TAINÁ GARCIA DA FONSECA

ENVIRONMENTAL RISK ASSESSMENT AND TOXICITY OF PHARMACEUTICALS IN COASTAL TROPICAL AND TEMPERATE ORGANISMS

Faculdade de Ciências e Tecnologia

Centro de Investigação Marinha e Ambiental

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Doutoramento em Ciências do Mar, Terra e Ambiente

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Trabalho efetuado sob a orientação da

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Faculdade de Ciências e Tecnologia

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Environmental Risk Assessment and toxicity of pharmaceuticals in coastal tropical and temperate organisms

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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GENERAL ABSTRACT

Demographic attributes of the urban society have prompted a milestone shift towards structural aging of global population and increase of life expectancy, crucial to the prominence of cancer as one of the non-communicable diseases leading mortality. Currently, cancer diseases were accountable for about 9.6 million deaths in 2018, with concerning increasing projections of cancer incidence and mortality, by 2030, at a greater proportion in developing countries. As a result, there is an increasing trend of production and consumption of pharmaceuticals applied in cancer treatments. Once administered and metabolized, such drugs are excreted into waterways following to waste water treatment plants (WWTPs), ending up in freshwater and coastal ecosystems, where they can be found at a sub ng to ng L^{-1} range. Anticancer drugs are designed to damage DNA and disrupt mechanisms of its transcription, replication and synthesis, as well as suppress the cell´s defense, ultimately causing cell death. In cancer therapy, drugs are typically administered in a combinatory cocktail, that covers different molecular targets, reducing the risk of clonal selection based upon cell resistance to a single drug. However, these chemicals may bring potential toxicity if discharged into the aquatic environment, both in temperate and tropical zones, particularly in the benthic compartment, where they are expected to accumulate.

In this sense, the present thesis aimed to assess the effects of anticancer agents of different classes, namely the cytotoxic platinum-based cisplatin (CisPt), the alkylating agent cyclophosphamide (CP) and the endocrine disruptor tamoxifen (TAM), on non-target benthic marine species under realistic environmental concentrations, at individual and combined exposures. For this purpose, a multi-biomarker approach was applied including the assessment of behavioural responses, oxidative stress, biotransformation, neurotoxicity, lipid peroxidation and genotoxicity in the temperate polychaete *Nereis diversicolor*, in addition to the evaluation of acute and chronic effects of drugs in tropical and representative benthic organisms: the sea urchin *Echinometra lucunter*, the polychaete *Scolelepis squamata* and the amphipod *Tiburonella viscana* from the Brazilian coast.

The main results revealed that single drug exposures caused alteration of AChE activity, oxidative stress and oxidative damage and, ultimately, DNA damage in *N.*

diversicolor. Besides, alterations in its burrowing behaviour also occurred, as an ecological outcome. These effects were more pronounced in organisms under CisPt at 100 ng Pt L^{-1}, CP at 1000 ng L^{-1} and TAM at 0.5 ng L^{-1}. Findings from bioassays with *N. diversicolor* conducted with drugs in tertiary mixtures indicated that each biomarker effect respond differently, according to the trends proposed by models of drugs´s interaction. Toxicity did not increase in a dose-response manner but showed different patterns of effects, in a way that the highest DNA damage was observed at the set of lowest concentrations (Mixture A), an absence of oxidative stress at the intermediary drug levels (Mix B and C), with a potential dominance of TAM´s MoA in organisms exposed to Mixture C and D, suggesting an antagonist interaction between the cytotoxic drugs. Therefore, the effects ruled by single-drug MoAs cannot provide estimations and protective measures for a prospective ERA regarding scenarios of their combination in the environment. Although evidence on estrogen receptors expression is still not disclosed for the herein selected biological models, determination of biochemical and genotoxic outcomes from TAM exposure are highlighted, since it is assumed to comprise a targeted therapy on nuclear estrogen receptors.

