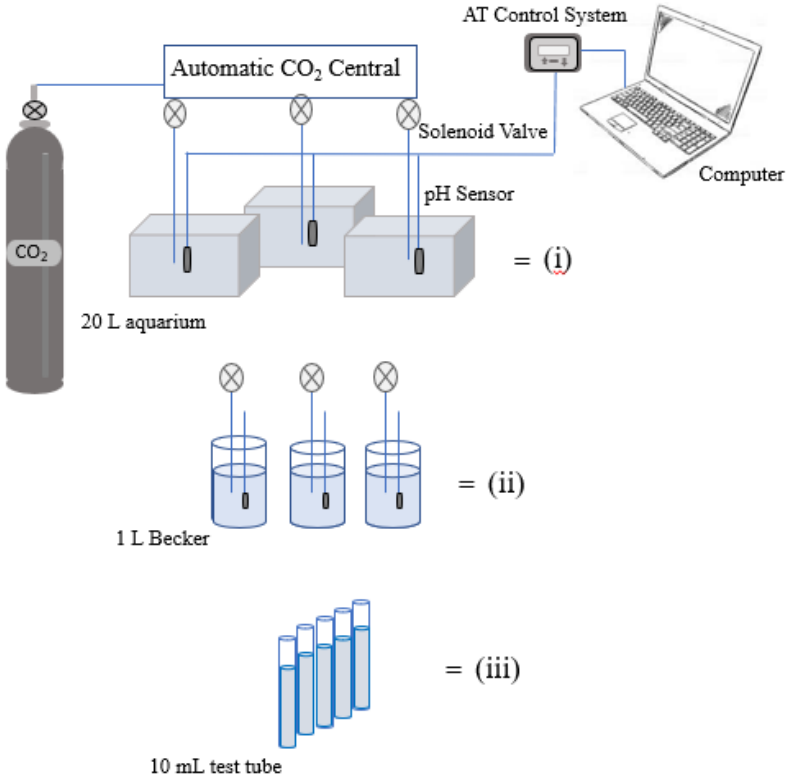

Figure 5: Schematic design of the CO_2 injection system used in this: (i) tests with adult mussel to assess DNA damage, NRRT and LPO; (ii) embryo larval development using sea urchin and mussel; (iii) fertilization rate using sea urchin and mussel.

The pH in each aquarium was monitored by an electronic system that allows monitoring and controlling the pH in aquariums. With this system it is possible to select the experimental pH in the tank and controlling the injection of CO_2 through the solenoid valve. The program achieves all options of desired pH values. The opening of the solenoid valve causes the injection of CO_2 in aquariums through a hose to the bottom of the tanks and provides with perforations.

The experiments design in the embryo larval development and fertilization rate assays were performed using aquarium of one liter in which the pH treatments were applied in triplicate. On the other hand, the NRRT, DNA damage and LPO assays were performed based in the adults' exposition to different concentrations of crack/cocaine in 25 liters aquariums. During the 96 hours of exposition, the pH was controlled and the water which was replaced each 48 hours were already in the desired pH, avoiding any kind of variation that could affect the results confidence.

1.4 Illicit Drugs and CO_2 Enrichment

Several studies described adverse effects in marine organisms submitted to acidification, including reduction of calcification rates, changes in metabolism functioning and increase of oxidative stress, among others (Allison et al., 2018; Esbaugh, 2017; Heuer and Grosell, 2014; Rato et al., 2017; Sekizawa et al., 2017). Different studies reported the presence of illicit drugs in in freshwater and marine environments worldwide (Edwards et al., 2017; Ferrey et al., 2015; Pereira et al., 2016; Thomaidi et al., 2015). In addition, adverse effects in aquatic organisms due to an exposure of cocaine and its by products have been reported (Capaldo et al., 2019; Maranho et al., 2017; Ortega et al., 2018; Parolini et al., 2018, 2017; Rosi-Marshall et al., 2015).

To the best of our knowledge, there is no information available about the influence of climate change on the accumulation, metabolization and depuration of illicit drugs in marine organisms. Highlighting the importance of this study by providing further understanding of the effect of warming and acidification in marine organisms and evidencing that acidification may hinder marine organisms' capacity to metabolize some contaminants, such as crack-cocaine.

2 Hypothesis and Objectives

The hypothesis of this work is that the acidification associated with enrichment of CO_2 in the marine environment will provoke a variation in the bioavailability and toxicity of cocaine and its byproducts.

In order to achieve results to verify the hypothesis, the main objective of this project is to assess adverse effects of crack-cocaine (CC) in marine ecosystems combined with ocean acidification. It was determined the effects of CC in different pH scenarios related to the increase of CO_2 by assessing biological responses of non-target marine organisms. ***This project is the first study*** on illicit drugs (cocaine and crack cocaine) ecotoxicity related to different acidification scenarios associated with enrichment of CO_2 focusing specifically and effects, to draw attention to these emerging contaminants in future scenarios of ocean acidification. It was also proposed tools for biomonitoring and environmental risk assessments of illicit drugs in marine environments. Therefore, the specific aims were:

- Evaluate the acute and chronic toxicity of crack-cocaine exposed to different pH levels (pH 8.5, 8.0, 7.5, 7.0, 6.5 and 6.0) employing different standardized species under controlled

laboratory conditions; The organisms selected for the toxicity test were: sea urchin (*Echinometra Lucunter*) and brown mussels (*Perna perna*)

- Evaluate the effect of pCO_2 on the lysosomal membrane stability, DNA damage and Lipid Peroxidation of *Perna perna* mussels exposed to acidified samples (pH 8.3; 8.0; 7.5; 7.0; 6.5 and 6.0)

- Assess the effect of CO_2 enrichment on the lysosomal membrane stability, DNA damage and Lipid Peroxidation of *Perna perna* mussels exposed to acidified samples with different crack-cocaine concentrations, using neutral red retention time assay, DNA stand-break an LPO.

- Provide information about adverse effects of an illicit drug (crack-cocaine) to support the stakeholders and policymakers in finding appropriate solutions for the conservation and protection of marine ecosystems

3 Thesis structure

This thesis is organized into six chapters plus annex. The first chapter includes a general introduction and the state-of-the-art of the research, hypotheses and objectives of the Thesis and the system used for mimicking the different scenarios of CO_2 enrichment.

The second chapter reviews and update the different lines of evidence used in the past to address the impact of the acidification by CO_2 in the environment, including behavior of contaminants, toxicity, alteration in situ, bioaccumulation etc. One paper was elaborated in this chapter, that was published in a high impact factor journal (Environmental Reviews), entitle:

"***Integrative assessment of sediment quality in acidification scenarios associated with carbon capture and storage operations***".

In the third chapter was analyzed the effects of CO_2 enrichment on the fertilization rate and embryo larval development applying two different organisms: sea urchin (*Echinometra lucunter)* and marine brown mussel (*Perna perna*). The results obtained in this chapter were shared in two papers depending on the organism selected for the study: one of them has been submitted for publication in the Chemosphere journal (manuscript number: CHEM61635) as "***Adverse effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios***". The second article of this chapter, with the obtained results of the experiments performed with marine mussels, is being elaborated to be submitted Environmental Science and Pollution Research and is entitled as "***Could ocean acidification intensify illicit drug effects on reproduction of marine mussels?***"

Fourth chapter includes the results obtained using different line of evidences (cytogenotoxicity and oxidative stress) addressing the risk of CO_2 enrichment and its association with the effects of crack-cocaine in marine mussels. The results obtained in this chapter were shared in two papers. The first article approached the adverse effects of the CO_2-enrichement itself and has been submitted for publication in the Marine Pollution Bulletin (manuscript number: MPB-D-19-00300) as "***Assessing CO_2-induced acidification lethal and sublethal effects on tropical mussels Perna perna (Linnaeus, 1758)***". The second article of this chapter related adverse effects of an illicit drug (crack-cocaine) in different scenarios of ocean acidification and is submitted Science of Total Environment, (manuscript number: STOTEN-D-19-14048) entitle "***Sub-lethal combined effects of illicit drug and decreased pH on marine mussels: A short-time exposure to crack cocaine in CO_2 enrichment scenarios***".

The fifth chapter contain the joint discussion of the obtain results in this Thesis. The objective of this chapter is to llink all the results obtained in the previous chapters using an integrative approach based on the use multivariant analysis. It allows to assess the effects of CC, CO_2 enrichment and the association of both factors. Already in the sixth chapter, it is assembled the main conclusions of the thesis derived from all the chapters in this Thesis, follow by the annexes, which includes the certificates achieved during the last four years and author's collaboration articles.

References

Allison, N., Cole, C., Hintz, C., Hintz, K., Rae, J., Finch, A., 2018. The effect of ocean acidification on tropical coral calcification: Insights from calcification fluid DIC chemistry. Chem. Geol. 497, 162–169. https://doi.org/10.1016/j.chemgeo.2018.09.004

Ankley, G.T., Brooks, B.W., Huggett, D.B., Sumpter, J.P., 2007. Repeating history: Pharmaceuticals in the environment. Environ. Sci. Technol. 41, 8211–8217. https://doi.org/10.1021/es072658j

Baker, D.R., Kasprzyk-Hordern, B., 2013. Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: New developments. Sci. Total Environ. 454–455, 442–456. https://doi.org/10.1016/j.scitotenv.2013.03.043

Baker, D.R., Očenášková, V., Kvicalova, M., Kasprzyk-Hordern, B., 2012. Drugs of abuse in wastewater and suspended particulate matter - Further developments in sewage epidemiology. Environ. Int. 48, 28–38. https://doi.org/10.1016/j.envint.2012.06.014

Basallote, M.D., Rodriguez-Romero, A., Blasco, J., DelValls, A., Riba, I., 2012. Lethal effects on different marine organisms, associated with sediment-seawater acidification deriving from CO_2 leakage. Environ. Sci. Pollut. Res. 19, 2550–2560. https://doi.org/10.1007/s11356-012-0899-8

Beardall, J., Stojkovic, S., Larsen, S., 2009. Living in a high CO_2 world: impacts of global climate change on marine phytoplankton. Plant Ecol. Divers. 2, 191–205. https://doi.org/10.1080/17550870903271363

Binelli, A., Marisa, I., Fedorova, M., Hoffmann, R., Riva, C., 2013. First evidence of protein profile alteration due to the main cocaine metabolite (benzoylecgonine) in a freshwater biological model. Aquat. Toxicol. 140–141, 268–278. https://doi.org/10.1016/j.aquatox.2013.06.013

Binelli, A., Pedriali, A., Riva, C., Parolini, M., 2012. Illicit drugs as new environmental pollutants: Cyto-genotoxic effects of cocaine on the biological model *Dreissena polymorpha*. Chemosphere 86, 906–911. https://doi.org/10.1016/j.chemosphere.2011.10.056

Bones, J., Thomas, K. V., Paull, B., 2007. Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals. J. Environ. Monit. 9, 701–707. https://doi.org/10.1039/b702799k

Borova, V.L., Maragou, N.C., Gago-Ferrero, P., Pistos, C., Thomaidis, N.S., 2014. Highly sensitive determination of 68 psychoactive pharmaceuticals, illicit drugs, and related human metabolites in wastewater by liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 406, 4273–4285. https://doi.org/10.1007/s00216-014-7819-3

Brodin, T., Fick, J., Jonsson, M., Klaminder, J., 2013. Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 339, 814–5. https://doi.org/10.1126/science.1226850

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J. Geophys. Res. 110, C09S04. https://doi.org/10.1029/2004JC002671

Capaldo, A., Gay, F., Laforgia, V., 2019. Changes in the gills of the European eel (*Anguilla anguilla*) after chronic exposure to environmental cocaine concentration. Ecotoxicol. Environ. Saf. 169, 112–119. https://doi.org/10.1016/j.ecoenv.2018.11.010

Castiglioni, S., Bagnati, R., Melis, M., Panawennage, D., Chiarelli, P., Fanelli, R., Zuccato, E., 2011. Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine. Water Res. 45, 5141–5150. https://doi.org/10.1016/j.watres.2011.07.017

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R., Bagnati, R., 2006. Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry. Anal. Chem. 78, 8421–8429. https://doi.org/10.1021/ac061095b

de Orte, M.R., Lombardi, A.T., Sarmiento, A.M., Basallote, M.D., Rodriguez-Romero, A., Riba, I., DelValls, T.A., 2014. Metal mobility and toxicity to microalgae associated with acidification of sediments: CO_2 and acid comparison. Mar. Environ. Res. 96, 136–144. https://doi.org/10.1016/j.marenvres.2013.10.003

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

Domènech, X., Peral, J., Muñoz, I., 2009. Predicted environmental concentrations of cocaine and benzoylecgonine in a model environmental system. Water Res. 43, 5236–5242. https://doi.org/10.1016/j.watres.2009.08.033

Doney, S.C., Schimel, D.S., 2007. Carbon and climate system coupling on timescales from the Precambrian to the Anthropocene. Annu. Rev. Environ. Resour. 32, 31–66. https://doi.org/10.1146/annurev.energy.32.041706.124700

Drury, B., Rosi-Marshall, E., Kelly, J.J., 2013. Wastewater treatment effluent reduces the abundance and diversity of benthic bacterial communities in urban and suburban rivers. Appl. Environ. Microbiol. 79, 1897–1905. https://doi.org/10.1128/AEM.03527-12

Edwards, Q.A., Kulikov, S.M., Garner-O'Neale, L.D., Metcalfe, C.D., Sultana, T., 2017. Contaminants of emerging concern in surface waters in Barbados, West Indies. Environ. Monit. Assess. 189, 1–6. https://doi.org/10.1007/s10661-017-6341-4

Esbaugh, A.J., 2017. Physiological implications of ocean acidification for marine fish: emerging patterns and new insights. J. Comp. Physiol. B 0, 0. https://doi.org/10.1007/s00360-017-1105-6

Fabry, V.J., Seibel, B.A., Feely, R.A., Orr, J.C., 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J. Mar. Sci. 65, 414–432.

Feely, R. a, Sabine, C.L., Lee, K., Berelson, W., Kleypas, J., Fabry, V.J., Millero, F.J., Anonymous, 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science. 305, 362–366. https://doi.org/10.1126/science.1097329

Ferrey, M.L., Heiskary, S., Grace, R., Hamilton, M.C., Lueck, A., 2015. Pharmaceuticals and other anthropogenic tracers in surface water: A randomized survey of 50 Minnesota lakes. Environ. Toxicol. Chem. 34, 2475–2488. https://doi.org/10.1002/etc.3125

Florence, A.T., Attwood, D., 2006. Physicochemical Principles of Pharmacy. Pharm. Press 286–290.

García-Cambero, J., García-Cortés, H., Valcárcel, Y., Catalá, M., 2015. Environmental concentrations of the cocaine metabolite benzoylecgonine induced sublethal toxicity in the development of plants but not in a zebrafish embryo–larval model. J. Hazard. Mater. 300, 866–872. https://doi.org/10.1016/j.jhazmat.2015.08.019

Gheorghe, A., Van Nuijs, A., Pecceu, B., Bervoets, L., Jorens, P.G., Blust, R., Neels, H., Covaci, A., 2008. Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry. Anal. Bioanal. Chem. 391, 1309–1319. https://doi.org/10.1007/s00216-007-1754-5

Global CCS Institute, 2017. Global Costs of Carbon Capture and Storage - 2017 Update 14.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17. https://doi.org/10.1016/S0378-4274(02)00041-3

Hernández, F., Ibáñez, M., Botero-Coy, A.-M., Bade, R., Bustos-López, M.C., Rincón, J., Moncayo, A., Bijlsma, L., 2015. LC-QTOF MS screening of more than 1,000 licit and illicit drugs and their metabolites in wastewater and surface waters from the area of Bogotá, Colombia. Anal. Bioanal. Chem. 407, 6405–6416. https://doi.org/10.1007/s00216-015-8796-x

Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. AJP Regul. Integr. Comp. Physiol. 307, R1061–R1084. https://doi.org/10.1152/ajpregu.00064.2014

Huerta-Fontela, M., Galceran, M.T., Martin-Alonso, J., Ventura, F., 2008. Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain. Sci. Total Environ. 397, 31–40. https://doi.org/http://dx.doi.org/10.1016/j.scitotenv.2008.02.057

Huerta-Fontela, M., Galceran, M.T., Ventura, F., 2007. Ultraperformance liquid chromatography tandem mass spectrometry analysis of stimulatory drugs of abuse in wastewater and surface waters. Anal. Chem. 79, 3821–3829. https://doi.org/10.1021/ac062370x

IBGE, 2015. INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA. Estimativas da população residente para os municípios e para as unidades da federação brasileiros com data de referência em 1º de julho de 2015. 1–8.

IEA, 2010. Carbon capture and storage: Model Regulatory Framework. JAMA 303, 1601; author reply 1601. https://doi.org/10.1001/jama.2010.513

IPCC, 2014. Summary for Policymakers, Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https://doi.org/10.1017/CBO9781107415324

Jonsson, M., Fick, J., Klaminder, J., Brodin, T., 2014. Antihistamines and aquatic insects: Bioconcentration and impacts on behavior in damselfly larvae (Zygoptera). Sci. Total Environ. 472, 108–111. https://doi.org/10.1016/j.scitotenv.2013.10.104

Kapsenberg, L., Okamoto, D.K., Dutton, J.M., Hofmann, G.E., 2017. Sensitivity of sea urchin fertilization to pH varies across a natural pH mosaic. Ecol. Evol. 7, 1737–1750. https://doi.org/10.1002/ece3.2776

Karolak, S., Nefau, T., Bailly, E., Solgadi, A., Levi, Y., 2010. Estimation of illicit drugs consumption by wastewater analysis in Paris area (France). Forensic Sci. Int. 200, 153–160. https://doi.org/10.1016/j.forsciint.2010.04.007

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res. 43, 363–380. https://doi.org/10.1016/j.watres.2008.10.047

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water Res. 42, 3498–3518. https://doi.org/10.1016/j.watres.2008.04.026

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2007. Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultraperformance liquid chromatography-positive electrospray ionization

tandem mass spectrometry. J. Chromatogr. A 1161, 132–145. https://doi.org/10.1016/j.chroma.2007.05.074

Kirchsteiger, C., 2008. Carbon capture and storage-desirability from a risk management point of view. Saf. Sci. 46, 1149–1154. https://doi.org/10.1016/j.ssci.2007.06.012

Lackner, K.S., Brennan, S., 2009. Envisioning carbon capture and storage: Expanded possibilities due to air capture, leakage insurance, and C-14 monitoring. Clim. Change 96, 357–378. https://doi.org/10.1007/s10584-009-9632-0

Leung, D.Y.C., Caramanna, G., Maroto-Valer, M.M., 2014. An overview of current status of carbon dioxide capture and storage technologies. Renew. Sustain. Energy Rev. 39, 426–443. https://doi.org/10.1016/j.rser.2014.07.093

Lüthi, D., Le Floch, M., Bereiter, B., Blunier, T., Barnola, J.-M., Siegenthaler, U., Raynaud, D., Jouzel, J., Fischer, H., Kawamura, K., Stocker, T.F., 2008. High-resolution carbon dioxide concentration record 650,000-800,000 years before present. Nature 453, 379–382. https://doi.org/10.1038/nature06949

Maranho, L.A., Fontes, M.K., Kamimura, A.S.S., Nobre, C.R., Moreno, B.B., Pusceddu, F.H., Cortez, F.S., Lebre, D.T., Marques, J.R., Abessa, D.M.S., Ribeiro, D.A., Pereira, C.D.S., 2017. Exposure to crack cocaine causes adverse effects on marine mussels *Perna perna*. Mar. Pollut. Bull. 0–1. https://doi.org/10.1016/j.marpolbul.2017.08.043

McNeil, B.I., Sasse, T.P., 2016. Future ocean hypercapnia driven by anthropogenic amplification of the natural CO_2 cycle. Nature 529, 383–386. https://doi.org/10.1038/nature16156

Metcalfe, C., Tindale, K., Li, H., Rodayan, A., Yargeau, V., 2010. Illicit drugs in Canadian municipal wastewater and estimates of community drug use. Environ. Pollut. 158, 3179–3185. https://doi.org/10.1016/j.envpol.2010.07.002

Millero, F.J., Pierrot, D., Lee, K., Wanninkhof, R., Feely, R., Sabine, C.L., Key, R.M., Takahashi, T., 2002. Dissociation constants for carbonic acid determined from eld measurements. Deep. Res. 49, 1705–1723.

Muñoz, I., López-Doval, J.C., Ricart, M., Villagrasa, M., Brix, R., Geiszinger, A., Ginebreda, A., Guasch, H., de Alda, M.J.L., Romaní, A.M., Sabater, S., Barceló, D., 2009. Bridging levels of pharmaceuticals in river water with biological community structure in the Llobregat River basin (northeast Spain). Environ. Toxicol. Chem. 28, 2706–14. https://doi.org/10.1897/08-486.1

Nogueira, L., Mello, D.F., Trevisan, R., Garcia, D., da Silva Acosta, D., Dafre, A.L., de Almeida, E.A., 2017. Hypoxia effects on oxidative stress and immunocompetence biomarkers in the mussel *Perna perna* (Mytilidae, Bivalvia). Mar. Environ. Res. 126, 109–115. https://doi.org/10.1016/j.marenvres.2017.02.009

Ortega, A. dos S.B., Maranho, L.A., Nobre, C.R., Moreno, B.B., Guimarães, R.S., Lebre, D.T., de Souza Abessa, D.M., Ribeiro, D.A., Pereira, C.D.S., 2018. Detoxification, oxidative stress, and cytogenotoxicity of crack cocaine in the brown mussel *Perna perna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-018-1600-7

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

Parolini, M., De Felice, B., Ferrario, C., Salgueiro-González, N., Castiglioni, S., Finizio, A., Tremolada, P., 2018. Benzoylecgonine exposure induced oxidative stress and altered swimming behavior and reproduction in *Daphnia magna*. Environ. Pollut. 232, 236–244. https://doi.org/10.1016/j.envpol.2017.09.038

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

Parolini, M., Pedriali, A., Riva, C., Binelli, A., 2013. Sub-lethal effects caused by the cocaine metabolite benzoylecgonine to the freshwater mussel *Dreissena polymorpha*. Sci. Total Environ. 444, 43–50. https://doi.org/10.1016/j.scitotenv.2012.11.076

Passarelli, M.C., Cesar, A., Riba, I., DelValls, T.A., 2017a. Comparative evaluation of sea-urchin larval stage sensitivity to ocean acidification. Chemosphere 184, 224–234. https://doi.org/10.1016/j.chemosphere.2017.06.001

Passarelli, M.C., Riba, I., Cesar, A., Serrano-Bernando, F., DelValls, T.A., 2017b. Assessing the influence of ocean acidification to marine amphipods: A comparative study. Sci. Total Environ. 595, 759–768. https://doi.org/10.1016/j.scitotenv.2017.04.004

Pereira, C.D.S., Maranho, L.A., Cortez, F.S., Pusceddu, F.H., Santos, A.R., Ribeiro, D.A., Cesar, A., Guimarães, L.L., 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Sci. Total Environ. 548–549, 148–154. https://doi.org/10.1016/j.scitotenv.2016.01.051

Pires, J.C.M., Martins, F.G., Alvim-Ferraz, M.C.M., Simões, M., 2011. Recent developments on carbon capture and storage: An overview. Chem. Eng. Res. Des. 89, 1446–1460. https://doi.org/10.1016/j.cherd.2011.01.028

Quinn, B., Gagné, F., Weber, J.P., Blaise, C., 2005. Ecotoxicological effects of a semi-submerged municipal dump (Castle harbour, Bermuda) on the *Calico scallop Argopecten gibbus*. Mar. Pollut. Bull. 51, 534–544. https://doi.org/10.1016/j.marpolbul.2005.07.019

Rato, L.D., Novais, S.C., Lemos, M.F.L., Alves, L.M.F., Leandro, S.M., 2017. *Homarus gammarus* (Crustacea: Decapoda) larvae under an ocean acidification scenario: responses across different levels of biological organization. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 203, 29–38. https://doi.org/10.1016/j.cbpc.2017.09.002

Riba, I.L., DelValls, T.A., Forja, J.M., Gómez-Parra, A., 2004. The influence of pH and salinity on the toxicity of heavy metals in sediment to the estuarine clam *Ruditapes philippinarum*. Environ. Toxicol. Chem. 23, 1100–1107. https://doi.org/10.1897/023-601

Rosi-Marshall, E.J., Kincaid, D.W., Bechtold, H.A., Royer, T. V., Rojas, M., Kelly, J.J., 2013. Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms. Ecol. Appl. 23, 583–593. https://doi.org/10.1890/12-0491.1

Rosi-Marshall, E.J., Royer, T. V., 2012. Pharmaceutical Compounds and Ecosystem Function: An Emerging Research Challenge for Aquatic Ecologists. Ecosystems 15, 867–880. https://doi.org/10.1007/s10021-012-9553-z

Rosi-Marshall, E.J., Snow, D., Bartelt-Hunt, S.L., Paspalof, A., Tank, J.L., 2015. A review of ecological effects and environmental fate of illicit drugs in aquatic ecosystems. J. Hazard. Mater. 282, 18–25. https://doi.org/10.1016/j.jhazmat.2014.06.062

Schmittner, A., Oschlies, A., Matthews, H.D., Galbraith, E.D., 2008. Future changes in climate, ocean circulation, ecosystems, and biogeochemical cycling simulated for a business-as-usual CO_2 emission scenario until year 4000 AD. Global Biogeochem. Cycles 22, 1–21. https://doi.org/10.1029/2007GB002953

Sekizawa, A., Uechi, H., Iguchi, A., Nakamura, T., Kumagai, N.H., Suzuki, A., Sakai, K., Nojiri, Y., 2017. Intraspecific variations in responses to ocean acidification in two branching coral species. Mar. Pollut. Bull. 122, 282–287. https://doi.org/10.1016/j.marpolbul.2017.06.061

Sui, Q., Huang, J., Deng, S., Chen, W., Yu, G., 2011. Seasonal variation in the occurrence and removal of pharmaceuticals and personal care products in different biological wastewater treatment processes. Environ. Sci. Technol. 45, 3341–3348. https://doi.org/10.1021/es200248d

Szalaj, D., De Orte, M.R., Goulding, T.A., Medeiros, I.D., DelValls, T.A., Cesar, A., 2017. The effects of ocean acidification and a carbon dioxide capture and storage leak on the early life stages of the marine mussel *Perna perna* (Linneaus, 1758) and metal bioavailability. Environ. Sci. Pollut. Res. 24, 765–781. https://doi.org/10.1007/s11356-016-7863-y

Taylor, P., Lichtschlag, A., Toberman, M., Sayer, M.D.J., Reynolds, A., Sato, T., Stahl, H., 2015. Impact and recovery of pH in marine sediments subject to a temporary carbon dioxide leak. Int. J. Greenh. Gas Control 38, 93–101. https://doi.org/10.1016/j.ijggc.2014.09.006

Thomaidi, V.S., Stasinakis, A.S., Borova, V.L., Thomaidis, N.S., 2015. Is there a risk for the aquatic environment due to the existence of emerging organic contaminants in treated domestic wastewater? Greece as a case-study. J. Hazard. Mater. 283, 740–747. https://doi.org/10.1016/j.jhazmat.2014.10.023

UNODC, 2014. World Drug Report 2014, United Nations publication. https://doi.org/10.1007/s12117-997-1166-0

UNODC, 2012. World Drug Report 2012, New Directions for Youth Development. https://doi.org/10.1002/yd.20002

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

Zhan, Y., Hu, W., Duan, L., Liu, M., Zhang, W., Chang, Y., Li, C., 2017. Effects of seawater acidification on early development of the sea urchin *Hemicentrotus pulcherrimus*. Aquac. Int. 25, 655–678. https://doi.org/10.1007/s10499-016-0064-3

Zuccato, E., Castiglioni, S., 2009. Illicit drugs in the environment. Philos. Trans. A. Math. Phys. Eng. Sci. 367, 3965–78. https://doi.org/10.1098/rsta.2009.0107

Zuccato, E., Castiglioni, S., Bagnati, R., Chiabrando, C., Grassi, P., Fanelli, R., 2008. Illicit drugs, a novel group of environmental contaminants. Water Res. 42, 961–968. https://doi.org/10.1016/j.watres.2007.09.010

Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S., Fanelli, R., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. Environ. Heal. A Glob. Access Sci. Source 4, 1–7. https://doi.org/10.1186/Received

Chapter II. Weight-of-evidence approach for assessing effects associated with CO_2 acidification and bioactive compounds in aquatic ecosystems

Introduction

Ocean acidification, a predictable consequence of rising atmospheric CO_2, and related changes in ocean carbonate chemistry will contribute to major changes in marine ecosystems. In chapter II, the main objective is to review the scientific and technological advances that have been developed in recent years to evaluate the behavior of contaminants, and their toxic effects, when subjected to different scenarios of CO_2 acidification. The main objective is not only reviewing the previous studies but update them to allow a well design of the risk assessment based on an integrated approach.

Annual anthropogenic greenhouse gases (GHG) emissions have increased by 10 GtCO2 eq between 2000 and 2010, with this increase directly coming from energy supply (47 %), industry (30 %), transport (11%) and buildings (3 %) sectors (IPCC, 2014b). Since 2000, GHG emissions have been growing in all sectors, except AFOLU. Of the 49 (±4.5) GtCO2 eq emissions in 2010, 35 % (17 GtCO2eq) of GHG emissions were released in the energy supply sector, 24 % (12 GtCO2 eq, net emissions) in AFOLU, 21 % (10 GtCO2 eq) in industry, 14 % (7.0 GtCO2 eq) in transport and 6.4 % (3.2 GtCO2eq) in buildings (Figure 1) (IPCC, 2014b).

Since industrial revolution, sustained absorption of anthropogenic derived CO_2 leads to ocean acidification (OA), which has already led to a reduction of 0.1 units in global surface seawater pH (Caldeira and Wickett, 2003). With continuous and increasing release of anthropogenic CO_2, ocean pH is projected to reduce by a further 0.3–0.5 units by 2100 (Gattuso and Lavigne, 2009)

Large-scale carbon dioxide capture and storage (CCS) in geological formations is a mitigation strategy that have been developed and proposed to reduce atmospheric CO_2 levels, trapping emissions of greenhouse gases (such as carbon dioxide, CO_2) from large point sources (such as fossil power plants and industrial sites) and pumping the gas to underground in order to keep them away safely instead of releasing them into the atmosphere. Therefore, concerns about the possibility of leakage and potential environmental impacts are still largely unknow (Keating et al., 2011).

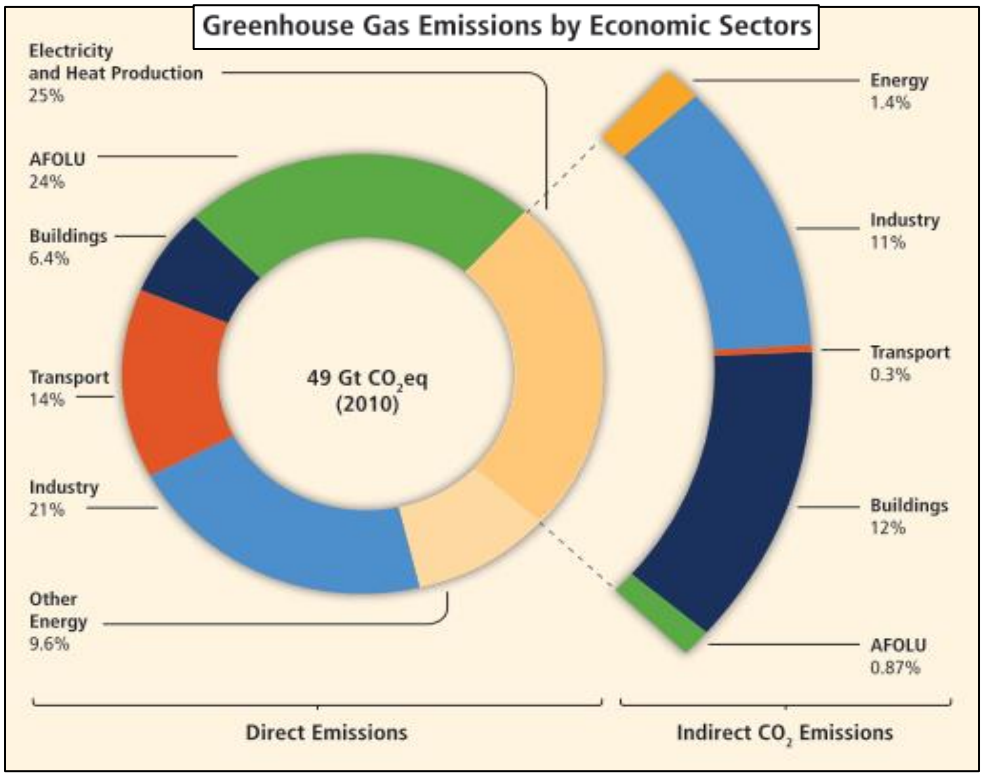

Figure 1: Total anthropogenic GHG emissions (GtCO2eq / yr) by economic sectors. Inner circle shows direct GHG emission shares (in % of total anthropogenic GHG emissions) of five economic sectors in 2010 (by IPCC, 2014)

Changes in seawater chemistry caused by ocean acidification could cause various effects on marine organisms (Clements and Chopin, 2017; M.C. Passarelli et al., 2018; Silva et al., 2016). In addition to biological changes, accumulation of toxic metals present in coastal waters is expected to be modified by ocean acidification through changes in physiological performance and/or elements availability (de Orte et al., 2014c; Payán et al., 2012; Stockdale et al., 2016).

The changes in bioaccumulation due to lowering pH are likely to be differently affected depending on the nature (essential or non-essential) and speciation of each element. Dorey et al. (2018) observed that CO_2 induced pH changes can modify the bioaccumulation rates of metals in sea urchin larvae and concluded that the relationship between the changes in speciation, bioaccumulation and toxicity are not straightforward and urgently requires more research. The current knowledge about the effect of *p*CO2-driven ocean acidification on the bioaccumulation of organic pollutants in marine species is still scarce, as only limited types of pollutants have been investigated (Su et al., 2019), mainly metals.

One scientific article is included in this chapter that was published in an international well recognized impact journal: *Environmental Reviews*. A literature review of CO_2 enrichment effects in the marine ecosystems were done in the article entitle ***Integrative assessment of sediment quality in acidification scenarios associated with carbon capture and storage operations***. Also, the article includes the updated related to some results not yet published to demonstrate the need of using the integrative approach. Besides, the article for the first time shows results related to the combined effects of Crack/Cocaine and CO_2 induced acidification. The article addressed not only the effects of acidification but also the combined effect of acidification on some of the contaminants known worldwide for their adverse effects on marine organisms.

Metals and ocean acidification are likely to exert interactive effects on the physiology of marine organisms (Bibby et al., 2008; Cao et al., 2018; Khosrovyan et al., 2014). The main proposed of this part is to distinguish between the biological adverse effects associated with the acidification itself compared to the increase of metals bioavailability associated with the acidification by the enrichment of CO_2. In general, studies considering different contaminated samples by metals (and metalloids) and in different matrix (sediment and water) have been used to expose different organisms to acidification conditions by enrichment of CO_2. A considering number of studies have address to this problematic

The effects of acidification by CO_2 enrichment on the behavior of organic pollutants are also discussed, although there is a lack of reported data in the bibliography that address the influence of aquatic ecosystems enriched by CO_2 associated with change in bioavailability of organic contaminants. Studies in this line of evidence (LOEs) will be presented in the later chapters of this thesis (chapter III, IV and V) where effects of CO_2 enrichment on the bioavailability of the illicit drug crack-cocaine will be presented.

Studies conducted by means of mesocosms and under field conditions, using macrobenthic community or caged animals, showed impacts on the diversity, richness and biomass of this community. Recovery of the community is reported in studies conducted under field conditions, at acidification scenarios, without considering the potential contamination of the sites studied. The difficulty of the adverse biological effect's evaluation resides on the measurement of 'in situ' effects. There are no easy ways to reproduce CO_2 leakage rates under field conditions and only big approaches allow this kind of surveys compared to laboratory surveys. The main propose of the

use of these LOEs is to distinguish between adverse effects associated with contaminants than those related to acidification by enrichment of CO_2 alone.

None of the previous studies have been designed or conducted under an integrative point of view. Neither, it has been proposed to employ these LOEs under a weight-of-evidence approach in risk characterization and management of CCS operations and other situations related to acidification by enrichment of CO_2 in the aquatic ecosystem. The combination of acidification and contamination must be solved under an integrative assessment. The best option is to use multiples lines of evidence, which will be linked based on a weight of evidence approach. This kind of research to solve the effects related to the combination of acidification and contamination is not only necessary, but urgent to be addressed under an integrated point of view.

References

Bibby, R., Widdicombe, S., Parry, H., Spicer, J., Pipe, R., 2008. Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. Aquat. Biol. 2, 67–74. https://doi.org/10.3354/ab00037

Caldeira, K., Wickett, M.E., 2003. Anthropogenic carbon and ocean pH. Nature 425, 365–365. https://doi.org/10.1038/425365a

Cao, R., Liu, Y., Wang, Q., Zhang, Q., Yang, D., Liu, H., Qu, Y., Zhao, J., 2018. The impact of ocean acidification and cadmium on the immune responses of Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol. 81, 456–462. https://doi.org/10.1016/j.fsi.2018.07.055

Clements, J.C., Chopin, T., 2017. Ocean acidification and marine aquaculture in North America: potential impacts and mitigation strategies. Rev. Aquac. 9, 326–341. https://doi.org/10.1111/raq.12140

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

Dorey, N., Martin, S., Oberhänsli, F., Teyssié, J.L., Jeffree, R., Lacoue-Labarthe, T., 2018. Ocean acidification modulates the incorporation of radio-labeled heavy metals in the larvae of the Mediterranean sea urchin *Paracentrotus lividus*. J. Environ. Radioact. 190–191, 20–30. https://doi.org/10.1016/j.jenvrad.2018.04.017

Gattuso, J.-P., Lavigne, H., 2009. Perturbation experiments to investigate the impact of ocean acidification: approaches and software tools. Biogeosciences Discuss. 6, 4413–4439. https://doi.org/10.5194/bgd-6-4413-2009

IPCC, 2014. Climate Change 2014: Mitigation of Climate Change. Summary for Policymakers and Technical Summary, Climate Change 2014: Mitigation of Climate Change. Part of the

Working Group III Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https://doi.org/10.1017/CBO9781107415416.005

Keating, E.H., Hakala, J.A., Viswanathan, H., Capo, R., Stewart, B., Gardiner, J., Guthrie, G., William Carey, J., Fessenden, J., 2011. The challenge of predicting groundwater quality impacts in a CO_2 leakage scenario: Results from field, laboratory, and modeling studies at a natural analog site in New Mexico, U.S.A. Energy Procedia 4, 3239–3245. https://doi.org/10.1016/j.egypro.2011.02.242

Khosrovyan, A., DelValls, T.A., Riba, I., 2014. Effects of simulated CO_2 escape from sediments on the development of midge *Chironomus riparius*. Aquat. Toxicol. 156, 230–239. https://doi.org/10.1016/j.aquatox.2014.09.005

Passarelli, M.C., Riba, I., Cesar, A., DelValls, T.A., 2018. What is the best endpoint for assessing environmental risk associated with acidification caused by CO_2 enrichment using mussels? Mar. Pollut. Bull. 128, 379–389. https://doi.org/10.1016/j.marpolbul.2018.01.055

Payán, M.C., Verbinnen, B., Galan, B., Coz, A., Vandecasteele, C., Viguri, J.R., 2012. Potential influence of CO_2 release from a carbon capture storage site on release of trace metals from marine sediment. Environ. Pollut. 162, 29–39. https://doi.org/10.1016/j.envpol.2011.10.015

Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E.J., Faria, A.M., 2016. Effects of ocean acidification on the swimming ability, development and biochemical responses of sand smelt larvae. Sci. Total Environ. 563–564, 89–98. https://doi.org/10.1016/j.scitotenv.2016.04.091

Stockdale, A., Tipping, E., Lofts, S., Mortimer, R.J.G., 2016. Effect of Ocean Acidification on Organic and Inorganic Speciation of Trace Metals. Environ. Sci. Technol. 50, 1906–1913. https://doi.org/10.1021/acs.est.5b05624

Su, W., Shi, W., Han, Y., Hu, Y., Ke, A., Wu, H., Liu, G., 2019. The health risk for seafood consumers under future ocean acidification (OA) scenarios: OA alters bioaccumulation of

three pollutants in an edible bivalve species through affecting the in vivo metabolism. Sci. Total Environ. 650, 2987–2995. https://doi.org/10.1016/j.scitotenv.2018.10.056

Integrative assessment of sediment quality in acidification scenarios associated with carbon capture and storage operations

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Abstract

Nowadays it has been applied a new technology to fight against the global change by decreasing the concentration of CO_2 in the atmosphere; it consists in its capture and storage (CCS) in stable geological structures. This paper shows the advance in the risk assessment related to potential acidification associated with this technology by CO_2 enrichment in the aquatic ecosystem. It reviews and updates the different lines of evidence (LOEs) used to characterize the effects of the

acidification and the combination of it with contamination of sediments in aquatic environments. It shows and discusses the effects of acidification on the LOEs: Contamination and mobility of contaminants in sediments, toxicity, macro-benthic community structure, in situ effects and bioaccumulation/biomagnification processes. Also, it is commented the results of the acidification on the toxicity of organic contaminants such us antibiotics or illicit drugs like crack/cocaine. The main propose of the use of these LOEs is to distinguish between adverse effects associated with contaminants than those related to acidification by enrichment of CO_2 alone. None of the previous studies have been designed or conducted under an integrative point of view. Neither, it has been proposed to employ these LOEs under a weight-of-evidence approach in risk characterization and management of CCS operations and other situations related to acidification by enrichment of CO_2 in the aquatic ecosystem.

Key-words**:** bioavailability; emerging pollutants; metal(loid)s; risk assessment; sediment assessment; Weight-of-Evidence.

1. Introduction

1.1. Aquatic ecosystem acidification and mitigative efforts

Carbon Capture and Storage (CCS) technology have a main purpose to mitigate the increase of CO_2 concentration in the atmosphere, balancing the thermodynamic and also the kinetic in the chemical process of organic matter combustion.

The CCS initiative has been developed in the last years and nowadays it is applied around the world with a well-established technology that implies the process of carbon sequestration (Global CCS Institute, 2016; IPCC, 2012) and its potential decreases the amount of CO_2 in the atmosphere. Reguera et al. (2009) reported the initiatives taken by different international conventions, such as London Convention, OSPAR etc., to regulate the use of this technology. These conventions and protocols have been amended to allow the storage of CO_2 in the sub-seabed geological formations and to elaborate comprehensive guidelines in Risk Assessment and Management of the storage areas. These regulations will ensure that the CCS activity does not lead to significant adverse consequences either for marine environment, human health or other legitimate use of the sea (London Convention, 2007, 2006; OSPAR Guidelines, 2007).

The Global Carbon Capture and Storage Institute identified 17 large-scale CCS facilities operating globally, with four more coming on stream in 2018 (Global CCS Institute, 2017). Most of these projects are conducted in countries signatories to different international conventions. Twelve of the 17-large scale facilities in operation are located in the United States and Canada and two of those came on stream in the past twelve months (Petra Nova and Illinois Industrial). In Europe, Middle East and Africa (EMEA), four large scale facilities are operating successfully (two in Norway and two in the Middle East), with two more in early development in the United Kingdom. In South America, there is only one CCS large scale system operating. It is located in the Santos Basin (Brazil) where the offshore facilities have injected over 4 million tones of CO_2 (Global CCS Institute, 2017).A significant number of these projects may impact natural ecosystems. However, political uncertainties have generated a slow rate in the regulatory development, including countries with sophisticated legal regimes for CCS, such as Australia, Canada, Denmark, United Kingdom and USA, which have been stalled since 2015. In addition,

the Global CCS Institute highlighted the need of establishing (region-relevant) public/private business models that better manage risk allocation between the capture, transport and storage elements of the CCS chain, thus reducing overall risks (Global CCS Institute, 2017). In this sense, most of the projects are under the regulations proposed by the different international conventions and are running different risk characterization and risk management approaches to minimize and monitoring them (Global CCS Institute, 2016).

1.2. Potential adverse effects associated with CCS in aquatic ecosystems

There are different natural and anthropogenic processes that can increase the acidity in aquatic ecosystems (marine and freshwater). For instance, the atmospheric depositions of CO_2, submarine volcanic activity, sulfate, and nitrate runoffs from watersheds, organic diagenesis, etc. These processes will produce an acute (short term) impact on the aquatic ecosystems since the release of CO_2 in it (sediment/water) will decrease the pH (acidification). These changes in pH are directly related to the partial pressure of CO_2 and the chemical buffering capacity of the seawater.

However, an accidental leakage of CO_2 gas from the geological structures used in CCS sites or during its storage operations may cause sudden and indefinitely acidification in the surrounding environments which will affect both onshore and offshore ecosystems besides water supplies (DelValls and Riba, 2007). The effects of CO_2 leakage will depend on the amount and/or rate of leakage; the transport and dispersion processes; and the chemical buffering capacity of the sedimentary or water system. For instance, it has been recently demonstrated in a controlled CO_2 gas release under ‘in situ’ conditions (QICS experiment - Quantifying and Monitoring Potential

Ecosystem Impacts of Geological Carbon Storage by Blackford et al. (2014)that after an event of leakage followed by gas supply stopped, the time that the pore water took to reach normal pH values in marine waters is about 3 weeks. In the experiment, the total dissolved inorganic carbon (DIC) within the sediment, mainly in interstitial and pore waters above the release zone, showed that up to 63% of the carbon dioxide released during the experiment could remain in the dissolved phase within the sediment pore water (Taylor et al. 2015). Thus, impacts associated with an acidification scenario must be addressed. Furthermore, the environment acidification might enhance the adverse biological effects of the existing contaminants and/or make them more bioavailable (Riba et al. 2003; Riba et al. 2004a). In the QICS experiment (Blackford et al. 2014)the selected area for the CO_2 release was low contaminated and neither the effects of the contamination combined with acidification, nor the potential adverse effects associated with changes in their bioavailability were evaluated.

Most of the CCS projects in execution include the regulation established in the International Conventions, performing different risk characterizations and management options. Six main steps are followed to improve the management: (a) problem recognition; (b) selection and site characterization ; (c) exposure assessment; (d) effects assessment; (e) risk characterization and its management (including monitoring and mitigation) (Reguera et al. 2009). Several impacts on biogeochemistry levels are characterized during projects execution and gas release in the field, such as in the QCIS experiment (Blackford et al. 2014), which requires a multiple and multivariate approaches, followed by a correct monitoring of these processes and their impacts (Blackford et al. 2015). However, in the recent studies the ecotoxicological impacts determined were focused exclusively on acidification "per se" and the interaction with contaminants was neither considered

nor fully addressed. Moreover, it was not used a weight of evidence approach to address the environmental and human risk.

The effects of CO_2 leakage in freshwater environments have been receiving little attention in recent years; nevertheless, these ecosystems can be affected by leaks from different sources: (a) depleted oil and gas fields; (b) Underground storages (since technical solutions include onshore storage facilities such as aquifers); (c) deep coal seams, and (d) unmineable coal seams. These processes may represent a leakage of CO_2 and its propagation an environmental and human risk, which must also be considered in the risk characterization.

We discuss in this article the progress made in the last years regarding to the evaluation in adverse effects associated with different scenarios of acidification caused mainly by potential leaks during CCS. In addition, some of these reported results will be discussed to get a clearer idea of the impact on acidification associated with CO_2 enrichment in aquatic environments. It will be conducted using different lines of evidence in the framework of the International convention guidelines that has been previously reported and recommends the use of a Weight-of-Evidence (WOE) approach for the sediment quality assessment in environments suffering acidification by CO_2. The effort of this paper is to highlight the feasibility and strengths of using WOE to environmental risk characterization in aquatic sediments affected by acidification related to enrichment of CO_2.

2. Materials and Methods

2.1.Weight-of-evidence (WOE) approach

The WOE implies the use of multiple lines of evidence (LOEs) to determine the sediment quality in different aquatic ecosystems affected by acidification from CO_2enrichment under an integrative approach. The typical lines of evidence used in the WOE approach includes: (a) Aquatic Contamination: chemical concentration (including the speciation of CO_2) in sediments, interstitial and overlaying waters; (b) Toxicity: laboratory experiments to evaluate the effects of the acidification and contaminants in the aquatic ecosystem; (c) Ecological integrity: in situ exposure experiments to determine de effects of the acidification and contaminants in the aquatic ecosystem; (d) Bioaccumulation/Biomagnification: to establish the effects of the contaminants mobility in organisms and related it with human health by consumption of contaminated species. The WOE can integrate the mentioned lines of evidence using different approaches previously reported by Riba et al. (2004a) which includes multivariate analysis, chart diagrams, pollution index and tabular decision matrix based on a professional best judgment (Riba et al. 2004a; DelValls and Riba 2007). A schematic representation of the WOEs is included in the Fig.1. The application of this method will be paramount importance; especially if it is suspected either the transposition of CO_2 above the CCS formation can get extended to the seafloor (or groundwater) or in cases where storage site is near to sensitive or endangered habitats and species. The WOE will allow monitoring the sediments and overlaid water (in both marine and freshwater ecosystems) to detect and measure possible leakage of CO_2 (and incidental associated substances) into the aquatic environment. In this context special attention should be given to wells that intersect

the storage formation; and monitoring biological communities to detect and measure the effects of leakages on aquatic organisms and to the ecosystems.

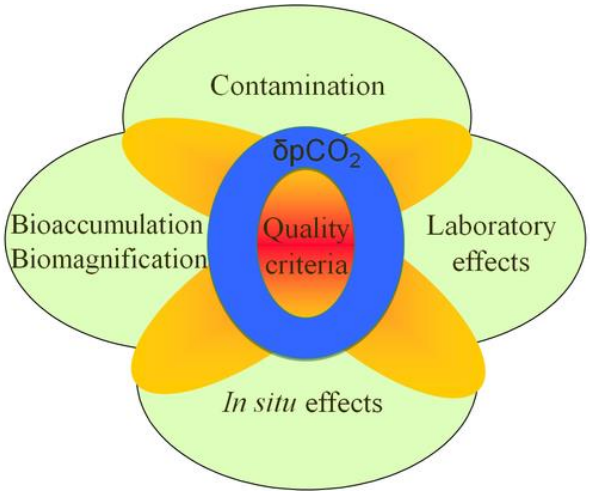

Fig.1. Schematic representation of a weight-of-evidence approach used to address the four questions in risk characterization (adapted of DelValls (2007): a) What contaminants? b) What level of contaminants? c) What biological effects? d) Does it exist a bioaccumulation/biomagnification of contaminants? These four questions must be addressed to risk characterization in scenarios of CO_2-induced acidification and contamination of environmental samples. The main aim is to distinguish the risk associated with the acidification, with the contamination and with the combination of these two processes.

The WOE approach will allow measuring the effect of small and diffuse leakage in both short or long term and the degree of contamination originated by this leakage (DelValls and Riba, 2007). Moreover, then the acidification impact on the aquatic ecosystem, the effects of the incidental associated substances and the existing substances mobilized by the injection and storage of CO_2 streams (e.g., brine) can also be assessed with this methodology.

3. Results and Discussion

In this section is included an upgrade of the different approaches and results recently used to establish the acidification impact on the aquatic ecosystems using different lines of evidence. These LOEs were individually used and no WOE was applied in any of the revised studies. It is also discussed if these LOEs are used or not in running projects of CCS.

3.1. Contamination

This line of evidence must address the acidification influence in variation of the water/sediment/benthic parameters to establish a potential bioavailability of contaminants, including emergent contaminants. Different environmental samples collected from potential CO_2 storage sites and surrounding areas will be exposed to CO2-induced acidification. It will be necessary to study the fluxes variations of the different contaminants as a function of the acidification by enrichment of CO_2, combined with the evolution of pH values and inorganic carbon concentration. This kind of simulation will help to predict the behavior and potential effects of the contaminants when they change their reactivity, mobility etc., relating to sediment acidification. Also, this kind of approaches will be helpful for the site selection during CCS operations.

There are different studies that have addressed the environmental impact of acidification by CO_2 leakages in the aquatic sediments considering the modification of the chemical equilibriums, moreover those that include the link between the acidification and the contamination in sediments, mainly by metals. Some of them are commented below.

Dewar et al. (2013) proposed a model to understand the dissolution of CO_2 in the form of bubbles/droplets, associated with a leakage. They model the behavior to fully dissolve before reach the water surface. It is well-known that the increase of CO_2 determines changes in most of the chemical equilibriums as a result of the acidification changes as outlined by Lichtschlag et al. (2015). These authors participated in a controlled release of CO_2 field experiment in a marine environmental ecosystem by means of monitoring during one year the physicochemical changes in sediment and water. They found different changes related to increases in concentrations of inorganic dissolved species of carbonic acid. These high concentrations were measured during the first 5 weeks of the experiment reaching a magnitude of concentration higher than the natural. Also, it was found changes in alkalinity and other carbonic acid parameters together with an increase in the dissolution of carbonate and silicate minerals. Three weeks after the CO_2 enrichment was stopped most of the chemical components return to the normal values. These authors did not monitor the influence of these changes in potential contaminants mobility, like metals or organic compounds, probably for inexistence of these compounds in the study area (Dewar et al. 2013; Lichtschlag et al. 2015).

There are cases where studies addressed the influence of CO_2 in physicochemical changes under laboratory conditions to contaminant mobility. Payán et al. (2012), for instance, reported the influence of CO_2 leakage on metals using standardized leaching tests. They used different type of waters and acidification conditions (deionized water, natural seawater and acidified seawater at pH 5.0, 6.0, and 7.0) obtained by CO_2 bubbling. They reported that at the different solutions tested all the metals increase mobility and bioavailability when the proton concentration increased (decrease in pH values).

De Orte et al. (2014a, 2014b, 2014c, 2018) have reported significant changes in the mobility and chemical speciation of different metals when reached by acidification processes associated with CO_2 enrichment. In general, all the metals analyzed (Fe, Al, Cr, Co, Ni, Cu, Zn, Pb), in different sediments, with different concentrations, showed an increase in mobility when increases concentration of protons. They also reported that the acidification by CO_2 enrichment produces an increase in concentration of some metals and metalloids in different stations relating it to potential environmental risk due to the mobility of metal(loid)s and the increase of their bioavailability. In some cases, like As, the concentration did not show increase with the acidification, but its chemical speciation has changed. These changes were associated with increases of chemical species more toxic of this metalloid, like As (III).

In summary, all these studies concluded that generally the increase in metal bioavailability was related to the increase of proton concentration by enrichment of CO_2. This effect was significantly intense when the metals were bioavailable in interstitial waters (de Orte et al. 2014b; De Schamphelaere et al. 2002; Riba et al. 2010). Similar results were reported by other authors related to different metals in interstitial and sea water (Millero et al. 2009; Wang et al. 2015).The mobility and chemical speciation of different contaminants is one of the LOEs included in the monitoring programs of practically 100% of the running projects (Global CCS Institute, 2016).

Nevertheless, there is a lack of reported data in the bibliography that address the influence of aquatic ecosystems enriched by CO_2 associated with change in bioavailability of organic contaminants. Sircar (2014) showed that antibiotics change net charge in different pH values. Based on that, the neutral form is considered most bioavailable and therefore most toxic than its metabolites. They postulate that in seawater with high concentration of CO_2 (acidic conditions) polar organic contaminants will be differently charged than in current seawater pH conditions.

3.2. Toxicity

The toxicity of the environmental samples around the CO_2 injection and storage sites is usually carried out by means of bioassays under 'in situ' and laboratory conditions. These bioassays are needed to quantify the exposure pathways of the contaminants and their negative effects by measuring different adverse biological effects (mortality, reproduction, biomarkers of effect, etc.). High CO_2 levels in the environment may cause asphyxiation (impair respiration in organisms) (Seibel, 2016), acidosis (lowering of pH in animal body fluids) (Esbaugh, 2017) and hypercapnia (increased concentrations of CO_2 in body fluids) (Esbaugh, 2017; Gutowska et al. 2010) among other adverse biological effects. In the last years it has been conducted an effort to increase the knowledge about effects associated with acidification and also in it combination with contaminated environmental samples. The main results obtained are summarized below.

Basallote et al. (2012) exposed different species of marine organisms to different scenarios of acidification by enrichment of CO_2. Polychaetes, fish larvae, mollusk and amphipods were exposed to water acidified by CO_2 in gradients of pH values from 5.5 to 8.0. Results show that all the organisms tested, except polychaete, were highly sensitive to increase of proton concentration from CO_2 enrichment. Thus, pH values of 7.0 or lower were associated with significant increase of toxicity in these species. Sokołowski et al.(2017) reproduced the high pressure and determined biological responses of the infaunal bivalve *Limecola balthica* to CO_2-induced seawater acidification through long term exposition and concluded that even the most acidic conditions (pH= 6.3) did not prove to be fatal. Recently, Bautista-Chamizo et al. (2016) and Passarelli et al. (2017b) have compared the effects of acidification by exposing benthic and pelagic organisms to different scenarios by means of acid mixtures and CO_2. Their results show that the effects of the acidification produced by CO_2 enrichment have significant differences compared to acidification

using mixtures of acid. In this sense, Bautista-Chamizo et al. (2016) showed that marine microalgae *Pleurochrysis roscoffensis* were growing in perfect shape ranging pH value among 8.0-6.5, being 6.5 the optimal pH value for the specie growth when the acidification was produced by CO_2 enrichment. Nevertheless, when HCl was used to reproduce the acidification condition, the toxicity of the protons on these microalgae was measured at values lower than 7.0. Both treatments at pH values lower than 6.5 produced significant toxicity on the population of microalgae, causing their collapse or death. The membrane permeability for protons favors that gaseous CO_2 transfer inside the cell producing different adverse effects in marine organisms. At the same time, this enrichment of CO_2 will benefit microalgae growth (photosynthesis).

There are recent and numerous studies that address the ocean acidification effects on marine organisms (i.e., 4th International Symposium on the Ocean in a High-CO_2 World). These studies have been a very significant advance to understand the impact of physicochemical changes in the carbonic acid system in the aquatic ecosystem (mainly marine). However, most of these studies do not consider the combination of acidification plus the existing contamination on the adverse biological effect assessment, in other words they were not able to identify the relationship between the effects of the acidification on the physicochemical changes of contaminants and their consequent toxicity to the organisms. Follow below a reviewed selection of studies that address the effects of CO_2 on trace metals and their effects using different organisms. The main propose of these studies were to distinguish between the biological adverse effects associated with the acidification itself compared to the increase of bioavailability of metals associated with the acidification by the enrichment of CO_2.

First attempts to address the acidification influence on toxicity of environmental contaminated samples used mixtures of acid. Riba et al. (2002; 2004a) pointed out the effects of

increasing acidification in mobility, bioavailability and toxicity of metals in contaminated samples in southern Spain. These authors show that changes in 0.5 units of pH value were associated with significant increase in mobility and bioavailability of metals as well as their toxicity on mollusks. Basallote et al. (2014) used benthic organism, amphipod *Ampelisca brevicornis*, to address the effects of the acidification by enrichment of CO_2 in sediments with different levels of metal contamination. These authors reported significant toxicity of metals bound to sediments when the pH decreased. Thus, for a given sample (contaminated with metals) it was not observed toxicity at pH values of 7.5 and 8.0. However, when the same sample was at pH of 7.0 or below the increase in the mobility of certain metals (Ni, Zn, and Cu, among others) was associated with the significant adverse biological effects measured in the exposed amphipods. Recently, Goulding et al. (2017) and Passarelli et al. (2017b) have conducted different research studies that distinguish between the effects of acidification by enrichment of CO_2 'per se' from those biological effects related to contamination of samples (mainly metals). These authors used different amphipods species from different sites in Brazil and Europe. Main results showed to be similar to those reported by Basallote et al. (2015), concerning acidification threshold values. Thus, for the species tested the values of pH lower than 6.5 produced lethal effects whereas those values between 7.0 - 6.5 showed adverse biological effects but not death. The authors also showed the relationship among adverse effects, acidification, mobility and concentration of metals, demonstrating that the amphipod species used (Tropical, *Hyaleyoungi* and European *Ampelisca brevicornis*) were sensitive and valid to address the impact of the process's combination (acidification combined with contamination). Other authors that exposed benthic species to different scenarios of acidification by enrichment of CO_2 reported similar results.Rodríguez-Romero et al.(2014) and Basallote et al.(2015) exposed bivalves (*Ruditapes philipinarum*) to different contaminated samples under

different scenarios of acidification produced by CO_2 enrichment. The authors reported similar results related to the increase of some metals mobility (Zn and Fe) and their increase in the toxicity on the organisms. A given sample of sediment demonstrated no toxicity to metal concentration when pH values were higher than 7.0. However, the same sample was toxic to the same organisms when pH values were lower than 7.0. It was concluded by these authors the relationship between the increase in metals mobility on interstitial water and the increase of their bioavailability with the adverse biological effect measured. Although these authors used the same species, the life stage was different, from juveniles to adults. Comparison of the results obtained by these authors show that juveniles were more sensitive to the combination of acidification and metal contamination than adults.

Rodríguez-Romero et al. (2014) exposed organisms of polychaeta *Hediste diversicolor* to different contaminated samples at different acidification scenarios by CO_2 enrichment. These authors used the same contaminated samples than those used for the exposure of other organisms in previous studies (like mollusks) (Rodríguez-Romero et al. 2014). Results showed that individuals of *H. diversicolor* were more tolerant to acidification effects than mollusks, resisting to pH values lower than 6.5 without any significant adverse effects. Besides, these organisms were more resistant to the combination of both effects (acidification and metal contamination) than mollusks. No significant effects were measured in contaminated samples with metals to pH values higher than 6.5.

Recent studies have used mixtures of acid (Riba et al. 2016) and enrichment of CO_2 (Passarelli et al. 2017a; Szalaj et al. 2017) to mimic acidification scenarios. Different species of sea urchin (*Paracentrotus lividus*, *Lytechinus variegatus*), oyster (*Crassostrea gigas*) and mussels (*Mytilus edulis*, *Mytilus galloprovincialis*) were used in these studies to distinguish between

adverse effects related to acidification and those associated with metals contamination. The contaminated sediments (mainly by metals) used in these studies were sampling from the same location, allowing a comparison of obtained responses (Passarelli et al. 2017a; Riba et al. 2016; Szalaj et al. 2017). The mobility was higher to Zn, Cu, Fe, As and Al, whose last three were unrelated directly with toxicity it selves, evidencing that the acidification conditions could provoke a reaction among non-toxic ligand species. In addition, mobility of metal(loid)s increases their bioavailability and consequently increase the toxicity for the species checked, as it was shown in the study by non-toxicity effects of the samples in these species to pH values between 8.0 and 7.5, reaching toxic effects when the pH value was 7.0 or lower. The link of the different data set concluded that the acidification and consequent metals mobility was the main cause of toxicity, showing sea urchins as the most sensitive species to acidification and mussels the more tolerant.

In general, studies considering different contaminated samples by metals (and metalloids) and in different matrix (sediment and water) have been used to expose different organisms to acidification conditions by enrichment of CO_2. Main results show that the increase of concentration of protons is toxic itself at pH values lower than 7.0. Besides, these studies also demonstrated that environmental non-toxic samples at environmental values of pH (7.5 - 8.0) became toxic when pH values decrease to 7.0, or lower, for all the invertebrates used in the experiments under laboratory conditions. In summary, the results of most revised marine/estuarine studies that acidification by adding CO_2 affects the mobility of metals present in marine sediments. Thus, at a normal marine ecosystem pH, most of metals are bound to the sediments and were not available to exposed organisms. However, it was demonstrated in these studies that some of metals became bioavailable and subsequently potentially toxic to the exposed biota when the proton concentration was increase as a result of CO_2 enrichment.

In Fig.2 it is shown a descriptive summarized gradient of the different organism's sensitivity exposed to combined effects of metal contamination and acidification by CO_2 enrichment. In general, early life stage was more sensitive to acidification effects or to combination of acidification combined with metal contamination, than adults of the same species. The figure allows an ordination of the species used in the discussed studies based on the tolerance and sensitivity responses of them to the different CO_2-induced acidification scenarios. It determines that sea urchins and amphipods are the most sensitive species to the conditions tested in the experiments followed by mollusks and finally the polychaete that was the more resistant species of those used in this kind of studies.

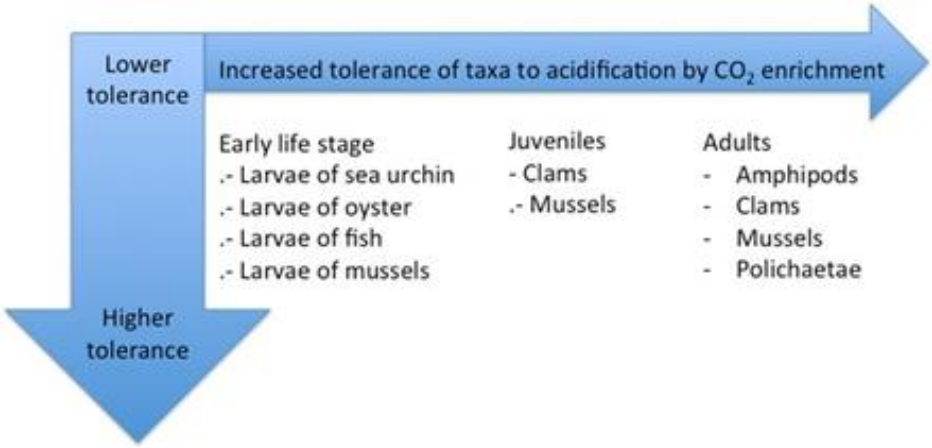

Fig.2. Diagram representation of the sensibility of different taxa to the combination effects of acidification and that associated with contaminant concentration in environmental samples acidified by CO_2 release that mimic different scenarios of enrichment of CO_2.

Although increasing toxicity studies combining acidification and contaminants effects using estuarine and marine species, there are only few attempts to determining effects related to CO_2 trace element aqueous geochemistry in freshwater aquifers and gas flux measurements in soil

(Jones et al. 2015). There is also limited information about metal mobilization caused by a potential CO_2 leak in freshwater sediment layer and its consequent effects on resident aquatic organisms. To the best of our knowledge, there are only two studies addressing acidification scenarios by enrichment of CO_2 and contamination in freshwater sediment samples. The first one used the sediment dweller *Chironomus riparius* (Khosrovyan et al. 2014)and the second the water column organism *Daphnia magna* (Khosrovyan et al. 2017). Results of these two studies show similar effects for acidification and its combination with metal contamination, which increases the toxicity when the proton concentration and the mobility of metals increase. Different endpoints, from survival to behavior, were used in the tests. *D. magna* showed to be sensitive to a gradual CO_2 enrichment, presenting adverse effects by acidic conditions. It was also identified that metal mobility, especially from Al ions, were related to an increase in mortality and decrease in reproduction of parental *D. magna*. Related to the midge *C. riparius*, results showed an impairment of reproductive functions of these organisms that may lead to population decline and disruption of their functions in the ecosystem. At pH values lower than 7.0, it was measured an increase in metal mobility and bioavailability associated with the toxicity identified in the experiment.

In recent years, the use of microorganism community to address the response of different acidification scenarios has been increased. Furthermore, there are few but significant studies addressing the combination of toxic effects of acidification and contamination in aquatic organisms. Tait et al. (2015) reported changes in the microphytobenthos and bacteria community during an in-situ experiment by release of CO_2. These authors have found changes related to the acidification conditions forced in the experiment in the relative abundance of bacterial taxa. They also discussed that some of the impacts measured could be related to the presence of metals in the sediments but the concentration related with their effects was not reported in the study. Liu et al.

(2017) studied the effects of acidification by enrichment of CO_2 in bacterial communities' dynamics, in a seawater mesocosm experiment. In the low CO_2 treatments, the consumption of the dissolved inorganic nitrogen the uptake ratios of N/P and N/Si increased significantly during the bloom. The observed responses suggest highly extensive and complex effects related to higher CO_2 concentrations on phytoplankton communities from coastal eutrophic environments.

Recently, Borrero-Santiago et al. (2016a) pointed out an underestimation within the use of bacterial population and also communities to the risk assessment of acidification by CO_2 enrichment effects. These authors conducted different research addressing the role of this kind of organisms as acidification effects indicator and in a combination with contaminants. Borrero-Santiago et al. (2016b) used populations of *Pseudomona stanieri* to optimize the protocol and to address the influence of acidification in these microorganisms. Further studies from the same group (Borrero-Santiago et al. 2016b; 2017a,b) showed responses of two marine strains to different scenarios of acidification by enrichment of CO_2. Strains of *P. litoralis* were more sensible to acidification than *Roseobacter* sp., representing the first attempts to show the diversity and resistance of marine bacteria and their capacity to adapt themselves under different acidification scenarios. Borrero-Santiago et al. (2017a,b) have been tested the stress responses in bacterial community exposed to combination of acidification and contaminants. These authors carried out a toxicity assessment using bacterial responses (total number of cells, respiring activity, changes in the bacterial community composition and diversity). The main results showed an impact on bacterial communities related to the acidification and measured by changes in respiring activity, community composition groups and diversity. These results suggested that an acidification by enrichment of CO_2 will remove contaminants from the sediment. In the same study, it was shown that bacterial communities used were able to adapt to the new acidic conditions by means of

changing the diversity and structure. Despite the adaptability of bacterial communities in the contaminated sediments used within the acidification experiments, a negative impact was observed on respiring activity under all pH treatments, showing that this kind of bacterial responses is a rapid and useful indicator for environmental risk assessments in polluted sediments exposed to acidification by enrichment of CO_2.

There are few studies that tested the influence of acidification in the toxicity of organic compounds (Freitas et al. 2016; Munari et al. 2016; Schiedek et al. 2007). As observed by Schiedek et al. (2007), new persistent organic contaminants, such as flame retardants and perfluorinated compounds, together with emerging contaminants (such as pharmaceuticals and personal care products) are being produced and released into aquatic ecosystems with limited understanding on their biological effects in different climate changes scenarios. The combined effects of emerging contaminants and pH alteration are scarcely reported in scientific literature. Freitas et al. (2016) found that toxicity of carbamazepine on *Scrobicularia plana* clams was synergistically increased under ocean acidification conditions (pH 7.1), the specimen's survival was reduced and oxidative stress was enhanced when compared to single exposures. Munari et al. (2016) investigated the combined effects of seawater acidification and diclofenac on survival, growth and oxidative stress-related parameters in the larvae of *Ruditapes philippinarum*. This study revealed that mortality was higher under reduced pH in the presence of the pharmaceutical, whereas shell morphology and larvae growth were negatively affected by both acidification and diclofenac, highlighting that acidification enhances the sensitivity of clam larvae to environmentally relevant concentrations of diclofenac.

Due the increase in global consumption of illicit drugs and consequently their occurrence in aquatic ecosystems, this class of bioactive compounds has been considered a potential new

environmental concern (Binelli et al. 2012). Recent studies have reported coastal water contamination by drugs as cocaine and its by-products (Klosterhaus et al. 2013; Pereira et al. 2016). However, few studies have focused on biological effects on marine organisms (Maranho et al. 2017). There is a new initiative that is testing the influence of acidification by enrichment of CO_2 in illicit drug crack/cocaine (Pereira et al. 2016). The system of crack-cocaine is a very interesting model to study the influence of proton concentration. The molecular difference between both compounds (crack and cocaine) is a proton defining a system similar to other inorganic compounds like NH_3/NH_4^+, etc.

Although originally the crack compound is considered more toxic based in its neutral molecule compared to cocaine, the first results obtained by these authors shows how the increase in proton concentration also produce an increase in the toxicity of these compounds. In their first results Souza et al. (2017) demonstrated different bioavailability of crack-cocaine exposed to different organisms when modifying the acidification conditions.

Fig.3 shows the first results of this kind of experiment that were discussed during the last SETAC Latin-American meeting (Souza et al. 2017) linking this concerning with ocean acidification scenario. Souza et al. (2017) performed a study on the interaction between toxic effects of illicit drugs and pH alterations in marine organisms. These authors showed that toxicity of crack-cocaine to *Perna perna* mussels' gametes and larvae increased due to acidification. The enhanced toxicity of contaminants of emerging concern as illicit drugs on quality of gametes and their interaction, as well as embryo-larval development under different pH values, shed light on more relevant adverse effects of bioactive compounds in ocean acidification scenarios.

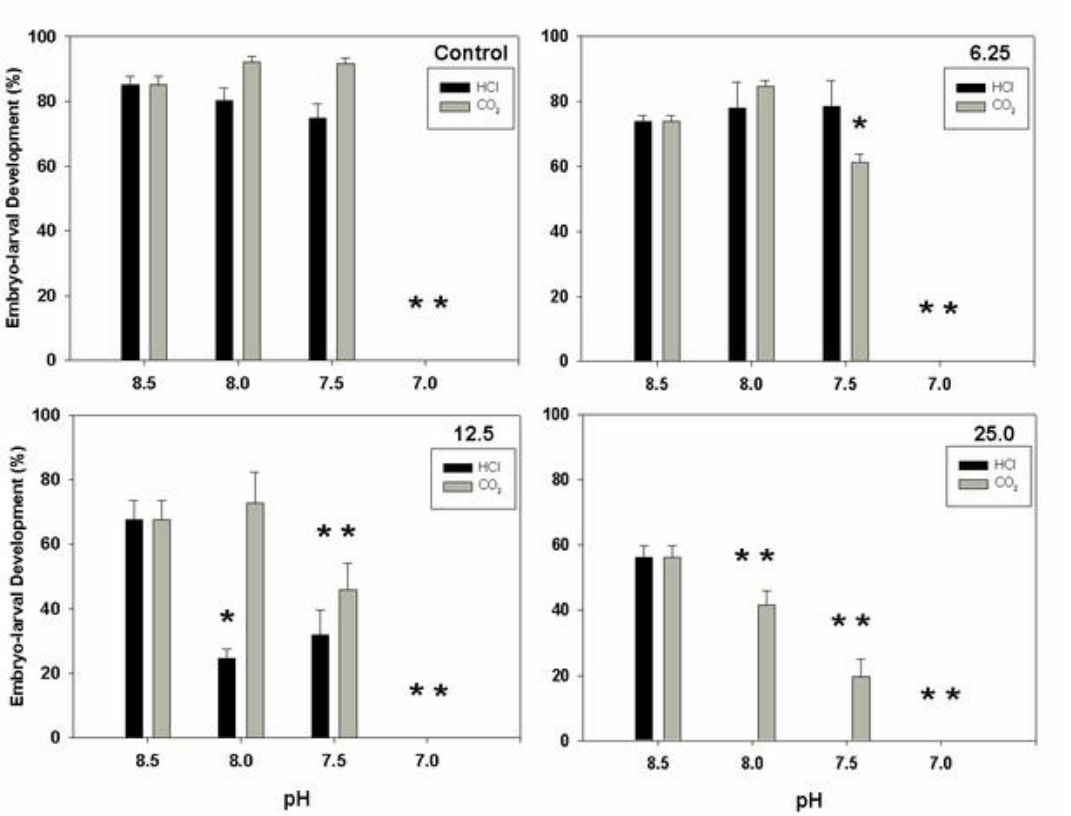

Fig.3. Results from larval development success of *Perna perna* mussel. The larvae were exposed to different concentrations of crack-cocaine (Control, 6.25, 12.5 and 25 mg/mL) at different scenarios of acidification defined by pH values (8.5; 8.0; 7.5 and 7.0). Asterisks (*) mean significant difference to the control ($p < 0.05$).

Sircar (2014) reported results of (acidification by enrichment of CO_2 and contamination of antibiotics) a combined effect of proton concentration and antibiotics was significantly observed to bacteria community. In general, the bacteria typically occurring in winter appeared more sensitive compared to the spring bloom community that is expected to be more diverse than that at winter time. The toxicity effect of amoxicillin was greater at higher concentration of protons. These results suggest that organic contaminants will be changing their toxicity effects on the organisms at different pH values.

3.3.Field studies and resident infauna

The difficulty of the adverse biological effects's evaluation resides on the measurement of 'in situ' effects. There are no easy ways to reproduce CO_2 leakage rates under field conditions and only big approaches allow this kind of surveys compared to laboratory surveys. The integrated method proposes different approaches: (a) In situ releases ofCO_2 streams to observe the behavior of the stream (Adams et al. 2013; Blackford et al. 2014; Brewer et al. 2006) and its effects on the marine environment; (b) Use natural analogues as to understand the consequences of the release of significant amounts of CO_2 (Benson, 1980); or (c) to realize the in situ experiments in either natural analogs or in environments with an specific pH variation (e.g., estuaries, acidic environments as Rio Tinto in Spain) (DelValls and Riba, 2007).

Regarding the use of macrobenthic community structure, there are few studies conducted under field and mesocosm conditions. Widdicombe et al. (2015) reported results of benthic macrofauna distribution exposed to an artificial release of CO_2 under field conditions. They collect macrofauna samples within the area of CO_2 release and in gradient separated areas of the source of CO_2 emission up to 450 m. Their results showed that macrofaunal community structure changed significantly. The authors only related the changes in macrofauna to CO_2 in the area closest to the emission of gas. They also showed that macrofaunal recovery was detected 18 days after the CO_2 gas injection had stopped. The authors concluded that short-term CO_2 enrichment events are likely to cause localized impacts on macrofaunal communities. Also, they pointed out that it is expected a rapid recovery to occur, depending on the characteristics of the communities and habitats impacted.

Almagro-Pastor et al.(2015) used a mesocosm experiment to mimic different scenarios of acidification by enrichment of CO_2 and expose macrobenthic community structures from a

contaminated area. Results showed that in general the increase in proton concentration was associated with the decrease in number of species, diversity, richness and abundance of macrobenthic community structure. They reported that abundance of each species was affected in different way by the increase of proton concentration. Their results pointed out the differential vulnerabilities of different species, as previously documented in similar mesocosm experiments (Hale et al. 2011; Widdicombe et al. 2009). They showed that polychaeta are the most tolerant taxa to acidification conditions reproduced in the mesocosms. These authors also reported a decrease in the biomass of the resident infauna related to the increase of proton concentration. Also, Passarelli et al. (2018b) show similar results comparing two different macrobenthic population from two different ecosystems (south and north hemisphere).

3.4. Bioaccumulation/Biomagnification

There are few studies that have been considered the bioaccumulation of metals at different scenarios of exposure by enrichment of CO_2 (Basallote et al. 2015; Riba et al. 2010; Rodríguez-Romero et al. 2014). In these studies, different species of polychaeta and mollusk were exposed to different values of pH (8.0 to 6.0) and to different contaminated samples. The main conclusions obtained from these few studies were that polychaeta are more tolerant to acidification than mollusks, then is considered a suitable species to be used in this kind of bioaccumulation experiments. Mollusks and polychaete did not show significant bioaccumulation of metals to pH values higher than 7.0. In general, none of these studies showed significant bioaccumulation of metals when exposed to different acidification scenarios. In recent studies (Passarelli et al. 2018a) for values among 7.0 and 6.0 there was proved a significant correlation between metal bioaccumulation and increase of acidification by enrichment of metals Fe, Ni and Zn. In general,

the bioaccumulation factor of the contaminants analyzed in samples exposed to different acidification scenarios by enrichment of CO_2 must be addressed in order to determine the risk of transference of contaminants through the trophic chain. There are not enough studies that assess this problem. These studies should be increased in the next future, mainly related to those potential contaminants that could suffer biomagnification processes like organic, metal and other substances. The results commented in previous sections of this paper clearly addressed the increase in the mobility of metals bound or tramped in environmental samples when the acidification increase. It is expected a potential increase in the bioaccumulation/biomagnification processes of certain contaminants. Also, it should be addressed in future studies to establish a correct risk evaluation not only at ecosystem health level but at human health too.

3.5. Weight of evidence

The weight-of-evidence approach will link the set of data from all the lines of evidence previously reported in the current study. There is under our knowledge none study in the bibliography that have integrated this kind of LOEs to distinguish between the adverse effects of acidification and those related to the changes in the contaminants physic-chemistry associated with it in the aquatic ecosystem. There are few studies that have used different LOEs to address the impact of acidification in the ecosystem by enrichment of CO_2. One of the recent studies (Blackford et al. 2014) reported an integration of different methods carried out to address the impact of a CO_2 release at a field conditions. They concluded that the impact of the release at geochemical and biological impacts of the enrichment of CO_2 (<1-ton CO_2 d^{-1}) is limited to the meters surrounding the source of gas. It has been a significant advance in the understanding of the impact related to the enrichment of CO_2 at marine environments. However, they were not linked

to the effects of the potential contaminants in the sediments or water to the biogeochemical processes.

The WOE must answer the four questions related to environment and human health (Fig.1) and furthermore in this application under acidification environments must be able to distinguish between the adverse biological effects associated with the acidification 'per se' and those related to changes in the physic-chemistry of contaminants presents in the area of study and suffering acidification.

The integration of the results can be conducted using different approaches. A classical use of statistical analysis by means of performing ANOVA and multivariate analysis (MAA) will allow the predictive identification of nonlinear and mechanistically unpredictable relationships among properties of natural complex systems like sediments (DelValls, 2007). The integration of the different set of data will allow quantifying the environmental quality of the studied ecosystems. Also, this integration will permit to establish the mobility of some of the contaminants in the environment, including their mobility through the food chain. The use of WOE by integrating all the results (chemistry, laboratory toxicity and field effects) using multivariate analysis as a tool for their link will allow to derive sediment quality values (SQGs) as recently reported by Passarelli, (2018). Using the same approach, it will be possible to derived tissue quality values (TQGs) linking the concentration of contaminants measured in the tissue of the organisms and the associated sublethal effects (for instance, using biomarkers of exposure and effects). These results will contribute in the risk characterization and management for the development of a decision-making framework of CO_2 storage sites. This will be based on the characterization of the sediment quality after the leakage and its relationship with the ecosystem health and the effects of leakage on the

marine/freshwater biological community. Furthermore, it will contribute to establish the risks associated with the bioaccumulation and/or biomagnifications processes.

Recent amendments to the London Protocol and OSPAR agreement established Guidelines for Risk Assessment and Management of Storage of CO_2 Streams in Geological formations (2007) (OSPAR Guidelines, 2007). These conventions ask for the *"improvement of impact prediction by gaining knowledge on the effects on species and ecosystems as a result of leakage of* CO_2" to improve the risk assessment and management. The use of WOE (as part of the guidelines) as recommended by International Conventions is shown in Fig.4. It shows the role of WOE during a risk characterization schema approved by the regulatory guidelines for the management of risk in CCS areas. This figure includes a brief scheme of the contents of this risk assessment and management proposed in the international conventions. The different LOEs included in the WOE are related to the cause, effects and exposure assessment within the risk characterization schema proposed. They are needed to characterize the risk and to design an appropriated management plan. Moreover, it is required that the link of LOEs under a WOE will be able to identify potential hazards related to the activity by estimating the probability of these hazards occurring and the severity of effects to exposed species and ecosystems. Also, it will be necessary to describe the risk estimate in the context of significance of any adverse effects and to identify and also summarize the uncertainties assumptions qualifying in the risk assessment. In the EU Directive for CO_2 capture and storage there is a specific article (art.13) about the requirements of the monitoring of the site selection for CCS, including the storage complex and the surrounding environment.

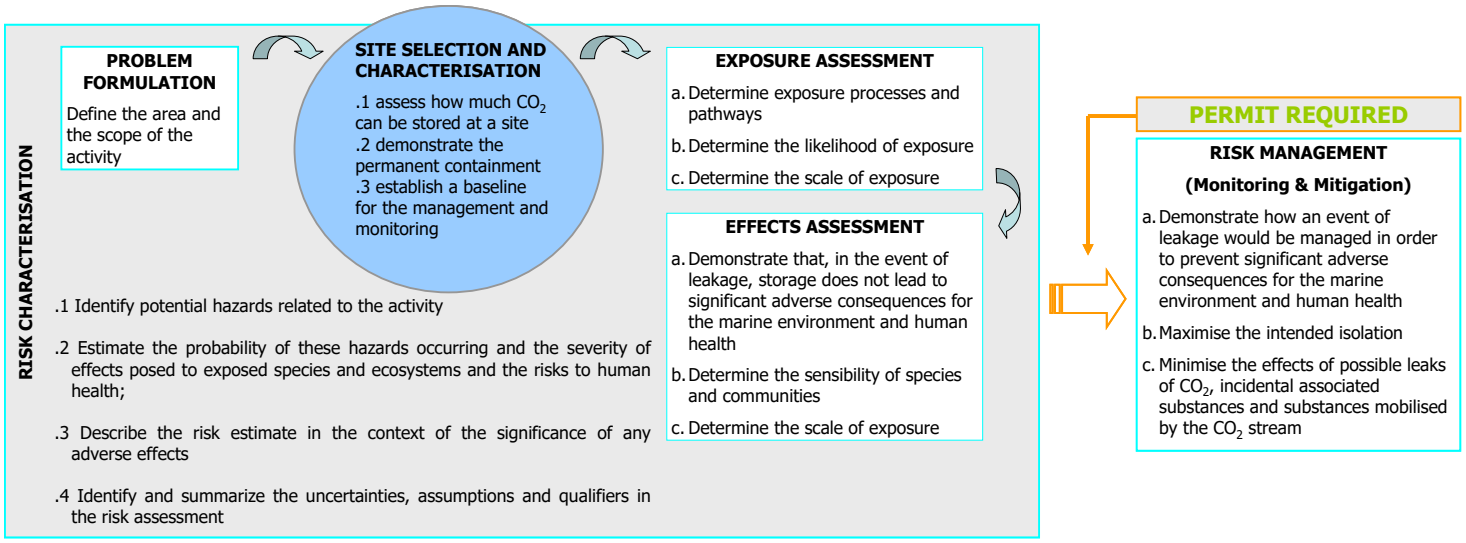

Fig.4 Schematic description of the contents included in the framework for risk assessment and management of CO_2 storage in sub-seabed geological formations established in different international regulations (adapted from Reguera et al. (2009) and DelValls (2007). The main role of the WOE approach is described in the figure and related to the characterization of the ecosystem and human risks.

The WOE includes the physicochemical and ecotoxicological characterization of sediments. Also, it includes the bioavailability of contaminants under laboratory and field surveys (bioassays) that will allow identifying biomagnification processes. All these lines of evidence (LOEs) will be carried out in a synoptic approach using field collected sediment and biological samples. This method will allow measuring the contamination and its effects related to the acidification associated with small and diffuse CO_2 leakages, including both short and long term (DelValls and Riba, 2007; Passarelli, 2018; Reguera et al. 2009)

4. Final Remarks

The physicochemical approach is one of the most used lines of evidence in risk assessment related to acidification and contamination combined effects. The equilibrium of carbon dioxide and its relationship with mobility and bioavailability (including speciation) of metal(loid)s is the most addressed issue in this kind of studies. Few studies addressing the effects of acidification in bioavailability of organic contaminant and its toxicity has been reported. In general, these studies concluded that the increase in metal bioavailability was related to the increase of proton concentration by enrichment of CO_2. Additional efforts must be conducted to address the influence of acidification in the behavior of organic contaminants and their bioavailability, including toxicity.

The toxicity results discussed in the present study showed that most of the revised marine/estuarine/freshwater studies established that the CO_2–induced acidification affects the mobility of metals bound to marine sediments. At a normal marine ecosystem pH value, those metals bounding to sediments that were not available to the exposed organisms became available and subsequently toxic to the exposed biota when the proton concentration increases as result of CO_2 enrichment. These results show a gradient of tolerance at different life stage and taxa, being those early life stages more sensible to acidification and to the combination effects with contaminant presence than adults. Regarding taxa, the sea urchin and amphipods were more sensitive to these effects than mollusks and polychaetae. These two last taxa are more recommendable to conduct bioaccumulation/biomagnification studies than sea urchin or amphipods, since they are considered more sensitive species.

There is little or limited information about metal mobilization and toxicological effects on resident aquatic organisms caused by a potential CO_2 leak from sediment layer in freshwater ecosystems. Although, the studies here discuss show a similar behavior of the acidification and contamination to the seawater ecosystems this kind of studies should be increased in the future.

Studies conducted by means of mesocosms and under field conditions, using macrobenthic community or caged animals, showed impacts on the diversity, richness and biomass of this community. Recovery of the community is reported in studies conducted under field conditions, at acidification scenarios, without considering the potential contamination of the sites studied. When linking both effects (acidification and contamination) at mesocosms studies, using macrobenthic community, significant adverse effects in the structure of the community was measured and related to metal mobility.

There are few and not enough studies that characterize the effects of acidification in the bioaccumulation/biomagnification of contaminants. The results here discussed addressed the increase in the mobility of metals, bound or tramped in environmental samples, when the acidification increases. It is expected a potential increase in the bioaccumulation/biomagnification of certain contaminants with the proton concentrations increase, however it is not clearly demonstrated, and additional efforts should be conducted in future studies to establish a potential risk evaluation, not only at ecosystem health level, but also at human health. Special efforts should be conducted considered substances able to suffer biomagnification (organic, emerging contaminants, etc.) at different acidification scenarios.

The integration of the multiple LOEs under a WOE approach is recommended in the different steps included in the guidelines for the risk characterization and management proposed by different

international regulations. Nowadays there are none study that have fully applied the WOE to determine the effects combining acidification and contamination effects. It is strongly recommended to increase the research and technological efforts to improve the use of WOE in the risk management and characterization of aquatic ecosystems affected by enrichment of CO_2 episodes (CCS, or other sources).

The combination of acidification and contamination must be solved under an integrative assessment. The best option is to use multiples lines of evidence, which will be linked based on a weight of evidence approach. We must solve this kind of wicked problem with competing risks and benefits. Although protection goals should be based on ecosystem services and not in ecosystem health, risk assessment and management must consider the ecosystem and human health. This kind of research to solve the effects related to the combination of acidification and contamination is not only necessary, but urgent to be addressed under an integrated point of view.

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References

Adams, M.B., DeWalle, D.R.., Hom, J.L., 2013. The furrow watershed acidification study, Journal of Chemical Information and Modeling. https://doi.org/10.1017/CBO9781107415324.004

Almagro-Pastor, V., Conradi, M., DelValls, T.A., Riba, I., 2015. Alterations in the macrobenthic fauna from Guadarranque River (Southern Spain) associated with sediment-seawater acidification deriving from CO_2 leakage. Mar. Pollut. Bull. 96, 65–75. https://doi.org/10.1016/j.marpolbul.2015.05.044

Basallote, M.D., De Orte, M.R., DelValls, T.A., Riba, I., 2014. Studying the Effect of CO_2 - Induced Acidification on Sediment Toxicity Using Acute Amphipod Toxicity Test. Environ. Sci. Technol. 48, 8864–8872. https://doi.org/10.1021/es5015373

Basallote, M.D., Rodriguez-Romero, A., Blasco, J., DelValls, A., Riba, I., 2012. Lethal effects on different marine organisms, associated with sediment-seawater acidification deriving from CO_2 leakage. Environ. Sci. Pollut. Res. 19, 2550–2560. https://doi.org/10.1007/s11356-012-0899-8

Basallote, M.D., Rodriguez-Romero, A., De Orte, M.R., DelValls, T.A., Riba, I., 2015. Evaluation of the threat of marine CO_2 leakage-associated acidification on the toxicity of sediment metals to juvenile bivalves. Aquat. Toxicol. 166, 63–71. https://doi.org/10.1016/j.aquatox.2015.07.004

Bautista-Chamizo, E., De Orte, M.R., DelValls, T.A., Riba, I., 2016. Simulating CO_2 leakages from CCS to determine Zn toxicity using the marine microalgae *Pleurochrysis roscoffensis*. Chemosphere 144, 955–965. https://doi.org/10.1016/j.chemosphere.2015.09.041

Benson, S.M., 1980. Carbon Dioxide Capture and Storage in Underground Geologic Formations. 10-50 Solut. Technol. Policies a Low-Carbon Futur. 1–19.

Binelli, A., Pedriali, A., Riva, C., Parolini, M., 2012. Illicit drugs as new environmental pollutants: Cyto-genotoxic effects of cocaine on the biological model *Dreissena polymorpha*. Chemosphere 86, 906–911. https://doi.org/10.1016/j.chemosphere.2011.10.056

Blackford, J., Bull, J.M., Cevatoglu, M., Connelly, D., Hauton, C., James, R.H., Lichtschlag, A., Stahl, H., Widdicombe, S., Wright, I.C., 2015. Marine baseline and monitoring strategies for carbon dioxide capture and storage (CCS). Int. J. Greenh. Gas Control 38, 221–229. https://doi.org/10.1016/j.ijggc.2014.10.004

Blackford, J., Stahl, H., Bull, J.M., Bergès, B.J.P., Cevatoglu, M., Lichtschlag, A., Connelly, D., James, R.H., Kita, J., Long, D., Naylor, M., Shitashima, K., Smith, D., Taylor, P., Wright, I., Akhurst, M., Chen, B., Gernon, T.M., Hauton, C., Hayashi, M., Kaieda, H., Leighton, T.G., Sato, T., Sayer, M.D.J., Suzumura, M., Tait, K., Vardy, M.E., White, P.R., Widdicombe, S., 2014. Detection and impacts of leakage from sub-seafloor deep geological carbon dioxide storage. Nat. Clim. Chang. 4, 1011–1016. https://doi.org/10.1038/nclimate2381

Borrero-Santiago, A.R., Bautista-Chamizo, E., DelValls, T.A., Riba, I., 2017a. A possible CO_2 leakage event: Can the marine microbial community be recovered? Mar. Pollut. Bull. 117, 380–385. https://doi.org/10.1016/j.marpolbul.2017.02.027

Borrero-Santiago, A.R., Carbú, M., DelValls, T.A., Riba, I., 2016a. CO_2 leaking from sub-seabed storage: Responses of two marine bacteria strains. Mar. Environ. Res. 121, 2–8. https://doi.org/10.1016/j.marenvres.2016.05.018

Borrero-Santiago, A.R., DelValls, T.A., Riba, I., 2016b. Carbon Capture and Storage (CCS): Risk assessment focused on marine bacteria. Ecotoxicol. Environ. Saf. 131, 157–163. https://doi.org/10.1016/j.ecoenv.2016.04.020

Borrero-Santiago, A.R., DelValls, T.A., Riba, I.L., 2017b. Bacterial community responses during a possible CO_2 leaking from sub-seabed storage in marine polluted sediments. Sci. Total Environ. 593–594, 116–123. https://doi.org/10.1016/j.scitotenv.2017.03.153

Brewer, P.G., Chen, B., Warzinski, R., Baggeroer, A., Peltzer, E.T., Dunk, R.M., Walz, P., 2006. Three-dimensional acoustic monitoring and modeling of a deep-sea CO_2 droplet cloud. Geophys. Res. Lett. 33, 1–5. https://doi.org/10.1029/2006GL027181

de Orte, M.R., Bonnail, E., Sarmiento, A.M., Bautista-Chamizo, E., Basallote, M.D., Riba, I., DelValls, T.A., Nieto, J.M., 2018. Metal fractionation in marine sediments acidified by

enrichment of CO_2: A risk assessment. Mar. Pollut. Bull. 131, 611–619. https://doi.org/10.1016/j.marpolbul.2018.04.072

de Orte, M.R., Lombardi, A.T., Sarmiento, A.M., Basallote, M.D., Rodriguez-Romero, A., Riba, I., DelValls, T.A., 2014a. Metal mobility and toxicity to microalgae associated with acidification of sediments: CO_2 and acid comparison. Mar. Environ. Res. 96, 136–144. https://doi.org/10.1016/j.marenvres.2013.10.003

de Orte, M.R., Sarmiento, A.M., Basallote, M.D., Rodríguez-Romero, A., Riba, I., DelValls, A., 2014b. Effects on the mobility of metals from acidification caused by possible CO_2 leakage from sub-seabed geological formations. Sci. Total Environ. 470–471, 356–363. https://doi.org/10.1016/j.scitotenv.2013.09.095

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014c. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

De Schamphelaere, K.A.C., Heijerick, D.G., Janssen, C.R., 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 133, 243–258. https://doi.org/10.1016/S1532-0456(02)00087-X

DelValls, T.A., 2007. Diseño y aplicación de modelos integrados de evaluación de la contaminación y sus efectos sobre los sistemas marinos y litorales y la salud humana. Ministerio de la Presidencia. Cent. para la Prevención y Lucha contra la Contam. Maritíma y Litoral.

DelValls, T.A., Riba, I., 2007. A weight of evidence approaches to assess sediment quality in the Guadalquivir estuary. Aquat. Ecosyst. Heal. Manag. 10, 101–106. https://doi.org/10.1080/14634980701213025

Dewar, M., Wei, W., McNeil, D., Chen, B., 2013. Small-scale modelling of the physiochemical impacts of CO_2 leaked from sub-seabed reservoirs or pipelines within the North Sea and surrounding waters. Mar. Pollut. Bull. 73, 504–515. https://doi.org/10.1016/j.marpolbul.2013.03.005

Esbaugh, A.J., 2017. Physiological implications of ocean acidification for marine fish: emerging patterns and new insights. J. Comp. Physiol. B 0, 0. https://doi.org/10.1007/s00360-017-1105-6

Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016. The impacts of pharmaceutical drugs under ocean acidification: New data on single and combined long-term effects of carbamazepine on *Scrobicularia plana*. Sci. Total Environ. 541, 977–985. https://doi.org/10.1016/j.scitotenv.2015.09.138

Global CCS Institute, 2017. Global Costs of Carbon Capture and Storage - 2017 Update 14.

Global CCS Institute, 2016. the Global Status of Ccs | 2016 "Time to Accelerate" 1–28.

Goulding, T.A., De Orte, M.R., Szalaj, D., Basallote, M.D., DelValls, T.A., Cesar, A., 2017. Assessment of the environmental impacts of ocean acidification (OA) and carbon capture and storage (CCS) leaks using the amphipod *Hyale youngi*. Ecotoxicology 26, 521–533. https://doi.org/10.1007/s10646-017-1783-6

Gutowska, M.A., Melzner, F., Langenbuch, M., Bock, C., Claireaux, G., Pörtner, H.O., 2010. Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. J. Comp. Physiol. B. 180, 323–335. https://doi.org/10.1007/s00360-009-0412-y

Hale, R., Calosi, P., Mcneill, L., Mieszkowska, N., Widdicombe, S., 2011. Predicted levels of future ocean acidification and temperature rise could alter community structure and biodiversity in marine benthic communities. Oikos 120, 661–674. https://doi.org/10.1111/j.1600-0706.2010.19469.x

IPCC, 2012. Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation, Special Re. ed. https://doi.org/10.1017/CBO9781139177245

Jones, D.G., Beaubien, S.E., Blackford, J.C., Foekema, E.M., Lions, J., De Vittor, C., West, J.M., Widdicombe, S., Hauton, C., Queirós, A.M., 2015. Developments since 2005 in

understanding potential environmental impacts of CO_2 leakage from geological storage. Int. J. Greenh. Gas Control 40, 350–377. https://doi.org/10.1016/j.ijggc.2015.05.032

Khosrovyan, A., DelValls, T.A., Luque, A., Riba, I., 2017. Effects of a hypothetical escape of CO_2 gas from subterranean storage sites on water flea *Daphnia magna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-017-0154-4

Khosrovyan, A., DelValls, T.A., Riba, I., 2014. Effects of simulated CO_2 escape from sediments on the development of midge *Chironomus riparius*. Aquat. Toxicol. 156, 230–239. https://doi.org/10.1016/j.aquatox.2014.09.005

Klosterhaus, S.L., Grace, R., Hamilton, M.C., Yee, D., 2013. Method validation and reconnaissance of pharmaceuticals, personal care products, and alkylphenols in surface waters, sediments, and mussels in an urban estuary. Environ. Int. 54, 92–99. https://doi.org/10.1016/j.envint.2013.01.009

Lichtschlag, A., James, R.H., Stahl, H., Connelly, D., 2015. Effect of a controlled sub-seabed release of CO_2 on the biogeochemistry of shallow marine sediments, their pore waters, and the overlying water column. Int. J. Greenh. Gas Control 38, 80–92. https://doi.org/10.1016/j.ijggc.2014.10.008

Liu, X., Li, Y., Wu, Y., Huang, B., Dai, M., Fu, F., Hutchins, D.A., Gao, K., 2017. Effects of elevated CO_2 on phytoplankton during a mesocosm experiment in the southern eutrophicated coastal water of China. Sci. Rep. 7, 1–14. https://doi.org/10.1038/s41598-017-07195-8

London Convention, 2007. Specific Guidelines for the Assessment of Carbon Dioxide Streams for Disposal into Sub-seabed Geological Formations.

London Convention, 2006. Risk Assessment and Management Framework for CO_2 Sequestration in Sub-seabed Geological Formations. London Conv. Prev. Mar. Pollut. by Dump. Wastes Other Matter 1972 1996.

Maranho, L.A., Fontes, M.K., Kamimura, A.S.S., Nobre, C.R., Moreno, B.B., Pusceddu, F.H., Cortez, F.S., Lebre, D.T., Marques, J.R., Abessa, D.M.S., Ribeiro, D.A., Pereira, C.D.S.,

2017. Exposure to crack cocaine causes adverse effects on marine mussels *Perna perna*. Mar. Pollut. Bull. 0–1. https://doi.org/10.1016/j.marpolbul.2017.08.043

Millero, F., Woosley, R., DiTrolio, B., Waters, J., 2009. Effect of Ocean Acidification on the speciation of metals in seawater. Oceanography 22, 72–85. https://doi.org/10.5670/oceanog.2009.98

Munari, M., Chemello, G., Finos, L., Ingrosso, G., Giani, M., Marin, M.G., 2016. Coping with seawater acidification and the emerging contaminant diclofenac at the larval stage: A tale from the clam *Ruditapes philippinarum*. Chemosphere 160, 293–302. https://doi.org/10.1016/j.chemosphere.2016.06.095

OSPAR Guidelines, 2007. Risk Assessment and Management of Storage of CO_2 Streams in Geological Formations. Conv. Prot. Mar. Environ. NORTH-EAST Atl.

Passarelli, M.C., 2018. Design and optimization of a weight-of-evidence approach to risk characterization in sediments affected by CO_2 acidification. Ph. D. Thesis - Dep. Química-Física Fac. Ciencias del Mar y Ambient. Univ. Cádiz 276.