Anticancer drugs´ concentrations that triggered the above-mentioned responses are potentially present in coastal environments, even more prone to be encountered in developing regions, where technologies of WWTPs are not efficient or are lacking. Accordingly, effects regarding the bioassays conducted with the tropical counterparts showed a non-monotonic reduction of viable pluteus larvae of *E. lucunter,* at CisPt and CP treatment respectively at the range from 0.1 to 10 ng Pt L^{-1}, and from 50 to 500 ng CP L^{-1}, in contrast to the increasing reduction of embryo larval development under TAM exposure, significant over the whole range of concentrations. In polychaetes *S. squamata,* a significant acute toxicity was exerted at the higher pharmaceutical doses (Mix C and D). In the amphipod *T. viscana* demonstrated a higher sensitivity to pharmaceuticals, presenting a significant non-linear reduction of survival in Mix B and D, as a potential outstanding effect of TAM when present as mixtures. By virtue of the non-monotonicity addressed in the dose-responses relationships reported in all bioassays, another concern stands out on the urgency to the development of a risk analysis and safety assessment designated to anticancer drugs.

Keywords: Anticancer drugs, cisplatin, cyclophosphamide, tamoxifen, sediment, mixture, toxicity, biochemical biomarkers, genotoxicity.

RESUMO GERAL

Atualmente, a dinamica demográfica global demonstra uma excepcionl tendência de transição regida pelo envelhecimento populacional e pelo aumento da esperança média de vida. Estes fatores, combinados a atributos sociais relacionados aos hábitos modernos e exposição a fatores de risco (e.g. dieta inapropriada, tabaco, sedentarismo, consumo de álcool, agentes infecciosos), aliados à hereditariedade, contribuem para um significativo aumento da incidência dos casos de cancro, classificado como a doença mais preocupante do século, após os distúrbios cardiovasculares. Atualmente, o cancro representa 21,7% dos casos de morte a nível mundial, com projeções preocupantes para 2030, e maiores proporções de incidência em países em desenvolvimento. Dessa forma, estima-se uma consequente expansão na necessidade de produção e consumo de medicamentos anticancerígenos em todo o mundo, com aumento anual entre 6 – 8% estimado para o ano de 2018, comparado com os 6,5% nos últimos 5 anos.

Após o consumo, via administração oral ou intravenosa, os fármacos anticancerígenos são excretados na sua forma parental e metabolizada através de efluentes hospitalares e industriais, bem como por descargas de esgotos provenientes de estações de tratamento de águas residuais (ETARs), as quais não possuem tecnologia adequada para a remoção de moléculas orgânicas complexas. Dessa forma, as águas superficiais e marinhas são os destinos finais desses medicamentos. Esta classe terapêutica atua sobre biomoléculas e componentes celulares críticos à proliferação celular, tais como DNA, RNA, proteínas, membrana fosfolipídica, microfilamentos do citoesqueleto e sinalização celular, a fim de causar danos irreversíveis e morte celular. Contudo, estes alvos não são específicos de células tumourais, pois, as drogas interferem em células proliferativas normais, como as dos organismos aquáticos.

Apesar da crescente quantidade de informação envolvendo fármacos no âmbito da ecotoxicologia aquática, verifica-se uma escassez de dados sobre o impacto de drogas anticancerígenas no meio marinho. Em países em desenvolvimento, onde são escassas as políticas de saneamento ambiental e a implementação de infraestruturas avançadas em ETARs, o cenário de risco ambiental torna-se ainda mais elevado frente à contaminação por

estes fármacos. Além disso, a investigação acerca dos efeitos de fármacos em organismos nativos de ambiente (sub)tropical é escassa, e ausente relativamente aos anticancerígenos, dentro de um âmbito em que os critérios de qualidade ambiental em países em desenvolvimento são baseados em extrapolações de respostas geradas em organismos de zonas temperadas da Europa e América do Norte. O sedimento consiste num compartimento de destino final das substâncias introduzidas nos ecossistemas aquáticos, capaz de acumulá-las em níveis muito mais elevados do que aqueles observados na coluna de água adjacente. Além de atuarem como repositório de compostos químicos, os sedimentos atuam também como fonte de poluição após eventos de remobilização. Portanto, por meio do contato com a água intersticial e material particulado, as espécies bentónicas estão constantemente expostas a misturas de compostos químicos.