Passarelli, M.C., Cesar, A., Riba, I., DelValls, T.A., 2017a. Comparative evaluation of sea-urchin larval stage sensitivity to ocean acidification. Chemosphere 184, 224–234. https://doi.org/10.1016/j.chemosphere.2017.06.001

Passarelli, M.C., Ray, S., Cesar, A., DelValls, T.A., Riba, I., 2018a. Effects of CO_2 enrichment on metal bioavailability and bioaccumulation using *Mytilus galloprovincialis*. Mar. Pollut. Bull. 133, 124–136. https://doi.org/10.1016/j.marpolbul.2018.05.021

Passarelli, M.C., Riba, I., Cesar, A., Newton, A., DelValls, T.A., 2018b. Using a mesocosm approach to evaluate marine benthic assemblage alteration associated with CO_2 enrichment in coastal environments. Ecotoxicol. Environ. Saf. 157, 29–39. https://doi.org/10.1016/j.ecoenv.2018.03.049

Passarelli, M.C., Riba, I., Cesar, A., Serrano-Bernando, F., DelValls, T.A., 2017b. Assessing the influence of ocean acidification to marine amphipods: A comparative study. Sci. Total Environ. 595, 759–768. https://doi.org/10.1016/j.scitotenv.2017.04.004

Payán, M.C., Verbinnen, B., Galan, B., Coz, A., Vandecasteele, C., Viguri, J.R., 2012. Potential influence of CO_2 release from a carbon capture storage site on release of trace metals from marine sediment. Environ. Pollut. 162, 29–39. https://doi.org/10.1016/j.envpol.2011.10.015

Pereira, C.D.S., Maranho, L.A., Cortez, F.S., Pusceddu, F.H., Santos, A.R., Ribeiro, D.A., Cesar, A., Guimarães, L.L., 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Sci. Total Environ. 548–549, 148–154. https://doi.org/10.1016/j.scitotenv.2016.01.051

Reguera, D.F., Riba, I., Forja, J.M., DelValls, T.A., 2009. An integrated approach to determine sediment quality in areas above CO_2 injection and storage in agreement with the requirements of the international conventions on the protection of the marine environment. Ecotoxicology 18, 1123–1129. https://doi.org/10.1007/s10646-009-0381-7

Riba, I.L., DelValls, T.A., Forja, J.M., Gómez-Parra, A., 2004a. The influence of pH and salinity on the toxicity of heavy metals in sediment to the estuarine clam *Ruditapes philippinarum*. Environ. Toxicol. Chem. 23, 1100–1107. https://doi.org/10.1897/023-601

Riba, I.L., DelValls, T.A., Forja, J.M., Gómez-Parra, A., 2002. Influence of the Aznalcóllar mining spill on the vertical distribution of heavy metals in sediments from the Guadalquivir estuary (SW Spain). Mar. Pollut. Bull. 44, 39–47. https://doi.org/10.1016/S0025-326X(01)00171-0

Riba, I.L., Forja, J.M., Gómez-Parra, A., DelValls, T.A., 2004b. Sediment quality in littoral regions of the Gulf of Cádiz: A triad approach to address the influence of mining activities. Environ. Pollut. 132, 341–353. https://doi.org/10.1016/j.envpol.2004.03.021

Riba, I.L., Gabrielyan, B., Khosrovyan, A., Luque, A., DelValls, T.A., 2016. The influence of pH and waterborne metals on egg fertilization of the blue mussel (*Mytilus edulis*), the oyster (*Crassostrea gigas*) and the sea urchin (*Paracentrotus lividus*). Environ. Sci. Pollut. Res. 23, 14580–14588. https://doi.org/10.1007/s11356-016-6611-7

Riba, I.L., García-Luquea, E., Blasco, J., DelValls, T.A., 2003. Bioavailability of heavy metals bound to estuarine sediments as a function of pH and salinity values. Chem. Speciat. Bioavailab. 15, 101–114. https://doi.org/10.3184/095422903782775163

Riba, I.L., Kalman, J., Vale, C., Blasco, J., 2010. Influence of sediment acidification on the bioaccumulation of metals in *Ruditapes philippinarum*. Environ. Sci. Pollut. Res. 17, 1519–1528. https://doi.org/10.1007/s11356-010-0338-7

Rodríguez-Romero, A., Jiménez-Tenorio, N., Basallote, M.D., Orte, M.R. De, Blasco, J., Riba, I., 2014. Predicting the impacts of CO_2 leakage from subsea bed storage: Effects of metal accumulation and toxicity on the model benthic organism *Ruditapes philippinarum*. Environ. Sci. Technol. 48, 12292–12301. https://doi.org/10.1021/es501939c

Schiedek, D., Sundelin, B., Readman, J.W., Macdonald, R.W., 2007. Interactions between climate change and contaminants. Mar. Pollut. Bull. 54, 1845–1856. https://doi.org/10.1016/j.marpolbul.2007.09.020

Seibel, B.A., 2016. Cephalopod susceptibility to asphyxiation via ocean incalescence, deoxygenation, and acidification. Physiology 31, 418–429. https://doi.org/10.1152/physiol.00061.2015

Sircar, T., 2014. Combined effect of antibiotics and ocean acidification on marine bacterial communities during winter and spring bloom conditions.

Sokołowski, A., Brulińska, D., Mirny, Z., Burska, D., Pryputniewicz-Flis, D., 2017. Differing responses of the estuarine bivalve *Limecola balthica* to lowered water pH caused by potential CO_2 leaks from a sub-seabed storage site in the Baltic Sea: An experimental study. Mar. Pollut. Bull. 127, 761–773. https://doi.org/10.1016/j.marpolbul.2017.09.037

Souza, L. da S., Pusceddu, F.H., Cortez, F.S., de Orte, M.R., Cesar, A., Ribeiro, D.A., DelValls, T.A., Pereira, C.D.S., 2017. Effects on fertilization rate and embryolarval development of *Perna perna* mussels exposed to crack cocaine in different pHs. SETAC Santos – SETAC Lat. Am. 12th Bienn. Meet.

Szalaj, D., De Orte, M.R., Goulding, T.A., Medeiros, I.D., DelValls, T.A., Cesar, A., 2017. The effects of ocean acidification and a carbon dioxide capture and storage leak on the early life stages of the marine mussel *Perna perna* (Linneaus, 1758) and metal bioavailability. Environ. Sci. Pollut. Res. 24, 765–781. https://doi.org/10.1007/s11356-016-7863-y

Tait, K., Stahl, H., Taylor, P., Widdicombe, S., 2015. Rapid response of the active microbial community to CO_2 exposure from a controlled sub-seabed CO_2 leak in Ardmucknish Bay (Oban, Scotland). Int. J. Greenh. Gas Control 38, 171–181. https://doi.org/10.1016/j.ijggc.2014.11.021

Taylor, P., Lichtschlag, A., Toberman, M., Sayer, M.D.J., Reynolds, A., Sato, T., Stahl, H., 2015. Impact and recovery of pH in marine sediments subject to a temporary carbon dioxide leak. Int. J. Greenh. Gas Control 38, 93–101. https://doi.org/10.1016/j.ijggc.2014.09.006

Wang, Z., Wang, Y., Zhao, P., Chen, L., Yan, C., Yan, Y., Chi, Q., 2015. Metal release from contaminated coastal sediments under changing pH conditions: Implications for metal mobilization in acidified oceans. Mar. Pollut. Bull. 101, 707–715. https://doi.org/10.1016/j.marpolbul.2015.10.026

Widdicombe, S., Dashfield, S.L., McNeill, C.L., Needham, H.R., Beesley, A., McEvoy, A., Oxnevad, S., Clarke, K.R., Berge, J.A., 2009. Effects of CO_2 induced seawater acidification on infaunal diversity and sediment nutrient fluxes. Mar. Ecol. Prog. Ser. 379, 59–75. https://doi.org/10.3354/meps07894

Widdicombe, S., McNeill, C.L., Stahl, H., Taylor, P., Queirós, A.M., Nunes, J., Tait, K., 2015. Impact of sub-seabed CO_2 leakage on macrobenthic community structure and diversity. Int. J. Greenh. Gas Control 38, 182–192. https://doi.org/10.1016/j.ijggc.2015.01.003

Chapter III. Determining toxicity of crack-cocaine concentrations at different CO_2-induced acidification scenarios using first life stages of marine organisms.

Approximately one-third of the CO_2 that has entered the atmosphere over the past 100 yr. has been absorbed into ocean surface waters and has resulted in the elevation of partial pressure of CO_2 (pCO_2) in seawater and reduction of seawater pH (Caldeira and Wickett, 2003). Currently, the average surface seawater pH is 8.1 (already 0.1 units lower than in 1750) (Bernstein et al., 2007; Raven et al., 2005). According to estimates based on the IPCC emission scenarios, pH will further decline between 0.3 and 0.5 units during the 21st century. The unrestricted burning of fossil fuels may cause a more extreme decrease, of 0.7 units from current values, by the year 2300 (Caldeira and Wickett, 2003). The reduction of 0.1 units on ocean pH corresponds to a 30% increase of [H+] in the seawater (Bindoff et al., 2007) and a pH downfall of 0.3 or 0.4 units is equivalent to a 150% increase of [H+] and 50% decrease of [CO_3^{2-}] (Doney et al., 2009; Orr et al., 2009).

The change in ocean pH generates intense impacts on marine fauna and flora. One of the most alarming effects associated with this phenomenon is the depth change of the lysocline layer. This layer corresponds to the oceanic depth at which carbonate compensation occurs, that is, the depth from which carbonate (CO_3^{2-}) dissolves in the water column, making it impossible to maintain structures formed by this compound (eg.: shells and skeletons). The composition of an adult bivalve shell consists of calcite, aragonite or both, however, all shells of bivalve larvae contain aragonite (Weiss et al., 2002), the most soluble form of $CaCO_3$. The segregation and mineralization of the first larvae shell is initiated during the trochophore larvae stage through a specialized group of ectodermic cells (Iwata, 1980).

In chapter III, the main objective is to assess the effects of CO_2 enrichment itself and its association with an illicit drug (crack-cocaine) on early life stages of two different organisms: sea

urchin – *Echinometra lucunter* (figure 1); and a marine mussel – *Perna perna* (figure 2); through responses as fertilization rate assay and embryo-larval development. It includes two different scientific papers addressing the mentioned objective. In the first paper, it was addressed the impact and effects related to the CC concentrations and two different methods of acidification (CO_2 and HCl) on sea urchin. Beyond this assessment an additional experimental design was proposed to determine the effects related to the combination of acidification and environmental relevant concentration of an illicit drug in early life stages of a marine bivalve (*Perna perna* mussel).

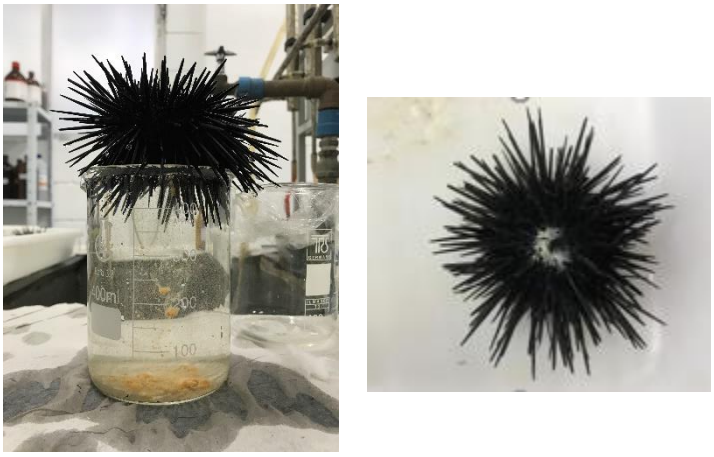

Figure 1: Image of the sea urchin gametes obtention. On the left picture a female specimen and on the right a male individual (*E. lucunter*).

Figure 2: Image of a marine mussel *Perna perna* specimen.

The methods used to address these effects were based on laboratory experimentation. The sea water used in the experiment was artificially done in the laboratory by dissolving natural salt from Red Sea (Red Sea salt®) in deionize water until 35 ppm, which is the optimum value for the specie, describe by Zaroni et al. (2005). The reconstituted seawater was used as control and also used to diluted the different CC treatments (6,25 $mg.L^{-1,}$, 12,5 $mg.L^{-1}$, 25 $mg.L^{-1}$, 50 $mg.L^{-1}$, and 100 $mg.L^{-1}$).

Two different methods were used to acidify the samples containing crack-cocaine (by Chloridric acid and CO_2 injection system). The pH values applied in our experiments ranged from 8.3 to 7.0. The CO_2 injection system (Fig.2) used for this experiment is an adaptation of the experimental set up described by de Orte et al. (2014b), patent process nº: P201200753, Cadiz University, Faculty of Marine and Environmental Sciences, Physical Chemistry Department (RNM 375). Controlled by Aqua Medic AT control hardware, the system injects CO_2 gas (Air Liquide) according to the previously established configuration.

Figure 2: Image of the CO_2 injection system used in this study for the embryo-larval exposition to crack-cocaine in different pHs.

The results obtained in the experiments assessing the effect of CC concentration combined to ocean acidification using sea urchins were showed in the first paper of this chapter published in Chemosphere Journal: *"Adverse effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios".*

The main objective of this paper was evaluating if ocean acidification would trigger enhanced toxicity of illicit drugs to non-target marine organisms due the combined effects of crack-cocaine and low pH (from 8.5 to 7.0 pH values) on the reproduction of the sea urchin *Echinometra lucunter*. Addressing this objective, it will be possible to delineate a baseline of effects associated with the concentrations of crack-cocaine and acidification that will be used later in this chapter to assess a marine mussel (second paper of this chapter).

The EC50 was defined as the crack-cocaine concentration that causes effects in 50% of the population exposed, in this case on the success of the embryo larval normal development. Results obtained by are shown in table 1. The control treatment (no HCl or CO_2 addition) presents an EC50 of crack/cocaine of 58.83 $mg.L^{-1}$. EC50 values decrease with decreasing pH revealing that acidification increases the toxicity of crack-cocaine compounds. This effect is higher with the HCl addition method when compared to CO_2 injection.

Table 1. Values of IC50 derived at the different treatments (control and concentrations) for both methodologies of acidification (HCl and CO_2). Values of EC50 at pH 8.5 show the results for the control without acidification method.

pH values	IC50	
	HCl	CO_2
8.5	58.83 (52.54 – 63.38)	58.83 (52.54 – 63.38)
8.0	10.67 (10.31 – 10.87)	23.28 (22.44 – 24.30)
7.5	11.58 (10.88 - 13.04)	12.5 (10.60 – 14.91)
7.0	-	-

The second article of this chapter related adverse effects of an illicit drug (crack-cocaine) on early life stage of the mussel *P. perna* exposed to scenarios of ocean acidification. It will be submitted to the Environmental Science and Pollution Research, entitle *"Could ocean acidification intensify illicit drug effects on reproduction of marine mussels?".* The main objective of this paper is to assess how ocean acidification will play with the toxicity of illicit drugs to non-target marine organisms due the combined effects of crack-cocaine and low pH (from 8.3 to 6.0 pH values) on the reproduction of the marine mussel *Perna perna*

Specimens of marine mussel (*Perna perna*) were purchased from an aquaculture facility (Cocanha beach at Caraguatatuba, SP/Brazil) and held in 500-liter tank filled clean aerated seawater until use in the tests, within 24 h of acquisition. The gametes were obtained according ASTM E724-98 (2012), with minor adaptations proposed by Zaroni et al. (2005). Four replicates were used for each CC concentrations (ranged from 0 to 100 mg/L) in the different pH values applied (8.3; 8.0; 7.5 and 7.0). The pH value of 8.3 was used as control, where no CO_2 or HCl was added (8.3 were the natural pH of the reconstitute water).

The IC50 was calculated from results of embryo-larval assay and presented in table 2. The different methods of acidification presented different values of IC50 associated with CC, evidencing HCl as most toxic than CO_2 when associated to the same concentrations of CC.

Table 2. Values of IC50 derived from CC concentrations in the different pH values for both methodologies of acidification (HCl and CO_2).

pH values	IC50 (mg. L^{-1})	
	CO_2	HCl
8.3	14.08 (12.66 - 15.30)	8.85 (8.64 - 9.01)
8.0	13.85 (12.50 - 14.60)	8.72 (8.44 - 8.95)
7.7	9.37 (4.66 - 16.15)	3.92 (3.73 - 4.14)
7.5	-	-

Regarding to pH effects, table 4 shows the EpH50 and E[H^+]50 derived at the different treatments, that is, the pH value that causes effects in 50% of the embryos after 44 h exposure. The HCl acidification method presented greater effect on the organisms (including control groups) when compared to CO_2 method. In addition, CC showed more severe toxic effects when associated with acidification by HCl, as observed in tables 3 and 4. This increase in toxicity may be related to the chemical reaction of the HCl acid, that release protons H^+ and ions of Cl^-, against the bicarbonate ($H_2CO_3^-$) released from CO_2.

Table 4. Values of EpH50 and (control and crack-cocaine concentrations) for both methodologies of acidification (HCl and CO_2).

CC Concentrations (mg. L^{-1})	CO_2		HCl	
	EpH50	$E[H^+]50$ (mol. kg^{-1})	EpH50	$E[H^+]50$ (mol. kg^{-1})
Control	7.34	4.56×10^{-8}	7.53	2.95×10^{-8}
6.25	7.55	2.81×10^{-8}	7.65	2.21×10^{-8}
12.5	7.61	2.41×10^{-8}	8.18	0.66×10^{-8}
25	7.58	2.61×10^{-8}	-	-
50	8.18	0.66×10^{-8}	-	-

Since the intracellular pH of sea urchin eggs is known to rise after insemination and trigger the initiation of embryonic development in addition to the impact on sperm motility, the low intra cellular egg pH may prevent fertilization and subsequent development (Kurihara, 2008). Different components of larval physiology are affected by different carbonate system parameters for the species (and perhaps life-stage) studied. However, it is important to note that failure to embryo-larval develop represents a significant bottleneck in population dynamics, and while other carbonate system parameters may act as stressors, saturation state (Ωarag) appears to matter most first for the rapid shell building of prodissoconch I phase in bivalve larvae (Waldbusser et al., 2015). Our results put in evidence that the gametes of *E. lucunter* and *P. perna* are affected by acidification when exposed to realistic pH reductions until the end of this century, and exposure to bioactive compounds as illicit drugs could be more toxic in such conditions.

References

ASTM E724-98, 2012. Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs [WWW Document]. ASTM Int. West Conshohocken, PA. https://doi.org/10.1520/E0724-98

Bernstein, L., Bosch, P., Canziani, O., Chen, Z., Crist, R., et al., 2007. Summary for policymakers of the synthesis report. In: allali, a. (ed.), climate change, 2007: fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, p. 22.

Bindoff, N.L., Willebrand, J., Artale, V., Cazenave, A., Gregory, J., et al., 2007. Observations: oceanic climate change and sea level. In: solomon, s., qin, d., et al. (eds.), climate change 2007: the physical science basis. Contribution of working group i to the fourth assessment report of the intergovernmental panel on climate change. CambridgeUniversity Press, Cambridge, United Kingdom (NewYork, NY, USA, 48pp.).

Caldeira, K., Wickett, M.E., 2003. Anthropogenic carbon and ocean pH. Nature 425, 365–365. https://doi.org/10.1038/425365a

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

Doney, S., Fabry, V., Feely, R., Kleyplas, J., 2009. Ocean acidification: the other CO_2 problem. Annu. Rev. Mar. Sci. 1, 169–192.

Iwata, K., 1980. Mineralization and architecture of the larval shell of *Haliotis discushannai* Ino, (Archaeogastropoda). J. Fac. Sci. Hokkaido University IV 19, 305–320.

Kurihara, H., 2008. Effects of CO_2 -driven ocean acidification on the early developmental stages of invertebrates. Mar. Ecol. Prog. Ser. 373, 275–284. https://doi.org/10.3354/meps07802

Orr, J.C., Caldeira, K., Fabry, V., Gattuso, J.-P., Haugan, P., et al., 2009. Research Priorities for Ocean Acidification, SCOR, UNESCO-IOC, IAEA, and IGBP (25 pp.)

Raven, J., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., et al., 2005. Ocean Acidification due to Increasing Atmospheric Carbon Dioxide. The Royal Society (68 pp.)

Waldbusser, G.G., Hales, B., Langdon, C.J., Haley, B.A., Schrader, P., Brunner, E.L., Gray, M.W., Miller, C.A., Gimenez, I., Hutchinson, G., 2015. Ocean acidification has multiple modes of action on bivalve larvae. PLoS One 10. https://doi.org/10.1371/journal.pone.0128376

Weiss, I., Tuross, N., Addadi, L., Weiner, S., 2002. Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite. J. Exp. Zool. 293, 478–491.

Zaroni, L.P., Abessa, D.M.S., Lotufo, G.R., Sousa, E.C.P.M., Pinto, Y.A., 2005. Toxicity testing with embryos of marine mussels: Protocol standardization for *Perna perna* (Linnaeus, 1758). Bull. Environ. Contam. Toxicol. 74, 793–800. https://doi.org/10.1007/s00128-005-0651-x

Harmful effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios

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Abstract

This study has as main objective assessing the toxicity of crack-cocaine combined with different scenarios of ocean acidification on fertilization rate and embryo-larval development of *Echinometra lucunter* sea urchin. Effects on early life stages were assessed at five different concentrations (6,25 mg.L^{-1}; 12,5 mg.L^{-1}; 25 mg.L^{-1}; 50 mg.L^{-1} and 100 mg.L^{-1}) of crack-cocaine at four different pH values (8.5; 8.0; 7.5; 7.0). The pH values were achieved using two different

methodologies: adding hydrochloric acid (HCl) and injecting carbon dioxide (CO_2). The fertilization test did not show significant differences ($p \leq 0.05$) compared with control sample at pH values 8.5; 8.0 and 7.5. Results of embryo-larval assays showed a half maximal effective concentration (EC50) of crack-cocaine at pH values tested (8.5, 8.0, 7.5) as 58.83, 10.67 and 11.58 mg/L^{-1} for HCl acidification and 58.83, 23.28 and 12.57 mg/L^{-1} for CO_2 enrichment. At pH 7.0 the effects observed in fertilization rate and embryo development were associated with the acidification. This study is the first ecotoxicological assessment of illicit drug toxicity in aquatic ecosystems at different ocean acidification scenarios.

Keywords: Illicit drugs; CO_2 enrichment; Crack-cocaine; Early life stages; Ocean acidification; Sea-urchin.

1. Introduction

As a recent environmental concern, illicit drugs have been targeted by some studies that have shown the occurrence and effects of this group of compounds in aquatic ecosystems (Binelli et al., 2013, 2012; Parolini et al., 2013). According to Zuccato and Castiglioni (2009) the global consumption of illicit drugs are comparable with those of therapeutic drugs, taking into account the number of individuals that are current users of illegal substances as cocaine, marijuana, amphetamine, heroin and others. In analogy with occurrence for therapeutic drugs, residues of illicit drugs that persist in a consumer's urine reach sewage networks through domestic wastewater and are also only partially removed by some sewage treatment plants (STPs) (Baker and Kasprzyk-Hordern, 2013; Borova et al., 2014; Pal et al., 2013).

The illicit drugs most widely used around the world are cannabis and cocaine (UNODC, 2014). United Nations Office on Drug and Crimes identified the increased use of stimulants, such as cocaine and its byproducts, in emerging nations as Brazil (UNODC, 2014). There are some factors which highlight this rise in consumption rate: (i) Brazil's geographic position, neighbouring the world's largest cocaine producers — Peru, Colombia and Bolivia; (ii) the socio-economic rise seen in the last decade in Brazil which represents higher purchasing power and (iii) the cheap price of cocaine in the country (UNODC, 2012).

With only about 1% excreted in the urine unchanged, cocaine is extensively metabolized, especially in the liver, where the metabolism is dominated by the ester hydrolytic cleavage, causing the metabolites eliminated to consist mainly of benzoylecgonine (BE), its main metabolite, and other significant metabolites in smaller amounts, such as ecgonine methyl ester (EME) and ecgonine (García-Cambero et al., 2015). Once cocaine and its metabolites are excreted, they reach inland waters directly by sewage outfalls. At best, cocaine and its metabolites reach a treatment plant (Castiglioni et al., 2011), nevertheless, conventional treatments are only able to remove part of these substances (Domènech et al., 2009; Zuccato et al., 2008), so, most of the cocaine and its metabolites will reach surface waters, contributing to an increased amount of pollution (Pereira et al., 2016).

It is interesting to note that even though cocaine is almost totally metabolized by the body; the compound is present in surface water. Recent studies have demonstrated the environmental concentration of these compounds in ranging from 35.3 to 572 $ng.L^{-1}$ in freshwater environments, (Castiglioni et al. 2006; Metcalfe et al. 2010; Baker et al. 2012; Baker and Kasprzyk-Hordern 2013) and from 7.8 to 400.5 $ng.L^{-1}$ in marine environments (Borova et al., 2014; Pereira et al., 2016). Drugs in general have a specific characteristic in relation to its pKa values (negative

logarithm of the ionization constant). When the drug has a pH similar to pKa, it is found at 50% in the ionic form and 50% in molecular form. The general rule is: Acidic drugs are favored, relative to their absorption, at an acid pH and their elimination is favored at a basic pH. Cocaine is a basic drug, when combined with sodium bicarbonate it converts into a free base form (crack-cocaine), an alkaloid compound, with the unusual characteristic of being either highly hydrophilic or lipophilic (Florence and Attwood, 2006).

The bioavailability of pharmaceuticals, as well as that of illicit drugs, is related to their pKa value and whether the alkaline pH of the coastal zones favor their bioavailability compared to freshwater environments (Pereira et al., 2016). To clarify, the contaminant that is the focus in this work (crack-cocaine), with a value of pKa = 8.5, tends to be partially found in its nonionic form at the pH of the sampling area (ranging from 7.9 to 8.3), this is due to the increase of the octanol-water partition of cocaine coefficient values (log Kow) from 0.10 (ionic form) to 2.30 (nonionic form) (EPISuite, 2012).

In the recent decades there has been significant progress in technology for numerical integration of models, such as, the mathematical representation of physical, chemical and biological processes in their complex interactions within the global climate system (Dameris and Jöckel, 2013; Randall et al., 2007). Thereby, the global and regional climate models have become a great tool available for the next generation considering possible scenarios in climate and environmental changes. According to the IPCC report (2014), the increase in CO_2 emissions due to anthropogenic activities tripled since 1970, representing 34.8 ± 2.9 $GtCO_2$/yr. The importance of fossil fuels as a source of electricity generation for the current society is undeniable, which points CO_2 as being the most important greenhouse gas (GHG) (Pires et al., 2011).

Since the pre-industrial era, the atmospheric concentration of CO_2 has increased from 280 ppm to currently over 410 ppm (Tans and Keeling, 2017). Due to physical and biological interactions between the ocean and the atmosphere ~ 30% of anthropogenic CO_2 is absorbed by the ocean leading to its acidification. As a consequence, the pH of the seawater has decreased 0.1 units since pre-industrial times and it is predicted that by the end of this century there will be a decline of more than 0.3-0.5 pH units (Caldeira and Wickett, 2005; Orr et al., 2005). Increases in the pCO_2 concentration in seawater also reduces the saturation state of calcium carbonate minerals (Marangoni et al., 2017). These changes in the carbonate chemistry of seawater are important drivers for biological and geochemical alterations in the marine ecosystem (Rost et al., 2008; Widdicombe et al., 2015; Zhan et al., 2017). For example, acidification increases the mobility of contaminants from sediments to the water column increasing their bioavailability and therefore their toxicity (de Orte et al., 2014; Nardi et al., 2018; Wang et al., 2015). The decreases in saturation state of carbonate minerals directly affects organisms that require carbonate to build their shells or skeletons, such as corals, gastropods and sea urchins (Bautista-Chamizo et al., 2016; Doney et al., 2009; Sekizawa et al., 2017).

Biological assays using reproductive parameters of sea urchin have been widely employed to assess toxicity of a variety of samples, including chemical compounds, effluents, water or sediment (elutriate, interstitial water, interface sediment-water) in a rapid, sensitive and cost-effective way (Cesar et al., 2004; Salco-Alvaréz et al., 2010). These experiments are internationally accepted as suitable for toxicity, hazard or risk assessments (Environment Canada, 1992; USEPA, 1995; OSPAR, 2007; Associação Brasileira de Normas Técnicas, 2012), and have been employed to better understand negative effects on reproduction (relevant endpoint linked to populational level) of an organism broadly recommended for water bioassays.

The hypothesis of this work is that ocean acidification will trigger enhanced toxicity of illicit drugs to non-target marine organisms due the combined effects of these bioactive substances and low pH. To verify this hypothesis, our main objective was assessing the acute toxicity of crack-cocaine at different scenarios of ocean acidification on fertilization rate and embryo-larval development of *Echinometra lucunter* (Echinodermata: Echinoidea).

2. Material and methods

2.1.Simulating Acidification Scenarios:

Two different methods were used to acidify the samples containing crack-cocaine (by Chloridric acid and CO_2 injection system). The pH values applied in our experiments ranged from 8.5 to 7.0 (8.5; 8.0; 7.5; 7.0); where 8.5 is the pH of natural water (no acidified methodology applied) collected in a reference area at Enseada Beach in Guarujá/SP – Brazil (CETESB, 2016).

The CO_2 injection system (Fig.1) used for this experiment is an adaptation of the experimental set up described by de Orte et al. (2014b), patent process nº: P201200753, Cadiz University, Faculty of Marine and Environmental Sciences, Physical Chemistry Department (RNM 375). Controlled by Aqua Medic AT control hardware, the system injects CO_2 gas (Air Liquide) according to the newly programmed configuration. Thereat, it is possible to have in real time the information from the pH electrodes (NBS balance) introduced in each one of the glass beckers used during the experiment. Solenoid valves are automatically triggered every time the pH changes from 0.01 units, opening when pH increases and closing the gas injection when the pH is predetermined is reached.

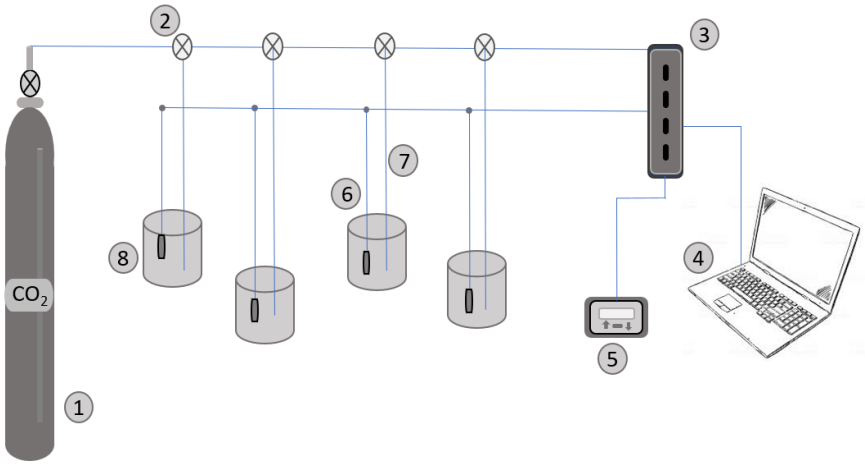

Fig. 1. CO_2 injection system (1. CO_2 gas bottle. 2. Solenoid valves for the electronic regulation of the CO_2 injection 3. Power strips and USB connectors. 4. Laptop with software (Aquamedic 8.0). 5. AT-Control system. 6. pH interface to connect the pH sensor to the AT- Control System. 7. CO_2 injection hose. 8. 1 L glass Becker flasks).

For the experiments conducted with HCl, a methodology adapted by Riba et al. (2010) from Gattuso and Lavigne (2009) was used, where a strong acid (HCl) was applied used in order to modify the total alkalinity. Thus, the different pH values were achieved by addition 2M HCl (37%) and pH measurements were taken every 5 hours, to verify the need of add more HCl to keep the pH constant throughout the toxicity test.

2.2.Chemical Characterization

The drug was donned as a courtesy by the Criminal Department of Limeira city, Sao Paulo State-Brazil, for research purposes. An aliquot of crack cocaine (100 mg) was analyzed by

LCMS/MS to quantify cocaine. It was analyzed by an HPLC Agilent 1260 (Agilent Technologies, CA, USA) combined with a 3200 QTRAP hybrid triple quadrupole/LIT (linear ion trap) mass spectrometer Sciex, Ontario (Canada), according procedure described by Shihomatsu (2015) and employed by Pereira et al. (2016). The cocaine primary standard was purchased from Cerilliant – Sigma Aldrich (Lot FE07271503).

A potentiometric titration system (Metrohm 794 Basic Titrino) with a glass electrode (Metrohm, ref. 6.0210.100) calibrated in NBS scale was used for total alkalinity (TA) measurements which was performed in triplicate. pH and TA values were used to calculate carbonate chemistry parameters in the software CO_2SYS (Pierrot et al., 2006) using dissociation constants by Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 according to Dickson (1990).

2.3.Toxicity Tests

Specimens of *Echinometra lucunter* were collected at Ilha das Palmas in Santos Bay, SP/Brazil. After the collection, the sea urchins were transported in cool boxes to the laboratory and placed in 500 liters tanks with aerated seawater before testing. The gametes were obtained by KCl (0.5 M) injection following ABNT NBR 15350 (Associação Brasileira de Normas Técnicas, 2012). As soon as the organisms started releasing the gametes, they were targeted for the fertilization procedure.

2.3.1. Fertilization bioassay

In order to obtain the fertilization rate, 50 µL sperm solution was added to test tubes with 10 mL of crack-cocaine solution at different pH values (four replicates per treatment). After one hour of sperm solution exposure to different treatments, an egg suspension containing 600 oocytes was added to each test tube. After 40 minutes of oocytes addition the experiment was finalized by the addition of 40% formaldehyde at pH 7.0 (pH was buffered by the addition of a 40 $g.L^{-1}$ solution of borax). After 100 eggs were counted in each replicate by using a light microscope with ×100 magnification, the fertilization success was assessed by the presence of fertilization polar body, fertilization membrane or first cellular divisions. The test was considered valid when ≥ 80 % eggs were successfully fertilized in the control (Cortez et al., 2012).

2.3.2. Embryo-larval development bioassay

The embryo-larval development assay followed the procedure described by ABNT NBR 15350 (Associação Brasileira de Normas Técnicas, 2012), where the fertilization was achieved by adding approximately 1.5 mL of sperm solution into 250 mL of the eggs suspension and left for 60 min at room temperature. Then approximately 25,000 embryos were introduced into 1.3 L glass chambers containing 500 mL of filtered (0.22 µm) seawater, at 25 °C and salinity of 35 ± 1 ppt, 7.5 $mg.L^{-1}$ dissolved oxygen, 12h light/dark photoperiod and the desired pH for each treatment. The exposure time was 42 h, where embryos remained in vessels with different pH and crack-cocaine concentrations and the assay was finished by adding 40% formaldehyde. After the process was finalized, 100 larvae were counted for each replicate and the larvae developed to the pluteus stage was considered as an endpoint for the embryo-larval toxicity test (Associação Brasileira de

Normas Técnicas, 2012). The test was considered valid when ≥ 80 % larvae were successfully developed in the control.

Aiming to provide an initial rapid approach and to identify acute effects, we have employed a short-term exposure to relatively high concentrations of cocaine and low pH values. The selection of the crack-cocaine concentrations used in this experiment to assess combined effects (drug and reduced pH) was based on previous effect concentrations reported by Maranho et al. (2017), and environmental studies reporting cocaine concentrations in aquatic ecosystem up to 5.0 $\mu g.L^{-1}$ (Thomas et al., 2014). Thus, five different concentrations of crack-cocaine (6,25 $mg.L^{-1,}$, 12,5 $mg.L^{-1}$, 25 $mg.L^{-1}$, 50 $mg.L^{-1}$, and 100 $mg.L^{-1}$) were used to determine the toxic effects at different acidification scenarios defined by the 4 different pH values: 8.5; 8.0; 7.5 and 7.0.

2.4.Data treatment (Statistical analysis)

The half maximal effective concentration (EC50) in this study refers to the concentration of crack cocaine capable of causing effects in 50% of the exposed population. Regarding the effects caused by the variation in the pH values or the H^+ concentration in 50% of the exposed population, the terms EpH 50 or E [H^+] 50 were used, according Basallote et al., (2017). Through the polynomial interpolation method, it was calculated the crack-cocaine concentrations, combined with different pH treatments, able to affect 50% of the fertilization rate or to inhibit the embryo larval development of *Echinometra lucunter* sea urchin (USEPA, 1995).

After the assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk's and Levene's tests, respectively, one-way ANOVAs followed by Tukey's post-hoc

tests were conducted using Statistical software SPSS 15.0 for Windows. Statistical analyzes were performed comparing of the results of the different pH values (protons) and crack-cocaine concentrations with the control (natural sea water, pH 8.5) and were classified according to their statistical significance, as follows: * ($p < 0.05$), ** ($p < 0.01$).

3. Results and discussion

3.1.Chemical analysis

The system maintained the nominated pH and temperature treatments (Table 1) throughout the experimental period with little variation and the mean values for the carbonate system speciation are shown in Table 2. As expected, the total inorganic carbon (TIC) and pCO_2 increased when the pH value decreased. In this sense and according to Fabry et al., (2008), as well as proven by our results, an increase in CO_2 concentrations decreases CO_3^{2-} rates, thereby lowering $CaCO_3$ saturation levels, that can be observed by an undersaturation in index for calcite (Ωcal) and aragonite (Ωarag).

Table 1. shows a summarized description of toxicity test and different water parameters (pH, Dissolved Oxygen; Temperature, and Salinity) measured in the different assays. For the pH average ± SD values were calculated from the pH data that were measured every 30 min during the 48 h of experiment exposure.

pH Treatment		pH Seawater	Salinity	D. O. (mg.L^{-1})	T (°C)
HCl					
8.5	Control	8.50 ± 0.02	35	6.0	25
	6.25	8.50 ± 0.03	35	6.2	25
	12.5	8.50 ± 0.04	35	6.4	25
	25	8.50 ± 0.01	35	6.1	25
8.0	Control	8.00 ± 0.02	35	6.6	25
	6.25	8.00 ± 0.04	35	6.9	25
	12.5	8.00 ± 0.05	35	5.8	25
	25	8.00 ± 0.02	35	6.3	25
7.5	Control	7.50 ± 0.07	35	6.8	25
	6.25	7.50 ± 0.03	35	6.1	25
	12.5	7.50 ± 0.08	35	6.4	25
	25	7.50 ± 0.01	35	6.2	25
7.0	Control	7.00 ± 0.02	35	6.8	25
	6.25	7.00 ± 0.03	35	6.4	25
	12.5	7.00 ± 0.01	35	6.3	25
	25	7.00 ± 0.02	35	6.5	25
CO2					
8.5	Control	8.48 ± 0.04	35	6.6	25
	6.25	8.50 ± 0.03	35	6.5	25
	12.5	8.52 ± 0.03	35	6.5	25
	25	8.51 ± 0.02	35	6.5	25
8.0	Control	8.00 ± 0.04	35	6.2	27
	6.25	8.00 ± 0.03	35	6.2	27
	12.5	8.01 ± 0.03	35	6.1	27
	25	7.99 ± 0.02	35	6.0	27
7.5	Control	7.50 ± 0.02	35	6.2	26
	6.25	7.51 ± 0.04	35	6.3	26
	12.5	7.52 ± 0.03	35	6.3	26
	25	7.50 ± 0.04	35	6.0	26
7.0	Control	7.01 ± 0.05	35	6.1	25
	6.25	6.99 ± 0.02	35	5.9	25
	12.5	6.99 ± 0.03	35	6.1	25
	25	7.00 ± 0.06	35	6.2	25

Table 2. Carbonate system speciation in assays exposed to the different pH treatments for both bioassays of fertilization and embryo-larval development.

Concentration ($mg.L^{-1}$)	pH treatment	TA (µmol/kg)	TIC/DIC (µmol/kg)	HCO_3^- (µmol/kg)	CO_3^{2-} (µmol/kg)	CO_2 (µmol/kg)	pCO_2 (µatm)	Ωcal	Ωarag
6.25	8.0	1820	1689	1573	96	20	725	2.31	1.53
12.5	8.0	1864	1734	1616	97	21	758	2.33	1.54
25	8.0	1883	1735	1609	107	19	678	2.59	1.71
6.25	7.5	1973	1982	1873	40	69	2488	0.95	0.63
12.5	7.5	1998	2010	1899	39	71	2578	0.95	0.63
25	7.5	2015	2030	1918	39	73	2660	0.94	0.62
6.25	7.0	1930	2113	1895	14	203	7367	0.33	0.22
12.5	7.0	1873	2049	1839	13	196	7111	0.32	0.21
25	7.0	1896	2079	1863	13	203	7367	0.32	0.21

Numerals studies had showed the differences in the carbonate speciation between HCl and CO_2 methodologies (Bautista-Chamizo et al., 2016; Kurihara and Shirayama, 2004; Sun et al., 2017, 2016), and according to Sun et al. (2016) the main difference between methodologies is how the chemical interacts in the sea water, where CO_2 enrichment showed an increase of H^+, HCO_3^-, and H_2CO_3 concentrations and the decrease of CO_3^{2-} concentration. On the other hand, the addition of HCl only increased the concentrations of H^+ and H_2CO_3 in seawater while decreased those of HCO_3^- and CO_3^{2-}.

The aliquot of crack cocaine analyzed by LC-MS/MS contained 37.99% of cocaine. It was not possible to measure the real concentrations of cocaine in the exposure aliquot, however, as reported by van Nuijs et al. (2009) to wastewater and by Maranho et al. (2017) to marine water, a low decrease in crack-cocaine (CC) concentrations during the assays is expected.

3.2.Toxicity bioassays

3.2.1. Fertilization rate assay

The results obtained by the fertilization tests are shown in figure 2 for both acidification methodologies (HCl and CO_2), control sample and the six concentrations of crack-cocaine (CC) used in the experiment. As it was demonstrated, there is no significant effect ($p \leq 0.05$) on the fertilization success of the sea urchin associated with the concentrations of crack-cocaine ranging from 6.25 to 100 $mg.L^{-1}$ when associated with pH values over to 7.5.

Decreases in pH values down to 7.5 did not affect fertilization rate of sea-urchins. However, when pH was lowered to 7.0 fertilization rates were significantly lower than the control ($p<0.01$). According to Bögner (2016), OA (pH> 7.5) leads the sperm to initiate a Na^+-dependent acid extrusion due to the Na^+ -H^+ exchangers (NHE) and Na^+ -K^+ -ATPase activities in the flagellar plasma membrane, lowering the ATP's production and hindering sperm mobility. In all concentrations of crack-cocaine tested, as well as all pH values, the results between both acidification methods used in the study (CO_2 and HCl) were similar.

The samples showed to be different about success for fertilization rate and differences in the state of cell division could be observed. That is, in samples with higher pH values, there was a larger number of cells provided with the division (6-12 cells). Nonetheless in lower pH values samples the division rate was lower (2-4 cells) or there was even only detected the presence of a polar body.

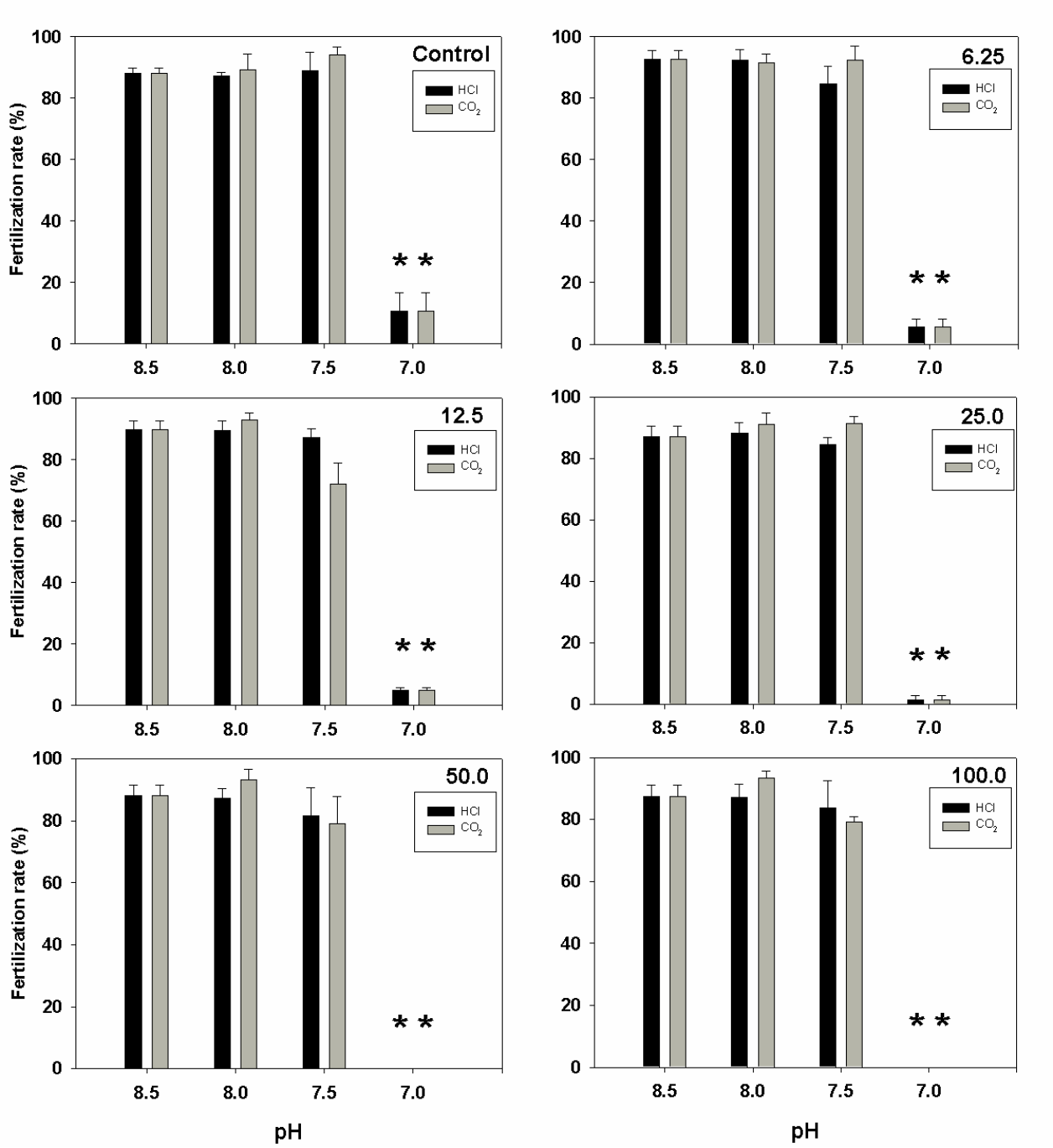

Fig. 2. *Echinometra lucunter* fertilization rate results obtained after exposure to different concentrations of crack-cocaine (6.25 mg.$L^{-1,}$, 12.5 mg.L^{-1}, 25 mg.L^{-1}, 50 mg.L^{-1}, and 100 mg.L^{-1}) at different scenarios of acidification defined by pH values. *means significant difference to the control ($p < 0,05$).

3.2.2. Embryo larval assay

Results of the embryo larval bioassay using *E. lucunter* are shown in figure 3, which only shows the values for the concentration 6.25; 12.50 and 25 (all in mg L^{-1}) for both methodologies used to mimic the acidification conditions (CO_2 and HCl), since the highest concentrations of crack-cocaine (50 mg.L^{-1} and 100 mg.L^{-1}) were highly toxic, culminating in 0% of normal larval development for both methodologies (CO_2 and HCl).

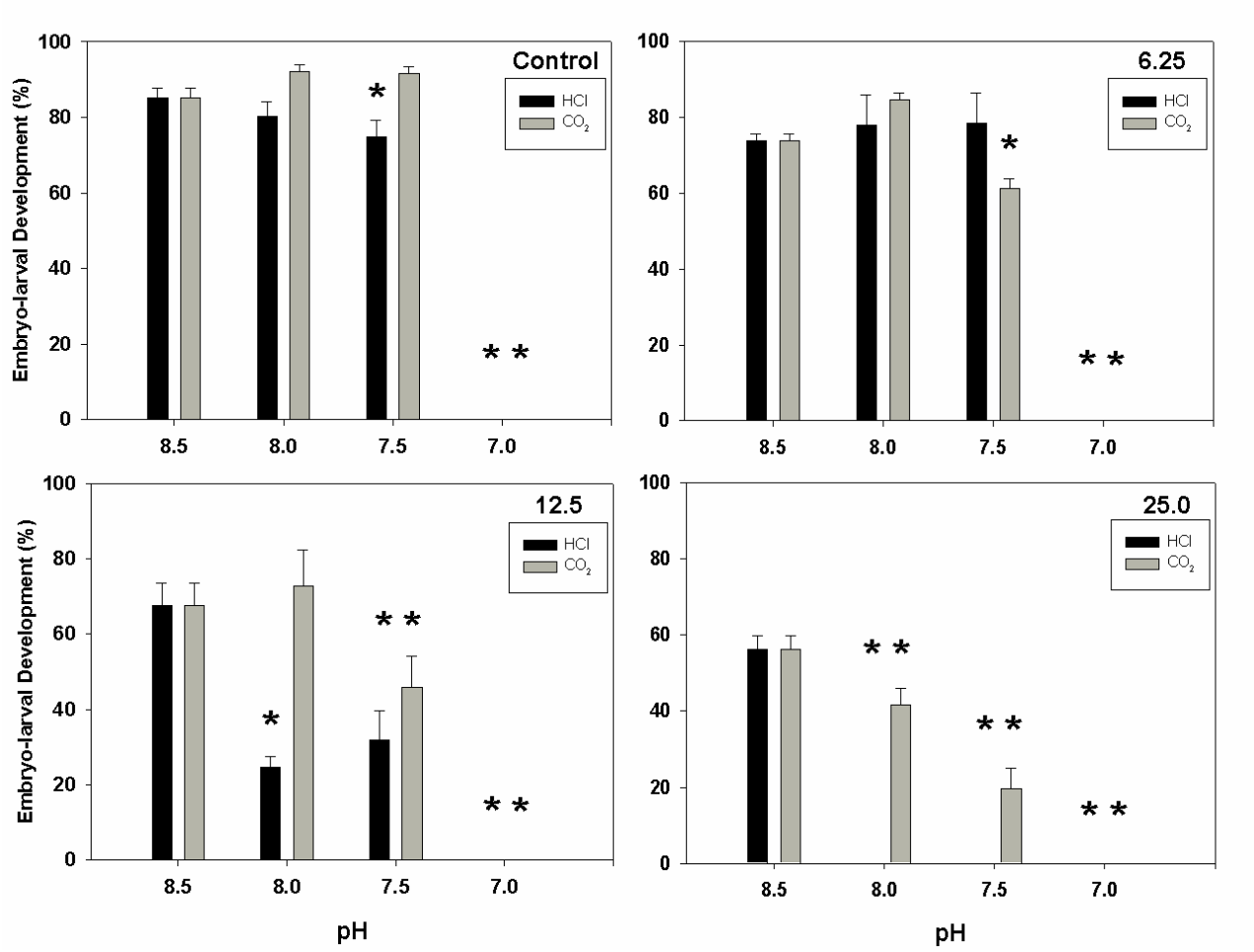

Fig 3**.** Results from larval development success of *Echinometra lucunter*. The larvae were exposed to different concentrations of crack-cocaine (6.25 mg.L^{-1}, 12.5 mg.L^{-1}, 25 mg.L^{-1}, 50 mg.L^{-1}, and 100 mg.L^{-1}) at different scenarios of acidification defined by pH values. *means significant difference to the control ($p < 0,05$).

The decrease in the pH value had a negative effect in embryo larval development when associated with the different crack-cocaine concentrations tested for both methodologies of acidification, showing a high significant reduction ($p < 0.05$) in the percentage of normally larvae developed in acidified medium (no crack-cocaine added). At the lowest pH tested, 0% of larvae were developed regardless of the tested concentration. Conversely, at the pHs 7.5 and pH 7.0 toxic effects were observed in all concentrations of crack-cocaine tested, with a significant decrease in percentage of normal larvae developed. Hence, the abnormalities finding in most of the larvae with some alteration includes the final development of arms not complete, the final shape different from the control and size of the arms were different between them. Also, regarding to pH treatments below 7.0, cell degradation processes were vastly observed. Thus, larvae with some abnormally shape were found or even in early stages of development as just a polar body or in the early stages of cell division.

As shown in figure 3, toxic responses of crack-cocaine differed depending on the acidification method. In the HCl bioassay there are significant decreases ($p<0.05$) on the development of larvae at concentration of 12.5 mg-L^{-1}. Whereas for CO_2 addition experiments, the effects can be observed from the lowest concentration of crack-cocaine used (6.25 mg-L-1) combined with a low variation at the pH value (7.5). The effects start in lower concentrations of CC when CO_2 is used than those detected in the HCl treatments. Regarding the values of pH higher than 7.5, a similar decrease could be observed, although starting at a concentration of crack-cocaine of 12.5 mgL^{-1}.

The EC50 was defined as the crack-cocaine concentration that causes effects in 50% of the population exposed and, in this case, on the success of the embryo larval normal development, as shown by the results obtained in table 3. The control treatment (no HCl or CO_2 addition) presents

an EC50 of crack/cocaine of 58.83. Therefore, EC50 values decrease with decreasing Ph, which reveals that acidification increases the toxicity of crack-cocaine compounds. This effect is higher with the HCl addition method when compared to CO_2 injection.

Table 3. Values of EC50 derived at the different treatments (control and concentrations) for both methodologies of acidification (HCl and CO_2). Values of EC50 at pH 8.5 show the results for the control without acidification method.

pH values	EC50	
	HCl	CO_2
8.5	58.83 (52.54 - 63.38)	58.83 (52.54 - 63.38)
8.0	10.67 (10.31 - 10.87)	23.28 (22.44 - 24.30)
7.5	11.58 (10.88 - 13.04)	12.5 (10.60 - 14.91)
7.0	-	-

To the best of our knowledge, there are no previous studies in the adverse effects of crack-cocaine to sea urchin. Maranho et al. (2017) reported crack cocaine toxicity for brown mussels gamete after exposure to 20 $mg.L^{-1}$ and estimated an IC50= 23.53 $mg.L^{-1}$. Indeed, mussels' embryo-larval development was impaired after 48h of exposure to concentrations up to 1.25 $mg.L^{-1}$ and an IC50= 16.31 $mg.L^{-1}$ was calculated as abnormal development. These results suggested a higher sensitivity of mussels when compared to sea urchin gametes and embryos exposed to pH 8.5 of the present study (EC50 of 58.83 $mg.L^{-1}$). Also within these premises, Parolini et al. (2016) employed zebrafish embryos as a model for cocaine toxicity and reported a significant increase in cell mortality after 96h of exposure to cocaine concentrations higher than 4 nM. The same authors reported that cocaine caused a significant increase in DNA fragmentation in embryo cells exposed

to 0.04 nM, confirming its capability to induce primary genetic lesions. In fact, apoptotic and necrotic significantly higher frequencies were noted after cocaine exposures to 0.4 nM and 40 nM, respectively (Parolini et al., 2015).

Crack-cocaine and cocaine cytogenotoxicity was also demonstrated by previous investigations on freshwater and marine invertebrates. Maranho et al. (2017) and Ortega et al. (2018) showed increases in DNA strand breaks for digestive glands of a marine mussel (*Perna perna)* exposed to different concentrations of crack-cocaine (500 $\mu g.L^{-1}$ and 0.5 to 50 $\mu g.L^{-1}$ respectively), besides cytotoxic effects after 168 h exposure. In freshwater mussel *Dreissena polymorpha*, Binelli et al. (2012) observed primary DNA damage with increases in micronucleate cells and a marked rise in apoptosis. In addition, cocaine short-term exposure is able to decrease the stability of lysosomal membranes of mussels haemocytes, highlighting its cytotoxicity (Binelli et al., 2012; Maranho et al., 2017). Furthermore, previous studies have related cocaine and its byproducts toxicity to non-target species with oxidative stress. Parolini et al. (2017) found a significant enhancement of reactive oxygen species (ROS) levels in zebra fish embryos exposed to cocaine in concentrations up to 0.4 nM.

The EpH50 refers to the pH value responsible for causing effect in 50% of the larvae analyzed in the embryo-larval assay. The values found in samples with no CO_2 added were used as control in the statistical analysis, having their results compared to each adjacent concentration. In this sense, Table 4 displays the results obtained in this study and points out the toxic effects on embryonal development increases when the concentration of crack-cocaine and protons increases. Although there is no significant difference, related to the toxic results, between both acidification methodologies (CO_2 and HCl), the treatment using HCl needs lower concentrations of protons to show toxicity, being considered more toxic for the organisms used in this study than the CO_2

treatment. In this regard, the two methods of acidification used in this study (CO_2 and HCl) affect differently the carbonate chemistry and consequently biological responses caused by them. The increase in pCO_2 concentration, for instance, directly increases H^+ concentration, facilitating the permeability of biological membranes causing intracellular acidosis (Sun et al., 2016; Vandenberg et al., 2017).

Table 4. Values of EpH50 derived at the different treatments (control and concentrations) for both methodologies of acidification (HCl and CO_2).

Concentrations ($mg.L^{-1}$)	HCl		CO_2	
	EpH50	$E[H^+]50$ ($mol.kg^{-1}$)	EpH50	$E[H^+]50$ ($mol.kg^{-1}$)
Control	7.21	6.16×10^{-8}	7.18	6.6×10^{-8}
6.25	7.18	6.6×10^{-8}	7.25	5.62×10^{-8}
12.5	8.04	9.1×10^{-9}	7.32	4.78×10^{-8}
25	8.18	6.6×10^{-9}	7.63	2.34×10^{-8}

Moreover, there are different previous studies that have demonstrated the sensitivity of the sea urchin embryonic stages to the marine acidification process to pH range between 7.4 and 6.8 (Havenhand et al., 2008; Kurihara and Shirayama, 2004; Moulin et al., 2011). As demonstrated in our study, although the test of fertilization successes has been considered to be a reliable to assess the presence of pollutants, it has been considered less sensitive than the embryo development test (Basallote et al., 2017; Geffard et al., 2003; Saco-Álvarez et al., 2010). These authors' findings agree with the results shown in our study.

Also, in accordance with the results obtained in the fertilization assay there are different previous studies that use sea urchin *Heliocidaris erythrogramma* fertilization success to indicate that increases in acidification and pCO_2 did not reduce the fertilization rate (Byrne et al., 2010; Havenhand et al., 2008)

The thresholds of proton concentration's (expressed as pH values) calculated for the embryo-larval assay by Basallote et al. (2017) ranged between 6.66 ± 0.03 and 7.16 ± 0.01 and are similar to those reported here for absence of CC concentration in the assays (7.21 and 7.18 for HCl and CO_2 acidification treatments respectively). However, there is no previous literature related to the EpH50 (or effective concentrations of protons) that affect the embryo larval normal development of the *Echinometra lucunter* used in this study. In this sense, these values are first reported data in the bibliography associated with toxic effects by acidification on this specie.

Unfavorable pH conditions related to enrichment of CO_2 will strongly affect early life stages, especially those of calcifying species, which are the most sensitive to elevated CO_2 (Pearce et al., 2014). Thus, when the toxic effects of the CO_2 concentration are assessed, it is recommendable to establish the pH values threshold to determine the concentration of protons that the species used in the different studies could tolerate. It will help to determine the pH range related to the potential chronic effects that should be assessed. There are different authors that have recently suggested (DelValls and Riba, 2007; Khosrovyan et al., 2017; Passarelli et al., 2017a, 2017b) developing new indices of sensitive species to elevated CO_2 on marine ecosystems, as well as the community's responses for the better understanding of the ecological impacts of carbon dioxide enrichment in marine environments.

Although previous results in experiments that combine acidification and metals are recently numerous in the bibliography (Dorey et al., 2018a, 2018b; Sezer et al., 2018; Su et al., 2019), there

is no previous data reported in the scientific bibliography related to the assess of combined effects of illicit drugs and acidification conditions. In this sense, the results obtained in this study are the first obtained and disseminated. Previous authors have reported a limited number of experiments where combined effects between pharmaceuticals or other emerging contaminants with acidification. Freitas et al. (2016) demonstrated that the effects of carbamazepine associated to acidification on survival and oxidative stress of the molluscum *Scrobicularia plana* increased significantly when pH values were lower than 7.1. Munari et al. (2016) showed that the bivalve *Ruditapes philippinarum*'s larvae had their sensitivity to diclofenac increased in the presence of lower pH, presenting anomalies and significant differences in the development of the shells. In another study with the same factors (pH variation and diclofenac), there were showed alterations in oxidative stress, DNA damage, and in the activities of superoxide dismutase, catalase and cyclooxygenase, when analyzed digestive gland from mussel *Mytilus galloprovincialis* and the clam *Ruditapes philippinarum* (Munari et al., 2018).

Sea urchins play essential functional roles in rocky shore ecosystems and constitute commercially important marine products. Consequently, studies regarding the effects of pH combined with drugs on these organisms are of major relevance. Thus, our findings on reproductive impairments after short-term exposure to cocaine in different scenarios of ocean acidification rising concern on bioavailability and toxicity of bioactive compounds in physiological process related to ecological fitness and resilience of key species.

4. Conclusions

The present study provides information on the consequences of changes in the seawater pH associated with different concentrations of crack-cocaine on the embryo-larval development and fertilization rate of sea-urchin *Echinometra lucunter*. The embryo-larval development showed to be more sensitive to acidified environment than fertilization rate. Additionally, regarding to the acidification methods used in this study to induce the different scenarios, the use of the acid HCl demonstrated greater toxicity than when used CO_2. Besides the difference in the toxicity of the acidification methods, both showed to lead an increase in the toxicity of the crack-cocaine.

Although the experiments have shown negative effects only in the lower pHs and relatively high concentrations of crack-cocaine, the organisms also could be impacted by chronic effects, which should be assessed in further studies. To the best of our knowledge, this is the first study demonstrating toxicity of illicit drugs to marine organisms at different acidification scenarios, becoming very important to expand data about ecological risks of ocean acidification associated with bioactive compounds.

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References

Associação Brasileira de Normas Técnicas, A., 2012. Ecotoxicologia aquática — Toxicidade crônica de curta duração — Método de ensaio com ouriço-do-mar (Echinodermata: Echinoidea) NBR 1530.

Baker, D.R., Kasprzyk-Hordern, B., 2013. Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: New developments. Sci. Total Environ. 454–455, 442–456. https://doi.org/10.1016/j.scitotenv.2013.03.043

Baker, D.R., Očenášková, V., Kvicalova, M., Kasprzyk-Hordern, B., 2012. Drugs of abuse in wastewater and suspended particulate matter - Further developments in sewage epidemiology. Environ. Int. 48, 28–38. https://doi.org/10.1016/j.envint.2012.06.014

Basallote, M.D., Rodríguez-Romero, A., De Orte, M.R., DelValls, T.A., Riba, I., 2017. CO_2 leakage simulation: effects of the pH decrease on fertilisation and larval development of *Paracentrotus lividus* and sediment metals toxicity. Chem. Ecol. 7540, 1–21. https://doi.org/10.1080/02757540.2017.1396319

Bautista-Chamizo, E., De Orte, M.R., DelValls, T.A., Riba, I., 2016. Simulating CO_2 leakages from CCS to determine Zn toxicity using the marine microalgae *Pleurochrysis roscoffensis*. Chemosphere 144, 955–965. https://doi.org/10.1016/j.chemosphere.2015.09.041

Binelli, A., Marisa, I., Fedorova, M., Hoffmann, R., Riva, C., 2013. First evidence of protein profile alteration due to the main cocaine metabolite (benzoylecgonine) in a freshwater biological model. Aquat. Toxicol. 140–141, 268–278. https://doi.org/10.1016/j.aquatox.2013.06.013

Binelli, A., Pedriali, A., Riva, C., Parolini, M., 2012. Illicit drugs as new environmental pollutants: Cyto-genotoxic effects of cocaine on the biological model *Dreissena polymorpha*. Chemosphere 86, 906–911. https://doi.org/10.1016/j.chemosphere.2011.10.056

Bögner, D., 2016. Life under Climate Change Scenarios: Sea Urchins' Cellular Mechanisms for Reproductive Success. J. Mar. Sci. Eng. 4, 28. https://doi.org/10.3390/jmse4010028

Borova, V.L., Maragou, N.C., Gago-Ferrero, P., Pistos, C., Thomaidis, N.S., 2014. Highly sensitive determination of 68 psychoactive pharmaceuticals, illicit drugs, and related human metabolites in wastewater by liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 406, 4273–4285. https://doi.org/10.1007/s00216-014-7819-3

Byrne, M., Soars, N., Selvakumaraswamy, P., Dworjanyn, S.A., Davis, A.R., 2010. Sea urchin fertilization in a warm, acidified and high pCO_2 ocean across a range of sperm densities. Mar. Environ. Res. 69, 234–239. https://doi.org/10.1016/j.marenvres.2009.10.014

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J. Geophys. Res. 110, C09S04. https://doi.org/10.1029/2004JC002671

Castiglioni, S., Bagnati, R., Melis, M., Panawennage, D., Chiarelli, P., Fanelli, R., Zuccato, E., 2011. Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine. Water Res. 45, 5141–5150. https://doi.org/10.1016/j.watres.2011.07.017

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R., Bagnati, R., 2006. Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry. Anal. Chem. 78, 8421–8429. https://doi.org/10.1021/ac061095b

Cesar, A., Marín-Guirao, L., Vita, R., Marín Atucha, A., 2004. Ensayos con anfípodos y erizos de mar para evaluar la toxicidad de sedimentos mediterráneos: El caso de la bahía de Portmán. Sci. Mar. 68, 205–213. https://doi.org/10.3989/scimar.2004.68s1205

CETESB, 2016. Companhia Ambiental do Estado de São Paulo - Qualidade das praias litorâneas no Estado de São Paulo.

CETESB, 1999. Água do mar: teste de toxicidade crônica de curta duração com *Lytechinus variegatus* LAMARCK, 1816 (echinodermata: echinoidea) - método de ensaio 126, 23.

Cortez, F.S., Seabra Pereira, C.D., Santos, A.R., Cesar, A., Choueri, R.B., Martini, G.D.A., Bohrer-Morel, M.B., 2012. Biological effects of environmentally relevant concentrations of

the pharmaceutical Triclosan in the marine mussel *Perna perna* (Linnaeus, 1758). Environ. Pollut. 168, 145–150. https://doi.org/10.1016/j.envpol.2012.04.024

Dameris, M., Jöckel, P., 2013. Numerical modeling of climate-chemistry connections: Recent developments and future challenges. Atmosphere (Basel). 4, 132–156. https://doi.org/10.3390/atmos4020132

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

DelValls, T.A., Riba, I., 2007. A weight of evidence approaches to assess sediment quality in the Guadalquivir estuary. Aquat. Ecosyst. Heal. Manag. 10, 101–106. https://doi.org/10.1080/14634980701213025

Dickson, A.G., 1990. Standard potential of the reaction: AgCl(s) +1/2H_2(g) = Ag(s) + HCl(aq), and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. Chem. Thermodynamics,22, 113-127.

Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res. Part A, Oceanogr. Res. Pap. 34, 1733–1743. https://doi.org/10.1016/0198-0149(87)90021-5

Domènech, X., Peral, J., Muñoz, I., 2009. Predicted environmental concentrations of cocaine and benzoylecgonine in a model environmental system. Water Res. 43, 5236–5242. https://doi.org/10.1016/j.watres.2009.08.033

Doney, S.C., Fabry, V.J., Feely, R.A., Kleypas, J.A., 2009. Ocean acidification: the other co_2 problem. Ann. Rev. Mar. Sci. 1, 169–192. https://doi.org/10.1146/annurev.marine.010908.163834

Dorey, N., Maboloc, E., Chan, K.Y.K., 2018a. Development of the sea urchin *Heliocidaris crassispina* from Hong Kong is robust to ocean acidification and copper contamination. Aquat. Toxicol. 205, 1–10. https://doi.org/10.1016/j.aquatox.2018.09.006

Dorey, N., Martin, S., Oberhänsli, F., Teyssié, J.L., Jeffree, R., Lacoue-Labarthe, T., 2018b. Ocean acidification modulates the incorporation of radio-labeled heavy metals in the larvae of the Mediterranean sea urchin *Paracentrotus lividus*. J. Environ. Radioact. 190–191, 20–30. https://doi.org/10.1016/j.jenvrad.2018.04.017

Environment Canada, 1992. Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars), Method Development and Applications Section.

Florence, A.T., Attwood, D., 2006. Physicochemical Principles of Pharmacy. Pharm. Press 286–290.

Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016. The impacts of pharmaceutical drugs under ocean acidification: New data on single and combined long-term effects of carbamazepine on *Scrobicularia plana*. Sci. Total Environ. 541, 977–985. https://doi.org/10.1016/j.scitotenv.2015.09.138

García-Cambero, J., García-Cortés, H., Valcárcel, Y., Catalá, M., 2015. Environmental concentrations of the cocaine metabolite benzoylecgonine induced sublethal toxicity in the development of plants but not in a zebrafish embryo–larval model. J. Hazard. Mater. 300, 866–872. https://doi.org/10.1016/j.jhazmat.2015.08.019

Gattuso, J.-P., Lavigne, H., 2009. Perturbation experiments to investigate the impact of ocean acidification: approaches and software tools. Biogeosciences Discuss. 6, 4413–4439. https://doi.org/10.5194/bgd-6-4413-2009

Geffard, O., Geffard, A., His, E., Budzinski, H., 2003. Assessment of the bioavailability and toxicity of sediment-associated polycyclic aromatic hydrocarbons and heavy metals applied to *Crassostrea gigas* embryos and larvae. Mar. Pollut. Bull. 46, 481–490. https://doi.org/10.1016/S0025-326X(02)00451-4

Havenhand, J.N., Buttler, F., Throndyke, M.C., Williamson, J.E., 2008. Near-future levels of ocean acidification reduce fertilization success in a sea urchin. Curr. Biol. 18, R651–R652. https://doi.org/10.1029/2004JC002671.6.Styan

Hernández, F., Ibáñez, M., Botero-Coy, A.-M., Bade, R., Bustos-López, M.C., Rincón, J., Moncayo, A., Bijlsma, L., 2015. LC-QTOF MS screening of more than 1,000 licit and illicit drugs and their metabolites in wastewater and surface waters from the area of Bogotá, Colombia. Anal. Bioanal. Chem. 407, 6405–6416. https://doi.org/10.1007/s00216-015-8796-x

IPCC, 2014. Climate Change 2014: Mitigation of Climate Change. Summary for Policymakers and Technical Summary, Climate Change 2014: Mitigation of Climate Change. Part of the Working Group III Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https://doi.org/10.1017/CBO9781107415416.005

Khosrovyan, A., DelValls, T.A., Luque, A., Riba, I., 2017. Effects of a hypothetical escape of CO_2 gas from subterranean storage sites on water flea *Daphnia magna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-017-0154-4

Kurihara, H., Shirayama, Y., 2004. Effects of increased atmospheric CO_2 on sea urchin early development. Mar. Ecol. Prog. Ser. 274, 161–169. https://doi.org/10.3354/meps274161

Marangoni, L.F. de B., Calderon, E.N., Marques, J.A., Duarte, G.A.S., Pereira, C.M., e Castro, C.B., Bianchini, A., 2017. Effects of CO_2-driven acidification of seawater on the calcification process in the calcareous hydrozoan *Millepora alcicornis* (Linnaeus, 1758). Coral Reefs 36, 1133–1141. https://doi.org/10.1007/s00338-017-1605-6

Maranho, L.A., Fontes, M.K., Kamimura, A.S.S., Nobre, C.R., Moreno, B.B., Pusceddu, F.H., Cortez, F.S., Lebre, D.T., Marques, J.R., Abessa, D.M.S., Ribeiro, D.A., Pereira, C.D.S., 2017. Exposure to crack cocaine causes adverse effects on marine mussels *Perna perna*. Mar. Pollut. Bull. 0–1. https://doi.org/10.1016/j.marpolbul.2017.08.043

Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicx, R.M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897–907. https://doi.org/10.4319/lo.1973.18.6.0897

Metcalfe, C., Tindale, K., Li, H., Rodayan, A., Yargeau, V., 2010. Illicit drugs in Canadian municipal wastewater and estimates of community drug use. Environ. Pollut. 158, 3179–3185. https://doi.org/10.1016/j.envpol.2010.07.002

Moulin, L., Catarino, A.I., Claessens, T., Dubois, P., 2011. Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816). Mar. Pollut. Bull. 62, 48–54. https://doi.org/10.1016/j.marpolbul.2010.09.012

Munari, M., Chemello, G., Finos, L., Ingrosso, G., Giani, M., Marin, M.G., 2016. Coping with seawater acidification and the emerging contaminant diclofenac at the larval stage: A tale from the clam *Ruditapes philippinarum*. Chemosphere 160, 293–302. https://doi.org/10.1016/j.chemosphere.2016.06.095

Munari, M., Matozzo, V., Gagné, F., Chemello, G., Riedl, V., Finos, L., Pastore, P., Badocco, D., Marin, M.G., 2018. Does exposure to reduced pH and diclofenac induce oxidative stress in marine bivalves? A comparative study with the mussel *Mytilus galloprovincialis* and the clam *Ruditapes philippinarum*. Environ. Pollut. 240, 925–937. https://doi.org/10.1016/j.envpol.2018.05.005

Nardi, A., Benedetti, M., Fattorini, D., Regoli, F., 2018. Oxidative and interactive challenge of cadmium and ocean acidification on the smooth scallop *Flexopecten glaber*. Aquat. Toxicol. 196, 53–60. https://doi.org/10.1016/j.aquatox.2018.01.008

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G.K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature. https://doi.org/10.1038/nature04095

Ortega, A. dos S.B., Maranho, L.A., Nobre, C.R., Moreno, B.B., Guimarães, R.S., Lebre, D.T., de Souza Abessa, D.M., Ribeiro, D.A., Pereira, C.D.S., 2018. Detoxification, oxidative stress, and cytogenotoxicity of crack cocaine in the brown mussel *Perna perna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-018-1600-7

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

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

Parolini, M., Magni, S., Castiglioni, S., Binelli, A., 2016. Genotoxic effects induced by the exposure to an environmental mixture of illicit drugs to the zebra mussel. Ecotoxicol. Environ. Saf. 132, 26–30. https://doi.org/10.1016/j.ecoenv.2016.05.022

Parolini, M., Magni, S., Traversi, I., Villa, S., Finizio, A., Binelli, A., 2015. Environmentally relevant concentrations of galaxolide (HHCB) and tonalide (AHTN) induced oxidative and genetic damage in *Dreissena polymorpha*. J. Hazard. Mater. 285, 1–10. https://doi.org/10.1016/j.jhazmat.2014.11.037

Parolini, M., Pedriali, A., Riva, C., Binelli, A., 2013. Sub-lethal effects caused by the cocaine metabolite benzoylecgonine to the freshwater mussel *Dreissena polymorpha*. Sci. Total Environ. 444, 43–50. https://doi.org/10.1016/j.scitotenv.2012.11.076

Passarelli, M.C., Cesar, A., Riba, I., DelValls, T.A., 2017a. Comparative evaluation of sea-urchin larval stage sensitivity to ocean acidification. Chemosphere 184, 224–234. https://doi.org/10.1016/j.chemosphere.2017.06.001

Passarelli, M.C., Riba, I., Cesar, A., Serrano-Bernando, F., DelValls, T.A., 2017b. Assessing the influence of ocean acidification to marine amphipods: A comparative study. Sci. Total Environ. 595, 759–768. https://doi.org/10.1016/j.scitotenv.2017.04.004

Pearce, J., Jones, D., Blackford, J., Beaubien, S., Foekema, E., Gemeni, V., Kirk, K., Lions, J., Metcalfe, R., Moni, C., Smith, K., Stevens, M., West, J., Ziogou, F., 2014. A guide for assessing the potential impacts on ecosystems of leakage from CO_2 storage sites. Energy Procedia 63, 3242–3252. https://doi.org/10.1016/j.egypro.2014.11.351

Pereira, C.D.S., Maranho, L.A., Cortez, F.S., Pusceddu, F.H., Santos, A.R., Ribeiro, D.A., Cesar, A., Guimarães, L.L., 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Sci. Total Environ. 548–549, 148–154. https://doi.org/10.1016/j.scitotenv.2016.01.051

Pierrot, D., Lewis, E., Wallace, D.W.R., 2006. Ms excel program developed for CO_2 system calculation. Carbon dioxide information anal. Center, Oak Ridge Natl. Lab. U.S. Dep. Energy. ORNL/CDIAC, 105.

Pires, J.C.M., Martins, F.G., Alvim-Ferraz, M.C.M., Simões, M., 2011. Recent developments on carbon capture and storage: An overview. Chem. Eng. Res. Des. 89, 1446–1460. https://doi.org/10.1016/j.cherd.2011.01.028

Randall, D.A., Wood, R.A., Bony, S., Colman, R., Fichefet, T., Fyve, J., Kattsov, V., Pitman, A., Shukla, J., Srinivasan, J., Stouffer, R.J., Sumi, A., Taylor, K.E., 2007. Climate Models and Their Evaluation. Clim. Chang. 2007 Phys. Sci. Basis. Contrib. Work. Gr. I to Fourth Assess. Rep. Intergov. Panel Clim. Chang. 591–662. https://doi.org/10.1016/j.cub.2007.06.045

Rost, B., Zondervan, I., Wolf-Gladrow, D., 2008. Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: Current knowledge, contradictions and research directions. Mar. Ecol. Prog. Ser. 373, 227–237. https://doi.org/10.3354/meps07776

Saco-Álvarez, L., Durán, I., Ignacio Lorenzo, J., Beiras, R., 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicol. Environ. Saf. 73, 491–499. https://doi.org/10.1016/j.ecoenv.2010.01.018

Sekizawa, A., Uechi, H., Iguchi, A., Nakamura, T., Kumagai, N.H., Suzuki, A., Sakai, K., Nojiri, Y., 2017. Intraspecific variations in responses to ocean acidification in two branching coral species. Mar. Pollut. Bull. 122, 282–287. https://doi.org/10.1016/j.marpolbul.2017.06.061

Sezer, N., Kılıç, Ö., Metian, M., Belivermiş, M., 2018. Effects of ocean acidification on ^{109}Cd, ^{57}Co, and ^{134}Cs bioconcentration by the European oyster (*Ostrea edulis*): Biokinetics and tissue-to-subcellular partitioning. J. Environ. Radioact. 192, 376–384. https://doi.org/10.1016/j.jenvrad.2018.07.011

Shihomatsu, H.M., 2015. Desenvolvimento e validação de metodologia spe-lc-ms/ms para a determinacão de fármacos e droga de abuso nas águas da represa guarapiranga-são Paulo/Sp, Brasil. Dissertação.

Su, W., Shi, W., Han, Y., Hu, Y., Ke, A., Wu, H., Liu, G., 2019. The health risk for seafood consumers under future ocean acidification (OA) scenarios: OA alters bioaccumulation of three pollutants in an edible bivalve species through affecting the in vivo metabolism. Sci. Total Environ. 650, 2987–2995. https://doi.org/10.1016/j.scitotenv.2018.10.056

Sun, T., Tang, X., Jiang, Y., Wang, Y., 2017. Seawater acidification induced immune function changes of haemocytes in *Mytilus edulis*: A comparative study of CO_2 and HCl enrichment. Sci. Rep. 7, 1–10. https://doi.org/10.1038/srep41488

Sun, T., Tang, X., Zhou, B., Wang, Y., 2016. Comparative studies on the effects of seawater acidification caused by CO_2 and HCl enrichment on physiological changes in *Mytilus edulis*. Chemosphere 144, 2368–2376. https://doi.org/10.1016/j.chemosphere.2015.10.117

Tans, P., Keeling, R., 2017. NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/) and Dr. Ralph Keeling, Scripps Institution of Oceanography (scrippsco2.ucsd.edu/).

UNODC, 2014. World Drug Report 2014, United Nations publication. https://doi.org/10.1007/s12117-997-1166-0

UNODC, 2012. World Drug Report 2012, New Directions for Youth Development. https://doi.org/10.1002/yd.20002

Usepa, 2004. The incidence and severity of sediment contamination in surface water of the united states national sediments quality survey: second edition 280.

van Nuijs, A.L.N., Pecceu, B., Theunis, L., Dubois, N., Charlier, C., Jorens, P.G., Bervoets, L., Blust, R., Neels, H., Covaci, A., 2009. Spatial and temporal variations in the occurrence of cocaine and benzoylecgonine in waste- and surface water from Belgium and removal during wastewater treatment. Water Res. 43, 1341–1349. https://doi.org/10.1016/j.watres.2008.12.020

Vandenberg, J.I., Metcalfe, J.C., Grace, A.A., 2017. Intracellular pH recovery during respiratory acidosis in perfused hearts. Am. J. Physiol. Physiol. 266, C489–C497. https://doi.org/10.1152/ajpcell.1994.266.2.c489

Wang, Z., Wang, Y., Zhao, P., Chen, L., Yan, C., Yan, Y., Chi, Q., 2015. Metal release from contaminated coastal sediments under changing pH conditions: Implications for metal mobilization in acidified oceans. Mar. Pollut. Bull. 101, 707–715. https://doi.org/10.1016/j.marpolbul.2015.10.026

Widdicombe, S., Beesley, A., Berge, J.A., 2015. Impact of elevated levels of CO_2 on animal mediated ecosystem function: The modification of sediment nutrient fluxes by burrowing urchins. https://doi.org/10.1016/j.marpolbul.2012.11.008

Zhan, Y., Hu, W., Duan, L., Liu, M., Zhang, W., Chang, Y., Li, C., 2017. Effects of seawater acidification on early development of the sea urchin *Hemicentrotus pulcherrimus*. Aquac. Int. 25, 655–678. https://doi.org/10.1007/s10499-016-0064-3

Zuccato, E., Castiglioni, S., 2009. Illicit drugs in the environment. Philos. Trans. A. Math. Phys. Eng. Sci. 367, 3965–78. https://doi.org/10.1098/rsta.2009.0107

Zuccato, E., Chiabrando, C., Castiglioni, S., Bagnati, R., Fanelli, R., 2008. Estimating community drug abuse by wastewater analysis. Environ. Health Perspect. 116, 1027–1032. https://doi.org/10.1289/ehp.11022

Could ocean acidification intensify illicit drug effects on reproduction of marine mussels?

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Abstract

In response to the increasing atmospheric burden of CO_2 and increasing oceanic uptake, the oceans are experiencing both physical and biogeochemical changes: surface and deep water warming, reduced subsurface oxygen, and a reduction in calcium carbonate saturation levels and pH. The changes of pH and seawater chemistry caused by increased CO_2 can modify the speciation of compounds, due to largely dependent element's speciation on physicochemical parameters (salinity, pH, redox potential). The hypothesis of this work is that ocean acidification will trigger enhanced toxicity of illicit drugs to non-target marine organisms due the combined effects of crack-cocaine and low pH (from 8.3 to 7.0 pH values) on the reproduction of the marine mussel *Perna perna*. Fertilization rate and embryo-larval development were conducted in order to assess the

effects of crack-cocaine concentrations (6.25; 12.5; 25; 50 and 100 $mg.L^{-1}$) and its association with pH values variation (8.3; 8.0; 7.5 and 7.0). The IC50 was calculated from results of embryo-larval assay in different methods of acidification (CO_2 and HCl) presented different values when associated with CC, evidencing HCl as most toxic than CO_2 when associated to the same concentrations of CC. Our results put in evidence that the gametes of *P. perna* react to acidification when exposed to crack-cocaine concentration and pH reductions.

Keywords: Illicit drugs; CO_2 enrichment; Crack-cocaine; Early life stages; Ocean acidification; Bivalve.

1. Introduction

Atmospheric CO_2 is at its highest concentration in 800 000 years (Lüthi et al., 2008) causing global environmental change that will continue in the future (IPCC, 2014). Rising CO_2 results in not only climate change but also a decreasing pH of surface seawater (ocean acidification) due to the exchange of CO_2 between the atmosphere and seawater (Cvijanovic and Caldeira, 2015). By 2300, surface ocean pH levels are predicted to decrease by 0.67 units compared to pre-industrial levels (Hartin et al., 2016).

In response to this increasing atmospheric burden of CO_2 and increasing oceanic uptake, the oceans are experiencing both physical and biogeochemical changes: surface and deep water warming, reduced subsurface oxygen, and a reduction in calcium carbonate saturation levels and pH (Doney, 2010). Marine biogeochemical dynamics is increasingly relevant to discussions of ecosystem health, climate impacts and mitigation strategies, and planetary sustainability (Hartin et al., 2016). Numerous experiments and observations indicate that ocean acidification will have

significant effects on calcifying marine organisms (Marangoni et al., 2017; Haley et al., 2018; Orr et al., 2005; Wang et al., 2018), marine bacteria (Borrero-Santiago et al., 2016; Sircar, 2014), amphipod (Goulding et al., 2017), macroalga (Bautista-Chamizo et al., 2016; de Orte et al., 2014a), as well as macrofauna (Passarelli et al., 2018b).

However, ocean acidification (OA) also has the potential to interact with other local and global stressors, as xenobiotics, but to date, interactions between OA and other environmental changes or contaminants are still much understudied (Huang et al., 2018). The changes of pH and seawater chemistry caused by increased CO_2 can modify the speciation of compounds, due to largely dependent element's speciation on physicochemical parameters (salinity, pH, redox potential) (Millero et al., 2009; Stockdale et al., 2016), and therefore their bioavailability for organisms (Dorey et al., 2018). Studies have shown the effect of OA on the speciation of metals (Haley et al., 2018; Passarelli et al., 2018a; Stockdale et al., 2016), however, this is still a poorly investigated research in contaminants as pharmaceuticals.

The occurrence of pharmaceuticals and personal care products (PPCPs) in the environment is increasingly recognized as an important issue (Edwards et al., 2017; Jiang et al., 2014; Maranho et al., 2015; Zuccato and Castiglioni, 2009). These substances, also including illicit drugs, are usually considered as emerging pollutants (Daughton and Ternes, 1999; Snyder et al., 2009). Pharmaceuticals and illicit drugs, once ingested, are partially metabolized and due to their physical–chemical characteristics, in particular their high solubility, enter the sewage stream (Domènech et al., 2009), which inefficient or incomplete treatment, can be considered the main cause of marine pollution.

Once in the environment, illicit drugs are pointed out as causing adverse effects in non-target organisms. Studies with cocaine its byproducts exposure showed genotoxicity, cytogenotoxicity, mutagenicity and oxidative stress on different organisms (Capaldo et al., 2019; Maranho et al., 2017; Ortega et al., 2018; Parolini and Binelli, 2013; Yujra et al., 2016). However, to the best of our knowledge, there is no studies showing the effects of an illicit drug in association with different environmental pH values in mollusks.

Understanding factors affecting survival and growth of juvenile mussels through vulnerable early life stages is critical both for aqua culture efforts and ecology equilibrium. The hypothesis of this work is that ocean acidification will trigger enhanced toxicity of illicit drugs to non-target marine organisms due the combined effects of crack-cocaine and low pH (from 8.3 to 6.0 pH values) on the reproduction of the marine mussel *Perna perna*.

2. **Material and methods**

2.1. Simulating Acidification Scenarios:

The sea water used in the experiment was artificially done in the laboratory by dissolving natural salt from Red Sea (Red Sea salt®) in deionize water until 35 ppm, which is the optimum value for the specie, describe by Zaroni et al. (2005). After the salinity was reach, the reconstituted seawater was filter through a 0.22 µm Millipore membrane. The reconstituted seawater was used as control and also used to diluted the different CC treatments (6,25 $mg.L^{-1,}$, 12,5 $mg.L^{-1}$, 25 $mg.L^{-1}$, 50 $mg.L^{-1}$, and 100 $mg.L^{-1}$).

Two different methods were used to acidify the samples containing crack-cocaine (by Chloridric acid and CO_2 injection system). The pH values applied in our experiments ranged from 8.3 to 7.0.

The CO_2 injection system (Fig.1) used for this experiment is an adaptation of the experimental set up described by de Orte et al. (2014b), patent process nº: P201200753, Cadiz University, Faculty of Marine and Environmental Sciences, Physical Chemistry Department (RNM 375). Controlled by Aqua Medic AT control hardware, the system injects CO_2 gas (Air Liquide) according to the previously established configuration. Through pH electrodes (NBS balance), inserted in each of the glass becker, it is possible to monitor in real time the information of each treatment during the experiment. The solenoid valves are automatically triggered every time the pH changes from 0.01 units, opening when pH increases and closing the gas injection when the predetermined pH is reached.

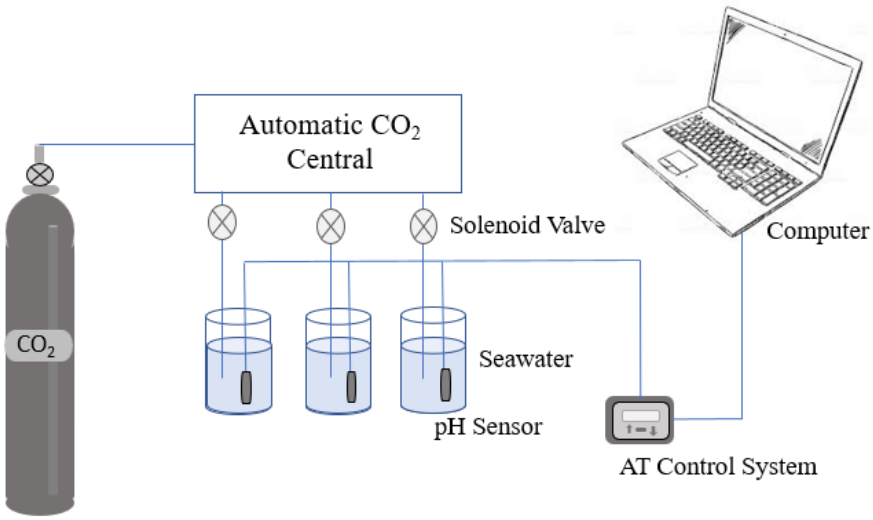

Figure 1: Schematic design of the CO_2 injection system used to implement the toxicity tests carried out in this study.

For the experiments conducted with HCl, a methodology adapted by from Gattuso and Lavigne (2009) was used, where a strong acid (HCl) was used in order to modify the total alkalinity. Thus, the different pH values were achieved by addition 2M HCl (37%) and pH measurements were taken every 5 hours, to verify the need of add more HCl to keep the pH constant throughout the toxicity test.

2.2. Chemical Characterization

The drug was donned as a courtesy by the Criminal Department of Limeira city, Sao Paulo State-Brazil, for research purposes. An aliquot of crack cocaine (100 mg) was analyzed by LCMS/MS to quantify cocaine. It was analyzed by an HPLC Agilent 1260 (Agilent Technologies, CA, USA) combined with a 3200 QTRAP hybrid triple quadrupole/LIT (linear ion trap) mass spectrometer Sciex, Ontario (Canada), according procedure described by Shihomatsu (2015) and employed by Fontes et al. (2019). The cocaine primary standard was purchased from Cerilliant – Sigma Aldrich (Lot FE07271503).

A potentiometric titration system (Metrohm 794 Basic Titrino) with a glass electrode (Metrohm, ref. 6.0210.100) calibrated in NBS scale was used for total alkalinity (TA) measurements which was performed in triplicate. pH and TA values were used to calculate carbonate chemistry parameters in the software CO_2SYS (Pierrot et al., 2006) using dissociation constants by Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 according to Dickson (1990).

2.3. Toxicity Tests

Specimens of marine mussel (*Perna perna*) were purchased from an aquaculture facility (Cocanha beach at Caraguatatuba, SP/Brazil) and held in 500-liter tank filled clean aerated seawater until use in the tests, within 24 h of acquisition. The gametes were obtained according ASTM E724-98 (2012), with minor adaptations proposed by Zaroni et al. (2005). Four replicates were used for each CC concentrations (ranged from 0 to 100 mg/L) in the different pH values applied (8.3; 8.0; 7.5 and 7.0). The pH value of 8.3 was used as control, where no CO_2 or HCl was added (8.3 were the natural pH of the reconstitute water).

2.4. Fertilization bioassay

The fertilization rate was conducted according to Zaroni et al., 2005 in test tubes with 10 mL of crack-cocaine solution at different pH value, besides a control with reconstituted seawater and no CC added was done in order to ensure the quality of the experiment. 50 µL sperm solution was added in each test tube and after one hour of sperm solution exposure to different treatments, oocytes were added. After 40 minutes 40% formaldehyde at pH 7.0 (pH was buffered by the addition of a 40 g.L-1 solution of borax) were added to finalize the assay. By using a light microscope with 100x magnification was evaluated the presence of the polar body of fertilization membrane or first cell divisions in 100 eggs. The test is considered valid when ≥ 80% of the eggs were successfully fertilized in the control (Cortez et al., 2012).

2.5. Embryo-larval development bioassay

The embryo-larval development assay followed the procedure described by ASTM E724-98, 2012, where adult individuals were induced to spawn by thermal stimulation, and the fertilization was achieved by adding 1.5 mL of sperm solution into 250 mL of the eggs suspension for 60 min at room temperature. Then approximately 25,000 embryos were introduced into 1.3 L glass chambers containing 500 mL of filtered (0.22 μm) reconstituted seawater, at 25 °C and salinity of 35 ± 1 ppt, 7.5 $mg.L^{-1}$ dissolved oxygen, 12h light/dark photoperiod, the desired pH and CC concentration for each treatment. After exposure time (42 h), the assay was finished by adding 40% formaldehyde and 100 larvae were counted for each replicate. The test was considered valid when ≥ 80 % larvae were successfully developed in the control.

The selection of the crack-cocaine concentrations used in our experiment with marine mussel was based on previous experiments by our group (Maranho et al., 2017; Souza et al., 2019). Five different concentrations of crack-cocaine (6,25 $mg.L^{-1,}$, 12,5 $mg.L^{-1}$, 25 $mg.L^{-1}$, 50 $mg.L^{-1}$, and 100 $mg.L^{-1}$) were used to determine the toxic effects at different acidification scenarios defined by the 6 different pH values: 8.3; 8.0; 7.7 and 7.5.

2.6. Statistical analysis

Regarding the effects caused by the variation in the pH values, the terms EpH50 was used. The IC50 and EpH50 were calculated through the polynomial interpolation method. After the

assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk's and Levene's tests, respectively, two-ways ANOVA followed by Tukey's post-hoc tests were conducted using Statistical software SPSS 15.0 for Windows. Statistical analyzes were performed comparing of the results of the different pH values (protons) and crack-cocaine concentrations with the control (reconstitute seawater, pH 8.3) and were classified according to their statistical significance, as follow: * ($p < 0.05$), ** ($p < 0.01$).

3. Results and Discussion

3.1.Chemical Analysis

Optimal water quality conditions, which are essential for the success of a toxicity test, was pre-determined and maintained by the CO_2 injection system. The small variation and the mean values in the parameters throughout the experimental period can be assess in table 1.

Table 1. Shows a summarized description of toxicity test and different water parameters (pH, Dissolved Oxygen; Temperature and Salinity) measured in the different assays. For the pH average ± SD values were calculated from the pH data that were measured every 30 min during the 48 h of experiment exposure.

pH Treatment/ CC concentration in µg.L^{-1}		pH Seawater	Salinity	D. O. (mg.L^{-1})	T (°C)
Reconstitute seawater	N	8.30 ± 0.03	34	5.14	25
	6.25	8.30 ± 0.02	34	5.78	25
	12.5	8.30 ± 0.01	34	5.78	25
	25	8.30 ± 0.04	34	5.35	25
	50	8.30 ± 0.03	34	5.14	25
CO_2					
8.0	Control	8.00 ± 0.05	34	5.80	25
	6.25	8.00 ± 0.03	34	5.82	25
	12.5	8.00 ± 0.02	34	6.60	25
	25	8.00 ± 0.03	34	5.91	25
	50	8.00 ± 0.05	34	5.80	25
7.5	Control	7.50 ± 0.01	34	5.99	25
	6.25	7.50 ± 0.06	34	5.74	25
	12.5	7.50 ± 0.03	34	5.32	25
	25	7.50 ± 0.08	34	5.66	25
	50	7.50 ± 0.01	34	5.99	25
7.0	Control	7.00 ± 0.03	33	5.67	25
	6.25	7.00 ± 0.06	34	6.03	25
	12.5	7.00 ± 0.03	34	6.00	25
	25	7.00 ± 0.01	33	5.91	25
	50	7.00 ± 0.03	33	5.67	25
HCl					
8.0	Control	8.00 ± 0.04	35	5.49	24
	6.25	8.00 ± 0.03	35	5.19	24
	12.5	8.00 ± 0.03	35	6.34	24
	25	8.00 ± 0.02	35	5.09	24
	50	8.00 ± 0.04	35	5.49	24
7.5	Control	7.50 ± 0.02	35	5.64	25
	6.25	7.50 ± 0.04	35	5.59	25
	12.5	7.50 ± 0.03	35	5.79	25
	25	7.50 ± 0.04	35	5.45	25
	50	7.50 ± 0.02	35	5.64	25
7.0	Control	7.00 ± 0.05	35	5.23	26
	6.25	7.00 ± 0.02	35	6.09	26
	12.5	7.00 ± 0.03	35	5.99	26
	25	7.00 ± 0.06	35	6.01	26
	50	7.00 ± 0.05	35	5.23	26

The values for the carbonate species, calculated by CO_2SYS software, are shown in Table 2. The decrease in seawater pH resulted in the reduction of the concentration of both hydroxide and carbonate (OH^- and CO_3^{2-}), as presented by our results, since was observed an increase in CO_2 concentrations and decrease in CO_3^{2-} rates. Higher the concentration of CO_2, the lower the CO_3^{2-}stability, which decrease the capacity to link with other chemical elements such as calcium, since the carbonate (CO_3^{2-}) has a higher chemical affinity for H^+ ions than for calcium (Ca^{2+}).

On the other hand, the addition of HCl only increased the concentrations of H^+ and H_2CO_3 in seawater while decreased those of HCO_3^- and CO_3^{2-}. There are studies that focus and explain the main difference in the carbonate speciation between HCl and CO_2 methodologies (Bautista-Chamizo et al., 2016; Kurihara and Shirayama, 2004; Sun et al., 2017, 2016).

The decrease in $CaCO_3$ saturation levels, confirmed in this study by an undersaturation in index for calcite (Ωcal) and aragonite (Ωarag), posing a major threat for marine organisms, particularly shell-forming and calcifying organisms (Hendriks et al., 2010), as in the study from Marangoni et al. (2017), that showed alteration in the calcification process in *Millepora alcicornis* (a calcareous hydrozoan) triggered by CO_2-driven acidification.

Table 2. Carbonate system speciation in assays exposed to the different pH by CO_2 enrichment treatments for both bioassays (fertilization and embryo-larval development).

pH Treatment	CC Concentration ($\mu g.L^{-1}$)	TA ($\mu mol.L^{-1}$)	TIC ($\mu mol.kg^{-1}$)	HCO_3^- ($\mu mol.kg^{-1}$)	CO_3^{2+} ($\mu mol.kg^{-1}$)	CO_2 ($\mu mol.kg^{-1}$)	pCO_2 (μatm)	Ωcal	Ωarag
8.0	Control	1641	1516	1413	85.6	18.1	655	2.06	1.36
	6.25	1654	1525	1419	88.3	17.7	646	2.13	1.41
	12.5	1312	1475	1291	7.5	176.2	6541	0.18	0.12
	25	1754	1650	1547	79.2	23.8	880	1.91	1.27
	50	1799	1794	1697	39.3	57.8	2152	0.95	0.63
	100	1862	1883	1776	33.6	74.2	2767	0.81	0.54
7.5	Control	1685	1604	1511	65.9	27.5	1030	1.59	1.06
	6.25	1672	1632	1545	48.1	39.1	1451	1.16	0.77
	12.5	1744	1713	1622	47.1	44.1	1641	1.14	0.75
	25	1717	1691	1600	44.6	45.6	1715	1.08	0.72
	50	1855	1888	1776	30.7	81.9	3083	0.74	0.49
	100	1874	1924	1803	27.5	93.3	3481	0.67	0.44
7.0	Control	1713	1707	1615	37.7	54.0	2007	0.92	0.61
	6.25	934	911	863	22.9	25.3	952	0.56	0.37
	12.5	1751	1800	1686	25.1	88.9	3333	0.61	0.41
	25	1793	1736	1641	58.9	36.0	1355	1.44	0.95
	50	1796	1846	1728	26.1	91.5	3473	0.63	0.42
	100	1925	1910	1808	46.0	56.1	2117	1.12	0.74

The aliquot of crack cocaine analyzed by LC-MS/MS contained 37.99% of cocaine. It was not possible to measure the real concentrations of cocaine in the exposure aliquot, however, as reported by van Nuijs et al. (2009) to wastewater and by Maranho et al. (2017) to marine water, a low decrease in crack-cocaine (CC) concentrations during the assays is expected.

3.2.Toxicity bioassays

3.2.1. Fertilization rate assay

Figure 2 shows results from fertilization rate assay using natural pH 8.3 and different concentrations of CC. The treatments did not present significative difference ($p < 0.05$) with N for this assay.

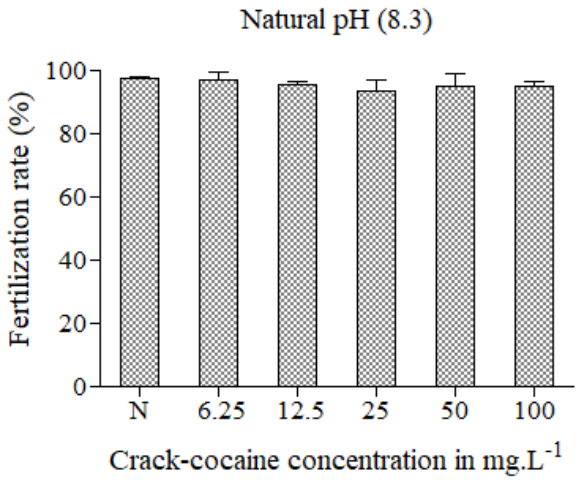

Fig. 2. *Perna perna* fertilization rate results obtained after exposure to different concentrations of crack-cocaine (6.25 $mg.L^{-1}$, 12.5 $mg.L^{-1}$, 25 $mg.L^{-1}$, 50 $mg.L^{-1}$ and 100 $mg.L^{-1}$) in Natural pH 8.3, with no acidification method applied.

Figure 3 present the results of fertilization rate for both acidification methodologies (HCl and CO_2). It was demonstrated for CO_2 acidification methodology that in pH 7.5 the highest concentrations of CC (50 $mg.L^{-1}$ and 100 $mg.L^{-1}$) presented a significant decrease ($p < 0.05$) in the fertilization success of *P. perna* mussel when compared with the control (N). In case of HCl acidification methodology, there was a significant decrease in fertilization rate in all pH and the CC concentration assessed compared to the control.

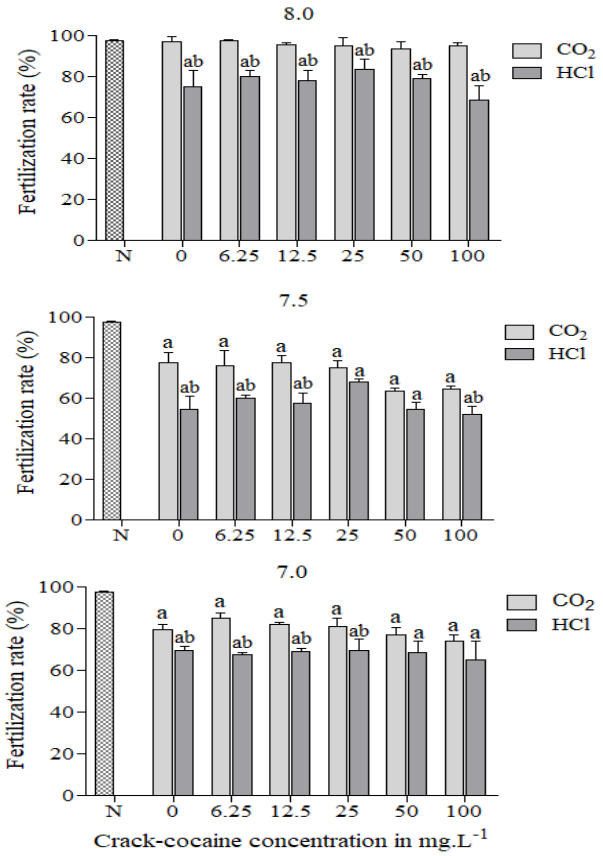

Fig. 3. *Perna perna* fertilization rate results obtained after exposure to different concentrations of crack-cocaine (6.25 mg.L^{-1}, 12.5 mg.L^{-1}, 25 mg.L^{-1}, 50 mg.L^{-1} and 100 mg.L^{-1}) at different scenarios of acidification defined by pH values (8.0; 7.5 e 7.0) in two different acidification methods (CO_2 and HCl). Concentration 0 meas acidified but with no CC. a - means significant difference to N (reconstituted seawater) ($p < 0,05$). b – means significant difference between CC concentrations in different acidified methods.

Agreeing with our results, Barros et al. (2013) showed that the percentage of fertilized eggs of *C. gingas* was substantially reduced when pH value was decrease in 0.7 from de original value compared to the other treatments. These findings could support the hypothesis proposed by Kurihara et al. (2009) that seawater acidification would affect intracellular pH of sperm and alter sperm motility, fertilization and embryo development.

Regarding with CC concentrations that the sperm was exposure during one hour, our results showed that even the highest concentration of CC (100 mg.L^{-1}) was not able to cause effect in the sperm ability to fertilize the oocyte. On the other hand, Maranho et al. (2017) presented the effects on fertilization rate of *P. perna* mussel already in the CC concentration of 1.25 mg.L^{-1}.

3.2.2. Embryo-larval assay

Results of embryo-larval assay using *Perna perna* in reconstitute seawater (N) with different CC concentrarions are presented in Figure 4. Significant differences were observed in CC concentratons of 12.5; 25 and 50 mg.L^{-1} when compared with N (no CC concentrarion added). Maranho et al. (2017) found significant differences in *P. perna* embryo-larval development when exposed to CC of 1.25 mg.L^{-1}.