Dessa forma, a presente tese teve como objetivo avaliar a toxicidade de fármacos anticancerígenos, globalmente administrados em coquetéis no tratamento contra o cancro, em organismos marinhos bentónicos representativos de zonas temperadas e tropicais. Estes organismos foram expostos a drogas individualmente e em misturas, a concentrações ambientalmente relevantes. Os fármacos selecionados para tal foram os citotóxicos cisplatina (CisPt – 0,1 a 100 ng Pt L^{-1}) e ciclofosfamida (CP – 10 a 1000 ng L^{-1}), aplicadas no tratamento de tumoures sólidos (*e.g.* ovário, pulmão e bexiga), e o disruptor endócrino tamoxifeno (TAM - 0,5 a 100 ng L^{-1}), mundialmente aplicado no tratamento do cancro da mama. Foram usados como modelos os poliquetas *Nereis diversicolor* de ambientes marinhos temperados e o ouriço-do-mar *Echinometra lucunter,* o anfípoda *Tiburonella viscana* e o poliqueta *Scolelepis squamata* característicos de ambientes tropicais. Nas experiências de exposição com *N. diversicolor,* que tiveram a duração de 14 dias, avaliaram-se as respostas de biomarcadores de estresse oxidativo (superóxido dismutase -SOD; catalase – CAT; enzima de fase II glutationa-S-transferase – GST; glutationa peroxidase - GPx), dano oxidativo (peroxidação lipídica - LPO), de efeito neurotóxico (atividade da enzima acetilcolinesterase - AChE), genotoxicidade por dano ao ADN, e alteração do comportamento de escavação. Relativamente aos organismos de ambientes bentônicos tropicais foram efetuados ensaios agudos (mortalidade) e crônicos (desenvolvimento embrio-larval).

Os resultados obtidos indicaram que as drogas anticancerígenas, expostas individualmente, causam alterações neutotóxicas significativas, estresse e dano oxidativo e genotoxicidade em *N. diversicolor*. Além disso, registram-se alterações no comportamento de escavação que podem comprometer a proteção da espécie aos predadores presentes na superfície do sedimento, com consequências a nível ecológico. Estas respostas foram mais significativas em organismos expostos a CisPt (100 ng Pt L^{-1}), CP (1000 ng L^{-1}) e TAM (0,5 ng L^{-1}). Relativamente ao bioensaio conduzido em misturas ternárias, constatou-se que o perfil de toxicidade não ocorreu de maneira monotónica. A mistura contendo os anticancerígenos em menor concentração (Mistura A) foi responsável pelo maior grau de dano em ADN, enquanto que nas concentrações intermedias (Misturas B e C) não se registou estresse oxidativo. Comparando-se com os resultados obtidos na exposição aos fármacos individualmente, as misturas C e D indicaram uma potencial predominância dos efeitos do TAM, com efeito antagônico sobre CisPt e CP. Assim, pode-se inferir que as respostas desencadeadas pelo modo de ação dos fármacos presentes individualmente não são consistentes para gerar estimativas de medidas de proteção de avaliação de risco ambiental relativamente a misturas destes compostos. Os efeitos causados pela concentração do anticancerígeno TAM (0,5 ng L^{-1}), individualmente ou em misturas, indicaram um modo de ação por meio da intrusão da pró-droga na membrana celular. Logo, apesar da ausência de evidências referente à expressão de recetores de estrogénio nos modelos biológicos selecionados, a terapia administrada com TAM é também potencialmente tóxica em organismos não alvos da droga.