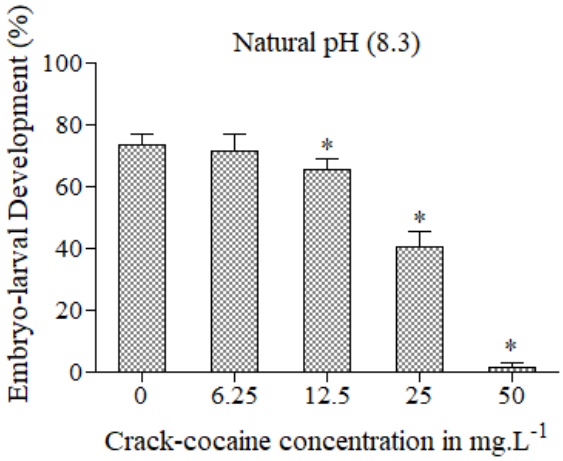

Fig. 4. *Perna perna* embryo-larval development results obtained after exposure to different concentrations of crack-cocaine (6.25 mg.L^{-1}, 12.5 mg.L^{-1}, 25 mg.L^{-1}, 50 mg.L^{-1}) in reconstitute seawater with natural pH 8.3, with no acidification method applied. * means significant difference to N (reconstitute seawater with no CC) ($p < 0,05$).

In the case of the results of embryo-larval success applying the two different methods of acidification (CO_2 and HCl) are showed in Figure 5. It was demonstrated that for CO_2 acidification method the assay have a significant decrease (compared to control treatment) ($p< 0.05$) in all CC concentration up to 12.5 $mg.L^{-1}$. The results obtained for HCl method showed significant differences in the CC concentration up to 6.25 $mg.L^{-1}$ on pH 7.7 in compared to control treatment. In the figure was present pH values of 8.3, 8.0 and 7.7, excluding pH 7.5 since this pH value were highly toxic by itself, presenting 0% of normal larval development for both methodologies.

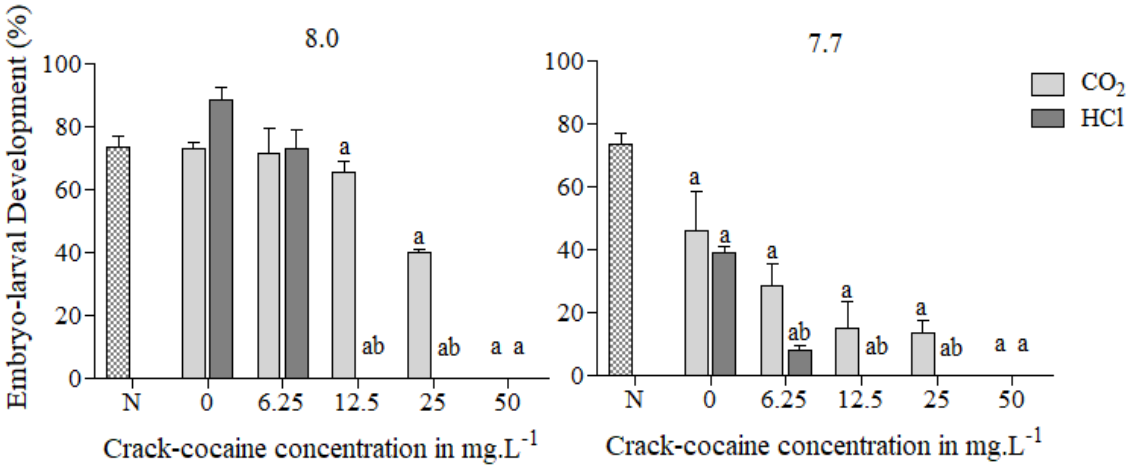

Fig.3. Results from larval development success of *P. perna* mussel. The larvae were exposed to different concentrations of crack-cocaine (6.25, 12.5, 25 and 50 $mg.L^{-1}$) at different scenarios of acidification defined by pH values (8.3; 8.0 and 7.5). a - means significant difference to N (reconstituted seawater) ($p < 0,05$). b – means significant difference between CC concentrations in different acidified methods.

The IC50 was calculated from results of embryo-larval assay and presented in table 3. The different methods of acidification presented different values of IC50 associated with CC, evidencing HCl as most toxic than CO_2 when associated to the same concentrations of CC.

Regarding to pH effects, table 4 shows the EpH50 and E[H^+]50 derived at the different treatments, that is, the pH value that causes effects in 50% of the ebyos after 44 h exposure. The HCl acidification method presented greater effect on the organisms (including control groups) when compared to CO_2 method. In addition, CC showed more severe toxic effects when associated with acidification by HCl, as observed in tables 3 and 4. This increase in toxicity may be related to the chemical reaction of the HCl acid, that release protons H^+ and ions of Cl^-, against the bicarbonate ($H_2CO_3^-$) realeased from CO_2.

Table 3. Values of IC50 derived from CC concentrations in the different pH values for both methodologies of acidification (HCl and CO_2).

pH values	IC50 (mg. L^{-1})	
	CO_2	HCl
8.3	14.08 (12.66 - 15.30)	8.85 (8.64 - 9.01)
8.0	13.85 (12.50 - 14.60)	8.72 (8.44 - 8.95)
7.7	9.37 (4.66 - 16.15)	3.92 (3.73 - 4.14)
7.5	-	-

Table 4. Values of EpH50 and (control and crack-cocaine concentrations) for both methodologies of acidification (HCl and CO_2).

CC Concentrations (mg. L^{-1})	CO_2		HCl	
	EpH50	E[H^+]50 (mol. kg^{-1})	EpH50	E[H^+]50 (mol. kg^{-1})
Control	7.34	4.56×10^{-8}	7.53	2.95×10^{-8}
6.25	7.55	2.81×10^{-8}	7.65	2.21×10^{-8}
12.5	7.61	2.41×10^{-8}	8.18	0.66×10^{-8}
25	7.58	2.61×10^{-8}	-	-
50	8.18	0.66×10^{-8}	-	-

According to Barros et al. (2013), the morphological abnormalities of the larvaes could be due to two possibilities: (a) damage to the embryonic ectodermic cells rendering them unable to produce sufficient amorphous calcium carbonate, which is crucial in the development of a strong and stable shell, or (b) dissolution of the shell due to corrosion by seawater. These evidences is thought to be due to an incomplete calcification process, with defaults on the Ca^+ transport process, enough not to cover the entire mantle of the larva (Kurihara, 2008). This feature may also decrease the swimming capability of the larva and therefore decrease its fitness (Beiras and His, 1994; Kurihara, 2008)

Different components of larval physiology are affected by different carbonate system parameters for the species (and perhaps life-stage) studied. However, it is important to note that failure to embryo-larval develop represents a significant bottleneck in population dynamics, and while other carbonate system parameters may act as stressors, saturation state (Ωarag) appears to matter most first for the rapid shell building of prodissoconch I phase in bivalve larvae

(Waldbusser et al., 2015). Our results put in evidence that the gametes of *P. perna* react to acidification when exposed to realistic pH reductions until the end of this century.

Besides the carbonate system parameters, according to Bayne (1976), in bivalves, destabilization of lysosomal membrane could promote nutrient unbalance during embryogenesis leading to disturbances on larval development, here evaluated as delayed growth and abnormal D-shape, since early stages of larval development represent a period of intense morphogenetic activity when there is complete dependence on the stored energy reserves acquired from the parental adult. Maranho et al. (2017) and Ortega et al. (2018) demonstrated that the lysosomal membrane stability of mussels is affected when exposed to CC concentrations, which could mean larval starvation affecting not only embryolval development, but also population growth.

4. Conclusions

HCl acidification method was found to be more toxic than CO_2 enrichment to early life stages of *P. perna* mussel. Our results put in evidence combined effects of a psychoative substance (crack-cocaine) in first life stages of *P. perna* exposed to ocean acidification scenarios. Considering the taxonomic position of the specie, it is quite possible that early development of other bivalves is similarly affected by high-CO_2 seawater, although further verification is necessary for other bivalves.

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References

ASTM E724-98, 2012. Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs [WWW Document]. ASTM Int. West Conshohocken, PA. https://doi.org/10.1520/E0724-98

Barros, P., Sobral, P., Range, P., Chícharo, L., Matias, D., 2013. Effects of sea-water acidification on fertilization and larval development of the oyster *Crassostrea gigas*. J. Exp. Mar. Bio. Ecol. 440, 200–206. https://doi.org/10.1016/j.jembe.2012.12.014

Bautista-Chamizo, E., De Orte, M.R., DelValls, T.A., Riba, I., 2016. Simulating CO_2 leakages from CCS to determine Zn toxicity using the marine microalgae *Pleurochrysis roscoffensis*. Chemosphere 144, 955–965. https://doi.org/10.1016/j.chemosphere.2015.09.041

Bayne, B.L., 1976. Marine Mussels: Their Ecology and Physiology., Inter-nat. ed, 1976. London.

Beiras, R., His, E., 1994. Effects of dissolved mercury on embryogenesis, survival, growth and metamorphosis of *Crassostrea gigas* oyster larvae. Mar. Ecol. Prog. Ser. 113, 95–103.

Borrero-Santiago, A.R., Carbú, M., DelValls, T.A., Riba, I., 2016. CO_2 leaking from sub-seabed storage: Responses of two marine bacteria strains. Mar. Environ. Res. 121, 2–8. https://doi.org/10.1016/j.marenvres.2016.05.018

Capaldo, A., Gay, F., Laforgia, V., 2019. Changes in the gills of the European eel (*Anguilla anguilla*) after chronic exposure to environmental cocaine concentration. Ecotoxicol. Environ. Saf. 169, 112–119. https://doi.org/10.1016/j.ecoenv.2018.11.010

Cortez, F.S., Seabra Pereira, C.D., Santos, A.R., Cesar, A., Choueri, R.B., Martini, G.D.A., Bohrer-Morel, M.B., 2012. Biological effects of environmentally relevant concentrations of the pharmaceutical Triclosan in the marine mussel *Perna perna* (Linnaeus, 1758). Environ. Pollut. 168, 145–150. https://doi.org/10.1016/j.envpol.2012.04.024

Cvijanovic, I., Caldeira, K., 2015. Atmospheric impacts of sea ice decline in CO_2 induced global warming. Clim Dyn 44, 1173–1186. https://doi.org/10.1007/s00382-015-2489-1

Daughton, C.G., Ternes, T. a., 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspect. 107, 907–938. https://doi.org/10.1289/ehp.99107s6907

de Orte, M.R., Lombardi, A.T., Sarmiento, A.M., Basallote, M.D., Rodriguez-Romero, A., Riba, I., DelValls, T.A., 2014a. Metal mobility and toxicity to microalgae associated with acidification of sediments: CO_2 and acid comparison. Mar. Environ. Res. 96, 136–144. https://doi.org/10.1016/j.marenvres.2013.10.003

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014b. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

Dickson, A.G., 1990. Standard potential of the reaction: $AgCl(s) + 1/2H_2(g) = Ag(s) + HCl(aq)$, and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. Chem. Thermodynamics,22, 113-127.

Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res. Part A, Oceanogr. Res. Pap. 34, 1733–1743. https://doi.org/10.1016/0198-0149(87)90021-5

Domènech, X., Peral, J., Muñoz, I., 2009. Predicted environmental concentrations of cocaine and benzoylecgonine in a model environmental system. Water Res. 43, 5236–5242. https://doi.org/10.1016/j.watres.2009.08.033

Doney, S.C., 2010. The growing human footprint on coastal and open-Ocean biogeochemistry. Science 328, 1512–1516. https://doi.org/10.1126/science.1185198

Dorey, N., Martin, S., Oberhänsli, F., Teyssié, J.L., Jeffree, R., Lacoue-Labarthe, T., 2018. Ocean acidification modulates the incorporation of radio-labeled heavy metals in the larvae of the Mediterranean sea urchin *Paracentrotus lividus*. J. Environ. Radioact. 190–191, 20–30. https://doi.org/10.1016/j.jenvrad.2018.04.017

Edwards, Q.A., Kulikov, S.M., Garner-O'Neale, L.D., Metcalfe, C.D., Sultana, T., 2017. Contaminants of emerging concern in surface waters in Barbados, West Indies. Environ. Monit. Assess. 189, 1–6. https://doi.org/10.1007/s10661-017-6341-4

Fontes, M.K., Campos, B.G., Cortez, F.S., Pusceddu, F.H., Moreno, B.B., Maranho, L.A., Lebre, D.T., Guimarães, L.L., Pereira, C.D.S., 2019. Seasonal monitoring of cocaine and benzoylecgonine in a subtropical coastal zone (Santos Bay, Brazil). Mar. Pollut. Bull. 149, 110545. https://doi.org/10.1016/j.marpolbul.2019.110545

Gattuso, J.-P., Lavigne, H., 2009. Perturbation experiments to investigate the impact of ocean acidification: approaches and software tools. Biogeosciences Discuss. 6, 4413–4439. https://doi.org/10.5194/bgd-6-4413-2009

Goulding, T.A., De Orte, M.R., Szalaj, D., Basallote, M.D., DelValls, T.A., Cesar, A., 2017. Assessment of the environmental impacts of ocean acidification (OA) and carbon capture and storage (CCS) leaks using the amphipod *Hyale youngi*. Ecotoxicology 26, 521–533. https://doi.org/10.1007/s10646-017-1783-6

Haley, B.A., Hales, B., Brunner, E.L., Kovalchik, K., Waldbusser, G.G., 2018. Mechanisms to Explain the Elemental Composition of the Initial Aragonite Shell of Larval Oysters. Geochemistry, Geophys. Geosystems 1064–1079. https://doi.org/10.1002/2017GC007133

Hartin, C.A., Bond-Lamberty, B., Patel, P., Mundra, A., 2016. Ocean acidification over the next three centuries using a simple global climate carbon-cycle model: Projections and sensitivities. Biogeosciences 13, 4329–4342. https://doi.org/10.5194/bg-13-4329-2016

Hendriks, I.E., Duarte, C.M., Álvarez, M., 2010. Vulnerability of marine biodiversity to ocean acidification: A meta-analysis. Estuar. Coast. Shelf Sci. 86, 157–164. https://doi.org/10.1016/j.ecss.2009.11.022

Huang, X., Liu, Z., Xie, Z., Dupont, S., Huang, W., Wu, F., Kong, H., Liu, L., Sui, Y., Lin, D., Lu, W., Hu, M., Wang, Y., 2018. Oxidative stress induced by titanium dioxide nanoparticles increases under seawater acidification in the thick shell mussel *Mytilus coruscus*. Mar. Environ. Res. 137, 49–59. https://doi.org/10.1016/j.marenvres.2018.02.029

IPCC, 2014. Summary for Policymakers, Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https://doi.org/10.1017/CBO9781107415324

Jiang, J.J., Lee, C.L., Fang, M. Der, 2014. Emerging organic contaminants in coastal waters: Anthropogenic impact, environmental release and ecological risk. Mar. Pollut. Bull. 85, 391–399. https://doi.org/10.1016/j.marpolbul.2013.12.045

Kurihara, H., 2008. Effects of CO_2 -driven ocean acidification on the early developmental stages of invertebrates. Mar. Ecol. Prog. Ser. 373, 275–284. https://doi.org/10.3354/meps07802

Kurihara, H., Asai, T., Kato, S., Ishimatsu, A., 2009. Effects of elevated pCO_2 on early development in the mussel *Mytilus galloprovincialis*. Aquat. Biol. 4, 225–233. https://doi.org/10.3354/ab00109

Kurihara, H., Shirayama, Y., 2004. Effects of increased atmospheric CO_2 on sea urchin early development. Mar. Ecol. Prog. Ser. 274, 161–169. https://doi.org/10.3354/meps274161

Lüthi, D., Le Floch, M., Bereiter, B., Blunier, T., Barnola, J.-M., Siegenthaler, U., Raynaud, D., Jouzel, J., Fischer, H., Kawamura, K., Stocker, T.F., 2008. High-resolution carbon dioxide concentration record 650,000-800,000 years before present. Nature 453, 379–382. https://doi.org/10.1038/nature06949

Marangoni, L.F. de B., Calderon, E.N., Marques, J.A., Duarte, G.A.S., Pereira, C.M., e Castro, C.B., Bianchini, A., 2017. Effects of CO_2-driven acidification of seawater on the calcification process in the calcareous hydrozoan *Millepora alcicornis* (Linnaeus, 1758). Coral Reefs 36, 1133–1141. https://doi.org/10.1007/s00338-017-1605-6

Maranho, L.A., Fontes, M.K., Kamimura, A.S.S., Nobre, C.R., Moreno, B.B., Pusceddu, F.H., Cortez, F.S., Lebre, D.T., Marques, J.R., Abessa, D.M.S., Ribeiro, D.A., Pereira, C.D.S., 2017. Exposure to crack cocaine causes adverse effects on marine mussels *Perna perna*. Mar. Pollut. Bull. 0–1. https://doi.org/10.1016/j.marpolbul.2017.08.043

Maranho, L.A., Garrido-Pérez, M.C., DelValls, T. a., Martín-Díaz, M.L., 2015. Suitability of Standardized Acute Toxicity Tests for Marine Sediment Assessment: Pharmaceutical Contamination. Water, Air, Soil Pollut. 226. https://doi.org/10.1007/s11270-014-2273-6

Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicx, R.M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897–907. https://doi.org/10.4319/lo.1973.18.6.0897

Millero, F., Woosley, R., DiTrolio, B., Waters, J., 2009. Effect of Ocean Acidification on the Speciation of Metals in Seawater. Oceanography 22, 72–85. https://doi.org/10.5670/oceanog.2009.98

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G.K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature. https://doi.org/10.1038/nature04095

Ortega, A. dos S.B., Maranho, L.A., Nobre, C.R., Moreno, B.B., Guimarães, R.S., Lebre, D.T., de Souza Abessa, D.M., Ribeiro, D.A., Pereira, C.D.S., 2018. Detoxification, oxidative stress, and cytogenotoxicity of crack cocaine in the brown mussel *Perna perna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-018-1600-7

Parolini, M., Binelli, A., 2013. Adverse effects induced by ecgonine methyl ester to the zebra mussel: A comparison with the benzoylecgonine. Environ. Pollut. 182, 371–378. https://doi.org/10.1016/j.envpol.2013.07.038

Passarelli, M.C., Ray, S., Cesar, A., DelValls, T.A., Riba, I., 2018a. Effects of CO_2 enrichment on metal bioavailability and bioaccumulation using *Mytilus galloprovincialis*. Mar. Pollut. Bull. 133, 124–136. https://doi.org/10.1016/j.marpolbul.2018.05.021

Passarelli, M.C., Riba, I., Cesar, A., Newton, A., DelValls, T.A., 2018b. Using a mesocosm approach to evaluate marine benthic assemblage alteration associated with CO_2 enrichment

in coastal environments. Ecotoxicol. Environ. Saf. 157, 29–39. https://doi.org/10.1016/j.ecoenv.2018.03.049

Pierrot, D., Lewis, E., Wallace, D.W.R., 2006. MS Excel Program Developed for CO_2 System Calculation. Carbon Dioxide Infromation Anal. Center, Oak Ridge Natl. Lab. U.S. Dep. Energy. ORNL/CDIAC, 105.

Shihomatsu, H.M., 2015. Desenvolvimento E Validação De Metodologia Spe-Lc-Ms/Ms Para a Determinacão De Fármacos E Droga De Abuso Nas Águas Da Represa Guarapiranga-São Paulo/Sp, Brasil. Dissertação.

Sircar, T., 2014. Combined Effect of Antibiotics and Ocean Acidification on Marine Bacterial Communities during winter and spring bloom conditions. Degree Project for Master of Science in Ecotoxicology.

Snyder, S., Lue-Hing, C., Cotruvo, J., Drewes, J.E., Eaton, A., Pleus, R.C., Schlenk, D., 2009. Pharmaceuticals in the Water Environment 38.

Souza, L. da S., Pusceddu, F.H., Cortez, F.S., de Orte, M.R., Seabra, A.A., Cesar, A., Ribeiro, D.A., Del Valls Casillas, T.A., Pereira, C.D.S., 2019. Harmful effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios. Chemosphere 236. https://doi.org/10.1016/j.chemosphere.2019.07.015

Stockdale, A., Tipping, E., Lofts, S., Mortimer, R.J.G., 2016. Effect of Ocean Acidification on Organic and Inorganic Speciation of Trace Metals. Environ. Sci. Technol. 50, 1906–1913. https://doi.org/10.1021/acs.est.5b05624

Sun, T., Tang, X., Jiang, Y., Wang, Y., 2017. Seawater acidification induced immune function changes of haemocytes in *Mytilus edulis*: A comparative study of CO_2 and HCl enrichment. Sci. Rep. 7, 1–10. https://doi.org/10.1038/srep41488

Sun, T., Tang, X., Zhou, B., Wang, Y., 2016. Comparative studies on the effects of seawater acidification caused by CO_2 and HCl enrichment on physiological changes in *Mytilus edulis*. Chemosphere 144, 2368–2376. https://doi.org/10.1016/j.chemosphere.2015.10.117

van Nuijs, A.L.N., Pecceu, B., Theunis, L., Dubois, N., Charlier, C., Jorens, P.G., Bervoets, L., Blust, R., Neels, H., Covaci, A., 2009. Spatial and temporal variations in the occurrence of cocaine and benzoylecgonine in waste- and surface water from Belgium and removal during wastewater treatment. Water Res. 43, 1341–1349. https://doi.org/10.1016/j.watres.2008.12.020

Waldbusser, G.G., Hales, B., Langdon, C.J., Haley, B.A., Schrader, P., Brunner, E.L., Gray, M.W., Miller, C.A., Gimenez, I., Hutchinson, G., 2015. Ocean acidification has multiple modes of action on bivalve larvae. PLoS One 10. https://doi.org/10.1371/journal.pone.0128376

Wang, Li, Zhao, L., Yang, G., Wang, Lianshun, Lu, Y., Cong, Y., 2018. Deciphering carbon sources of mussel shell carbonate under experimental ocean acidification and warming. Mar. Environ. Res. 142, 141–146. https://doi.org/10.1016/j.marenvres.2018.10.007

Yujra, V.Q., Moretti, E.G., Claudio, S.R., Silva, M.J.D., Oliveira, F. de, Oshima, C.T.F., Ribeiro, D.A., 2016. Genotoxicity and mutagenicity induced by acute crack cocaine exposure in mice. Drug Chem. Toxicol. 39, 388–391. https://doi.org/10.3109/01480545.2015.1126843

Zaroni, L.P., Abessa, D.M.S., Lotufo, G.R., Sousa, E.C.P.M., Pinto, Y.A., 2005. Toxicity testing with embryos of marine mussels: Protocol standardization for *Perna perna* (Linnaeus, 1758). Bull. Environ. Contam. Toxicol. 74, 793–800. https://doi.org/10.1007/s00128-005-0651-x

Zuccato, E., Castiglioni, S., 2009. Illicit drugs in the environment. Philos. Trans. A. Math. Phys. Eng. Sci. 367, 3965–78. https://doi.org/10.1098/rsta.2009.0107

Chapter IV. Assessing lethal and sublethal effects of crack-cocaine and proton concentrations on adult mussels *Perna perna*

Introduction

The illicit drugs, considered a class of emerging environmental contaminants, are used in enormous quantities worldwide. Illicit drugs are mainly excreted in urine; the efficiency with which they are removed from sewage effluent in sewage treatment plants is variable since it depends upon the technology used (Pal et al., 2013b). Therefore, many illicit drugs and their breakdown products are detected in surface waters (Karolak et al., 2010; Mendoza et al., 2014; Ondarza et al., 2019) and in seawater (Pereira et al., 2016) throughout the world. Although the environmental fate and ecological effects of illicit drugs need further investigation, the first studies showed toxic effects to the aquatic organisms, as expected for a constant exposure to bioactive substances. Even at low concentrations, these compounds may cause sublethal effects in non-target organisms.

The concentration of carbon dioxide (CO_2) in the atmosphere has grown by more than 40% since pre-industrial times (IPCC, 2014) exceeding 400 ppm, the highest level in recorded history (Monastersky, 2013). The IPCC, RCP 8.5 scenario, predicts a further rise in pCO_2 to 1000 ppm by 2100 (IPCC, 2014). This is likely to lead to a sea surface temperature increase of about 0.2°C per decade and a further decrease of 0.3–0.4 pH units by 2100 (IPCC 2014).

The consequences of ocean acidification has been mostly devoted to organisms that depend on the availability of carbonate ions in seawater, especially bivalves (Goncalves et al., 2018; Lemasson et al., 2017; Zhao et al., 2018) but little is known on the capacity of bivalves to modulate their biochemical parameters to cope with ocean acidification. Filter feeding organisms with sessile habits and wide distribution have been used as sentinel organisms in ecotoxicological studies and marine biomonitoring (Gerges, 1994). In Brazil, the brown mussel *Perna perna* has

been widely used both as seafood and as sentinel organisms in monitoring of anthropogenic pollution trends in coastal water (Abessa et al., 2005; Cortez et al., 2019, 2018; Ortega et al., 2018).

In chapter IV, the main objective is to assess the effects of CO_2 enrichment itself and its association with an illicit drug (crack-cocaine) on adults *Perna perna* mussels through responses of different biomarker systems such as: lysosomal membrane stability, lipid peroxidation and primary damages in DNA. It includes two different papers addressing the mentioned objective. First, it was addressed the impact and effects related to the CO_2-induced acidification on biochemical systems using adult mussels. Beyond this assessment an additional experimental design was proposed to determine the effects related to the combination of acidification and environmental relevant concentration of an illicit drug.

The methods used to address these effects were based on laboratory experimentation. Both experimental designs commented above used a system to mimic CO_2-induced acidification that consisted in a different set of aquarium replicates. These aquariums were filled with 20 liters water, previously collected and filtered, from Enseada Beach in Guarujá, SP/Brazil (CETESB, 2016) (Figure 1). A control was used with no induced CO_2 acidification - pH 8.3 (original pH from the area collected). The different pH values (8.0; 7.5; 7.0; 6.5 and 6.0) were adjusted and controlled by the CO_2 injection system (de Orte et al., 2014b) that is shown in detail in Chapter 1 (section 1.3). In summary, the CO_2 injection system proposed seeks to provide a laboratory-based simulation of the acidification process by CO_2 enrichment in the marine environment.

Figure 1: Image of the CO_2 injection system used in this study for the adult mussel's exposition to crack-cocaine in different pHs.

The laboratory conditions were controlled: seawater temperature at 20 ± 2 °C, salinity 35 ppt and dissolved oxygen 8.0 mg L^{-1} and photoperiod 12:12. All the system was renewed every 48 hours to maintain the water quality, avoiding organisms to stay in touch with its excreta. The mussels were acquired from an aquaculture farm located within a reference area (Cocanha Beach, Caraguatatuba, Brazil). The animals were acclimated to the laboratory conditions for 3 days.

During both experimental designs different toxic responses were measured, both lethal and sublethal. After 48 and 96 h of the beginning exposure, survival was recorded and ten mussels from each pH value have the hemolymph withdrawn before being dissected, and its gills frozen at − 80 °C. The hemolymph was used to assess the lysosomal membrane stability through Neutral Red Retention Time assay (NRRT) and the gills to assess lipid peroxidation and DNA damage (strand breaks).

The results obtained in the experiments assessing the effect of CO_2 acidification using adult mussels were showed in the first paper of this chapter submitted to Marine Pollution Bulletin: *Assessing CO_2-induced acidification lethal and sublethal effects on tropical mussels Perna perna (Linnaeus, 1758).*

The main objective of this paper was to address the effects of different CO_2-induced acidification in the biochemical systems using individuals of *Perna perna* mussel. Addressing this objective, it will be possible to delineate a baseline of effects associated with the acidification that will be used later in this chapter to discriminate the effects associated with other stressors like the concentrations of crack-cocaine (second paper of this chapter).

The percentage of mussel mortality for each treatment assay is shown in Table 1. In general, the mortality increases as pH values decrease. pH values upper to 7.5 presented no mortality, while pH values under 7.0 presented minimum 20% of mortality after 96 hours.

Table 1. *Perna perna* mortality percentages measured during mimic CO2-induced acidification after 48- and 96-hours exposure to 5 different pH levels plus and control (8.3 with no CO_2 added).

pH value	Mortality (%)	
	48 hours	96 hours
8.3	0	0
8.0	0	0
7.5	0	0
7.0	0	20
6.5	10	20
6.0	0	30

These data are the first ones determined on the effects associated with CO_2-induced acidification showed by different pH values in the brown mussel *P. perna*.

The biochemical responses showed different trends. Thus, the range in NRRT values observed for lysosomes from control mussels (n=10; no CO_2 added) at time zero, 48 h and 96 h of assay were 81 min ± 26; 93 min ± 23 and 77 min ± 24 respectively. In the first 48 h, the retention time decreased significantly (Dunn's test $p < 0.05$ and $p < 0.01$) at pH 7.0 and 6.5 when compared with control. After 96 h, the organisms exposed to different pH values showed significant decrease ($p < 0.01$) in the dye retention time in lysosomes only at pH 6.0. There was no statistically significant difference between retention time of the dye in control and the highest pH values (8.0 and 7.5). The NRRT values presented are in accordance with those presented by Cortez et al. (2012), where the retention time ranged from 55 to 80 minutes in a study on *P. perna*.

DNA strand breaks exhibited significant higher values ($p<0.05$) than T0 in gills of *Perna perna* mussels exposed to a pH value of 6.0 in both exposure time. Similarly, Conradi et al. (2016) demonstrated that an increase in DNA damage observed in *Scrobicularia plana* exposed to different CO_2-acidic sediments. Although the LPO presented a significant difference in relation to the control only in pH 6.0 (96 hours exposure), there was an increasing tendency for LPO in time 96 hours comparing to 48 hours at pH values < 8.0. The same tendency was presented by Conradi et al. (2016), where LPO values significant increased when the time increase and the pH value decrease.

The presented results corroborate with the toxic effects are observed from a decrease in pH value ≤6.5, while from pH values ≤7.5 a stress showed to be produced by activating enzymatic systems maintained at the lowest pH values. In summary, the stress produced by increasing protons

concentration can be noticed in the enzymatic systems of the mussels (NRRT and LPO) in pH values lower than 7.5, being accentuated when the value of the concentration of protons is higher, which produces toxic effects related to pH 6.5 and 6.0.

The second article of this chapter related adverse effects of an illicit drug (crack-cocaine) to individuals of the mussel *P. perna* using different scenarios of ocean acidification. It will be submitted to the journal Science of Total Environment, entitle *"Sub-lethal combined effects of illicit drug and decreased pH on marine mussels: A short-time exposure to crack cocaine in CO_2 enrichment scenarios".* The main objective of this paper is to discriminate the effects associated with the acidification, crack-cocaine concentrations and those related to the combination of both causes.

This study produced the first data on the molecular and cellular effects associated with CO_2-induced acidification combined with an illicit drug in marine organisms. The experimental design used in this paper is similar to that described for the first study and showed above.

Regarding the results obtained, during the first 48 h exposure the lysosomal membrane stability (LMS) showed a significant decrease in retention time response at 0.5; 5 and 50 µg/L of CC in pH 8.0; 7.5; 7.0; 6.5 and 6.0. At pH values 7.0; 6.5 and 6.0 a significant decrease can be observed in the control samples (low pH and no CC added), where the effect its associated to the increase in H^+. After 96 h exposure the NRRT significant decreased in organisms exposed to 0.5; 5 and 50 µg/L of CC in pH 8.3; 8.0; 7.0; 6.5 and 6.0.sample. pH 7.5 did not present statistically difference to control sample after 96 h.

Lipid peroxidation (LPO) differed significantly in mussels exposed to crack-cocaine in this study, decreasing in all exposure concentrations (0.5; 5 and 50 µg/L) at pH 7.0 after 48 hours and

increasing in 5 µg/L of crack-cocaine at pH 6.0 in 96 hours exposure. DNA damage was observed in gills after 48 h of exposure in 0.5 and 50 µg / L crack ($p < 0.05$) at pH 6.5. In 96 h significant differences were found in the concentration of 5 pH 8.3; 0.5, 5 and 50 at pH 7.5; 0.5 and 5 at pH 6.5 which demonstrates the compound's potential to cause primary genetic damage; at pH 6.0 the effect it is associated with increase in proton concentration.

A multivariate analysis was used to identify and distinguish the measured effects and their causes (acidification, illicit drug and / or combination). Even through evaluating the individual and combined effects of acidification and crack-cocaine highlighted the need to use integrated inputs to discriminate the cause of the measured effects, well due to acidification, the concentrations of the compounds and the effects produced by the combination of both causes. The application of the Factor Analysis to the original 52 variables indicates that they can be grouped in two new factors (Table 2).

Table 2. Sorted rotated factor loadings of 52 variables for the two principal factors.

Variables		Components	
		Factor 1	**Factor 2**
NRRT 48h	Control	---	0.70
	0.5	0.30	0.57
	5	---	0.89
	50	---	0.91
NRRT 96h	Control	-0.53	0.71
	0.5	---	0.60
	5	---	---
	50	-0.45	---
DNA 48h	Control	0.59	---
	0.5	0.67	0.28
	5	0.43	---
	50	0.70	---
DNA 96h	Control	0.83	---
	0.5	0.75	0.32
	5	---	0.79
	50	---	0.74

LPO 48h	Control	-0.46	0.54
	0.5	0.77	0.58
	5	0.65	
	50	0.95	
LPO 96h	Control	0.78	-0.34
	0.5	0.45	0.25
	5	---	0.38
	50	-0.48	0.49
Mortality 48h	Control	---	-0.79
	0.5	---	-0.79
	5	---	-0.79
	50	0.40	---
Mortality 96h	Control	0.69	-0.42
	0.5	0.87	-0.38
	5	-0.32	-0.76
	50	0.40	-0.06
TA	Control	0.88	-0.44
	0.5	0.86	-0.48
	5	0.87	-0.45
	50	0.87	-0.46
DIC	Control	0.82	-0.50
	0.5	0.85	-0.49
	5	0.87	-0.46
	50	0.87	-0.44
Ω cal	Control	-0.33	0.81
	0.5	-0.34	0.87
	5	-0.50	0.80
	50	-0.53	0.80
Ω arag	Control	-0.33	0.81
	0.5	-0.34	0.87
	5	-0.50	0.80
	50	-0.64	0.29
pCO_2	Control	0.79	-0.48
	0.5	0.86	-0.47
	5	0.86	-0.46
	50	0.86	-0.46
Variance (%)		18.9	16.3
Cumulative (%)		0.36	0.31

The first factor (F1) could be defined as the effects associated with the CO_2-induced acidification based on the correlations between the biological adverse effects and the concentration

of protons. The second factor (F2) is defined as the effects related to the concentrations of Crack-cocaine in the individuals of *Perna perna* used in the study. To confirm these definitions and to address the relationship between the effects and the causes related to them a representation of the score of these two factors is shown in figure 2.

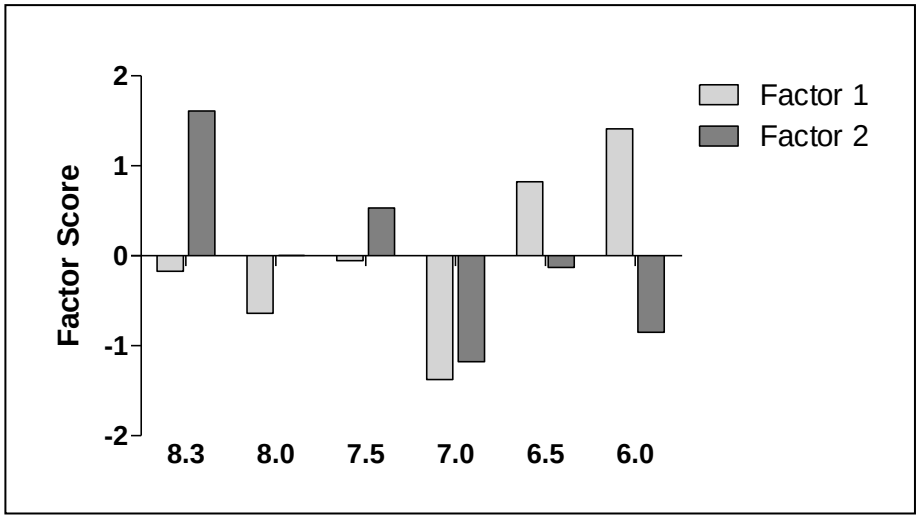

Figure 2. Estimated factors score in relation to the different pH treatments used in the study

The figure 2 shows the score of F1 as positive at pH values of 6.5 and negative at lower pH, having an uncertainty area between 7.0 and 6.5 that confirms the definition of this factor previously reported. Factor 2 is positive at higher pH (7.5 and 8.0) in which the effects measured are significantly associated with the concentration of crack-cocaine and not with the acidification. Thus, at higher pH the effects are directly related to the contaminant concentration (CC), whereas at lower pH values (6.5 and 6.0) are more related to the acidification than to the concentration of CC. At pH 7.0 it is shown that both causes are responsible of the effects measured, which could

be considered as a combination between the acidification and crack-cocaine increasing concentration.

The results obtained, in these two papers and included in this chapter, showed that uptake and sub-lethal effects of compounds, as crack-cocaine, can vary in a tissue-specific manner when combined with acidification. Lysosomal membrane stability of hemocytes showed the first signs of effects, followed by DNA and lipid oxidative damages in gills triggered by acidified conditions and after combination with an illicit drug. These findings should be taken into account when environmental monitoring approaches are performed in tropical marine areas receiving petroliferous or other activities employing Carbone dioxide capture and storage systems (CCS) and the need of additional studies to elucidate the impact of multiple stressors, particularly in species with elevated ecological and/or commercial importance.

References:

Abessa, D.M. de S., Zaroni, L.P., De Sousa, E.C.P.M., Gasparro, M.R., Pereira, C.D.S., De Figueredo Rachid, B.R., Depledge, M., King, R.S., 2005. Physiological and cellular responses in two populations of the mussel *Perna perna* collected at different sites from the Coast of São Paulo, Brazil. Brazilian Arch. Biol. Technol. 48, 217–225. https://doi.org/10.1590/S1516-89132005000200008

CETESB, 2016. Companhia Ambiental do Estado de São Paulo - Qualidade das praias litorâneas no Estado de São Paulo.

Conradi, M., Riba, I., Almagro-Pastor, V., DelValls, T.A., 2016. Lethal and sublethal responses in the clam *Scrobicularia plana* exposed to different CO_2-acidic sediments. Environ. Res. 151, 642–652. https://doi.org/10.1016/j.envres.2016.08.032

Cortez, F.S., Seabra Pereira, C.D., Santos, A.R., Cesar, A., Choueri, R.B., Martini, G.D.A., Bohrer-Morel, M.B., 2012. Biological effects of environmentally relevant concentrations of the pharmaceutical Triclosan in the marine mussel *Perna perna* (Linnaeus, 1758). Environ. Pollut. 168, 145–150. https://doi.org/10.1016/j.envpol.2012.04.024

Cortez, F.S., Souza, L. da S., Guimarães, L.L., Pusceddu, F.H., Maranho, L.A., Fontes, M.K., Moreno, B.B., Nobre, C.R., Abessa, D.M. de S., Cesar, A., Pereira, C.D.S., 2019. Marine contamination and cytogenotoxic effects of fluoxetine in the tropical brown mussel *Perna perna*. Mar. Pollut. Bull. 141, 366–372. https://doi.org/10.1016/j.marpolbul.2019.02.065

Cortez, F.S., Souza, L.D.S., Guimarães, L.L., Almeida, J.E., Pusceddu, F.H., Maranho, L.A., Mota, L.G., Nobre, C.R., Moreno, B.B., Abessa, D.M.D.S., Cesar, A., Santos, A.R., Pereira, C.D.S., 2018. Ecotoxicological effects of losartan on the brown mussel *Perna perna* and its occurrence in seawater from Santos Bay (Brazil). Sci. Total Environ. 637–638. https://doi.org/10.1016/j.scitotenv.2018.05.069

de Orte, M.R., Sarmiento, A.M., Basallote, M.D., Rodríguez-Romero, A., Riba, I., DelValls, A., 2014. Effects on the mobility of metals from acidification caused by possible CO_2 leakage

from sub-seabed geological formations. Sci. Total Environ. 470–471, 356–363. https://doi.org/10.1016/j.scitotenv.2013.09.095

Gerges, M.A., 1994. Marine Pollution Monitoring, Assessment and Control: UNEP's Approach and Strategy. Mar. Pollut. Bull. 28, 199–210.

Goncalves, P., Anderson, K., Raftos, D.A., Thompson, E.L., 2018. The capacity of oysters to regulate energy metabolism-related processes may be key to their resilience against ocean acidification. Aquac. Res. 49, 2059–2071. https://doi.org/10.1111/are.13663

IPCC, 2014. Climate Change 2014: Mitigation of Climate Change. Summary for Policymakers and Technical Summary, Climate Change 2014: Mitigation of Climate Change. Part of the Working Group III Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https://doi.org/10.1017/CBO9781107415416.005

Karolak, S., Nefau, T., Bailly, E., Solgadi, A., Levi, Y., 2010. Estimation of illicit drugs consumption by wastewater analysis in Paris area (France). Forensic Sci. Int. 200, 153–160. https://doi.org/10.1016/j.forsciint.2010.04.007

Lemasson, A.J., Fletcher, S., Hall-Spencer, J.M., Knights, A.M., 2017. Linking the biological impacts of ocean acidification on oysters to changes in ecosystem services: A review. J. Exp. Mar. Bio. Ecol. 492, 49–62. https://doi.org/10.1016/j.jembe.2017.01.019

Mendoza, A., López de Alda, M., González-Alonso, S., Mastroianni, N., Barceló, D., Valcárcel, Y., 2014. Occurrence of drugs of abuse and benzodiazepines in river waters from the Madrid Region (Central Spain). Chemosphere 95, 247–255. https://doi.org/10.1016/j.chemosphere.2013.08.085

Monastersky, R., 2013. Global carbon dioxide levels near worrisome milestone. Nature 497, 13–14. https://doi.org/10.1038/497013a

Ondarza, P.M., Haddad, S.P., Avigliano, E., Miglioranza, K.S.B., Brooks, B.W., 2019. Pharmaceuticals, illicit drugs and their metabolites in fish from Argentina: Implications for

protected areas influenced by urbanization. Sci. Total Environ. 649, 1029–1037. https://doi.org/10.1016/j.scitotenv.2018.08.383

Ortega, A. dos S.B., Maranho, L.A., Nobre, C.R., Moreno, B.B., Guimarães, R.S., Lebre, D.T., de Souza Abessa, D.M., Ribeiro, D.A., Pereira, C.D.S., 2018. Detoxification, oxidative stress, and cytogenotoxicity of crack cocaine in the brown mussel *Perna perna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-018-1600-7

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

Pereira, C.D.S., Maranho, L.A., Cortez, F.S., Pusceddu, F.H., Santos, A.R., Ribeiro, D.A., Cesar, A., Guimarães, L.L., 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Sci. Total Environ. 548–549, 148–154. https://doi.org/10.1016/j.scitotenv.2016.01.051

Zhao, L., Yang, F., Milano, S., Han, T., Walliser, E.O., Schöne, B.R., 2018. Transgenerational acclimation to seawater acidification in the Manila clam *Ruditapes philippinarum*: Preferential uptake of metabolic carbon. Sci. Total Environ. 627, 95–103. https://doi.org/10.1016/j.scitotenv.2018.01.225

Assessing CO_2-induced acidification lethal and sublethal effects on tropical mussels *Perna perna* (Linnaeus, 1758).

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Abstract

Rising atmospheric carbon dioxide (CO_2) concentration as such as leakages from CO_2 capture and storage systems (CCS) are able to cause significant ocean acidification leading to adverse biological effect in marine species. The aim of this study was to assess the effects of CO_2 enrichment on adults *Perna perna* mussels through responses of lysosomal membrane, lipid peroxidation and primary damages in DNA. Adult mussels were exposed to a pH range varying from 8.3 to 6.0 during 96 h. The results pointed out cytogenotoxic effects in haemolymph and gills after 48 and 96 h of exposure, respectively. Our results suggest that mortality and reduced heath status can occur with the tropical mussel *Perna perna* exposed to short periods at pH levels lower than 7.5. These findings should be considered when environmental monitoring approaches are performed in tropical marine areas employing Carbone dioxide capture and storage systems (CCS).

Keywords: CO_2 enrichment; Mollusks; *Perna perna*; Ocean acidification; Biomarkers; Carbon Capture Storage.

1. Introduction

Rising atmospheric carbon dioxide (CO_2) concentration is causing global warming and ocean acidification (Schmittner et al. 2008; Caldeira and Wickett 2005; Feely et al. 2004), which increasingly are recognized as important drivers of change in biological systems (Barros et al., 2013; Harvey et al., 2013; Rato et al., 2017; Wei et al., 2015). In the aquatic environment, the CO_2 undergoes a series of chemical transformations, and its elements recombine giving rise to new compounds (Fabry et al., 2008). First, the carbonic gas mixes with the water, producing aqueous

CO_2 and carbonic acid (H_2CO_3). This compound, classified as a weak acid, quickly dissociates producing bicarbonate (HCO_3^-) and protons (H^+), which react with other molecules present in the aquatic environment. Bicarbonate ions can also undergo chemical reactions, transforming into carbonate ions (CO_3^{2-}) and releasing hydrogen in the water column (H^+) lowering the pH (Rost et al., 2008; Rodríguez et al., 2017).

Since the beginning of the industrial era, oceanic uptake of CO_2 has resulted in acidification of the ocean; and the pH of ocean surface water has decreased by 0.1, corresponding to a 26% increase in acidity (IPCC, 2014a). In order to reduce atmospheric CO_2 levels, many mitigation strategies have been developed and proposed. One such strategy is large-scale carbon dioxide capture and storage (CCS) in geological formations. According to the International Energy Agency (IEA, 2010), it could contribute to a reduction of 19% of CO_2 emissions by 2050. This technology consists of trapping CO_2 from industrial and energy related sources, transporting it to a storage site, injecting and storing it for a long time instead of releasing this gas into the atmosphere (Kirchsteiger, 2008).

Considering that oceans have the largest capacity for CO_2 storage, sub-seabed geological formations, such as depleted oil and gas reservoirs and saline aquifers, have been designated as potential storage locations for CO_2 sequestration. The complexity in foresee the location and magnitude of possible seepages turns difficult the evaluation of potential effects on aquatics ecosystems. Two main sources of CO_2 escape are from transport facilities and storage areas (Leung et al., 2014). The effects of CO_2 leakage will depend on the amount and/or rate of leakage, the transport, dispersion processes and the chemical buffering capacity of the sedimentary or water system, contributing with the imbalance of seawater's chemistry. There are also natural sources of CO_2 enrichment, such as natural CO_2 vents (Hall-Spencer et al., 2008; McGinnis et al., 2011)

bacterial organic matter degradation and diagenesis process (Canfield, 1993) and submarine eruptions like in the Canary Islands (Spain) where pH values between 5.13 and 8.04 have been measured (Santana-Casiano et al., 2013).

In this context, many studies have been performed in order to assess the impacts from changes in the marine carbonate system as well as pH reduction by CO_2 enrichment to the organisms (Bibby et al., 2008; Blackford et al., 2014; Jones et al., 2015; Tait et al., 2015; Steve Widdicombe et al., 2015). Several authors mimicked a CO_2 leakage in lab, assessing different responses related to decreases in pH values in diversified organisms, for instance: growth rate of bacteria (*Roseobacter sp.* CECT 7117 and *Pseudomonas litoralis* CECT 7670) (Borrero-Santiago et al. 2016); mortality rate applied to amphipods (*Ampelisca brevicornis* and *Hyale youngi*) (Passarelli, et al. 2017); impact on the early life stages of marine mussel (*Perna perna*) (Szalaj et al. 2017) and effects on growth, cell viability and oxidative stress using three microalgae species (*Tetraselmis chuii, Phaeodactylum tricornutum* and *Nannochloropsis gaditana*) (Bautista-Chamizo et al., 2019). Histopathological effects and lysossomal membrane stability were also assessed in mollusks as clams (*Ruditapes philippinarum) and mussels (Mytilus edulis)* (Rodríguez-Romero et al., 2014; Sun et al., 2017) apart from a biomarkers battery using the clam *Scrobicularia plana* (Conradi et al., 2016)

Ocean acidification has also impact in other aspects of marine organisms physiology including acid-base balance, energy metabolism, redox balance and behavior as well (Ishimatsu et al., 2008; Sokolova et al., 2016). Acidification of ocean surface waters is a currently developing scenario that warrants a broadening of research foci in the study of acid–base physiology ensuring a strong basis for the physiological interactions of ocean acidification with pollutants that affect the same molecular and physiological pathways (Heuer and Grosell, 2014).

The cellular mechanisms of CO_2-induced changes, in the physiology of mollusks, are not yet fully understood but are likely to involve multiple pathways of metabolism, biomineralization and acid–base balance (Fabry, 2008; Pörtner, 2008). Metabolic effects of elevated pCO_2 vary between different species (Dupont et al., 2010; Hendriks et al., 2010) and depend on the CO_2 concentration in seawater.

For decades bivalve filter feeders have been used for environmental assessments (Cortez et al. 2012; Maranho et al. 2012; Moschino, Delaney, and Da Ros 2012). Due to their sedentary habits, low metabolic transformation rates and their ability to bioconcentrate pollutants, bivalves have been used as bioindicators suitable for monitoring studies in coastal areas such as useful bioindicators of persistent pollutants (Moore et al., 1989). Studies using clams (Liu et al., 2007; Maranho et al., 2015; Riba et al., 2004a), mussels (de Lafontaine Y et al., 2000; Gagné et al., 2007; Parolini et al., 2013; Pereira et al., 2007) and oysters (Edge et al., 2012; Patterson et al., 2014; Zanette et al., 2006) have been performed worldwide.

It has been reported that the early life stages of marine invertebrates are more susceptible to environmental toxicants than are the adult forms (His et al., 1999). However, mechanism of action oriented toxicity assays has been performed with adult organisms and the results are sensitive to environmentally relevant concentration (Marigómez et al., 2013; Tangwancharoen and Burton, 2014). The use of biological endpoints (biomarkers) has been advocated as an important tool for assessing the bioavailability of contaminants and the general health of individual organisms (Viarengo et al., 2007). These responses have been taken into consideration for monitoring different sources of anthropogenic contamination in coastal areas using bivalves as bioindicator species (Maranho et al., 2012).

The aim of this study is to assess adverse effects of CO_2-induced acidification on individuals of mussel *Perna perna* using a battery of sub-cellular effect biomarkers (lipid peroxidation, DNA primary damage and the lysosomal membrane stability). Also, the mortality of the organisms was used to establish the toxicity related to the increase of proton concentration associated with the enrichment of CO_2. For this purpose, organisms were exposed during five days to different acidification scenarios with pH values ranging from 8.3 to 6.0., and the influence of acidification associated with CO_2 enrichment was analyzed.

2. Material and methods

2.1.Mussel Exposure

2.1.2. Simulating CO_2 leakage

A CO_2 injection system was adapted from the experimental set up described by de Orte et al., (2014b), (patent nº: P201200753) in order to simulate the acidification process in a CO_2 enrichment event like CCS. Then, for each concentration of protons tested (pH from 8.0 to 6.0), replicates tanks of 20 L containing 10 mussels per aquarium were used. The experiment was developed using clean natural sea water from Enseada Beach in Guarujá, SP/Brazil (CETESB, 2016) that was used as control (no induced CO_2 acidification pH 8.3). The different pH values (8.0; 7.5; 7.0; 6.5 and 6.0) were adjusted and controlled by the CO_2 injection system (de Orte et al., 2014b). Briefly, electrodes (NBS scale) were placed in each aquarium and connected to the computer system. When the pH value was 0.01 higher than the pH value selected, a solenoid valve was opened, and the gas injected in the system; when the target pH value was reached the valves were closed, preventing the entry of the gas in the system. CO_2 gas was injected through a silicon

hose which connects the solenoid valve with the aquariums; the gas was provided by CO_2 bottles (Air Liquid).

The laboratory conditions were controlled: seawater temperature at 20 ± 2 °C, salinity 35 ppt and dissolved oxygen 8.0 mg L^{-1} and photoperiod 12:12. All the system was renewed every 48 hours to maintain the water quality, avoiding organisms to stay in touch with its excreta. The mussels were acquired from an aquaculture farm located within a reference area (Cocanha Beach, Caraguatatuba, Brazil). The animals were acclimated to the laboratory conditions for 3 days.