As concentrações das drogas, responsáveis por desencadear as respostas de toxicidade aguda, foram detetadas em ambientes temperados, e são encontradas em zonas costeiras tropicais. Significativa redução do desenvolvimento embrio-larval do ouriço *E. lucunter* foi verificada nos bioensaios conduzidos com CisPt e CP, respectivamente em concentrações entre 0.1 a 10 ng Pt L^{-1} e 50 a 500 ng CP L^{-1}, de maneira não-monotónica. Em contrapartida, o ensaio com TAM indicou uma resposta monotónica de redução significativa da viabilidade larval com o aumento da concentração da droga. O ensaio conduzido com misturas e poliquetas *S. squamata* demonstrou significativa toxicidade aguda com o aumento da concentração (Misturas C e D), apesar de uma sensibilidade inferior aos

anfípodas *T. viscana*, os quais demonstraram uma redução significativa e não-linear da sobrevivência nas misturas B e D, como potencial efeito da ação do TAM.

Devido aos perfis de toxicidade e dose-resposta não-monotónicos reportados em todos os bioensaios conduzidos com concentrações-traço ambientalmente relevantes, destaca-se a vulnerabilidade de espécies costeiras às misturas desses contaminantes emergentes, bem como à urgência de medidas preventivas à entrada desses compostos no meio marinho e ao desenvolvimento ferramentas de avaliação de risco ambiental especialmente dirigida a esses fármacos de expressiva toxicidade.

Palavras-chave: Anticancerígenos, cisplatina, ciclofosfamida, sedimento, toxicidade, misturas, biomarcadores bioquímicos, genotoxicidade.

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ABBREVIATIONS

4-HNE	4-hydroxyalkenals
ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
ATC	Acetylcholine
BCF	Bioconcentration factor
BHT	Butylated hydroxy toluene
BSA	Bovine albumin serum
CAGR	Compound annual growth rate
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
CETESB	Companhia Ambiental do Estado de São Paulo
CHMP	Committee for Medicinal Products for Human Use
CisPt	Cisplatin
CNPq	National council for scientific and technological development
CP	Cyclophosphamide
CT	Control
CTR 1	Copper transporter 1
CYP P450	Cytochrome P450 superfamily
DAPI	4,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTNB	5,5-dithiobis-2-nitrobenzoic acid
DTT	1,4-dithiothreitol
EC	Emerging contaminant
EC_{50}	Half maximal effective concentration
EDC	Endocrine disrupting compounds
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ER	Estrogen receptor

ERA	Environmental risk assessment
FDA	Food and Drug Administration
FMO	Flavin-containing monooxygenases
FW	Freshwater
GPx	Glutathione peroxidase
GSH	Glutathione
GSH-T	Total glutathione
GSSG	Glutathione disulphide
GSSH	Oxidized glutathione
GST	Glutatione-S-transferase
H_2O_2	Hydrogen peroxide
IARC	International agency for research on cancer
IC50	Half maximal inhibitory concentration
ICL	Inter-strand crosslink
KH	Henry´s coefficient
Koc	Organic carbon partition coefficient
LC50	Median lethal concentration
LECZ	Low-elevation coastal zones
LMA	Low melting point agarose
LPO	Lipid peroxidation
LOAEC	Lowest observable adverse effect concentration
LOD	Limit of detection limit
LOEC	Lowest effect concentration
LOQ	Limit of quantification
MBR	Membrane bioreactors
MDA	Malondialdehyde
MEC	Measured environmental concentration
MET	Methotrexate
MoA	Modes of action
MT	Metallothionein
MTLP	Metallothionein-like protein
NAT	N-acetyltransferase

NCD	Non-communicable diseases
NMA	Normal melting point agarose
NOAEC	No observed adverse effect concentration
NOEC	No observed effect concentration
$O_2^{\bullet-}$	Superoxide anion radical
PAM	Phosphoramide mustard
PCA	Principal component analysis
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
Pt	Platinum
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	Reactive oxygen species
ROW	Rest of the world
SDG	Sustainable development goals
Se-GPx	Glutathione peroxidase selenium-dependent
SERM	Selective estrogen receptor modulators
SOD	Superoxide dismutase
SPE	Solid phase extraction
SR	Sarcoplasmic reticulum
SRV	Sarcoplasmic reticulum vesicles
SULT	Sulfotransferase
SW	Seawater
TAM	Tamoxifen
TBARS	Tiobarbituric acid reactive substances
T-GPx	Total GPx
TP	Transformation products
UGT	Uridine 5´- diphospho (UDP)-glucoronosyltransferase
USEPA	United States Environmental Protection Agency
WHO	World Health Organization
WWTP	Waste water treatment plant

Chapter 1

General Introduction

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The polychaete *Nereis diversicolor* as a biomonitor species of emergent contaminants in the marine environment: a critical review of pharmaceuticals and nanomaterials.

1.1 World trends of pharmaceuticals production and usage

The global demographic outlook demonstrates the fast pace to which population have increased 1.1% per year), reaching a record of 7.7 billion people in 2017, with projections to a further mark of 8.6 billion in 2030 (United Nations, 2017). Moreover, the current scenario of an increasingly interconnected world indicates that over half of this population lives in urban areas. By 2030, 43 cities around the world will have 10 million or more inhabitants (*i.e.* megacities), which correspond to an increase of around 30% compared to 2016 (United Nations, 2016) (Figure 1.1). Another feature of the exceptional urbanization transition addressed in the 21st century is that two thirds of these megacities are established in zones near the coast, where 41% of humankind is settled (Burke *et al*., 2001; Martínez *et al*., 2007; Von Glasow *et al*., 2013).

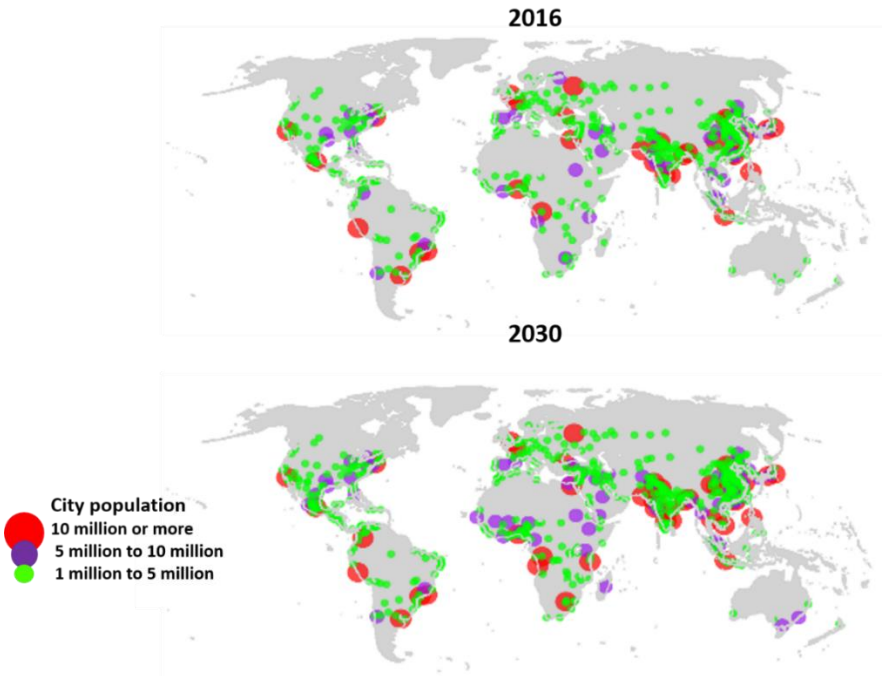


Figure 1.1: Cities with one million or more inhabitants, in 2016 and projections for 2030. Adapted from United Nations (2016).

Low-elevation coastal zones (LECZ) (those with 10 m above sea level) represent about 2% of total land area where 13% of global population inhabit (Barbier, 2015). LECZ are expanding faster than any other area through immigration and demographic changes

(Figure 1.2). The milestone shift of global demographic profiles associated to population aging, projected to continue and even accelerate, mainly driven by declining fertility rates and remarkable increases in life expectancy (WHO, 2014; Ferlay *et al.*, 2015).