After 48 and 96 h of the beginning exposure, survival was recorded and ten mussels from each pH value have the hemolymph withdrawn before being dissected, and its gills frozen at − 80 °C. The hemolymph was used to assess the lysosomal membrane stability through Neutral Red Retention Time assay (NRRT) and the gills to assess DNA damage (strand breaks) and lipid peroxidation.

2.1. Chemical characterization

The values of pH and Total alkalinity (TA) were measured by a potentiometric titration system (Metrohm 794 Basic Titrino) with a combined glass electrode (Metrohm, ref. 6.0210.100) calibrated for total pH scale using hydrochloric acid (HCl) 0.1M. The volume of each sample was 50mL. Samples were analyzed in replicate. Dissolved inorganic carbon (DIC) was determined by the experimental values of TA and pH values, considering the constants of dissociation described by Mehrbach (Mehrbach et al., 1973) and modified by Dickson and Millero (1987) for the total scale of pH. TA and pH values were also used to determine the seawater carbonate system speciation such as bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), carbon dioxide (CO_2), calcite

saturation index (Ω Cal), aragonite saturation index (Ω Arag) and partial pressure of carbon dioxide (pCO_2) using the CO_2SYS program (Pierrot et al., 2006).

2.2. Neutral red retention time assay (NRRT)

NRRT assay was carried out following the method described by Lowe and Pipe (1994), where specimens of adult mussels (n = 20) were placed in replicate into 20 L natural seawater aquarium (n = 10 each) with the water replacement each 48 hours. Concomitant to the water changed, 5 mussels were removed from each aquarium to perform the extraction of hemolymph.

For the NNRT assay 0.5 ml hemolymph was withdrawn from the adductor muscle of the mussels utilizing a hypodermic syringe containing 0.5 ml of physiological solution. The material collected was transferred to 2 ml eppendorf tubes. It was pipetted 40 uL of hemolymph solution on each slide, previously treated with poly-L-lysine, incubating in dark and humid chamber for 15 minutes. After this period, 40 uL of neutral red solution was added to hemolymph present in each slide, and the set was incubated for another 15 minutes. The slides were observed in a light microscope (400x) each 15 minutes until 50% or more of the total number of cells on the slide reach a stress symptom.

Cells were examined for both structural abnormalities and NR probe retention time. Conditions were recorded in a table at each time increment. The retention time of the NR probe by the lysosomes was recorded by estimating the proportion of cells displaying leakage from the lysosomes into the cytosol and/or exhibiting abnormalities in lysosomal size and color. Cell shape may also change as a consequence of a contaminant impact.

2.3. Lipid Peroxidation and DNA strand breaks

For LPO and DNA damage, mussel's gills were homogenized with a buffer solution containing Tris-HCl (50 Mm), EDTA (1 mM), dithiothreitol (DTT) (1 mM), sucrose (50 mM), KCl (150 mM), and phenylmethylsulfonyl fluoride (PMSF) (100 mM). DNA strand breaks and lipid peroxidation (LPO) were determined in the homogenized samples.

Oxidative damage was determined by LPO assay according to Wills (1987). The samples reactions with thiobarbituric acid (TBARS) were determined by fluorescence at 530-590 nm filters. Results were expressed as μM TBARS mg^{-1} min^{-1} protein. Mitochondrial DNA damage was evaluated according to Olive (1988) alkaline precipitation assay. A standard calibration curve was used with salmon sperm DNA and Hoescht solution. DNA damage was measured by fluorescence at 360-450 nm. Results were expressed as μg DNA strands mg^{-1} proteins.

LPO and DNA damage were normalized by the total protein content determined in the extract following the Bradford methodology (Bradford, 1976), using bovine serum for the calibration curve.

2.4. Data treatment (Statistical analysis)

Assumptions of a normal distribution and equal variance among groups of data collected from the experiment were confirmed by means of the Shapiro-Wilk's test and the Levene's F-test, respectively. One-way analysis of variance (One-way ANOVA) followed by the Dunn's test were used to identify treatments significantly different from the controls. The differences were calculated comparing the results from the different values of pH (protons) with the control (natural

seawater, pH 8.3) and were classified according to their statistical significance, as follows: *($p < 0.05$), **($p < 0.01$). ANOVA and post-hoc analysis were performed using Statistical software SPSS 15.0 for Windows.

3. Results and discussion

Data had enough homogeneity of variance for ANOVA. There were no significant differences between mussel holding tanks in any treatment.

3.1. Chemical Analysis

The average values for the carbonate system speciation are presented in Table 1. This data refers to the carbon parameters measured and calculated by the end of the experiments. The salinity parameter used was 35, and the temperature was 23.5±0.5 °C. As expected, the total inorganic carbon (TIC) was higher as pH was reduced and the saturation index for calcite (Ωcal) and aragonite (Ωarag) decreased as pH decreased and pCO_2 increased.

Table 1. Carbonate system speciation in assays exposed to the different pH treatments for both bioassays of fertilization and embryo-larval development

pH Treatment	TA in (µmol/kg)	TCO_2 in (µmol/kg)	TIC/DIC (µmol/kg)	HCO_3^- (µmol/kg)	CO_3^{-2} (µmol/kg)	CO_2 (µmol/kg)	pCO_2 (µatm)	Ωcal	Ωarag
8.3	1644	1404	1404	1249	147	8	262	3,53	2,31
8.0	1662	1511	1511	1397	99	14	472	2,38	1,56
7.5	1731	1718	1718	1629	39	50	1672	0,93	0,61
7.0	1682	1828	1828	1650	12	165	5451	0,29	0,19
6.5	2483	3249	3249	2469	6	774	25996	0,14	0,09
6.0	3172	5996	5996	3167	3	2827	97130	0,06	0,04

As mussel shells are made of up to 83% aragonite (Hubbard et al., 1981) and aragonite is more soluble than calcite, shell dissolution is going to be a considerable problem faced by mussels in acidified seawater (Beesley et al., 2008). ΩArag and remains favorable for calcification when >1 (Thomsen et al., 2018). The present study shows (table 1) a decrease in Ω value related to an increase in protons H^+, being a critical value for calcification when pH is ≤ 7.5. According to (Thomsen et al., 2018) it is of high ecological relevance the calcification kinetics for bivalve shell formation, and the reduced and dissolution depend on Ωarag/Ωcal or lowered substrate availability and inhibition by $[H^+]$.

3.2. Biological Responses

The percentage of mussel mortality for each treatment assay is shown in Table 2. In general, the mortality increases as pH values decrease. pH values upper to 7.5 presented no mortality, while pH values under 7.0 presented minimum 20% of mortality after 96 hours. The results compeer with those presented by Freitas et al. (2015), where the clam *Scrobicularia plana* showed a mortality of 20% at the pH value of 7.1 after 96 hours exposure.

Table 2. *Perna perna* mortality percentage after 48- and 96-hours exposure to 5 different pH levels plus and control (8.3 with no CO_2 added).

pH value	Mortality (%)	
	48 hours	96 hours
8.3	0	0
8.0	0	0
7.5	0	0
7.0	0	20
6.5	10	20
6.0	0	30

Lysosomes are central to many key biological processes, including reproduction, digestion and immune response; thus, any deleterious changes to their function can have serious consequences for those processes. The general health status of the mussels was therefore considered in terms of changes to the lysosomal system of the blood cells (Beesley et al., 2008).

Neutral red retention time assay is based on the principle that only lysosomes in healthy cells take up and retain the vital dye neutral red. Lysosomal membrane damage caused by the impact of xenobiotics (Dailianis et al., 2003) or by CO_2-induced seawater acidification (Beesley et al., 2008) can decrease the NRRT by inducing the leaking of lysosomal components (Binelli et al., 2009).

This study produced the first data on the effects associated with CO_2-induced acidification showed by different pH values in the brown mussel *P. perna*. The results of the biomarker of exposure neutral red retention time (NRRT) assay for *Perna perna* involving acidified sea water are shown in Figure 1. The range in NRRT values observed for lysosomes from control mussels (n=10; no CO2 added) at time zero, 48 h and 96 h of assay were 81 min ± 26; 93 min ± 23 and 77 min ± 24 respectively. In the first 48 h, the retention time decreased significantly (Dunn's test $p < 0.05$ and $p < 0.01$) at pH 7.0 and 6.5 when compared with control. After 96 h, the organisms exposed to different pH values showed significant decrease ($p < 0.01$) in the dye retention time in lysosomes only at pH 6.0. There was no statistically significant difference between retention time of the dye in control and the highest pH values (8.0 and 7.5).

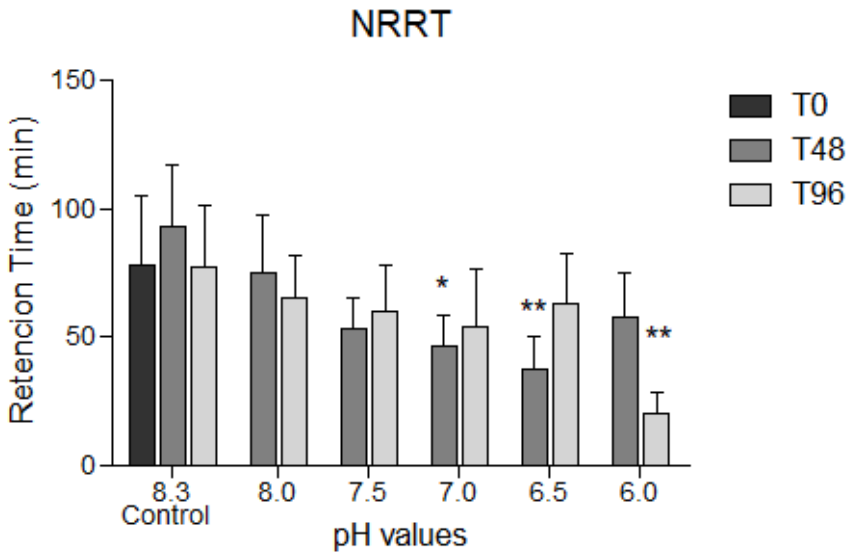

Figure 1. Mean and standard deviation of lysosomal membrane stability (LMS) assessed through neutral red retention time (NRRT) assay in the hemocytes of the mussel *P. perna* exposed to different pH levels for 96 hours. *reflects values that differed significantly from the control ($p < 0.05$). **reflects values that differed significantly from the control ($p < 0.01$).

The results presented in this study for the control sample (81 min ± 26) are reliable and fits with those obtained in previous studies reporting a NRRT ranging from 60 min to 90 min in a study on healthy or non-exposed *P. perna* (Ortega et al., 2018) and 60 min in a study using hemolymph of *Perna viridis* (Nicholson, 2003). Furthermore, Martin-Diaz et al. 2009 have reported NRRTs of 90 min by using *M. galloprovincialis* hemolymph and 106 min in the Aguirre-Martínez et al. 2013 study using clam *Ruditapes philippinarum* hemolymph.

According the criteria established by OSPAR (2013) the cytochemical method, animals are considered to be stressed but compensating if <120 but ≥ 50 minutes. However, studies have demonstrated that lysosomal membrane stability differs in tropical zones compared to temperate zones, as higher temperatures in the tropics leads the neutral red dye to be retained in the lysosomes for a shorter period of time (Pereira et al., 2011), agreeing the results of this work to previous

studies on NRRT of *P. perna* populations from Brazilian coast (Abessa et al., 2005; Cortez et al., 2012; Pereira et al., 2014; Souza et al., 2016). Thereat, this present study may also contribute as a health index for tropical mussels.

Lysosomal integrity is directly correlated with physiological scope for growth (SFG) and is also mechanistically linked in terms of the processes of protein turnover (Allen and Moore, 2004). Ringwood et al. (2004) have shown that lysosomal stability in parent oysters is also correlated with larval viability. The integrity or stability of this membrane is considered an indicator of "wellbeing" of the cell, acting as an important cellular and nonspecific biomarker of stress (Moore et al., 2007) once its activity is directly related to the ions transportation through the cells membrane (Marigómez et al., 2013).

The significant reduction in lysosomal health observed in the current study, from pH 7.0 (48 h) down, may be related to the increase in the calcium ions (Ca^{2+}) concentrations in the haemolymph (Beesley et al., 2008) which can cause lysosomal destabilization (Marchi et al., 2004). According to Bibby et al. (2008), decreasing pH levels promote the dissolution of mussel shells, consequently increasing concentration of Ca^{2+} ions in the hemolymph, altering cellular metabolism and reducing immune function. According to Jones and Waufeh (1982), extracellular calcium enter in the cells via pinocytosis and once in the cytosol, Ca^{2+} can activate calcium-dependent phospholipase A2, destabilizing the lysosome membrane.

The compensation of acid-base imbalance is also of great importance, since it is related to the ion's transportation through the cell's membrane. The CO_2 that is produced in the cells during routine metabolism is typically hydrated to form bicarbonate and H^+. The H^+ ions are buffered, while the bicarbonate is transported out of the cell in exchange for Cl^- via ion transport proteins.

Freitas et al., (2017) and Velez et al., (2016) indicated that mussels responded to maintain acid-base balance in a study where the carbonic anhydrase activity was significantly higher in mussels exposed to low pH in comparison to control individuals. As observed in the present study, the increase in environmental [H^+], once more may be associated to a decrease in the lysosome capacity to retain the dye as a healthy cell.

DNA strand breaks values and the LPO results are shown in figure 2. In the present study DNA strand breaks exhibited significant higher values ($p<0.05$) than T0 in gills of *Perna perna* mussels exposed to a pH value of 6.0 in both exposure time. Although there was significant difference in relation to the control only in pH 6.0 (96 hours exposure), there was an increasing tendency for LPO in time 96 hours comparing to 48 hours at pH values < 8.0.

Oxidative stress occurs due to an imbalance between the production of reactive oxygen species (ROS) and their elimination by the antioxidant system defense, leading to protein degradation and enzyme inactivation (Halliwell and Gutteridge, 2007). If continuously generated, ROS can damage important biomolecules such as DNA, proteins, and lipids DNA damage and lipid peroxidation (LPO) (Regoli and Winston, 1999). Leading to the results obtained in this study, which may be related to the increase in the ROS production in scenarios of oceanic acidification, as agreed with the study conducted by Cao et al. (2018), where bivalve *Crassostrea gigas* showed a significant increase in the production of ROS in hemocytes, as well as DNA damage (comet assay) in different scenarios of ocean acidification (pH 7.6 and 7.8).

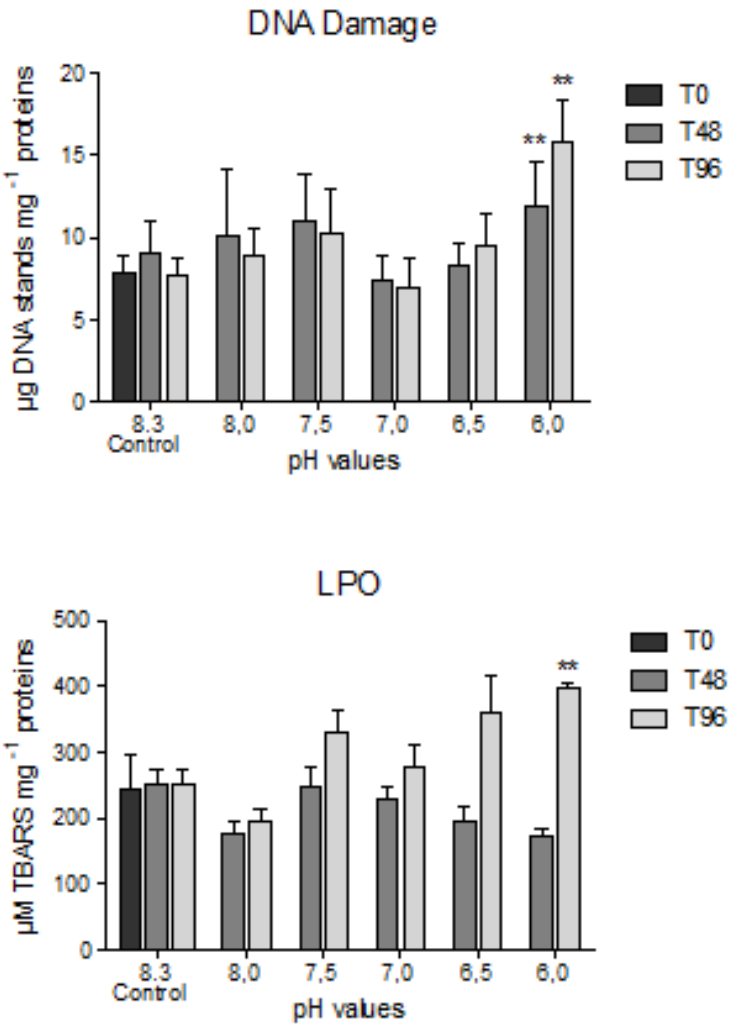

Figure 2. Mean and standard deviation of DNA damage (stand break) and Lipid peroxidation (LPO) assessed in *P. perna* mussels exposed to different pH levels for 96 hours. *reflects values that differed significantly from the control ($p < 0.05$). **reflects values that differed significantly from the control ($p < 0.01$)

Timmins-Schiffman et al. (2014) reported that low pH caused a decrease in electron supply from NADH to the electron transport chain in the oyster *Crassostrea gigas* and this response mechanism changed the cellular balance between resource supply and oxidative stress. As a protective system against the consequences of oxidative stress it is considering the role of

lysosomal autophagy by breaking down longer lived proteins and organelles, and recycling the products into protein-synthesis and energy production pathways (Cuervo, 2004).

3.3. Principal components analysis (PCA)

A multivariate analysis approach was applied to all data to help discriminate the main variables responsible for the variance of biological effects detected in mussels (Table 3, Figure 3). The application of PCA to the original 13 variables at the pH bioassay indicates that they can be grouped in three new factors. These new variables explain more than 96% of the total variance in the original data set. In the present study, we selected to interpret a group of variables as those associated with a particular component where loadings were 0.4 or higher, corresponding to an associated explained variance of more than 30%.

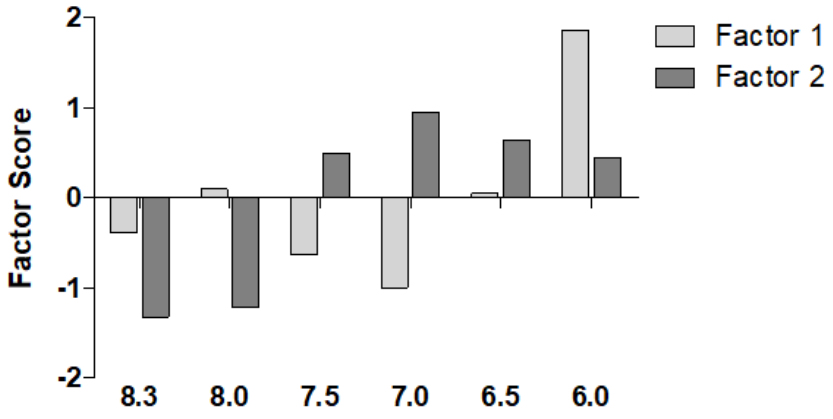

Figure 3. Estimated factor scores in relation to the pH treatments used in the study.

The first factor (61%), include the enzymatic systems, DNA damage and mortality of *P. perna* mussel. According to Velez et al. (2016), the negative LPO value after 96 hours exposure may demonstrated a relationship between increase of antioxidant enzymes and LPO levels since these antioxidant defense mechanisms were possibly overwhelmed by ROS produced in organism tissues in the first 48 hours, inducing LPO. It was more representative for pH 6.0 with higher positive values (Figure 3).

Table 3. Sorted rotated factor loadings of 13 variables for the three principal factors.

Variables	Components	
	Factor 1	Factor 2
NRRT 48h	---	-0,92
NRRT 96h	-0,76	-0,52
DNA 48h	0,69	---
DNA 96h	0,91	0,24
LPO 48h	-0,76	---
LPO 96h	0,51	0,72
Mortality 48h	---	---
Mortality 96h	0,55	0,68
TA	0,88	0,40
DIC	0,89	0,41
Ω cal	-0,26	-0,94
Ω arag	-0,26	-0,94
pCO_2	0,91	---

The second factor (21%) showed the relationship among NRRT, DNA damage, LPO, mortality and pH reduction. This factor could be related with the acidification by CO_2 enrichment in the effects on lysosome membrane stability (NRRT), increases in DNA damage, oxidative stress (LPO), besides mortality. Factor three (89%) represents the stress factor, showed by effects in NRRT and DNA damage in both exposure times analyzed. LPO was slightly responsive in 48 hours with no activation at 96 hours.

The presented results corroborate with the toxic effects are observed from a decrease in pH value ≤6.5, while from pH values ≤7.5 a stress showed to be produced by activating enzymatic systems maintained at the lowest pH values. In summary, the stress produced by increasing protons concentration can be noticed in the enzymatic systems of the mussels (NRRT and LPO) in pH values lower than 7.5, being accentuated when the value of the concentration of protons is higher, which produces toxic effects related to pH 6.5 and 6.0

4. Conclusions

The results obtained in this study suggest that mortality and reduced heath status can occur with the tropical mussel *Perna perna* exposed to short periods at pH levels lower than 7.5. Lysossomal membrane stability of haemocytes showed the first signs of effects, followed by DNA and lipid oxidative damages in gills triggered by acidified conditions. These findings should be taken in account when environmental monitoring approaches are performed in tropical marine areas receiving petroliferous or other activities employing Carbone dioxide capture and storage systems (CCS).

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References

Abessa, D.M. de S., Zaroni, L.P., De Sousa, E.C.P.M., Gasparro, M.R., Pereira, C.D.S., De Figueredo Rachid, B.R., Depledge, M., King, R.S., 2005. Physiological and cellular responses in two populations of the mussel *Perna perna* collected at different sites from the Coast of São Paulo, Brazil. Brazilian Arch. Biol. Technol. 48, 217–225. https://doi.org/10.1590/S1516-89132005000200008

Allen, J.I., Moore, M.N., 2004. Environmental prognostics: Is the current use of biomarkers appropriate for environmental risk evaluation? Mar. Environ. Res. 58, 227–232. https://doi.org/10.1016/j.marenvres.2004.03.119

Barros, P., Sobral, P., Range, P., Chícharo, L., Matias, D., 2013. Effects of sea-water acidification on fertilization and larval development of the oyster *Crassostrea gigas*. J. Exp. Mar. Bio. Ecol. 440, 200–206. https://doi.org/10.1016/j.jembe.2012.12.014

Bautista-Chamizo, E., Sendra, M., De Orte, M.R., Riba, I., 2019. Comparative effects of seawater acidification on microalgae: Single and multispecies toxicity tests. Sci. Total Environ. 649, 224–232. https://doi.org/10.1016/j.scitotenv.2018.08.225

Beesley, A., Lowe, D.M., Pascoe, C.K., Widdicombe, S., 2008. Effects of CO_2-induced seawater acidification on the health of *Mytilus edulis*. Clim. Res. 37, 215–225. https://doi.org/10.3354/cr00765

Bibby, R., Widdicombe, S., Parry, H., Spicer, J., Pipe, R., 2008. Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. Aquat. Biol. 2, 67–74. https://doi.org/10.3354/ab00037

Binelli, A., Cogni, D., Parolini, M., Riva, C., Provini, A., 2009. In vivo experiments for the evaluation of genotoxic and cytotoxic effects of Triclosan in Zebra mussel hemocytes. Aquat. Toxicol. 91, 238–244. https://doi.org/10.1016/j.aquatox.2008.11.008

Blackford, J., Stahl, H., Bull, J.M., Bergès, B.J.P., Cevatoglu, M., Lichtschlag, A., Connelly, D., James, R.H., Kita, J., Long, D., Naylor, M., Shitashima, K., Smith, D., Taylor, P., Wright, I., Akhurst, M., Chen, B., Gernon, T.M., Hauton, C., Hayashi, M., Kaieda, H., Leighton, T.G.,

Sato, T., Sayer, M.D.J., Suzumura, M., Tait, K., Vardy, M.E., White, P.R., Widdicombe, S., 2014. Detection and impacts of leakage from sub-seafloor deep geological carbon dioxide storage. Nat. Clim. Chang. 4, 1011–1016. https://doi.org/10.1038/nclimate2381

Borrero-Santiago, A.R., Carbú, M., DelValls, T.A., Riba, I., 2016. CO_2 leaking from sub-seabed storage: Responses of two marine bacteria strains. Mar. Environ. Res. 121, 2–8. https://doi.org/10.1016/j.marenvres.2016.05.018

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

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J. Geophys. Res. 110, C09S04. https://doi.org/10.1029/2004JC002671

Canfield, D.E., 1993. Organic Matter Oxidation in Marine Sediments. Interact. C, N, P S Biogeochem. Cycles Glob. Chang. I, 333–363. https://doi.org/10.1007/978-3-642-76064-8_14

Cao, R., Liu, Y., Wang, Q., Zhang, Q., Yang, D., Liu, H., Qu, Y., Zhao, J., 2018. The impact of ocean acidification and cadmium on the immune responses of Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol. 81, 456–462. https://doi.org/10.1016/j.fsi.2018.07.055

CETESB, 2016. Companhia Ambiental do Estado de São Paulo - Qualidade das praias litorâneas no Estado de São Paulo.

Conradi, M., Riba, I., Almagro-Pastor, V., DelValls, T.A., 2016. Lethal and sublethal responses in the clam *Scrobicularia plana* exposed to different CO_2-acidic sediments. Environ. Res. 151, 642–652. https://doi.org/10.1016/j.envres.2016.08.032

Cortez, F.S., Seabra Pereira, C.D., Santos, A.R., Cesar, A., Choueri, R.B., Martini, G.D.A., Bohrer-Morel, M.B., 2012. Biological effects of environmentally relevant concentrations of the pharmaceutical Triclosan in the marine mussel *Perna perna* (Linnaeus, 1758). Environ. Pollut. 168, 145–150. https://doi.org/10.1016/j.envpol.2012.04.024

Cuervo, A.M., 2004. Autophagy: In sickness and in health. Trends Cell Biol. 14, 70–77. https://doi.org/10.1016/j.tcb.2003.12.002

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M., Dimitriadis, V.K., 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Mar. Environ. Res. 56, 443–470. https://doi.org/10.1016/S0141-1136(03)00005-9

de Lafontaine Y, Gagné, F., Blaise, C., Costan, G., Gagnon, P., Chan, H., 2000. Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). Aquat. Toxicol. 50, 51–71.

de Orte, M.R., Sarmiento, A.M., Basallote, M.D., Rodríguez-Romero, A., Riba, I., DelValls, A., 2014a. Effects on the mobility of metals from acidification caused by possible CO_2 leakage from sub-seabed geological formations. Sci. Total Environ. 470–471, 356–363. https://doi.org/10.1016/j.scitotenv.2013.09.095

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014b. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res. Part A, Oceanogr. Res. Pap. 34, 1733–1743. https://doi.org/10.1016/0198-0149(87)90021-5

Dupont, S., Dorey, N., Thorndyke, M., 2010. What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? Estuar. Coast. Shelf Sci. 89, 182–185. https://doi.org/10.1016/j.ecss.2010.06.013

Edge, K.J., Johnston, E.L., Roach, A.C., Ringwood, A.H., 2012. Indicators of environmental stress: Cellular biomarkers and reproductive responses in the Sydney rock oyster (Saccostrea glomerata). Ecotoxicology 21, 1415–1425. https://doi.org/10.1007/s10646-012-0895-2

Fabry, V.J., 2008. Marine Calcifiers in a High-CO_2 Ocean. Science (80-.). 320, 1020–1022. https://doi.org/10.1126/science.1157130

Fabry, V.J., Seibel, B.A., Feely, R.A., Orr, J.C., 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J. Mar. Sci. 65, 414–432.

Feely, R. a, Sabine, C.L., Lee, K., Berelson, W., Kleypas, J., Fabry, V.J., Millero, F.J., Anonymous, 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science 280. 305, 362–366. https://doi.org/10.1126/science.1097329

Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2015. How life history influences the responses of the clam *Scrobicularia plana* to the combined impacts of carbamazepine and pH decrease. Environ. Pollut. 202, 205–214. https://doi.org/10.1016/j.envpol.2015.03.023

Freitas, R., De Marchi, L., Bastos, M., Moreira, A., Velez, C., Chiesa, S., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2017. Effects of seawater acidification and salinity alterations on metabolic, osmoregulation and oxidative stress markers in *Mytilus galloprovincialis*. Ecol. Indic. 79, 54–62. https://doi.org/10.1016/j.ecolind.2017.04.003

Gagné, F., Blaise, C., André, C., Gagnon, C., Salazar, M., 2007. Neuroendocrine disruption and health effects in *Elliptio complanata* mussels exposed to aeration lagoons for wastewater treatment. Chemosphere 68, 731–743. https://doi.org/10.1016/j.chemosphere.2006.12.101

Hall-Spencer, J.M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S.M., Rowley, S.J., Tedesco, D., Buia, M.C., 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. Nature 454, 96–99. https://doi.org/10.1038/nature07051

Harvey, B.P., Gwynn-Jones, D., Moore, P.J., 2013. Meta-analysis reveals complex marine biological responses to the interactive effects of ocean acidification and warming. Ecol. Evol. 3, 1016–1030. https://doi.org/10.1002/ece3.516

Hendriks, I.E., Duarte, C.M., Álvarez, M., 2010. Vulnerability of marine biodiversity to ocean acidification: A meta-analysis. Estuar. Coast. Shelf Sci. 86, 157–164. https://doi.org/10.1016/j.ecss.2009.11.022

Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. AJP Regul. Integr. Comp. Physiol. 307, R1061–R1084. https://doi.org/10.1152/ajpregu.00064.2014

His, E., Beiras, R., Seaman, M.N.L., 1999. The assessment of marine pollution-bioassays with bivalve embryos and larvae, advances in marine biology. Elsevier Masson SAS. https://doi.org/10.1016/S0065-2881(08)60428-9

Hubbard, F., McManus, J., Al-Dabbas, M., 1981. Environmental influences on the shell mineralogy of *Mytilus edulis*. Geo-Marine Lett. 1, 267–269. https://doi.org/10.1007/BF02462445

IEA, 2010. Carbon capture and storage: Model Regulatory Framework. JAMA 303, 1601; author reply 1601. https://doi.org/10.1001/jama.2010.513

IPCC, 2014. Summary for Policymakers, Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https://doi.org/10.1017/CBO9781107415324

Ishimatsu, A., Hayashi, M., Kikkawa, T., 2008. Fishes in high-CO_2, acidified oceans. Mar. Ecol. Prog. Ser. 373, 295–302. https://doi.org/10.3354/meps07823

Jones, D.G., Beaubien, S.E., Blackford, J.C., Foekema, E.M., Lions, J., De Vittor, C., West, J.M., Widdicombe, S., Hauton, C., Queirós, A.M., 2015. Developments since 2005 in understanding potential environmental impacts of CO_2 leakage from geological storage. Int. J. Greenh. Gas Control 40, 350–377. https://doi.org/10.1016/j.ijggc.2015.05.032

Jones, R.G., Waufeh, I., 1982. Calcium-containing lysosomes in the outer mantle epithelial cells of Amblema, a fresh-water mollusc - Jones - 2005 - The Anatomical Record - Wiley Online Library 343, 337–343.

Kirchsteiger, C., 2008. Carbon capture and storage-desirability from a risk management point of view. Saf. Sci. 46, 1149–1154. https://doi.org/10.1016/j.ssci.2007.06.012

Leung, D.Y.C., Caramanna, G., Maroto-Valer, M.M., 2014. An overview of current status of carbon dioxide capture and storage technologies. Renew. Sustain. Energy Rev. 39, 426–443. https://doi.org/10.1016/j.rser.2014.07.093

Liu, C.W., Liang, C.P., Lin, K.H., Jang, C.S., Wang, S.W., Huang, Y.K., Hsueh, Y.M., 2007. Bioaccumulation of arsenic compounds in aquacultural clams (Meretrix lusoria) and assessment of potential carcinogenic risks to human health by ingestion. Chemosphere 69, 128–134. https://doi.org/10.1016/j.chemosphere.2007.04.038

Lowe, D.M., Pipe, R.K., 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an in vitro study. Aquat. Toxicol. https://doi.org/10.1016/0166-445X(94)00045-X

Maranho, DelValls, T.A., Martín-Díaz, M.L., 2015. Assessing potential risks of wastewater discharges to benthic biota: An integrated approach to biomarker responses in clams (*Ruditapes philippinarum*) exposed under controlled conditions. Mar. Pollut. Bull. 92, 11–24. https://doi.org/10.1016/j.marpolbul.2015.01.009

Maranho, L.A., Pereira, C.D.S., Choueri, R.B., Cesar, A., Gusso-Choueri, P.K., Torres, R.J., Abessa, D.M.D.S., Morais, R.D., Mozeto, A.A., Delvalls, T.A., Martín-Díaz, M.L., 2012. The application of biochemical responses to assess environmental quality of tropical estuaries: Field surveys. J. Environ. Monit. 14, 2608–2615. https://doi.org/10.1039/c2em30465a

Marchi, B., Burlando, B., Moore, M.N., Viarengo, A., 2004. Mercury- and copper-induced lysosomal membrane destabilization depends on [Ca_2^+] independent phospholipase A2 activation. Aquat. Toxicol. 66, 197–204. https://doi.org/10.1016/j.aquatox.2003.09.003

Marigómez, I., Garmendia, L., Soto, M., Orbea, A., Izagirre, U., Cajaraville, M.P., 2013. Marine ecosystem health status assessment through integrative biomarker indices: A comparative study after the Prestige oil spill "mussel Watch." Ecotoxicology 22, 486–505. https://doi.org/10.1007/s10646-013-1042-4

Martin-Diaz, L., Franzellitti, S., Buratti, S., Valbonesi, P., Capuzzo, A., Fabbri, E., 2009. Effects of environmental concentrations of the antiepilectic drug carbamazepine on biomarkers and

cAMP-mediated cell signaling in the mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 94, 177–185. https://doi.org/10.1016/j.aquatox.2009.06.015

McGinnis, D.F., Schmidt, M., Delsontro, T., Themann, S., Rovelli, L., Reitz, A., Linke, P., 2011. Discovery of a natural CO_2 seep in the German North Sea: Implications for shallow dissolved gas and seep detection. J. Geophys. Res. Ocean. 116, 1–12. https://doi.org/10.1029/2010JC006557

Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicx, R.M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897–907. https://doi.org/10.4319/lo.1973.18.6.0897

Moore, M.T., Lizotte, R.E., Smith, S., 2007. Responses of Hyalella azteca to a pyrethroid mixture in a constructed wetland. Bull. Environ. Contam. Toxicol. 78, 245–248. https://doi.org/10.1007/s00128-007-9135-5

Moschino, V., Delaney, E., Da Ros, L., 2012. Assessing the significance of *Ruditapes philippinarum* as a sentinel for sediment pollution: Bioaccumulation and biomarker responses. Environ. Pollut. 171, 52–60. https://doi.org/10.1016/j.envpol.2012.07.024

Nicholson, S., 2003. Lysosomal membrane stability, phagocytosis and tolerance to emersion in the mussel *Perna viridis* (Bivalvia: Mytilidae) following exposure to acute, sublethal, copper. Chemosphere 52, 1147–1151. https://doi.org/10.1016/S0045-6535(03)00328-X

Olive, P.L., 1988. DNA precipitation assay: A rapid and simple method for detecting DNA damage in mammalian cells. Environ. Mol. Mutagen. 11, 487–495. https://doi.org/10.1002/em.2850110409

Ortega, A. dos S.B., Maranho, L.A., Nobre, C.R., Moreno, B.B., Guimarães, R.S., Lebre, D.T., de Souza Abessa, D.M., Ribeiro, D.A., Pereira, C.D.S., 2018. Detoxification, oxidative stress, and cytogenotoxicity of crack cocaine in the brown mussel *Perna perna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-018-1600-7

OSPAR, 2013. Background document and technical annexes for biological effects monitoring, Update 2013 Monitoring and Assessment Series.

Parolini, M., Pedriali, A., Riva, C., Binelli, A., 2013. Sub-lethal effects caused by the cocaine metabolite benzoylecgonine to the freshwater mussel *Dreissena polymorpha*. Sci. Total Environ. 444, 43–50. https://doi.org/10.1016/j.scitotenv.2012.11.076

Passarelli, M.C., Riba, I., Cesar, A., Serrano-Bernando, F., DelValls, T.A., 2017. Assessing the influence of ocean acidification to marine amphipods: A comparative study. Sci. Total Environ. 595, 759–768. https://doi.org/10.1016/j.scitotenv.2017.04.004

Patterson, H.K., Boettcher, A., Carmichael, R.H., 2014. Biomarkers of dissolved oxygen stress in oysters: A tool for restoration and management efforts. PLoS One 9. https://doi.org/10.1371/journal.pone.0104440

Pereira, C.D.S., Abessa, D.M.D.S., Bainy, A.C.D., Zaroni, L.P., Gasparro, M.R., Bícego, M.C., Taniguchi, S., Furley, T.H., De Sousa, E.C.P.M., 2007. Integrated assessment of multilevel biomarker responses and chemical analysis in mussels from São Sebastião, São Paulo, Brazil. Environ. Toxicol. Chem. 26, 462–469. https://doi.org/10.1897/06-266r.1

Pereira, C.D.S., Martin-Díaz, M.L., Zanette, J., Cesar, A., Choueri, R.B., Abessa, D.M. de S., Catharino, M.G.M., Vasconcellos, M.B.A., Bainy, A.C.D., de Sousa, E.C.P.M., DelValls, T.A., 2011. Integrated biomarker responses as environmental status descriptors of a coastal zone (São Paulo, Brazil). Ecotoxicol. Environ. Saf. 74, 1257–1264. https://doi.org/10.1016/j.ecoenv.2011.02.019

Pierrot, D., Lewis, E., Wallace, D.W.R., 2006. MS Excel Program Developed for CO_2 System Calculation. Carbon Dioxide Infromation Anal. Center, Oak Ridge Natl. Lab. U.S. Dep. Energy. ORNL/CDIAC, 105.

Pörtner, H.O., 2008. Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view. Mar. Ecol. Prog. Ser. 373, 203–217. https://doi.org/10.3354/meps07768

Rato, L.D., Novais, S.C., Lemos, M.F.L., Alves, L.M.F., Leandro, S.M., 2017. *Homarus gammarus* (Crustacea: Decapoda) larvae under an ocean acidification scenario: responses across different levels of biological organization. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 203, 29–38. https://doi.org/10.1016/j.cbpc.2017.09.002

Riba, I.L., DelValls, T.A., Forja, J.M., Gómez-Parra, A., 2004. The influence of pH and salinity on the toxicity of heavy metals in sediment to the estuarine clam *Ruditapes philippinarum*. Environ. Toxicol. Chem. 23, 1100–1107. https://doi.org/10.1897/023-601

Ringwood, A.H., Hoguet, J., Keppler, C., Gielazyn, M., 2004. Linkages between cellular biomarker responses and reproductive success in oysters - *Crassostrea virginica*. Mar. Environ. Res. 58, 151–155. https://doi.org/10.1016/j.marenvres.2004.03.010

Rodríguez-Romero, A., Jiménez-Tenorio, N., Basallote, M.D., Orte, M.R. De, Blasco, J., Riba, I., 2014. Predicting the impacts of CO_2 leakage from sub-seabed storage: Effects of metal accumulation and toxicity on the model benthic organism *Ruditapes philippinarum*. Environ. Sci. Technol. 48, 12292–12301. https://doi.org/10.1021/es501939c

Rodríguez, A., Hernández, J.C., Brito, A., Clemente, S., 2017. Effects of ocean acidification on juvenile's sea urchins: Predator-prey interactions. J. Exp. Mar. Bio. Ecol. 493, 31–40. https://doi.org/10.1016/j.jembe.2017.04.005

Rost, B., Zondervan, I., Wolf-Gladrow, D., 2008. Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: Current knowledge, contradictions and research directions. Mar. Ecol. Prog. Ser. 373, 227–237. https://doi.org/10.3354/meps07776

Santana-Casiano, J.M., González-Dávila, M., Fraile-Nuez, E., De Armas, D., González, A.G., Domínguez-Yanes, J.F., Escánez, J., 2013. The natural ocean acidification and fertilization event caused by the submarine eruption of El Hierro. Sci. Rep. 3, 5–12. https://doi.org/10.1038/srep01140

Schmittner, A., Oschlies, A., Matthews, H.D., Galbraith, E.D., 2008. Future changes in climate, ocean circulation, ecosystems, and biogeochemical cycling simulated for a business-as-usual CO_2 emission scenario until year 4000 AD. Global Biogeochem. Cycles 22, 1–21. https://doi.org/10.1029/2007GB002953

Sokolova, I.I., Matoo, O.B., Dickinson, G.H., Beniash, E., 2016. Physiological and ecological responses, in: Stressors in the Marine Environment. Oxford Scholarship. https://doi.org/10.1093/acprof:oso/9780198718826.001.0001

Souza, A., Moreno, B.B., Almeida, J.E., Rogero, S.O., Pereira, C.D.S., Rogero, J.R., 2016. Cytotoxicity evaluation of Amoxicillin and Potassium Clavulanate in *Perna perna* musselss. Ecotoxicol. Environ. Contam. 11, 21–26. https://doi.org/10.5132/eec.2016.01.04

Stefanoni, M.F., Abessa, D.M. de S., 2011. Physiological esponses of the brown mussel *Perna perna* (mollusca, bivalvia) exposed to the anionic surfactante linear alkylbenzene sulphonate (LAS) 8634, 44–53.

Sun, T., Tang, X., Jiang, Y., Wang, Y., 2017. Seawater acidification induced immune function changes of haemocytes in *Mytilus edulis*: A comparative study of CO_2 and HCl enrichment. Sci. Rep. 7, 1–10. https://doi.org/10.1038/srep41488

Szalaj, D., De Orte, M.R., Goulding, T.A., Medeiros, I.D., DelValls, T.A., Cesar, A., 2017. The effects of ocean acidification and a carbon dioxide capture and storage leak on the early life stages of the marine mussel *Perna perna* (Linneaus, 1758) and metal bioavailability. Environ. Sci. Pollut. Res. 24, 765–781. https://doi.org/10.1007/s11356-016-7863-y

Tait, K., Stahl, H., Taylor, P., Widdicombe, S., 2015. Rapid response of the active microbial community to CO_2 exposure from a controlled sub-seabed CO_2 leak in Ardmucknish Bay (Oban, Scotland). Int. J. Greenh. Gas Control 38, 171–181. https://doi.org/10.1016/j.ijggc.2014.11.021

Tangwancharoen, S., Burton, R.S., 2014. Early life stages are not always the most sensitive: Heat stress responses in the copepod *Tigriopus californicus*. Mar. Ecol. Prog. Ser. 517, 75–83. https://doi.org/10.3354/meps11013

Thomsen, J., Ramesh, K., Sanders, T., Bleich, M., Melzner, F., 2018. Calcification in a marginal sea - Influence of seawater [Ca_2^+] and carbonate chemistry on bivalve shell formation. Biogeosciences 15, 1469–1482. https://doi.org/10.5194/bg-15-1469-2018

Timmins-Schiffman, E., Coffey, W.D., Hua, W., Nunn, B.L., Dickinson, G.H., Roberts, S.B., 2014. Shotgun proteomics reveals physiological response to ocean acidification in *Crassostrea gigas*. BMC Genomics 15, 1–18. https://doi.org/10.1186/1471-2164-15-951

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. Native and introduced clams' biochemical responses to salinity and pH changes. Sci. Total Environ. 566–567, 260–268. https://doi.org/10.1016/j.scitotenv.2016.05.019

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 146, 281–300. https://doi.org/10.1016/j.cbpc.2007.04.011

Wei, L., Wang, Q., Wu, H., Ji, C., Zhao, J., 2015. Proteomic and metabolomic responses of Pacific oyster *Crassostrea gigas* to elevated pCO_2 exposure. J. Proteomics 112, 83–94. https://doi.org/10.1016/j.jprot.2014.08.010

Widdicombe, S., Beesley, A., Berge, J.A., 2015. Impact of elevated levels of CO_2 on animal mediated ecosystem function: The modification of sediment nutrient fluxes by burrowing urchins. https://doi.org/10.1016/j.marpolbul.2012.11.008

Wills, E.D., 1987. Evaluation of lipid peroxidation in lipids and biological membranes., in: Biochemical Toxicology: A Practical Approach. pp. 127–150.

Zanette, J., Monserrat, J.M., Bianchini, A., 2006. Biochemical biomarkers in gills of mangrove oyster *Crassostrea rhizophorae* from three Brazilian estuaries. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 143, 187–195. https://doi.org/10.1016/j.cbpc.2006.02.001

Sub-lethal combined effects of illicit drug and decreased pH on marine mussels: A short-time exposure to crack cocaine in CO_2 enrichment scenarios

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ABSTRACT

With the increase in greenhouse gases emissions in the last years, the ocean CO_2 uptake leads to a decrease in the general seawater pH (ocean acidification). Once dissolved in seawater, CO_2 as a weak acid generates several changes to seawater chemistry and the effects of ocean acidification are also expected to interact with contaminants of environmental concern, such as illicit drugs in the coastal zones. The mussels play essential ecological roles and constitute commercially important marine products, consequently, studies regarding the effects of pH

combined with drugs and its by products on these organisms are of major relevance. Thus, the aim of the present work was to evaluate the impacts of pH decrease and crack/cocaine exposure, acting alone and in combination, on the marine mussel *Perna perna* through biomarker responses (Lysosomal membrane stability, lipid peroxidation and DNA strand-breaks). The organisms were exposed to different crack-cocaine concentrations (0.5; 5 and 50 µg L^{-1}) combined with different pH values (8.3; 8.0; 7.5; 7.0; 6.5 and 6.0) for 96 hours. Crack-cocaine in the different acidification scenarios triggered cyto-genotoxicity, which affected the overall health of mussels exposed to cocaine environmentally relevant concentration. This study produced the first data on biomarker responses associated with CO_2-induced acidification and illicit drugs (crack-cocaine) in marine organisms.

Keywords: Illicit drug; ocean acidification; marine mussel; CO_2 enrichment.

1. Introduction

Global climate and environmental changes caused by continuously excessive carbon dioxide (CO_2) emission have become a topic of concern worldwide. The increase to approximately 400 ppm of CO_2 now in the atmosphere is one of the factors contributing to this rapid change (Cvijanovic and Caldeira, 2015). Due to its solubility, CO_2 is absorbed by the ocean. Once dissolved in seawater, CO_2 as a weak acid generates a number of changes to seawater chemistry and subsequently carbonate ion concentration and calcium carbonate saturation (DelValls, 2007).

Under the IPCC emission scenarios (IPCC, 2014a) average surface ocean pH could decrease by 0.3–0.4 pH units from the pre-industrial values by the end of this century.

Factors such as the decrease in pH value, the sediment buffering capacity and the transport and dispersions processes can have negative effects on marine organisms, including reduction of calcification rates, changes in metabolism functioning and increase of oxidative stress (Beesley et al., 2008; M.C. Passarelli et al., 2018; Szalaj et al., 2017; Wang et al., 2016). Besides to the direct effects on organisms' physiology, climate change impacts are also expected to influence the behavior of chemical contaminants in aquatic systems (Schiedek et al., 2007). Thereby, warming and acidification may alter the way that organisms interact with contaminants present in the environment and in their potential to accumulate them. Previous studies revealed changes in contaminants accumulation, like metals, in bivalve species under warming and acidification (Braga et al., 2018; Rodríguez-Romero et al., 2014)

Thus, in addition to ocean acidification, there is an increasing concern about large amounts of contaminants of emerging concern (CECs) entering aquatic ecosystem, which includes pharmaceuticals, steroid hormones and illicit drugs present in domestic wastewater released into coastal ecosystems (Bijlsma et al.. 2016; Edwards et al.. 2017; Skees et al.. 2018; Zuccato and Castiglioni. 2009).

As world population grows and concentrates in coastal areas, the number of marine ecosystems that become exposed to the influence of both treated and untreated wastewater discharges increases. Sewage inputs may compromise the environmental quality of receiving waters as thousands of different chemicals are releasing by this way. Among these chemicals, there

has been a growing interest over the last decade on illicit drugs (Klosterhaus et al. 2013; Borova et al., 2014; Pereira et al., 2016).

According to United Nations Office on Drugs and Crime (2017) an estimated quarter of a billion people, or around 5 per cent of the global adult population, used drugs at least once in 2015, matching the consumption levels of pharmaceuticals used to therapeutic purposes (Santos et al., 2010). This fact leads the scientific community to focus in the possible adverse effects of illicit drugs on non-target organisms (Binelli et al., 2012; Capaldo et al., 2018; Maranho et al., 2017; Parolini et al., 2016, 2013), since studies have shown the presence of illicit drugs not only in sewage treatment plants but also in receiving surface waters, sediment and tissues (Huerta-Fontela et al., 2008b; Moslah et al., 2017; Pereira et al., 2016; Zuccato et al., 2008a).

Pereira et al. (2016) assessed the occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone, and the results showed that the amount of cocaine was similar to that founded for caffeine (12.5-537 ng/L and 84.4-648 ng/L respectively). Based on this previous data, Maranho et al. (2017) were the first group to assess the effects of an illicit drug (crack/cocaine) to marine biota, highlighting the importance to study of this compound on the aquatic fauna which, till now, was unrecognized. To the best of our knowledge, as world population grows and concentrates in coastal areas, the number of marine ecosystems that become exposed to the influence of both treated and untreated wastewater discharges increases. Sewage inputs may compromise the environmental quality of receiving waters as thousands of different chemicals are releasing by this way. Among these chemicals, there has been a growing interest over the last decade on illicit drugs there is no data in the literature that contemplates effect of illicit drug in the marine environment ally to decrease in pH values (ocean acidification), since de variation of H^+ ions could affect the dynamic and toxicity of these compounds.

To assess the impacts caused in aquatic organisms, by natural and anthropogenic environmental changes, biomarkers can provide an indication of the sub-lethal impacts of stressors, as well as the underlying biochemical mechanisms, and an "early warning" of possible population impacts (Maranho et al., 2012; Martín-Díaz et al., 2007). The neutral red retention assay (NRRA) is applied for the measurement of lysosomal membrane stability as a global health status indicator (Moore et al., 2006) and has been employed as a tier-1 approach in large-scale biomonitoring programs. Lipid peroxidation and DNA damages have been recognized as reliable biomarkers of cyto-genotoxicity and understood as pre-pathological alterations in tissues of mussels (Danellakis et al., 2011).

Mussels play essential functional roles in coastal ecosystems and constitute commercially important marine products. Consequently, studies regarding the effects of pH combined with cocaine and its by products on these organisms are of major relevance. Thus, the aim of the present work was to evaluate the impacts of pH decrease and crack/cocaine exposure, acting alone and in combination, on the marine mussel *Perna perna*. For this purpose, it was assessed the lysosome membrane stability in haemocytes DNA damage (stand breaks) and lipid peroxidation in gills haemocytes of *Perna perna* mussels after short-term exposure to environmental relevant concentrations of cocaine in different scenarios of ocean acidification.

2. Materials and Methods

2.1 Experimental set-up

A CO_2 injection system (Figure 1) was adapted from the experimental set up described by De Orte et al., (2014), patent process n^{o}: P201200753, Cadiz University, Faculty of Marine and

Environmental Sciences, Physical Chemistry Department (RNM 375) to simulate the acidification process in a CCS leakage.