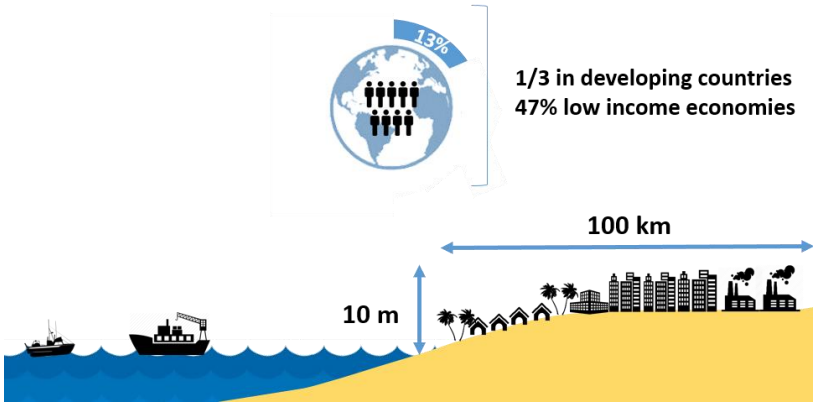


Figure 1.2: Representation of urban settlement in low-elevation coastal zones (LECZ), where 13% of the world population lives.

In 2010, it was estimated that 524 million people worldwide were aged 65 or older, depicting 8% of global population. By 2050, this number is expected to nearly triple to about 1.5 billion, thus represents 16% of the total. Although there are variations in patterns of population growth and levels of urbanization across regions, gains in longevity have been especially dramatic in cities of developing countries (WHO, 2016) (Figure 1.3). These trends have profound implications in society, once a transition to older populations on the demographic structure will occur, followed by health problems associated to chronic non-infectious and non-communicable diseases (NCD). NCDs mainly comprise cardiovascular and respiratory chronic illnesses, as well as cancer diseases, accounting for 67.9% of total deaths that occurred worldwide, in 2012, and currently represent the greatest burden on health of low, middle- and higher- income countries. However, the present rise of NCDs has shown to derive not only from older health status but also from the improvement of living standards and broaden socioeconomic, cultural and environmental underlying determinants, fitted by risk factors individually managed (e.g. unappropriated diet, tobacco, physical inactivity, harmful use of alcohol, infectious agents) (Jemal *et al.*, 2011; American Cancer Society, 2014). A rising rate of diseases burden will drive up production and consumption

of pharmaceuticals required for different therapeutic applications in a way that global medicines spending will reach 1.4 trillion dollars by 2020, an increase of 29-32% compared to 2015, in which 63% of the spending will be led by developed nations (Aitken and Kleinrock, 2015) (Figure 1.4).