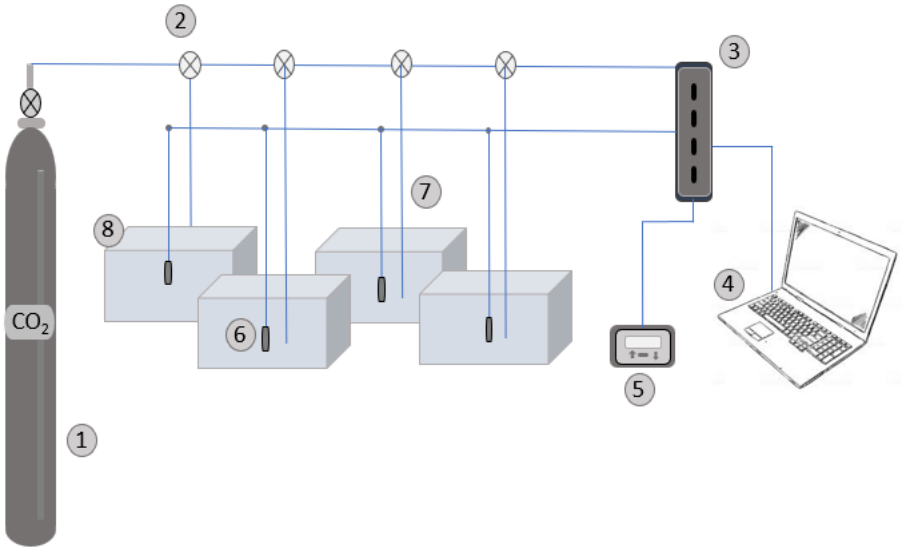

Figure 1. CO_2 injection system (1. CO_2 gas bottle. 2. Solenoid valves for the electronic regulation of the CO_2 injection 3. Power strips and USB connectors. 4. Laptop with software (Aquamedic 8.0). 5. AT-Control system. 6. pH interface to connect the pH sensor to the AT- Control System. 7. CO_2 injection hose. 8. Aquariums). Adapted from De Orte et al. (2014)

The experiments were realized in duplicate, where two 20 liters aquariums for each crack/cocaine concentration (0.5. 5.0 and 50 µg L^{-1}) plus the control (n = 20 per treatment) were arranged. Six pH values were tested, being 8.3 the control (natural pH, no CO_2 added), 8.0. 7.5. 7.0. 6.5 and 6.0. The experiment was developed with natural sea water from Enseada Beach in Guarujá, SP/Brazil. A pH control, as well as the crack-cocaine concentration tested, were fulfilled with this natural sea water (pH 8.3 - natural pH from the collected area), in this way was possible to assay the crack-cocaine effect to marine mussel itself.

The different pH values (8.0; 7.5; 7.0; 6.5 and 6.0) were adjusted and controlled by the Aqua Medic AT control hardware. Electrodes (NBS scale) were placed in each aquarium and connected to the computer system. When the pH value was 0.01 higher than the previously value selected, a solenoid valve was open injecting CO_2 in the system; when the target pH value was reached the valves were closed, preventing the entry of the gas in the system. CO_2 gas is injected through a silicon hose which connects the solenoid valve with the aquariums; the gas is provided by CO_2 bottles (Air Liquid).

The tested solutions were changed every 24 h to maintain the initial crack-cocaine concentration and the quality of the water, avoiding the organisms to stay in touch with its excreta. The laboratory conditions were controlled: temperature at 20 ± 2 °C, photoperiod 12:12 h, salinity 35 ppt and dissolved oxygen 8.0 mg/L. After 48 and 96 h of exposure, ten mussels from each pH value had the hemolymph extracted and used to assess the lysosomal membrane stability through neutral red retention time (NRRT). After hemolymph extracted the organisms were dissected and the gills were frozen at − 80 °C. The mussels were acquired from an aquaculture farm located within a reference area (Cocanha Beach, Caraguatatuba, Brazil). The animals were acclimated to the laboratory conditions for 3 days before the experiments started.

2.2. *Chemical characterization:*

Total alkalinity (TA) was measured by a potentiometric titration system (Metrohm 794 Basic Titrino) with a combined glass electrode (Metrohm, ref. 6.0210.100) calibrated for total pH scale using hydrochloric acid (HCl) 0.1M. The volume of each sample was 50mL and they were analyzed in triplicate. Dissolved inorganic carbon (DIC) was determined by the experimental values of TA and pH values, considering the constants of dissociation which were described by

(Mehrbach et al., 1973) and modified by Dickson and Millero (1987) for the total scale of pH. TA and pH were also used to determine the seawater carbonate system speciation such as bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), carbon dioxide (CO_2), calcite saturation index (Ω Cal), aragonite saturation index (Ω Arag) and partial pressure of carbon dioxide (pCO_2) by CO_2SYS program (Pierrot et al., 2006)

2.3. Neutral red retention time assay (NRRT):

Lysosomes are central to many key biological processes, including reproduction, digestion and immune response; thus, any deleterious changes to their function can have serious consequences for those processes. The general health status of the mussels was therefore considered in terms of changes to the lysosomal system of the hemolymph cells (Moore et al., 2006; Beesley et al., 2008). Neutral red retention time assay (NNRT) is based on the principle that only lysosomes in healthy cells take up and retain the vital dye neutral red. Lysosomal membrane damage caused by the impact of xenobiotics can decrease the NRRT by inducing the leaking of lysosomal components (Dailianis et al., 2003). NRRT assay was carried out following the method described by Lowe and Pipe (1994)

For the NNRT assay 0.5 ml hemolymph was extracted from the adductor muscle utilizing a hypodermic syringe containing 0.5 ml of physiological solution. The material collected was transferred to 2 ml eppendorf tubes. It was pipetted 40 uL of hemolymph solution on each treated slide previously with poly-L-lysine, which was incubated in dark and humid chamber for 15 minutes. After this period, 40 uL of neutral red solution was added to hemolymph present in each slide, and the set was incubated for another 15 minutes. The slides were observed in a light

microscope (400x) each 15 minutes until 50% or more of the total number of cells on the slide reach a stress symptom.

2.4. Lipid Peroxidation and DNA strand breaks

The mussel's gills were homogenized with a buffer solution containing Tris-HCl (50 Mm), EDTA (1 mM), dithiothreitol (DTT) (1 mM), sucrose (50 mM), KCl (150 mM), and phenylmethylsulfonyl fluoride (PMSF) (100 mM). DNA strand breaks and lipid peroxidation (LPO) were determined in the homogenized samples. All the biomarker responses were normalized by the total protein content determined in the homogenized following the Bradford methodology (Bradford, 1976), using bovine serum for the calibration curve.

Oxidative damage was determined by LPO according to Wills (1987). The samples reactions with thiobarbituric acid (TBARS) were determined by fluorescence at 530-590 nm filters. Results were expressed as μM TBARS mg^{-1} min^{-1} protein. Mitochondrial DNA damage was evaluated according to Olive (1988) alkaline precipitation assay. Salmon sperm DNA and Hoescht solution were used to calibrate the standard curve. DNA damage was measured by fluorescence at 360-450 nm. Results were expressed as μg DNA strands mg^{-1} proteins.

2.5. *Data treatment (Statistical analysis)*

Assumptions of a normal distribution and equal variance among groups of data collected from the experiment were confirmed by means of the Shapiro-Wilk's test and the Levene's F-test, respectively. One-way analysis of variance (One-way ANOVA) followed by the Dunn's test were used to identify treatments significantly different from the controls. The differences were

calculated comparing the results from the different values of pH (protons) with the control (natural seawater, pH 8.3) and were classified according to their statistical significance, as follows: *($p < 0.05$); **($p < 0.01$). ANOVA and post-hoc analysis were performed using Statistical software SPSS 15.0 for Windows.

Contamination and toxicity data were analyzed by factor analysis, by using principal components analysis as the ex- traction procedure, which is a multivariate statistical technique to explore variable distributions. The original data set used in the analysis included five pH values (8.0; 7.5; 7.0; 6.5 and 6.0); tree crack-cocaine concentrations (0.5. 5.0 and 50 µg L^{-1}) and the data from carbonate system speciation, which totalize 52 variables. Factor analysis was performed on the correlation matrix, that is, the variables were auto scaled (standardized) to be treated with equal importance. The principal component analysis (PCA) performed with the STATISTICA software, version 13.2.

3. Results and Discussion

3.1.Chemical Analysis

The mean values for the carbonate system speciation in the different treatments used in this study are shown in Table 1. The total inorganic carbon (TIC) shows to increase with the pH reduction, which is explained by the chemical equilibrium in the carbonate system in sea-water inducing a change in pCO_2 (McNeil and Sasse, 2016). Saturation index for calcite (ΩCal) and aragonite (Ωara) decreased as pH decreased and pCO_2 increased with lower pH. Aragonite and calcite are the two common $CaCO_3$ mineral forms and are generally precipitated in the ocean in highly saturated microenvironments created by marine organisms (Caldeira and Wickett, 2005)

Aragonite and calcite has an important role in shell calcification of mollusks implying that reduction of the carbonate saturation state below a threshold value will lead to large decreases in calcification rates, even when saturation is >1 (Feely et al., 2004). Wang et al. (2018) showed *R. philippinarum* shell carbonate decreased from 73% to 55% with reduced seawater pH from 8.1 to 7.7. Evidence in support of this assumption also comes from the observations of decreased Na^+/HCO_3^- transporter and HCO_3^-/Cl^- exchanger activities in the outer mantle epithelium when subjected to reduced seawater pH (Alves and Oliveira, 2013).

Table 1. Carbonate system speciation in assays exposed to the different pH treatments.

pH Treatment	Concentration ($\mu g.L^{-1}$)	TA ($\mu mol.L^{-1}$)	TIC ($\mu mol.kg^{-1}$)	HCO_3^- ($\mu mol.kg^{-1}$)	CO_3^{2+} ($\mu mol.kg^{-1}$)	CO_2 ($\mu mol.kg^{-1}$)	pCO_2 (μatm)	Ωcalc	Ωarag
8	Control	1663	1633	1549	42.8	40.8	1346	1	0.7
	0.5	1838	1796	1704	51.8	40.4	1310	1.2	0.8
	5	1951	1976	1867	32.8	75.9	2422	0.8	0.5
	50	1853	1903	1787	25.4	90.7	2935	0.6	0.4
7.5	Control	1673	1731	1600	27.4	103.4	3394	0.7	0.4
	0.5	1799	1840	1712	33.2	95	3139	0.8	0.5
	5	1849	1927	1789	23.3	115	3721	0.6	0.4
	50	1794	1895	1745	18.9	131	4281	0.5	0.3
7	Control	1683	1829	1651	11.9	166	5442	0.3	0.2
	0.5	1759	1885	1721	14.5	150	4968	0.4	0.2
	5	1747	1879	1711	13.8	154	5020	0.3	0.2
	50	1736	1887	1703	12.4	171	5626	0.3	0.2
6.5	Control	2428	3913	2409	7.3	1496.6	51288	0.2	0.1
	0.5	2457	3860	2436	8.6	1416	48672	0.2	0.1
	5	2740	3949	2717	9.2	1223	42089	0.2	0.1
	50	2695	4323	2675	7.8	1640	56223	0.2	0.1
6	Control	3172	5997	3167	2.7	2827.1	97133	0.1	0
	0.5	3156	5835	3150	2.8	2682	92375	0.1	0
	5	3732	6019	3722	4.5	2292	79157	0.1	0.1
	50	3653	6759	3647	3.2	3109	106819	0.1	0.1

The aliquot of crack cocaine analyzed by LC-MS/MS contained 37.99% of cocaine. It means a nominal concentration of cocaine of 0.2; 2.0 and 20 μg L^{-1} for each treatment, respectively. It was not possible to measure the real concentrations of cocaine in the exposure vessels. However, as reported by van Nuijs et al. (2009) to wastewater and by Maranho et al. (2017) to marine water, a low decrease in crack-cocaine (CC) concentrations during the assays is expected.

3.2.Biological responses

The percentage of mussel mortality for each treatment assay is shown in Table 2. In general, the mortality increases as pH values decrease. pH values upper to 7.5 presented no mortality, while pH values under 7.0 presented mortality after 96 hours. The results concur with those presented by Freitas et al. (2015) and Souza et al. (2019), where bivalves (*Scrobicularia plana* and *Perna perna*, respectively) showed a mortality of 20% at the pH value of 7.1 after 96h.

Table 2. *Perna perna* mortality percentage after 48- and 96-hours exposure to 5 different pH levels and three different crack-cocaine concentration.

pH Treatment	Concentration (µg.L^{-1})	Mortality (%)	
		48 hours	96 hours
8	Control	0	0
	0.5	0	0
	5	0	0
	50	0	0
7.5	Control	0	0
	0.5	0	0
	5	0	0
	50	0	0
7	Control	0	0
	0.5	0	0
	5	0	20
	50	0	0
6.5	Control	10	0
	0.5	0	0
	5	0	10
	50	30	20
6	Control	0	10
	0.5	0	10
	5	0	10
	50	0	0

This study produced the first data on the effects associated with the combination of CO_2-induced acidification and different concentrations of an illicit drug (crack-cocaine) in adults marine organism.

The results of the NRRT assay for *Perna perna* involving acidified sea water and CC exposure are shown in Figure 2. The range in NRRT values observed for lysosomes from control mussels (n=10; no CO_2 added) at time zero, 48 h and 96 h of assay were 81 min ± 26; 93 min ± 23 and 77 min ± 24 respectively. In the first 48 h exposure the lysosomal membrane stability (LMS) showed a significant decrease in retention time response at 0.5; 5 and 50 µg L^{-1} of CC in pH 8.0;

7.5; 7.0; 6.5 and 6.0. At pH values 7.0; 6.5 and 6.0 a significant decrease can be observed in the control samples (low pH and no CC added), where the effects are associated with the increase in concentrations of H^+. After 96 h exposure the NRRT significant decreased in organisms exposed to 0.5; 5 and 50 µg L^{-1} of CC in pH 8.3; 8.0; 7.0; 6.5 and 6.0.sample. pH 7.5 did not present statistically difference to control sample after 96 h.

Ortega et al. (2018) indicated that cytotoxicity evaluated through NRRT significantly decreased after 96 and 168 h since 0.5 µg L^{-1} of crack-cocaine, while Maranho et al. (2017) showed adverse effects (cyto-genotoxicity) in the order of 5 µg L^{-1} to 500 µg L^{-1} of crack cocaine, both assessing health of the brown mussel *Perna perna*. Similar results were found by Binelli et al. (2012) for *D. polymorpha* exposed to cocaine concentrations up to 220 ng/L. Parolini et al. (2013) also showed that 96 h of exposure to 1 µg L^{-1} of benzoylecgonine (the main human metabolite of cocaine) affected the lysosome membrane stability of *D. polymorpha* haemocytes. The results suggest risks to marine environments, since 0.537 µg L^{-1} of cocaine was found in surface seawater from Santos Bay, Brazil (Pereira et al., 2016).

Studies have shown a decrease in NRRT in different organisms affecting by ocean acidification from pH 7.0 in different bivalves (Beesley et al., 2008; Bibby et al., 2008; Nardi et al., 2018, 2017; M.C. Passarelli et al., 2018). As lysosomes play an important role in the defense system, by storing hydrolytic enzymes involved in intra cellular degradation, a reduction in lysosome health would contribute to the disruption of cellular pathways and increase in membrane fragility due to acidified seawater.

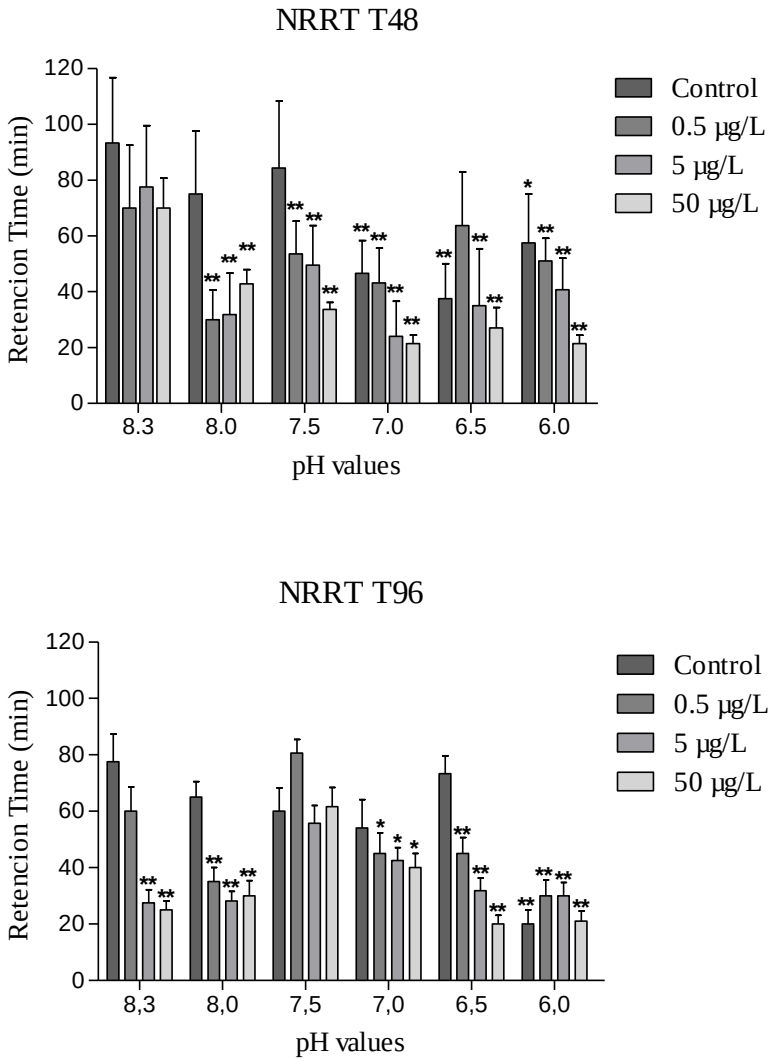

Figure 2. Mean and standard deviation of lysosomal membrane stability (LMS) assessed through neutral red retention time (NRRT) assay in the hemocytes of the mussel *P. perna* exposed to different crack-cocaine and pH treatments for 96 hours. *reflects values that differed significantly from the control ($p < 0.05$). **reflects values that differed significantly from the control ($p < 0.01$).

The LPO as well as the DNA damage in the gills' tissues are shown in Fig. 3. Marine organisms, such as bivalves, may undergo different alterations, such as oxidative stress, caused by anthropic and environmental pressures (Livingstone, 2001). Regarding to oxidative stress, its

incidence is relative to the inhibition of antioxidant enzymes (such as SOD, CAT and COX) bound to reactive oxygen species (ROS) produced in organism tissues (Soldatov et al., 2007). In bivalves, increased ROS levels may result in either the induction or inhibition of antioxidant enzymes. The reduction in antioxidant defenses may result in damage to the cell membrane, lipids, DNA and proteins. The measurement of antioxidant enzyme activities and the evaluation of potential damages can be helpful to reveal oxidative stress induced by deleterious conditions, such as seawater acidification and the exposure to environmental contaminants (Munari et al., 2018).

Lipid peroxidation (LPO) differed significantly in mussels exposed to crack-cocaine in this study, decreasing in all exposure concentrations (0.5; 5 and 50 µg L^{-1}) at pH 7.0 after 48 hours and increasing in 5 µg L^{-1} of crack-cocaine at pH 6.0 in 96 hours exposure. Parolini et al. (2013) showed increase in LPO levels in tissues of *D. polymorpha* exposed to 1 µg/L of benzoylecgonine and Ortega et al. (2018) results for Lipid peroxidation (LPO) did not differ significantly in mussels exposed to crack-cocaine. Moreira et al. (2018) observed increase of LPO levels in oysters (*C. gigas*) exposed to pH 7.4 and Freitas et al. (2016) reported significantly higher LPO for polychaetes (*D. neapolitana*) exposed to pH 7.5 than organisms exposed to pH 7.3 and 7.1.

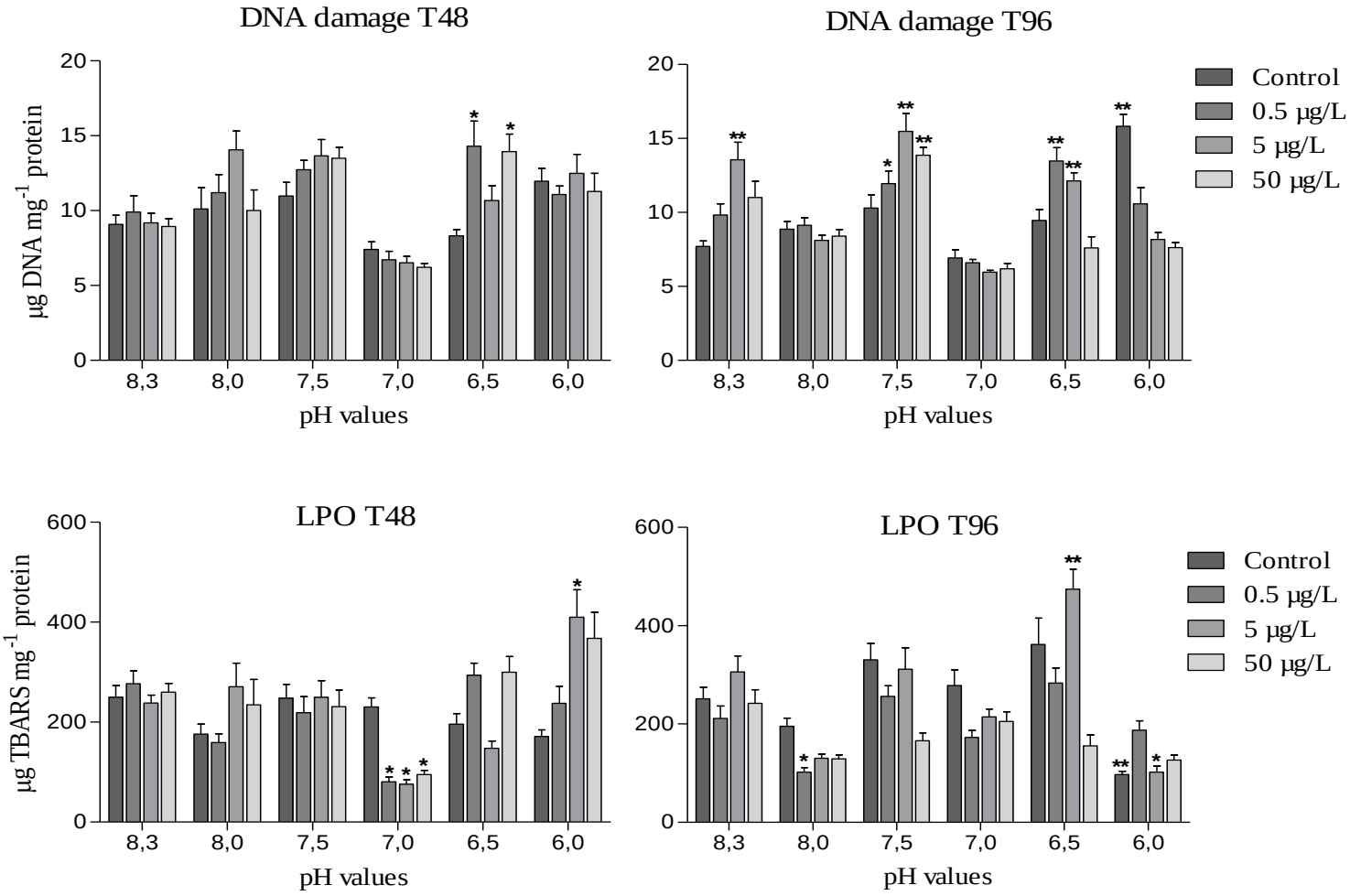

Figure 3. Mean and standard deviation of DNA damage (stand break) and Lipid peroxidation (LPO) assessed in *P. perna* mussels exposed to different crack-cocaine and pH treatments for 96 hours. *reflects values that differed significantly from the control ($p < 0.05$). **reflects values that differed significantly from the control ($p < 0.01$).

DNA damage was observed in gills after 48 h of exposure in 0.5 and 50 µg L^{-1} crack ($p < 0.05$) at pH 6.5. In 96 h significant differences were found in the concentration of 5 µg L^{-1} at pH 8.3; 0.5, 5 and 50 µg L^{-1} at pH 7.5; 0.5 and 5 µg L^{-1} at pH 6.5 which demonstrates the compound's

potential to cause primary genetic damage; at pH 6.0 the effect it is associated with increase in proton concentration. Our results corroborate previous studies describing the primary and fixed effects in DNA of invertebrates and vertebrates exposed to cocaine and metabolites. Maranho et al. (2017) observed an increase in DNA damage in the digestive glands of *P. perna* after 48 h of exposure to 500 μg L^{-1} of crack. Binelli et al. (2012) and Parolini et al. (2013) observed an increase in micronucleated cells and DNA damage (comet assay) in *D. polymorpha* exposed to benzoylecgonine and cocaine, respectively. Parolini et al. (2017) observed increased DNA damage in the cells of zebrafish (*D. rerio*) embryos exposed to cocaine and its metabolites (benzoylecgonine and ecgonine methyl ester). Yujra et al. (2016) and Moretti et al. (2016) presented studies where rat were exposed to concentrations of crack-cocaine (0.45, 9, and 18 mg kg^{-1}), however only the study of Moretti et al. (2016) presented a significant increase of micronucleated cells in the bone marrow in at the highest concentration and genetic damage in the liver cells and peripheral blood of rats exposed to 9 and 18 mg kg^{-1} of crack. Conradi et al. (2016) showed that an increase in acidification of seawater caused an increase in DNA damage in digestive gland tissues, of *Scrobicularia plana*, following a significant acidification-response correlation in pH 7.0 and 6.5.

3.3.Principal components analysis (PCA)

A multivariate analysis approach was applied to all data to discriminate the cause (CO_2-induced acidification, Crack-cocaine concentrations and the combination of both) responsible for the variance of biological effects detected in mussels (Table 3). The application of PCA to the original 52 variables indicates that they can be grouped in two new factors.

Table 3. Sorted rotated factor loadings of 52 variables for the two principal factors.

Variables		Components	
		Factor 1	**Factor 2**
NRRT 48h	Control	---	0.7
	0.5	0.3	0.57
	5	---	0.89
	50	---	0.91
NRRT 96h	Control	-0.53	0.71
	0.5	---	0.6
	5	---	---
	50	-0.45	---
DNA 48h	Control	0.59	---
	0.5	0.67	0.28
	5	0.43	---
	50	0.7	---
DNA 96h	Control	0.83	---
	0.5	0.75	0.32
	5	---	0.79
	50	---	0.74
LPO 48h	Control	-0.46	0.54
	0.5	0.77	0.58
	5	0.65	
	50	0.95	
LPO 96h	Control	0.78	-0.34
	0.5	0.45	0.25
	5	---	0.38
	50	-0.48	0.49
Mortality 48h	Control	---	-0.79
	0.5	---	-0.79
	5	---	-0.79
	50	0.4	---
Mortality 96h	Control	0.69	-0.42
	0.5	0.87	-0.38
	5	-0.32	-0.76
	50	0.4	-0.06
TA	Control	0.88	-0.44
	0.5	0.86	-0.48
	5	0.87	-0.45
	50	0.87	-0.46
DIC	Control	0.82	-0.5
	0.5	0.85	-0.49
	5	0.87	-0.46
	50	0.87	-0.44
Ω cal	Control	-0.33	0.81
	0.5	-0.34	0.87
	5	-0.5	0.8
	50	-0.53	0.8
Ω arag	Control	-0.33	0.81
	0.5	-0.34	0.87
	5	-0.5	0.8
	50	-0.64	0.29
pCO_2	Control	0.79	-0.48
	0.5	0.86	-0.47
	5	0.86	-0.46
	50	0.86	-0.46

The study indicates that the original variables can be grouped in two new factors: the first factor (F1) could be defined as the effects associated with the CO_2-induced acidification. It accounts for a 48.66% of the total variance and showed the relationship among DNA damage, LPO, mortality and pH reduction variables. This factor could be related with the effects produced by an increase in pCO_2 on several adverse effects defined by genotoxicity (DNA damage), oxidative stress (LPO), besides mortality variables. The second factor (F2) is related to the effects associated with the concentrations of crack-cocaine in the individuals of *Perna perna* used in the study and groups variables of adverse effects (biomarkers and mortality) with concentrations of crack-cocaine in different assays used in this work. Factor two accounts for a 18.95% of total variance and shows relationship among NRRT, DNA damage, LPO, crack-cocaine concentrations and calcium carbonate minerals (Ω cal and Ω arag).

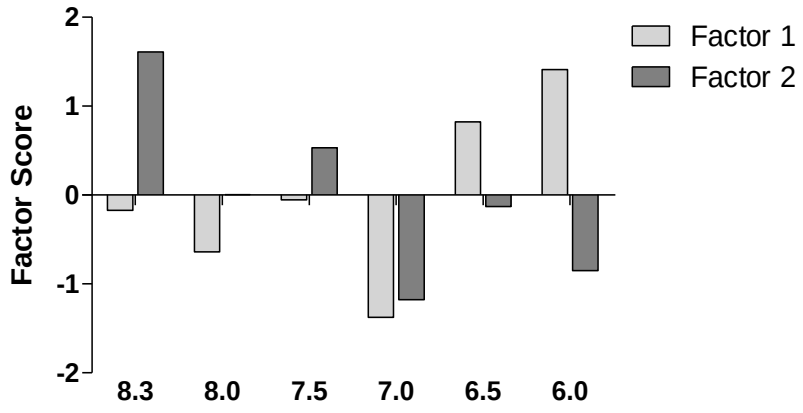

Figure 4. Estimated factors score in relation to the different pH treatments used in the study.

The figure 4 shows the score of F1 as positive at pH values of 6.5 and negative at lower pH, having an uncertainty area between 7 and 6.5 that confirms the definition of this factor previously reported. Factor 2 is positive at higher pH (7.5 and 8.0) in which the effects measured are

significantly associated with the concentration of crack-cocaine and not with the acidification. Thus, at higher pH the effects are directly related to the contaminant concentration (CC), whereas at lower pH values (6.5 and 6.0) are more related to the acidification than to the concentration of CC. At pH 7 it is shown that both causes are responsible of the effects measured, which could be considered as a combination between the acidification and the concentration of crack-cocaine.

4. Conclusions

Based on the results obtained it has been demonstrated that organisms exposed to lower pH values (pH <6.5) showed adverse effects (lethal and sublethal) related to the increase in the concentration of protons (CO_2-induced acidification). However, when the organisms were exposed to the pH higher than 7.5 (8.3; 8.0 and 7.5) it was showed that the measured adverse effects, including lethal response (mortality), and sublethal responses (biomarkers of exposure, biomarkers of effects, etc.) were directly related to the concentration of crack-cocaine that was used in the different bioassays used in this study. At pH values between 6.5 and 7.5 (pH 7.0) the adverse effects (lethal and sublethal) measured in the mussels *Perna perna* were related to the combination of both stressors, proton concentration from the CO_2-induced acidification and with the different crack-cocaine concentrations used in the experiments described here.

This is the first work that used both combinations of stressors proton concentration from CO_2-induced acidification and combination of concentration of Crack-Cocaine to address the biological effects in adult mollusks such as the mussels used in this study. The results obtained in this study will be useful for future works in relation to both effects associated with the acidification and the illicit drug concentrations using aquatic organisms.

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References

Alves, M.G., Oliveira, P.F., 2013. Effects of non-steroidal estrogen diethylstilbestrol on pH and ion transport in the mantle epithelium of a bivalve *Anodonta cygnea*. Ecotoxicol. Environ. Saf. 97, 230–235. https://doi.org/10.1016/j.ecoenv.2013.07.024

Beesley, A., Lowe, D.M., Pascoe, C.K., Widdicombe, S., 2008. Effects of CO_2-induced seawater acidification on the health of *Mytilus edulis*. Clim. Res. 37, 215–225. https://doi.org/10.3354/cr00765

Bibby, R., Widdicombe, S., Parry, H., Spicer, J., Pipe, R., 2008. Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. Aquat. Biol. 2, 67–74. https://doi.org/10.3354/ab00037

Bijlsma, L., Botero-Coy, A.M., Rincón, R.J., Peñuela, G.A., Hernández, F., 2016. Estimation of illicit drug use in the main cities of Colombia by means of urban wastewater analysis. Sci. Total Environ. 565, 984–993. https://doi.org/10.1016/j.scitotenv.2016.05.078

Binelli, A., Pedriali, A., Riva, C., Parolini, M., 2012. Illicit drugs as new environmental pollutants: Cyto-genotoxic effects of cocaine on the biological model *Dreissena polymorpha*. Chemosphere 86, 906–911. https://doi.org/10.1016/j.chemosphere.2011.10.056

Borova, V.L., Maragou, N.C., Gago-Ferrero, P., Pistos, C., Thomaidis, N.S., 2014. Highly sensitive determination of 68 psychoactive pharmaceuticals, illicit drugs, and related human metabolites in wastewater by liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 406, 4273–4285. https://doi.org/10.1007/s00216-014-7819-3

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

Braga, A.C., Camacho, C., Marques, A., Gago-Martínez, A., Pacheco, M., Costa, P.R., 2018. Combined effects of warming and acidification on accumulation and elimination dynamics of paralytic shellfish toxins in mussels *Mytilus galloprovincialis*. Environ. Res. 164, 647–654. https://doi.org/10.1016/j.envres.2018.03.045

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J. Geophys. Res. 110, C09S04. https://doi.org/10.1029/2004JC002671

Capaldo, A., Gay, F., Lepretti, M., Paolella, G., Martucciello, S., Lionetti, L., Caputo, I., Laforgia, V., 2018. Effects of environmental cocaine concentrations on the skeletal muscle of the European eel (*Anguilla anguilla*). Sci. Total Environ. 640–641, 862–873. https://doi.org/10.1016/j.scitotenv.2018.05.357

Conradi, M., Riba, I., Almagro-Pastor, V., DelValls, T.A., 2016. Lethal and sublethal responses in the clam *Scrobicularia plana* exposed to different CO_2-acidic sediments. Environ. Res. 151, 642–652. https://doi.org/10.1016/j.envres.2016.08.032

Cvijanovic, I., Caldeira, K., 2015. Atmospheric impacts of sea ice decline in CO_2 induced global warming. Clim Dyn 44, 1173–1186. https://doi.org/10.1007/s00382-015-2489-1

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M., Dimitriadis, V.K., 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Mar. Environ. Res. 56, 443–470. https://doi.org/10.1016/S0141-1136(03)00005-9

Danellakis, D., Ntaikou, I., Kornaros, M., Dailianis, S., 2011. Olive oil mill wastewater toxicity in the marine environment: Alterations of stress indices in tissues of mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 101, 358–366. https://doi.org/10.1016/j.aquatox.2010.11.015

de Orte, M.R., Lombardi, A.T., Sarmiento, A.M., Basallote, M.D., Rodriguez-Romero, A., Riba, I., DelValls, T.A., 2014a. Metal mobility and toxicity to microalgae associated with acidification of sediments: CO_2 and acid comparison. Mar. Environ. Res. 96, 136–144. https://doi.org/10.1016/j.marenvres.2013.10.003

de Orte, M.R., Sarmiento, A.M., DelValls, T.A., Riba, I., 2014b. Simulation of the potential effects of CO_2 leakage from carbon capture and storage activities on the mobilization and speciation of metals. Mar. Pollut. Bull. 86, 59–67. https://doi.org/10.1016/j.marpolbul.2014.07.042

DelValls, T.A., 2007. Diseño y aplicación de modelos integrados de evaluación de la contaminación y sus efectos sobre los sistemas marinos y litorales y la salud humana. Ministerio de la Presidencia. Cent. para la Prevención y Lucha contra la Contam. Maritíma y Litoral.

Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res. Part A, Oceanogr. Res. Pap. 34, 1733–1743. https://doi.org/10.1016/0198-0149(87)90021-5

Edwards, Q.A., Kulikov, S.M., Garner-O'Neale, L.D., Metcalfe, C.D., Sultana, T., 2017. Contaminants of emerging concern in surface waters in Barbados, West Indies. Environ. Monit. Assess. 189, 1–6. https://doi.org/10.1007/s10661-017-6341-4

Feely, R. a, Sabine, C.L., Lee, K., Berelson, W., Kleypas, J., Fabry, V.J., Millero, F.J., Anonymous, 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science (280). 305, 362–366. https://doi.org/10.1126/science.1097329

Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2015. How life history influences the responses of the clam *Scrobicularia plana* to the combined impacts of carbamazepine and pH decrease. Environ. Pollut. 202, 205–214. https://doi.org/10.1016/j.envpol.2015.03.023

Freitas, R., Pires, A., Velez, C., Almeida, Â., Moreira, A., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2016. Effects of seawater acidification *on Diopatra neapolitana* (Polychaete, Onuphidae): Biochemical and regenerative capacity responses. Ecol. Indic. 60, 152–161. https://doi.org/10.1016/j.ecolind.2015.06.032

Grujić, S., Vasiljević, T., Lausević, M., 2009. Determination of multiple pharmaceutical classes in surface and ground waters by liquid chromatography-ion trap-tandem mass spectrometry. J. Chromatogr. A 1216, 4989–5000. https://doi.org/10.1016/j.chroma.2009.04.059

Huerta-Fontela, M., Galceran, M.T., Ventura, F., 2008. Stimulatory drugs of abuse in surface waters and their removal in a conventional drinking water treatment plant. Environ. Sci. Technol. 42, 6809–6816. https://doi.org/10.1021/es800768h

IPCC, 2014. Summary for Policymakers, Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https://doi.org/10.1017/CBO9781107415324

Lara-Martín, P. a., González-Mazo, E., Petrovic, M., Barceló, D., Brownawell, B.J., 2014. Occurrence, distribution and partitioning of nonionic surfactants and pharmaceuticals in the urbanized Long Island Sound Estuary (NY). Mar. Pollut. Bull. 1–10. https://doi.org/10.1016/j.marpolbul.2014.01.022

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666. https://doi.org/10.1016/S0025-326X(01)00060-1

Lowe, D.M., Pipe, R.K., 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an in vitro study. Aquat. Toxicol. https://doi.org/10.1016/0166-445X(94)00045-X

Maranho, L.A., Fontes, M.K., Kamimura, A.S.S., Nobre, C.R., Moreno, B.B., Pusceddu, F.H., Cortez, F.S., Lebre, D.T., Marques, J.R., Abessa, D.M.S., Ribeiro, D.A., Pereira, C.D.S., 2017. Exposure to crack cocaine causes adverse effects on marine mussels *Perna perna*. Mar. Pollut. Bull. 0–1. https://doi.org/10.1016/j.marpolbul.2017.08.043

Maranho, L.A., Pereira, C.D.S., Choueri, R.B., Cesar, A., Gusso-Choueri, P.K., Torres, R.J., Abessa, D.M.D.S., Morais, R.D., Mozeto, A.A., Delvalls, T.A., Martín-Díaz, M.L., 2012. The application of biochemical responses to assess environmental quality of tropical estuaries: Field surveys. J. Environ. Monit. 14, 2608–2615. https://doi.org/10.1039/c2em30465a

Martín-Díaz, M.L., Blasco, J., Sales, D., Delvalls, T. a., 2007. Biomarkers study for sediment quality assessment in spanish ports using the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*. Arch. Environ. Contam. Toxicol. 53, 66–76. https://doi.org/10.1007/s00244-006-0121-4

McNeil, B.I., Sasse, T.P., 2016. Future ocean hypercapnia driven by anthropogenic amplification of the natural CO_2 cycle. Nature 529, 383–386. https://doi.org/10.1038/nature16156

Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicx, R.M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897–907. https://doi.org/10.4319/lo.1973.18.6.0897

Moore, M.N., Icarus Allen, J., McVeigh, A., 2006. Environmental prognostics: An integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. 61, 278–304. https://doi.org/10.1016/j.marenvres.2005.10.005

Moreira, A., Figueira, E., Pecora, I.L., Soares, A.M.V.M., Freitas, R., 2018. Native and exotic oysters in Brazil: Comparative tolerance to hypercapnia. Environ. Res. 161, 202–211. https://doi.org/10.1016/j.envres.2017.10.035

Moretti, E.G., Yujra, V.Q., Claudio, S.R., Silva, M.J.D., Vilegas, W., Pereira, C.D.S., de Oliveira, F., Ribeiro, D.A., 2016. Acute crack cocaine exposure induces genetic damage in multiple organs of rats. Environ. Sci. Pollut. Res. 23, 8104–8112. https://doi.org/10.1007/s11356-016-6141-3

Moslah, B., Hapeshi, E., Jrad, A., Fatta-Kassinos, D., Hedhili, A., 2017. Pharmaceuticals and illicit drugs in wastewater samples in north-eastern Tunisia. Environ. Sci. Pollut. Res. 1–16. https://doi.org/10.1007/s11356-017-8902-z

Munari, M., Matozzo, V., Gagné, F., Chemello, G., Riedl, V., Finos, L., Pastore, P., Badocco, D., Marin, M.G., 2018. Does exposure to reduced pH and diclofenac induce oxidative stress in marine bivalves? A comparative study with the mussel *Mytilus galloprovincialis* and the clam *Ruditapes philippinarum*. Environ. Pollut. 240, 925–937. https://doi.org/10.1016/j.envpol.2018.05.005

Nardi, A., Benedetti, M., Fattorini, D., Regoli, F., 2018. Oxidative and interactive challenge of cadmium and ocean acidification on the smooth scallop *Flexopecten glaber*. Aquat. Toxicol. 196, 53–60. https://doi.org/10.1016/j.aquatox.2018.01.008

Nardi, A., Mincarelli, L.F., Benedetti, M., Fattorini, D., d'Errico, G., Regoli, F., 2017. Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*. Chemosphere 169, 493–502. https://doi.org/10.1016/j.chemosphere.2016.11.093

Olive, P.L., 1988. DNA precipitation assay: A rapid and simple method for detecting DNA damage in mammalian cells. Environ. Mol. Mutagen. 11, 487–495. https://doi.org/10.1002/em.2850110409

Ortega, A. dos S.B., Maranho, L.A., Nobre, C.R., Moreno, B.B., Guimarães, R.S., Lebre, D.T., de Souza Abessa, D.M., Ribeiro, D.A., Pereira, C.D.S., 2018. Detoxification, oxidative stress, and cytogenotoxicity of crack cocaine in the brown mussel *Perna perna*. Environ. Sci. Pollut. Res. 1–10. https://doi.org/10.1007/s11356-018-1600-7

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

Parolini, M., Magni, S., Castiglioni, S., Binelli, A., 2016. Genotoxic effects induced by the exposure to an environmental mixture of illicit drugs to the zebra mussel. Ecotoxicol. Environ. Saf. 132, 26–30. https://doi.org/10.1016/j.ecoenv.2016.05.022

Parolini, M., Pedriali, A., Riva, C., Binelli, A., 2013. Sub-lethal effects caused by the cocaine metabolite benzoylecgonine to the freshwater mussel *Dreissena polymorpha*. Sci. Total Environ. 444, 43–50. https://doi.org/10.1016/j.scitotenv.2012.11.076

Passarelli, M.C., Riba, I., Cesar, A., DelValls, T.A., 2018. What is the best endpoint for assessing environmental risk associated with acidification caused by CO_2 enrichment using mussels? Mar. Pollut. Bull. 128, 379–389. https://doi.org/10.1016/j.marpolbul.2018.01.055

Pereira, C.D.S., Maranho, L.A., Cortez, F.S., Pusceddu, F.H., Santos, A.R., Ribeiro, D.A., Cesar, A., Guimarães, L.L., 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Sci. Total Environ. 548–549, 148–154. https://doi.org/10.1016/j.scitotenv.2016.01.051

Pierrot, D., Lewis, E., Wallace, D.W.R., 2006. MS Excel Program Developed for CO_2 System Calculation. Carbon Dioxide Information Anal. Center, Oak Ridge Natl. Lab. U.S. Dep. Energy. ORNL/CDIAC, 105.

Rodríguez-Romero, A., Jiménez-Tenorio, N., Basallote, M.D., Orte, M.R. De, Blasco, J., Riba, I., 2014. Predicting the impacts of CO_2 leakage from sub-seabed storage: Effects of metal accumulation and toxicity on the model benthic organism *Ruditapes philippinarum*. Environ. Sci. Technol. 48, 12292–12301. https://doi.org/10.1021/es501939c

Santos, L.H.M.L.M., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard. Mater. 175, 45–95. https://doi.org/10.1016/j.jhazmat.2009.10.100

Schiedek, D., Sundelin, B., Readman, J.W., Macdonald, R.W., 2007. Interactions between climate change and contaminants. Mar. Pollut. Bull. 54, 1845–1856. https://doi.org/10.1016/j.marpolbul.2007.09.020

Skees, A.J., Foppe, K.S., Loganathan, B., Subedi, B., 2018. Contamination profiles, mass loadings, and sewage epidemiology of neuropsychiatric and illicit drugs in wastewater and river waters from a community in the Midwestern United States. Sci. Total Environ. 631–632, 1457–1464. https://doi.org/10.1016/j.scitotenv.2018.03.060

Soldatov, A.A., Gostyukhina, O.L., Golovina, I. V., 2007. Antioxidant enzyme complex of tissues of the bivalve *Mytilus galloprovincialis* Lam. under normal and oxidative-stress conditions: A review. Appl. Biochem. Microbiol. 43, 556–562. https://doi.org/10.1134/s0003683807050092

Szalaj, D., De Orte, M.R., Goulding, T.A., Medeiros, I.D., DelValls, T.A., Cesar, A., 2017. The effects of ocean acidification and a carbon dioxide capture and storage leak on the early life stages of the marine mussel *Perna perna* (Linneaus, 1758) and metal bioavailability. Environ. Sci. Pollut. Res. 24, 765–781. https://doi.org/10.1007/s11356-016-7863-y

United Nations Office on Drugs and Crime, 2017. Executive summary. Conclusion and policy implications of the world drug report 2017, World Drug Report 2017.

van Nuijs, A.L.N., Pecceu, B., Theunis, L., Dubois, N., Charlier, C., Jorens, P.G., Bervoets, L., Blust, R., Neels, H., Covaci, A., 2009. Spatial and temporal variations in the occurrence of cocaine and benzoylecgonine in waste and surface water from Belgium and removal during

wastewater treatment. Water Res. 43, 1341–1349. https://doi.org/10.1016/j.watres.2008.12.020

Wang, Li, Zhao, L., Yang, G., Wang, Lianshun, Lu, Y., Cong, Y., 2018. Deciphering carbon sources of mussel shell carbonate under experimental ocean acidification and warming. Mar. Environ. Res. 142, 141–146. https://doi.org/10.1016/j.marenvres.2018.10.007

Wang, X., Wang, M., Jia, Z., Wang, H., Jiang, S., Chen, H., Wang, L., Song, L., 2016. Ocean acidification stimulates alkali signal pathway: A bicarbonate sensing soluble adenylyl cyclase from oyster *Crassostrea gigas* mediates physiological changes induced by CO_2 exposure. Aquat. Toxicol. 181, 124–135. https://doi.org/10.1016/j.aquatox.2016.11.002

Wills, E.D., 1987. Evaluation of lipid peroxidation in lipids and biological membranes., in: Biochemical Toxicology: A Practical Approach. pp. 127–150.

Yujra, V.Q., Moretti, E.G., Claudio, S.R., Silva, M.J.D., Oliveira, F. de, Oshima, C.T.F., Ribeiro, D.A., 2016. Genotoxicity and mutagenicity induced by acute crack cocaine exposure in mice. Drug Chem. Toxicol. 39, 388–391. https://doi.org/10.3109/01480545.2015.1126843

Zuccato, E., Castiglioni, S., Bagnati, R., Chiabrando, C., Grassi, P., Fanelli, R., 2008. Illicit drugs, a novel group of environmental contaminants. Water Res. 42, 961–968. https://doi.org/10.1016/j.watres.2007.09.010

Chapter V. Combined effects of an illicit drug and proton concentrations: An integrative approach

The potential impact of CO_2 enrichment in the marine environment, and the possible bioavailability variation in the toxicity of cocaine its byproducts, were studied in the present Thesis. The objective is to design the risk characterization associated with crack-cocaine (CC) in marine ecosystems combined with ocean acidification (enrichment of CO_2 and then increase concentration of protons in the marine environment). Many are the factors that must to be evaluated in an environmental risk assessment study such as: environmental contamination, the toxic effects of the contaminants to the biota and human health, potential reactions of changes in the natural conditions and others.

In this sense, different approaches and lines of evidence have been considered in this Thesis that were assessed in separated and jointed ways. The factors, or lines, selected to design this environment risk characterization related to CO_2 enrichment were showed in table 1, and include effects on mollusk and sea urchin at different levels of the life cycle.

The hypothesis was that acidification, associated with enrichment of CO_2 in the marine environment, will provoke a variation in the bioavailability and toxicity of cocaine and its byproducts. The use of different lines of evidence can significantly improve the environment risk characterization in marine environments exposed to this scenario (CC + CO_2). The main goal of this Thesis was to assess the adverse effects of CC in marine ecosystems combined with ocean acidification by assessing biological responses on non-target marine organisms. ***This thesis is the first study*** on illicit drugs (cocaine and crack cocaine) ecotoxicity related to different acidification scenarios associated with enrichment of CO_2 focusing specifically in effects, to draw attention to these emerging contaminants in future scenarios of ocean acidification. The main task after the application and evaluation of all these techniques will be further than the evaluation of the effects

associated with the acidification and the contamination by CC but to distinguish it and to address the effects observed as produced by two different stressors, CO_2 and/or CC.

Table 1. Summarized description of the different toxicity tests and organisms applied in this thesis. *LMS means lysosomal membrane stability.

Organism	Endpoint	Duration	Type (nature)
Mussel	Mortality	10 days	Lethal
Perna perna	D-phase	48h	Sub lethal
	Fertilization rate	1h	Sub lethal
	LMS*	0, 48 and 96h	Sub lethal
	DNA damage	0, 48 and 96h	Sub lethal
	LPO	0, 48 and 96h	Sub lethal
Sea Urchin	Pluteus stage	42h	Sub lethal
Echinometra lucunter	Fertilization rate	1h	Sub lethal

The development of the methodology proposed in this Thesis has obtained different results by means of applying two main lines of evidence: The concentration of stressors (CO_2, HCl, H^+ and CC) and the toxicity of them itself and in combination with the others using different organisms and toxicity tests. The first approaches, shown in chapter II, were based on a general review and showed that there is few information related to the biological adverse effect of acidification by CO_2 enrichment. Furthermore, there is no previous work addressing the observed effects of combination of the mentioned stressors. Thus, in the mentioned chapter were included the first tests conducted using different sources of protons (HCl and CO_2) together with different concentration of CC using larvae of mussels. The results showed the first evidence of toxic effects that, later, will be used to establish the effects of the different stressors.

The aim of the first tests performed in this thesis, was to confirm the results obtained in the previous assays using mussel, besides, to address the effect of the combination of acidification and CC concentrations using different organisms (sea urchin and mussels). For this, two different experiments were applied, fertilization rate and embryo larval assays, with a marine mussel (*Perna perna*) and sea urchin (*Echinometra lucunter*). The results, presented in the chapter III of this Thesis, put in evidence that the gametes of *E. lucunter* and *P. perna* are affected by acidification when exposed to realistic pH reductions until the end of this century, and exposure to bioactive compounds as illicit drugs could be more toxic in such conditions.

Table 2. Values of EC50 for Crack-Cocaine derived at the different concentrations of CC for both methodologies of acidification (HCl and CO_2). Values of EC50 at pH 8.5 show the results for the control without acidification method.

	pH values	EC50 ($mg.L^{-1}$)	
		HCl	CO_2
Perna perna	8.5	14.0 (12.6 - 15.3)	8.8 (8.6 - 9.0)
	8	13.8 (12.5 - 14.6)	8.7 (8.4 - 8.9)
	7.5	9.3 (4.6 - 16.1)	3.9 (3.7 - 4.1)
	7	-	-
Echinometra lucunter	8.5	58.8 (52.5 - 63.3)	58.8 (52.5 - 63.3)
	8	10.6 (10.3 - 10.8)	23.2 (22.4 - 24.3)
	7.5	11.5 (10.8 - 13.0)	12.5 (10.6 - 14.9)
	7	-	-

Once, the evaluation of adverse effects on the early life stages of the different organisms was conducted, new assays were designed, but at this time, using adults of marine organisms. It was the first time that a study was carried out based on the effects of proton concentration on adults of

Perna perna mussel. In these tests, the endpoints measured were beyond the mortality data, with more sensible endpoints selected to address the adverse effects of the protons on the organisms, through responses of different biomarker systems such as: lysosomal membrane stability, lipid peroxidation and primary damages in DNA.

The stress produced by increasing protons concentration could be noticed in the enzymatic systems of the mussels (NRRT and LPO) in pH values lower than 7.5, being accentuated when the value of the concentration of protons is higher, which produces toxic effects related to pH 6.5 and 6.0.

Once the baseline of proton effects was designed, a new and more complex tests were applied using the same organisms. In this case, not only protons were assessed as stressors, but also the combination with different concentrations of CC. The endpoints selected included the biomarkers previously mentioned (NRRT, LPO and DNA damage), allowing the comparison between the effects from CO_2 enrichment and CC. The results presented, at higher pH the effects are directly related to the contaminant concentration (CC), whereas at lower pH values (6.5 and 6.0) are more related to the acidification than to the concentration of CC. At pH 7.0 it is shown that both causes are responsible of the effects measured, which could be considered as a combination between the acidification and CC increasing concentration. In summary, the results obtained showed that uptake and sub-lethal effects of CC can vary in a tissue-specific manner when combined with acidification. Lysossomal membrane stability of haemocytes showed the first signs of effects, followed by DNA and lipid oxidative damages in gills triggered by acidified conditions and after combination with the drug.

Finally, aiming to address the effects of the combination of the all different stressors on the organisms used, and besides, to distinguish the effect observed related to each of the stressors, it was developed an integrated, and more precise, interpretation of the risks associated with CO_2 enrichment in the marine environment: the multivariate analysis tools. Specifically, the factor analysis method, with extraction of the variable, selected to link all the results obtained in this thesis was the Principal Component Analysis (PCA). Using this multivariate analysis is possible to understand the correlation between the biological effects measured in laboratory associated with the protons and CC.

To conduct the analysis of all the variables observed and obtained in this thesis different new variables or factors were calculated. These factors were rotated using the varimax normalized procedure. Also, the factor scores for each treatment and assay were calculated (factor scores), following protocols outlined (Del Valls and Chapman, 1998; Morales-Caselles et al., 2008; Riba et al., 2004) and using the STATISTICA® software package (version 13.2). The principal factors were extracted and the eigenvalues above 1.0 considered. Variables having loadings ≥0.40 to a factor were considered associated to the respective factor, following Tabachnick and Fidell (1996)

In this Chapter V a multivariate analysis approach was applied to all data to discriminate the cause (CO_2-induced acidification, Crack-cocaine concentrations and the combination of both) responsible for the variance of biological effects detected in two non-target organisms (sea urchin and mussels). The application of PCA to the original variables indicates that they can be grouped in two new factors (table 2).

Table 2. Sorted rotated factor loadings of 100 variables for the two main factors that includes toxicological effects in early life stages and adults of sea urchin and mussel, lethal and sublethal endpoints. Also, chemical concentrations of the CO_2 system in the aquatic environments used in the experiments are considered as variable in the principal component analysis.

Variables		Components	
		Factor 1	**Factor 2**
NRRT 48h	Control	0.89	--
	0.5	--	--
	5	0.77	--
	50	0.91	0.38
NRRT 96h	Control	0.46	0.64
	0.5	--	0.7
	5	-0.68	0.58
	50	-0.48	0.8
DNA 48h	Control	--	--
	0.5	--	--
	5	--	--
	50	--	--
DNA 96h	Control	--	-0.64
	0.5	--	--
	5	--	0.34
	50	--	0.61
LPO 48h	Control	--	0.66
	0.5	0.47	-0.39
	5	--	-0.35
	50	0.32	-0.66
LPO 96h	Control	-0.45	-0.64
	0.5	--	--
	5	--	--
	50	0.4	0.37
Mortality 48h	Control	-0.87	--
	0.5	-0.87	--
	5	-0.87	--
	50	--	-0.43
Mortality 96h	Control	--	-0.73
	0.5	--	-0.92
	5	-0.51	-0.2
	50	--	-0.43
Sea Urchin (embrio. dev. CO_2)	Control	0.59	0.72
	6.25	0.5	0.76
	12.5	0.79	0.53
	25	0.87	--

	50	0.87	--
	100	0.87	--
Sea Urchin (embrio. dev. HCl)	Control	0.5	0.76
	6.25	0.6	0.69
	12.5	0.67	0.65
	25	0.84	0.51
	50	0.87	--
	100	0.87	--
Sea Urchin (fert. rate CO_2)	Control	0.5	0.79
	6.25	0.56	0.75
	12.5	0.53	0.76
	25	0.54	0.75
	50	0.56	0.73
	100	0.55	0.74
Sea Urchin (fert. Rate HCl)	Control	0.48	0.8
	6.25	0.52	0.77
	12.5	0.61	0.72
	25	0.51	0.76
	50	0.58	0.72
	100	0.57	0.72
Mussel (embrio. dev. CO_2)	Control	0.76	0.56
	6.25	0.85	0.38
	12.5	0.87	--
	25	0.87	--
	50	0.87	--
	100	0.87	--
Mussel (embrio. dev. HCl)	Control	0.7	0.64
	6.25	0.78	0.54
	12.5	0.82	0.45
	25	0.8	0.5
	50	0.87	--
	100	0.87	--
Mussel (fert. rate CO2)	Control	0.87	--
	6.25	0.81	--
	12.5	0.84	--
	25	0.81	--
	50	0.76	-0.45
	100	0.8	-0.34
Mussel (fert. Rate HCl)	Control	0.58	-0.78
	6.25	0.65	-0.72
	12.5	0.64	-0.75
	25	0.58	-0.63
	50	0.53	-0.68
	100	0.47	-0.84
TA	Control	--	-0.92

	0.5	--	-0.9
	5	--	-0.9
	50	--	-0.91
DIC	Control	--	-0.89
	0.5	--	-0.91
	5	--	-0.92
	50	--	-0.92
Ω cal	Control	0.88	0.46
	0.5	0.86	0.49
	5	0.71	0.69
	50	0.71	0.7
Ω arag	Control	0.88	0.46
	0.5	0.86	0.49
	5	0.72	0.69
	50	--	0.83
pCO_2	Control	--	-0.86
	0.5	--	-0.92
	5	--	-0.92
	50	--	-0.92

The link among all the variables considered in this study could be grouped in two main factors: the first factor (F1) is defined with the effects associated with the effect of crack-cocaine in the organisms used in the study and explain more than 50% of the variance. F1 (50.08%) showed the relationship among NRRT, embryo larval development and the concentrations of CC. In this case higher the pH, higher the factor values that are associated to the cocaine concentration, predominating variables with positive weight, as NRRT and embryo larval development.

The first factor (F1) could be defined as the effects associated with the CO_2-induced acidification based on the correlations between the biological adverse effects and the concentration of protons. The second factor (F2) is defined as the effects related to the concentrations of Crack-cocaine in the individuals of *Perna perna* used in the study. To confirm these definitions and to

address the relationship between the effects, and the causes related to them, a representation of the score of these two factors is shown in figure 2.

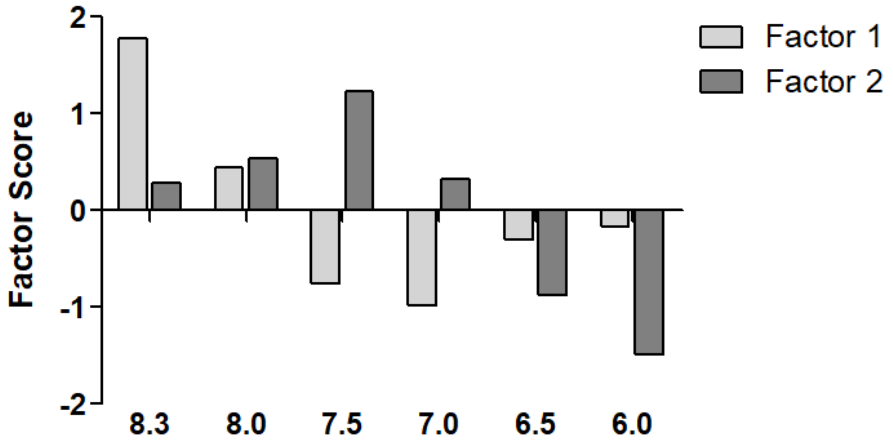

Figure 1. Estimated factors score in relation to the different pH treatments used in the study.

The figure 2 shows the score of F1 identifying relationship processes between variables and weight of the factors with positive / negative values. Thus, at higher pH values higher factor values occur, predominating positive weight variables, that is, NRRT and embryo larval development becomes greater when cocaine concentration increases. In this case, the toxicity it is associated with an increase in cocaine concentrations. On the other hand, there are variables explained by F1, with negative value, as mortality, NRRT (96 h) and slightly pCO_2 values, which is influenced by the acidification (being both HCl and CO_2). Therefore, it can be seen at pH values greater than 7.5 the influence on the results (due to the positive weight of the factor) is related to the toxicity produced by cocaine. Subsequently, from pH values lower than 7.5, the influence of the increase

in protons produced by acidification becomes more predominant and it is related to the toxicity of acidification and cocaine together.

The second factor (F2) could be defined as the effects associated with the CO_2-induced acidification. This factor has positive values for NRRT, DNA damage and LPO (48 h), and negative values for pCO_2, mortality, embryo larval development and LPO (96 h). In this sense, the positive values of this factor explain that there is a predominance of effect only by acidification (since occurs in pH values 7.0 and 6.5), being explained mainly in embryo larval development, LPO and mortality. On the other hand, higher pH values presented NRRT opposing the pCO_2 values, also pointing out the effect by acidification in this variable. In summary, it is a factor presenting toxic effect by acidification, that according to our results is significant in the space of the results studied from pH 7.

The new grouping of the total variables used in this study can be used to address the main conclusions of this work associated with the general and specific objectives proposed in this Thesis. These conclusions will be defined in the next chapter of this work.

Finally, it is expected a potential increase in the bioaccumulation/biomagnification of certain contaminants with the proton concentrations increase, however it is not clearly demonstrated, and additional efforts should be conducted in future studies to establish a potential risk evaluation, not only at ecosystem health level, but also at human health. Special efforts should be conducted considered substances able to suffer biomagnification (organic, emerging contaminants, etc.) at different acidification scenarios.

References

Del Valls, T.A., Chapman, P.M., 1998. Site-specific sediment quality values for the Gulf of Cadiz (Spain) and San Francisco Bay (USA), using the sediment quality triad and multivariate analysis. Ciencias Mar. 24, 313–336.

Morales-Caselles, C., Riba, I., Sarasquete, C., Ángel DelValls, T., 2008. Using a classical weight-of-evidence approach for 4-years' monitoring of the impact of an accidental oil spill on sediment quality. Environ. Int. 34, 514–523. https://doi.org/10.1016/j.envint.2007.11.007

Riba, I.L., Forja, J.M., Gómez-Parra, A., DelValls, T.A., 2004. Sediment quality in littoral regions of the Gulf of Cádiz: A triad approach to address the influence of mining activities. Environ. Pollut. 132, 341–353. https://doi.org/10.1016/j.envpol.2004.03.021

Tabachnick, G.B., Fidell, L.S., 1996. Using Multivariate Statistics. Harper Collins Publishers, New York.

Chapter VI - Conclusions

There are few and not enough studies addressing the influence of acidification in the behavior of organic contaminants and their bioavailability, including toxicity. The results here discussed addressed the increase in the toxicity of an organic compound when the acidification increases. The main conclusions obtained in this study are here summarized:

1) It has been determined the acute toxicity of crack-cocaine under different scenarios of acidification (pH values from 6.0 to 8.0) using different induced acidification methods (CO_2-induced and HCl-induced acidification) by means of fertilization rate and embryo-larval development of sea-urchin *Echinometra lucunter* and marine mussel *Perna perna*. The embryo-larval development was significantly more sensitive to acidified environment than fertilization rate in both cases. Regarding to the acidification methods used in this study to induce the different scenarios, the use of the acid HCl demonstrated higher toxicity than when used CO_2 for the same concentration of crack-cocaine and the same pH value. Besides the difference in the toxicity of the acidification methods, both showed to lead an increase in the toxicity of the crack-cocaine.

2) The lethal toxicity addressed by the endpoint mortality has been significantly measured in treatments with pH values lower than 6 for all the species and experiments used in this Thesis. The mortality shows a pattern similar to the acute endpoints used in the embryo-larval and fecundation tests for both stressors, proton concentration (acidification) and concentration of crack-cocaine, and for the combination of them.

4) A reduction in health status of adult mussels occurs when exposed to short periods at pH levels lower than 7.5. Lysossomal membrane stability of haemocytes showed the first signs of effects, followed by DNA and lipid oxidative damages in gills triggered by acidified conditions. These findings should be taken in account when environmental monitoring approaches are

performed in tropical marine areas receiving petroliferous or other activities employing Carbone dioxide capture and storage systems (CCS).

5) When exposes to an association between acidification by CO_2 and CC concentrations, the treatments with lower pH values (pH <6.5) associated adverse effects (lethal and sublethal) related to the proton's concentration (CO_2-induced acidification). However, at higher pH values (8.3; 8.0 and 7.5) the adverse effects were directly related to the concentration of crack-cocaine. These adverse effects were significantly determined using lysossomal membrane stability of haemocytes, DNA damage and lipid oxidation in *P. perna*.

6) At pH values of 7.0 the adverse biological effects (lethal and sublethal) measured in the mussels *P. perna* were related to the proton concentration associated with the CO_2-induced acidification besides the increase in the crack-cocaine concentrations used in the bioassays.

7) The experiments have shown negative effects mainly associated with high concentration of protons (lower pH values) and relatively high concentrations of crack-cocaine for all the organisms used in the different assays. This thesis demonstrate that acidification of coastal ecosystems will trigger enhanced adverse effects on marine organisms exposed to drugs.

8) To the best of our knowledge, this is the first study demonstrating toxicity of illicit drugs to marine organisms at different acidification scenarios induced by CO_2. These findings will become very important to expand the set of data about ecological risks of ocean acidification associated with bioactive compounds.

9) It has been successfully designed and applied an integrative method that uses multiple lines of evidence (LOE) under a weight of evidence (WOE) approach to characterize the risk associated with the combination of two different stressors such as the concentration of Crack-Cocaine and

the proton concentration related to the CO_2-induced acidification. It is the first study that has addressed this approach using different marine organisms. The integration of the different LOEs has allowed distinguishing between the effects associated with the CC and those related to proton concentration. Furthermore, it has characterized the effects and the combination of them.

10) These results under an integrative approach will provide validated information about adverse effects of an illicit drug (crack-cocaine) to support stakeholders and policymakers interested in finding solutions for marine ecosystems conservation.

Annexes

Annex A

(A i) Certificate of State in Other Institution (University of Algarve)

UAlg
UNIVERSIDADE DO ALGARVE
GABINETE DE RELAÇÕES INTERNACIONAIS E MOBILIDADE

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DECLARAÇÃO

Declara-se para os devidos efeitos, que **Lorena da Silva Souza**, portadora do passaporte Nº FS434067, natural do Brasil, efetuou um período de estudos de 9 meses, entre novembro de 2017 e junho de 2018, na Faculdade de Ciências e Tecnologia, ao abrigo do Programa *Erasmus Mundus*, Ação 1 - Doutoramento Erasmus Mundus em Gestão Marinha Costeira (MACOMA), na Faculdade de Ciências e Tecnologia.

DECLARATION

To whom it may concern, we hereby declare that **Lorena da Silva Souza**, bearer of the passport Nº FS434067, born in Brazil, carried out a study period of 9 months, started on November 2017 until June 2018, in the Faculty of Sciences and Technologies, under the Erasmus Mundus Programme Action 1 - Erasmus Mundus Joint Doctorate Programme in Marine and Coastal Management (MACOMA).

Faro, 02-09-2019

Dra. Dina Jesus

Annex A

(A ii) Certificate of State in Other Institution (University of Bologna)

Erasmus Mundus Joint Doctorate Marine and Coastal Management

Mobility Declaration

Name of Candidate (as on passport): **Lorena Da Silva Souza**

Hosting institution: University of Bologna – Campus de Ravenna

This is to confirm that **Lorena Da Silva Souza**, doctoral researcher of the Erasmus Mundus Joint Doctorate Marine and Coastal Management carried out research at University of Bologna, Campus of Ravenna for the following periods:

From	To
2018/26/06	2018/30/07

During her stay she interacted with the researchers of my laboratory, and with other PhD candidate (enrolled in Italian programs as well as in Macoma), and participated to some activities in the laboratory regarding biomarker responses to marine pollutants. She also gave a successful seminar on the research she is performing.

Signed 
Elena Fabbri Institutional Coordinator of EMJD MACOMA
Date 2018/30/07

Joint PhD in Marine and Coastal Management
Institutional Coordinator

To whom it may concern

I hereby attest that Lorena Da Silva Souza, PhD candidate enrolled in the 3rd year Joint PhD Course in Marine and Coastal Management, on July 16th 2018 presented the seminar entitled "Adverse effects of crack/cocaine to marine organisms affected by acidification conditions", topic of her studies.
The seminar was attended by researchers and Master students in Environmental Analysis and Management of the University of Bologna, Campus of Ravenna.

Best Regards

Yours Sincerely

July 18th, 2018

Prof. Elena Fabbri

Host Coordinator

Prof. Elena Fabbri
Università di Bologna, sede di Ravenna
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48123 Ravenna
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Annex B. Europass CV

europass Curriculum vitae

PERSONAL INFORMATION

Lorena da Silva Souza

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JOB APPLIED FOR

University Research

WORK EXPERIENCE

01/08/2009–01/12/2011 **trainee**
Laboratório de Ecotoxicologia Universidade Santa Cecília, Santos (Brazil)

1 Mar 2012–31 Aug 2012 **Supervisor of Groundwater Collection**
Planejar Ambiental, Sorocaba (Brazil)

1 May 2013–31 Aug 2013 **Supervisor of Groundwater Collection**
Planejar Ambiental, Sorocaba (Brazil)

EDUCATION AND TRAINING

1 Feb 2008–1 Dec 2011 **Bachelor in Marine Biology**
Santa Cecilia University, Santos (Brazil)

1 Sep 2013–31 Jul 2015 **Master in Water and Coastal Management**
University of Cadiz, Cadiz (Spain)

9 Nov 2015–9 Nov 2019 **PhD in Marine and Coastal Management**
University of Cadiz, Cadiz (Spain)

PERSONAL SKILLS

Mother tongue(s): Portuguese

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B2	B2	B2	B2	C1
Spanish	C2	C1	B2	B2	B1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills: Good communication skills gained through my experience in recent years within the university and lectures given.

Digital skills

SELF-ASSESSMENT

europass Curriculum vitae Lorena da Silva Souza

Information processing	Communication	Content creation	Safety	Problem-solving
Proficient user	Proficient user	Proficient user	Proficient user	Independent user

Digital skills - Self-assessment grid

Other skills: Open Water Diving - PADI

Driving licence: A, B

ADDITIONAL INFORMATION

Publications: da Silva Souza, L., Pusceddu, F.H., Cortez, F.S., de Orte, M.R., Seabra, A.A., Cesar, A., Ribeiro, D.A., Del Valls Casillas, T.A., Pereira, C.D.S., 2019. Harmful effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios. Chemosphere 236. https://doi.org/10.1016/j.chemosphere.2019.07.015

Publications: DelValls, Á., Souza, L. da S., Bonnail, E., de Seabra, A.A., Riba, I., Seabra Pereira, C.D., 2018. Integrative assessment of sediment quality in acidification scenarios associated with carbon capture and storage operations. Environ. Rev. er-2018-0084. https://doi.org/10.1139/er-2018-0084

Publications: Cortez, F.S., Souza, L. da S., Guimarães, L.L., Pusceddu, F.H., Maranho, L.A., Fontes, M.K., Moreno, B.B., Nobre, C.R., Abessa, D.M. de S., Cesar, A., Pereira, C.D.S., 2019. Marine contamination and cytogenotoxic effects of fluoxetine in the tropical brown mussel Perna perna. Mar. Pollut. Bull. 141, 366–372. https://doi.org/10.1016/j.marpolbul.2019.02.065

Cortez, F.S., Souza, L.D.S., Guimarães, L.L., Almeida, J.E., Pusceddu, F.H., Maranho, L.A., Mota, L.G., Nobre, C.R., Moreno, B.B., Abessa, D.M.D.S., Cesar, A., Santos, A.R., Pereira, C.D.S., 2018. Ecotoxicological effects of losartan on the brown mussel Perna perna and its occurrence in seawater from Santos Bay (Brazil). Sci. Total Environ. 637–638. https://doi.org/10.1016/j.scitotenv.2018.05.069

Conferences: International Symposium on Toxicity Assessment (ISTA 18) - Limeira/SP - Brazil

Conferences: 12th SETAC Latin America Biennial Meeting - Santos/SP - Brazil

Seminars: "Adverse effects of crack/cocaine to marine organisms affected by acidification conditions" - University of Bologna, Campus of Ravenna

Courses: Herramientas para la Evaluación de la Calidad Ambiental en Ecosistemas Litoraneos (100h).

Courses: Scientific Diving in Marine and Coastal Research. (Carga horária: 100h).
Universidad de Cádiz, UCA, Espanha.

Courses: Água de Lastro e seus Riscos Ambientais. (Carga horária: 9h).
Universidade Santa Cecília, UNISANTA, Brasil.

Courses: Técnica de Diagnóstico Não Invasivo. (Carga horária: 10h).
Universidade Santa Cecília, UNISANTA, Brasil.

Ecotoxicologia Aquática. (Carga horária: 9h).
Universidade Santa Cecília, UNISANTA, Brasil.

Courses	Biologia de Organismos Marinhos Venenosos e Peçonh. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
Courses	Psicologia Evolutiva. (Carga horária: 8h). Universidade Estadual de Campinas, UNICAMP, Brasil.
Courses	Recifes de Corais: Formação, Biologia, Preservação. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
Courses	Neurobiologia dos Sistemas Sensoriais. (Carga horária: 8h). Universidade Estadual de Campinas, UNICAMP, Brasil.
Courses	Bioindicadores e Biomarcadores de Impacto Ambienta. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
Courses	
Projects	Avaliação da Toxicidade do Hormônio Sintético 17a-Etinilestradiol Empregando-se o Mexilhão Marinho Perna perna (Linnaeus, 1758) Situação: Concluído; Natureza: Pesquisa. Integrantes: Lorena da Silva Souza - Coordenador / Fernando Sanzi Cortez - Integrante / Camilo Dias Seabra Pereira - Integrante. Financiador(es): Fundação de Amparo à Pesquisa do Estado de São Paulo - Bolsa.
Projects	Avaliação dos Efeitos Tóxicos do Fármaco Omeprazol Sobre Mexilhões Perna perna (LINNAEUS, 1758) Situação: Em andamento; Natureza: Pesquisa. Integrantes: Lorena da Silva Souza - Integrante / Fernando Sanzi Cortez - Integrante / Camilo Dias Seabra Pereira - Integrante / Arthur Juan Costa Mathias - Coordenador. Financiador(es): Fundação de Amparo à Pesquisa do Estado de São Paulo - Outra.
Seminars	Poluição aquática, ecotoxicologia e diagnóstico de qualidade ambiental. 2016. (Curso de curta duração ministrado).
Honours and awards	Menção honrosa, Universidade Federal de São Carlos, campi Sorocaba. Congresso Aberto aos Estudantes de Biologia. 2009
ATTACHMENTS	■ Currículo do Sistema de Currículos Lattes (Lorena da Silva Souza).pdf

Currículo do Sistema de Currículos Lattes (Lorena da Silva Souza).pdf

Lorena da Silva Souza

Endereço para acessar este CV: http://lattes.cnpq.br/7378492023216457
Última atualização do currículo em 23/07/2019

Bacharelada e Licenciada em Ciências Biológicas (Biologia Marinha) pela Universidade Santa Cecília - SP (2011). Trabalho FAPESP de Iniciação Científica em farmacologia, experimentos citotóxicos e fisiológicos (2011). Mestre em Gerenciamento Costeiro e Água (Water and Coastal Management ? Erasmus Mundus) no Departamento de Física e Química da Universidade de Cádiz ? Espanha (2015), onde hoje realiza seu Doutorado pelo programa ERASMUS MUNDUS em Gerenciamento Costeiro e Marinho (Marine and Coastal Management - MACOMA). Tem experiência na área de ecotoxicologia aquática, toxicidade de fármacos e bioacumulação. **(Texto informado pelo autor)**

Identificação

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Nome em citações bibliográficas SOUZA, L. S.;SOUZA, LORENA DA SILVA;DA SILVA SOUZA, LORENA

Endereço

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Formação acadêmica/titulação

2015
Doutorado em andamento em Water and Coastal Management (MACOMA).
Universidad de Cádiz, UCA, Espanha.
Título: Advers Effects of Crack-cocaine to Marine Organisms Affected by Acidification Conditions,
Orientador: Camilo Dias Seabra Pereira.
Coorientador: Tomas Angel del Valls.
Bolsista do(a): Erasmus Mundus, MACOMA, Espanha.
Palavras-chave: Emerging Contaminants; Ecotoxicity; Biomarkers.
Grande área: Outros
Grande Área: Ciências Biológicas / Área: Farmacologia / Subárea: Toxicologia.
Setores de atividade: Pesquisa e desenvolvimento científico.

2013 - 2015
Mestrado em Water and Coastal Management.
Universidad de Cádiz, UCA, Espanha.
Título: Bioaccumulation and Effects of Priority and Emerging Organic Contaminants in Ruditapes philippinarum,Ano de Obtenção: 2015.
Orientador: Pablo Antonio Lara Martin.
Coorientador: Maria Laura Martin Diaz.
Bolsista do(a): Erasmus Mundus Master Courses, EMMCS, Espanha.
Palavras-chave: Biomarkers; Bioaccumulation; Emerging Contaminants; Ecotoxicity.
Grande área: Ciências Biológicas
Grande Área: Ciências Biológicas / Área: Biologia Geral / Subárea: Biologia Marinha.
Setores de atividade: Pesquisa e desenvolvimento científico.

2008 - 2011
Graduação em Ciências Biológicas.
Universidade Santa Cecília, UNISANTA, Brasil.
Título: Efeitos de concentrações ambientais do hormônio sintético 17 Alfa-etinilestradiol na integridade da membrana lisossômica de mexilhões Perna perna (LINNAEUS, 1758).
Orientador: Camilo Dias Seabra Pereira.
Bolsista do(a): Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, Brasil.

Formação Complementar

2014 - 2014	Herramientas para la Evaluación de la Calidad Ambiental en Ecosistemas Lito. (Carga horária: 100h). Universidad de Cádiz, UCA, Espanha.
2014 - 2014	Scientific Diving in Marine and Coastal Research. (Carga horária: 100h). Universidad de Cádiz, UCA, Espanha.
2012 - 2013	Estudo da lingua Inglesa. (Carga horária: 460h). Orlando Language School, OLS, Estados Unidos.
2010 - 2010	Água de Lastro e seus Riscos Ambientais. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
2010 - 2010	Técnica de Diagnóstico Não Invasivo. (Carga horária: 10h). Universidade Santa Cecília, UNISANTA, Brasil.
2010 - 2010	Ecotoxicologia Aquática. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
2009 - 2009	Biologia de Organismos Marinhos Venenosos e Peçonh. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
2009 - 2009	Psicologia Evolutiva. (Carga horária: 8h). Universidade Estadual de Campinas, UNICAMP, Brasil.
2009 - 2009	Recifes de Corais: Formação, Biologia, Preservação. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
2009 - 2009	Neurobiologia dos Sistemas Sensoriais. (Carga horária: 8h). Universidade Estadual de Campinas, UNICAMP, Brasil.
2008 - 2008	Bioindicadores e Biomarcadores de Impacto Ambienta. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.
2008 - 2008	Conservação de Recifes de Coral. (Carga horária: 9h). Universidade Santa Cecília, UNISANTA, Brasil.

Atuação Profissional

Planejar Ambiental, PLANEJAR, Brasil.

Vínculo institucional

2012 - 2012	Vínculo: Bióloga, Enquadramento Funcional: Surpervisora de Coleta, Carga horária: 45, Regime: Dedicação exclusiva.
Outras informações	Supervisora de coleta de águas subterraneas, e acompanhamento na expedição de laudos ambientais.

Universidade Santa Cecília, UNISANTA, Brasil.

Vínculo institucional

2010 - 2011	Vínculo: estágiaria, Enquadramento Funcional: Laboratório de Ecotoxicologia, Carga horária: 20

Projetos de pesquisa

2011 - 2012	Avaliação da Toxicidade do Hormônio Sintético 17a-Etinilestradiol Empregando-se o Mexilhão Marinho Perna perna (Linnaeus, 1758) Situação: Concluído; Natureza: Pesquisa. Integrantes: Lorena da Silva Souza - Coordenador / Fernando Sanzi Cortez - Integrante / Camilo Dias Seabra Pereira - Integrante. Financiador(es): Fundação de Amparo à Pesquisa do Estado de São Paulo - Bolsa.
2011 - 2011	Avaliação dos Efeitos Tóxicos do Fármaco Omeprazol Sobre Mexilhões Perna perna (LINNAEUS, 1758) Situação: Em andamento; Natureza: Pesquisa. Integrantes: Lorena da Silva Souza - Integrante / Fernando Sanzi Cortez - Integrante / Camilo Dias Seabra Pereira - Integrante / Arthur Juan Costa Mathias - Coordenador. Financiador(es): Fundação de Amparo à Pesquisa do Estado de São Paulo - Outra.

Áreas de atuação

1.	Grande área: Ciências Biológicas / Área: Farmacologia / Subárea: Toxicologia.
2.	Grande área: Ciências Biológicas / Área: Biologia Geral / Subárea: Biologia Marinha.

Idiomas

Inglês	Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Português	Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Espanhol	Compreende Bem, Fala Bem, Lê Bem, Escreve Razoavelmente.

Prêmios e títulos

2011	Menção honrosa, Universidade Federal de São Carlos, campi Sorocaba.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. CORTEZ, FERNANDO SANZI ; **SOUZA, LORENA DA SILVA** ; GUIMARÃES, LUCIANA LOPES ; PUSCEDDU, FABIO HERMES ; MARANHO, LUCIANE ALVES ; FONTES, MAYANA KAROLINE ; MORENO, BEATRIZ BARBOSA ; NOBRE, CAIO RODRIGUES ; ABESSA, DENIS MOLEDO DE SOUZA ; CESAR, AUGUSTO ; PEREIRA, CAMILO DIAS SEABRA . Marine contamination and cytogenotoxic effects of fluoxetine in the tropical brown mussel Perna perna. MARINE POLLUTION BULLETIN JCR, v. 141, p. 366-372, 2019.
2. **DA SILVA SOUZA, LORENA**; PUSCEDDU, FABIO HERMES ; CORTEZ, FERNANDO SANZI ; DE ORTE, MANOELA ROMANO ; SEABRA, ALESSANDRA ALOISE ; CESAR, AUGUSTO ; RIBEIRO, DANIEL ARAKI ; DEL VALLS CASILLAS, TOMÁS ANGEL ; PEREIRA, CAMILO DIAS SEABRA . Harmful effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios. CHEMOSPHERE JCR, v. 236, p. 124284, 2019.
3. CORTEZ, FERNANDO SANZI ; **SOUZA, LORENA DA SILVA** ; GUIMARÃES, LUCIANA LOPES ; ALMEIDA, JOÃO EMANOEL ; PUSCEDDU, FABIO HERMES ; MARANHO, LUCIANE ALVES ; MOTA, LUCIANA GONÇALVES ; NOBRE, CAIO RODRIGUES ; MORENO, BEATRIZ BARBOSA ; ABESSA, DENIS MOLEDO DE SOUZA ; CESAR, AUGUSTO ; SANTOS, ALDO RAMOS ; PEREIRA, CAMILO DIAS SEABRA . Ecotoxicological effects of losartan on the brown mussel Perna perna and its occurrence in seawater from Santos Bay (Brazil). SCIENCE OF THE TOTAL ENVIRONMENT JCR, v. 637-638, p. 1363-1371, 2018.
4. DELVALLS, ÁNGEL ; **SOUZA, LORENA DA SILVA** ; DE SEABRA, ALESSANDRA ALOISE ; SEABRA PEREIRA, CAMILO DIAS ; BONNAIL, ESTEFANÍA ; RIBA, INMACULADA . Integrative assessment of sediment quality in acidification scenarios associated with carbon capture and storage operations. ENVIRONMENTAL REVIEWS JCR, v. 1, p. 1, 2018.

Apresentações de Trabalho

1. **SOUZA, L. S.**; PUSCEDDU, F. H. ; CORTEZ, F. S. ; DelValls, T. A. C. ; PEREIRA, C. D. S. . Effects on fertilization rate and embryolarval development of Perna perna mussels exposed to crack cocaine in different pHs. 2017. (Apresentação de Trabalho/Congresso).
2. **SOUZA, L. S.**; PUSCEDDU, F. H. ; CORTEZ, F. S. ; RIBEIRO, D. A. ; DelValls, T. A. C. ; PEREIRA, C. D. S. . Adverse effects of cocaine byproduct in the reproduction of sea urchin in an ocean acidification scenario. 2017. (Apresentação de Trabalho/Simpósio).
3. CORTEZ, F. S. ; ALEMIDA, J. E. ; PUSCEDDU, F. H. ; SANTOS, A. R. ; **SOUZA, L. S.** ; ROSA, J. L. ; NOBRE, C. R. ; MORENO, B. B. ; MARANHO, L. A. ; FONTES, M. K. ; CESAR, A. ; ABESSA, D. M. S. ; PEREIRA, C. D. S. . Efeitos sub-letais dos fármocos losartan, metformina e fluoxetina no molusco bivalve Perna perna (LINNAEUS, 1758). 2016. (Apresentação de Trabalho/Congresso).
4. MATHIAS, A. J. C. ; **SOUZA, L. S.** ; CORTEZ, F. S. ; PEREIRA, C. D. S. . Avaliação dos efeitos tóxicos do fármaco omeprazol sobre o mexilhão Perna perna (Linnaeus, 1758). 2012. (Apresentação de Trabalho/Congresso).
5. **SOUZA, L. S.**; MATHIAS, A. J. C. ; CORTEZ, F. S. ; PEREIRA, C. D. S. . Efeitos fisiológicos de concentrações ambientais do hormônio sintético 17-a etinilestradiol no mexilhão marinho Perna perna (Linnaeus, 1758). 2012. (Apresentação de Trabalho/Congresso).

6. **SOUZA, L. S.**; MATHIAS, A. J. C. ; CORTEZ, F. S. ; PEREIRA, C. D. S. . Efeitos de concentrações ambientais do hormônio sintético 17α-etinilestradiol na integridade da membrana lisossômica de mexilhões Perna perna (LINNAEUS, 1758). 2011. (Apresentação de Trabalho/Congresso).
7. **SOUZA, L. S.**; MATHIAS, A. J. C. ; CORTEZ, F. S. ; PEREIRA, C. D. S. . Avaliação da toxicidade do hormônio sintético 17a-etinilestradiol empregando-se o mexilhão marinho Perna perna (LINNAEUS, 1758). 2011. (Apresentação de Trabalho/Congresso).
8. MATHIAS, A. J. C. ; **SOUZA, L. S.** ; CORTEZ, F. S. ; PEREIRA, C. D. S. . Avaliação dos efeitos tóxicos do fármaco Omeprazol em mexilhão Perna perna (Linnaeus, 1758). 2011. (Apresentação de Trabalho/Congresso).

Demais tipos de produção técnica

1. **SOUZA, L. S.**. Adverse effects of crack/cocaine to marine organisms affected by acidification conditions. 2018. (Curso de curta duração ministrado/Outra).
2. **SOUZA, L. S.**. Poluição aquática, ecotoxicologia e diagnóstico de qualidade ambiental. 2016. (Curso de curta duração ministrado/Outra).

Eventos

Participação em eventos, congressos, exposições e feiras

1. 18th International Symposium on Toxicity Assessment.Adverse effects of cocaine byproduct in the reproduction of sea urchin in an ocean acidification scenario. 2017. (Simpósio).
2. Society of Environmental Toxicology and Chemistry. Effects on fertilization rate and embryolarval development of Perna perna mussels exposed to crack cocaine in different pHs. 2017. (Congresso).
3. XIV Congresso Brasileiro de Ecotoxicologia. Efeitos sub-letais dos fármocos losartan, metformina e fluoxetina no molusco bivalve Perna perna (LINNAEUS, 1758). 2016. (Congresso).
4. XVIII Simpósio de Biologia Marinha.Poluição aquática, ecotoxicologia e diagnóstico de qualidade ambiental. 2016. (Simpósio).
5. XII Congresso Brasileiro de Ecotoxicologia. Avaliação dos efeitos tóxicos do fármaco Omeprazol em mexilhão Perna perna (Linnaeus, 1758).. 2012. (Congresso).
6. XII Congresso Brasileiro de Ecotoxicologia. Efeitos fisiológicos de concentrações ambientais do hormônio sintético 17-a etinilestradiol no mexilhão marinho Perna perna (Linnaeus, 1758). 2012. (Congresso).
7. 11º Congresso Nacional de Iniciação Científica CONIC-SEMESP. Avaliação da toxicidade do hormônio sintético 17a-etinilestradiol empregando-se o mexilhão marinho Perna perna (LINNAEUS, 1758). 2011. (Congresso).
8. 13º Simposio de Biologia Marinha. 2010. (Simpósio).
9. 12º Simpósio de Biologia Marinha. 2009. (Simpósio).
10. Congresso Aberto aos Estudantes de Biologia. 2009. (Congresso).
11. Meeting Vida Marinha. 2009. (Encontro).
12. 11º Simpósio de Biologia Marinha. 2008. (Simpósio).

Orientações

Orientações e supervisões em andamento

Trabalho de conclusão de curso de graduação

1. Júlia Alves Luzzi. Efeitos adversos do crack na reprodução de mexilhões marinhos em diferentes cenários de acidificação oceânica. Início: 2018. Trabalho de Conclusão de Curso (Graduação em Ciências Biológicas) - Universidade Santa Cecília. (Orientador).

Educação e Popularização de C & T

Cursos de curta duração ministrados

1. **SOUZA, L. S.**. Adverse effects of crack/cocaine to marine organisms affected by acidification conditions. 2018. (Curso de curta duração ministrado/Outra).
2. **SOUZA, L. S.**. Poluição aquática, ecotoxicologia e diagnóstico de qualidade ambiental. 2016. (Curso de curta duração ministrado/Outra).

Página gerada pelo Sistema Currículo Lattes em 13/08/2019 às 16:49:11

Annex C

Passport Mobility

MACOMA PASSPORT OF RESEARCH MOBILITY

Joint Ph.D. Programme in Marine and Coastal Management

1. This MACOMA Passport of Research Mobility is awarded to

1.Surname: **da Silva Souza** 2. First name: **Lorena**
3.Address: **111, Calle Alejandro Sanchez. 28019. Madrid. Spain**
4.Date of birth: **20/01/1989** 5. Nationality: **Brazilian** 6. Signature of the holder:

3. The Partner Organization of the MACOMA Research Mobility Experience Nº 1.
First and Second Academic year 2015-17

13. Host partner:
University of Cadiz, Spain
1st Year: 120 ECTS

14. Name, type and address of host partner:
University of Cadiz, Spain
Address: Faculty of Marine and Environmental Sciences
Campus Puerto Real. 11519. Puerto Real. Cádiz. Spain

15. Surname(s) and first names of coordinator: **Mr Miguel Ángel Pendón Meléndez**
16.Title/position: **Vice-Rector of Planning and Postgraduate Studies**
17.Telephone: **+34 956016794**
18.e-mail: agua.mundus@uca.es,;

Duration of the MACOMA Research Mobility:
19. From: **01/10/2015** 20. To: **30/10/2017**

21. Score of the MACOMA Research Mobility (ECTS scale): **120 ECTS**
22. Additional requirement or conditions (special equipment, field work, observation data etc) or 22. Grade (please use ECTS scale: A –F):

23. Any comments from host partner:

24. Signature of the host coordinator: 24.a. Signature of the Ph.D. candidate's supervisor:

Mr Miguel Ángel Pendón Meléndez
25. Date of issue:

Explanatory note

MACOMA Passport of Research mobility is Ph.D. programme standard document, which records detail of the contents and the results – in terms of skills and competences or of academic achievements –of period that a Ph.D. student has spent in Institutions another that host University (other University, Enterprises, Research Center and others) for learning and research purpose. MACOMA Passport of Research Mobility designed on the base of ideas of EUROPASS facility.

3.b Description of the MACOMA Research Mobility experience Nº 2
Third Academic Year 2017-18. Research Period

26. Host partner: **University of Algarve**
27. Name, type and address of host partner:
University of Cadiz, Spain
Campus de Gambelas – 8005-139 Faro – Portugal

28. Surname(s) and first names of coordinator: **Dr. Alice Newton**
29.Title/position:
30.Telephone: +351 289 800 003
31.e-mail: international@ualg.pt
Duration of the MACOMA Research Mobility:
32. From: **01/11/2017**
33. To**: 26/06/2018**

34. Score of the MACOMA Research Mobility (ECTS scale): **40 ECTS**

35. Additional requirement or conditions (special equipment, field work, observation data etc.):
or 36. Grade (please use ECTS scale: A –F):

37. Any comments from host partner:

38. Signature of the host coordinator: 38.a. Signature of the Ph.D. candidate's supervisor:

Dr. Prof. Alice Newton **Camilo Dias Seabra Pereira**

39. Date of issue: 12th, September 2019

Objective(s) of the MACOMA Research Mobility:

To write thesis manuscript and prepare scientific papers for publication.

40. Activities/research carried out during MACOMA Research Mobility:
- Theoretical research
- Bibliography research
- Write article

41. Study visits (if possible, where):

42. Participation in Conferences, workshops, seminars without presentation (please indicate title, host institutions, duration):

43. Participation in Conferences, workshops, seminars with presentation(s) (please indicate title; host institutions; title of presentation(s); indicate: oral or poster; others):

44. Publications preparing during MACOMA Research Mobility (please indicate title; where and when publication is planning to be published; others):
(2019) Harmful effects of cocaine byproduct in the reproduction of sea urchin in different ocean acidification scenarios. Chemosphere 236 (2019) 124284.
https://doi.org/10.1016/j.chemosphere.2019.07.015

45. Other information:

3.c Description of the MACOMA Research Mobility experience Nº3
Third Academic Year 2016-17. Research Period

26. Host partner: **University of Bologna**
27. Name, type and address of host partner: **University of Bologna. Italy**
Address: **via Sant'Alberto 163, 48123 Ravenna, Italia**

28. Surname(s) and first names of coordinator: **Dr. Prof. Elena Fabbri**
29.Title/position: **Professore Ordinario di Fisiologia**
Presidente del Campus di Ravenna
Coordinator of the Erasmus Mundus Wacoma (2017-2022)
30.Telephone: +39 3346113833
31.e-mail: elena.fabbri@unibo.it

Duration of the MACOMA Research Mobility:
32. From: **26/06/2018**
33. To**: 30/07/2018**

34. Score of the MACOMA Research Mobility (ECTS scale): ***10 ECTS***

35. Additional requirement or conditions (special equipment, field work, observation data etc.):
or 36. Grade (please use ECTS scale: A –F):

37. Any comments from host partner:

38. Signature of the host coordinator: 38.a. Signature of the Ph.D. candidate's supervisor:

Dr. Prof. Elena Fabbri **Camilo Dias Seabra Pereira**

39. Date of issue: 12th, September 2019

Objective(s) of the MACOMA Research Mobility:

To write thesis manuscript and prepare scientific papers for publication.

40. Activities/research carried out during MACOMA Research Mobility:
- Theoretical research
- Bibliography research
- Write article

41. Study visits (if possible, where):

42. Participation in Conferences, workshops, seminars without presentation (please indicate title, host institutions, duration):

43. Participation in Conferences, workshops, seminars with presentation(s) (please indicate title; host institutions; title of presentation(s); indicate: oral or poster; others):

on July 16th, 2018 presented the seminar entitled "Adverse effects of crack/cocaine to marine organisms affected by acidification conditions",

44. Publications preparing during MACOMA Research Mobility (please indicate title; where and when publication is planning to be published; others):

Title: Assessing CO_2-induced acidification lethal and sublethal effects on tropical mussels *Perna perna* (Linnaeus, 1758).
Manuscript Number: MPB-D-19-00300

45. Other information:

3. The Partner Organization of the MACOMA Research Mobility Experience Nº 4.
First and Second Academic year 2015-17

13. Host partner:
University of Cadiz, Spain
Last Year: 10 ECTS

14. Name, type and address of host partner:
University of Cadiz, Spain
Address: Faculty of Marine and Environmental Sciences

Campus Puerto Real. 11519. Puerto Real. Cádiz. Spain

15. Surname(s) and first names of coordinator: **Mr Miguel Ángel Pendón Meléndez**
16.Title/position: **Vice-Rector of Planning and Postgraduate Studies**
17.Telephone: +34 956016794
18.e-mail: agua.mundus@uca.es,;

Duration of the MACOMA Research Mobility:

19. From: **31/08/2018** 20. To: **09/11/2018**

21. Score of the MACOMA Research Mobility (ECTS scale): **10 ECTS**

22. Additional requirement or conditions (special equipment, field work, observation data etc) or 22. Grade (please use ECTS scale: A –F):

23. Any comments from host partner:

24. Signature of the host coordinator: 24.a. Signature of the Ph.D. candidate's supervisor:

Mr Miguel Ángel Pendón Meléndez **Camilo Dias Seabra Pereira**

25. Date of issue: **12th, September 2019**

4. The Partner Organization of the MACOMA Research Mobility experience.

a. (Annex: Certificate of stay for the mobility Nº 1).

b. (Annex: Certificate of stay for the 2nd year of the mobility Nº 2a/b)

c. (Annex: Certificate of stay for the 3rd year of the mobility Nº 3a/b)

4.a Description of the MACOMA Research Mobility experience

Research mobility to Faro meant a scientific knowledge where I had the opportunity to learn different areas of study. This experience let me improve my knowledge in relation to the integrated methods approach that has been widely used in environmental risk assessment.

4.b Description of skills and Competences Acquired During the MACOMA Research Mobility experience.

The research mobility to Bologna also meant a very important scientific knowledge where I had the support in the elaboration of one of the papers published in the Thesis. Besides, it was good to introduce new cultures and exchange experiences with other researchers.

Annex D. Congresses Attendance Certificates

CERTIFICATE

The Organizing Committee of the 18th International Symposium on Toxicity Assessment (ISTA 18), hereby declares that:

Lorena da Silva Souza

attended the 18th International Symposium on Toxicity Assessment, held in Limeira, Brazil, from July 16th to 21st, 2017.

ISTA 18

Gisela Umbuzeiro
Chair

12th SETAC Latin America Biennial Meeting | 7-10 September 2017 Santos, São Paulo, Brazil | SETAC

This is to certify that

Lorena Souza

attended the

12th SETAC Latin America Biennial Meeting

7–10 September 2017
Santos, São Paulo, Brazil

Society of Environmental Toxicology and Chemistry
Environmental Quality Through Science

Helena C. de Silva de Assis

Helena Silva de Assis

President, SETAC Latin America

Annex E. Co-authored articles

Marine Pollution Bulletin 141 (2019) 366–372

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Marine contamination and cytogenotoxic effects of fluoxetine in the tropical brown mussel *Perna perna*

Fernando Sanzi Cortez[a,b], Lorena da Silva Souza[c], Luciana Lopes Guimarães[a], Fabio Hermes Pusceddu[a], Luciane Alves Maranho[a], Mayana Karoline Fontes[b], Beatriz Barbosa Moreno[d], Caio Rodrigues Nobre[b], Denis Moledo de Souza Abessa[b], Augusto Cesar[a,d], Camilo Dias Seabra Pereira[a,d,*]

[a] *Universidade Santa Cecília, Rua Oswaldo Cruz 266, Santos, SP CEP:11045-907, Brazil*
[b] *Universidade Estadual Paulista Júlio de Mesquita, Pr. Infante Dom Henrique, s/n, São Vicente CEP: 11330-900, Brazil*
[c] *Universidad de Cádiz, Polígono Río San Pedro, s/n, Puerto Real CP: 11510, Spain*
[d] *Universidade Federal de São Paulo, Rua Maria Máximo 168, Santos, SP CEP 11030-100, Brazil*

ARTICLE INFO

Keywords:
Antidepressants
Contaminants of emerging concern
Tropical ecotoxicology
Bivalves
Brazil

ABSTRACT

Concerns are growing about the presence of fluoxetine (FLX) in environmental matrices, as well as its harmful effects on non-target organisms. FLX in aquatic ecosystems has been detected in a range varying from pg/L to ng/L, while adverse effects have been reported in several organisms inhabiting freshwater and marine environments. The present study quantifies FLX concentrations in seawater samples from Santos Bay, Brazil and assesses metabolic responses and sublethal effects on the tropical brown mussel *Perna perna*. Levels of ethoxyresorufin-O-deethylase, dibenzylfluorescein dealkylase, glutathione S-transferase, glutathione peroxidase, cholinesterase, lipoperoxidation, and DNA damage were assessed in the gills and digestive gland of these animals, and lysosomal membrane stability was also assessed in hemocytes. FLX altered phase I and II enzyme activities, caused cytogenotoxic effects, and negatively impacted the overall health of mussels exposed to environmentally relevant concentrations. These findings contribute to characterize the risks of introducing this drug into the marine environment.

Science of the Total Environment 637–638 (2018) 1363–1371

Contents lists available at ScienceDirect

Science of the Total Environment

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

Ecotoxicological effects of losartan on the brown mussel *Perna perna* and its occurrence in seawater from Santos Bay (Brazil)

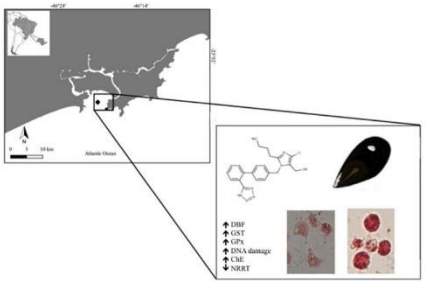
Fernando Sanzi Cortez [a,b], Lorena da Silva Souza [c], Luciana Lopes Guimarães [a], João Emanoel Almeida [d], Fabio Hermes Pusceddu [a], Luciane Alves Maranho [a,b], Luciana Gonçalves Mota [d], Caio Rodrigues Nobre [b], Beatriz Barbosa Moreno [d], Denis Moledo de Souza Abessa [b], Augusto Cesar [a,d], Aldo Ramos Santos [a], Camilo Dias Seabra Pereira [a,d,*]

[a] *Unisanta - Universidade Santa Cecília, Santos, SP, Brazil*
[b] *Unesp - Universidade Estadual Paulista Julio de Mesquita, São Vicente, SP, Brazil*
[c] *UCA - Universidad de Cádiz, Spain*
[d] *Unifesp - Universidade Federal de São Paulo, Santos, SP, Brazil*

HIGHLIGHTS

- Losartan concentrations in seawater from Santos bay ranged from 0.2 to 8.6 ng/L.
- Reproductive parameters were altered after acute exposure up to 75 mg/L.
- Cyto-genotoxic effects observed after short-term exposure (48–96 h) to ng/L
- *Perna perna* is a sensitive model for assessing losartan toxicity.
- Lysosomal membrane stability was the most sensitive endpoint.

GRAPHICAL ABSTRACT



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ABSTRACT

The antihypertensive losartan (LOS) has been detected in wastewater and environmental matrices, however further studies focused on assessing the ecotoxicological effects on aquatic ecosystems are necessary. Considering the intensive use of this pharmaceutical and its discharges into coastal zones, our study aimed to determine the environmental concentrations of LOS in seawater, as well as to assess the biological effects of LOS on the marine bivalve *Perna perna*. For this purpose, fertilization rate and embryolarval development were evaluated through standardized assays. Phase I (ethoxyresorufin O deethylase EROD and dibenzylfluorescein dealkylase DBF) and II (glutathione S-transferase GST) enzymes, glutathione peroxidase (GPx), Cholinesterase (ChE), lipoperoxidation (LPO) and DNA damage were used to analyze sublethal responses in gills and digestive gland of adult individuals. Lysosomal membrane stability was also assessed in hemocytes. Our results showed the occurrence of LOS in 100% of the analyzed water samples located in Santos Bay, Sao Paulo, Brazil, in a range of 0.2 ng/L–8.7 ng/L. Effects on reproductive endpoints were observed after short-term exposure to concentrations up to 75 mg/L. Biomarker responses demonstrated the induction of CYP450 like activity and GST in mussel gills exposed to 300 and 3000 ng/L of LOS, respectively. GPx activity was also increased in concentration of exposure to 3000 ng/L of LOS. Cyto-genotoxic effects were found in gills and hemocytes exposed in concentrations up to 300 ng/L. These results highlighted the concern of introducing this class of contaminants into marine

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E-mail address: camilo.seabra@pq.cnpq.br (C.D.S. Pereira).

https://doi.org/10.1016/j.scitotenv.2018.05.069