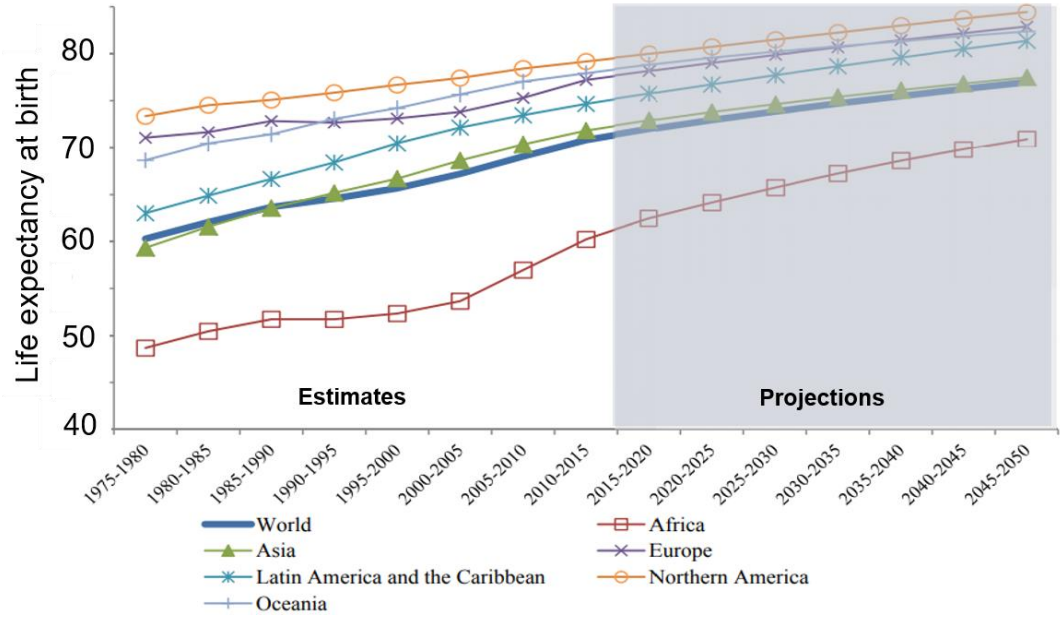


Figure 1.3: Life expectancy (years) by region: estimates 1975-2015 and projections 2015-2050. Adapted from World Population Prospects: The 2017 Revision (WHO, 2017).

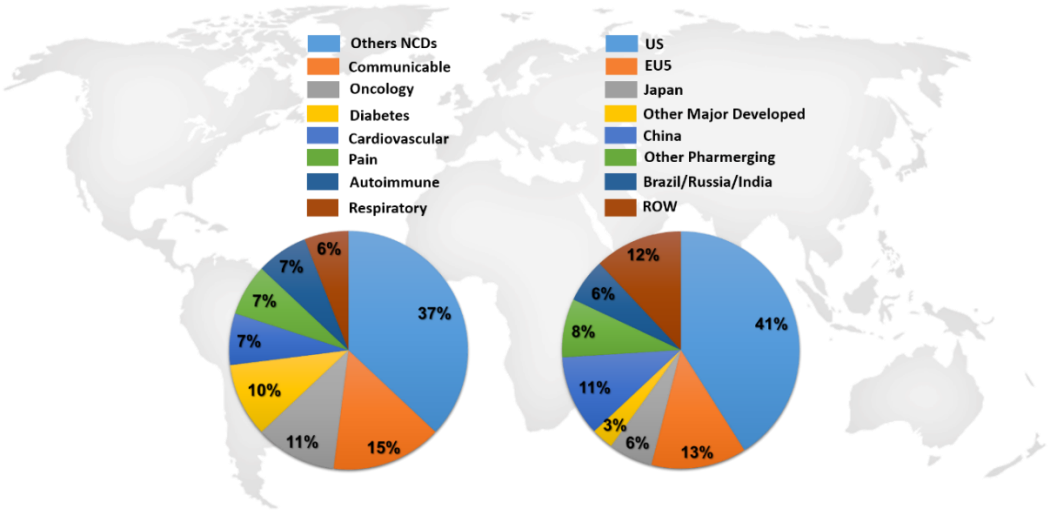


Figure 1.4: Projections of global spending (US$) in pharmaceuticals in 2020, by therapeutic application and countries. Adapted from Aitken and Kleinrock (2015).

1.2 The global scenario of cancer diseases

Neoplastic or cancer diseases depict a leading concern in global health condition responsible for an estimated 9.6 million deaths in 2018, with worrying projections of cancer incidence and mortality, by 2030 (Figure 1.5) (WHO, 2014; Ferlay *et al.*, 2015; WHO, 2017). In 2015, 8.8 million people died from cancer, which represents one in six deaths globally reaching countries of all income levels. However, actions aiming to bring cancer rates under control are better managed in developed nations through decreasing the prevalence of risk factors, early detection measures and prevention action, whereas economically less developed countries continue to bear disproportionate burden of infection-related cancers cases and a rise in exposure to hazard external determinants associated to lifestyle (Torre *et al*., 2015). Therefore, approximately two thirds of global cancer deaths are in less developed countries, especially due to late-stage detection and less accessible treatment (WHO, 2017).

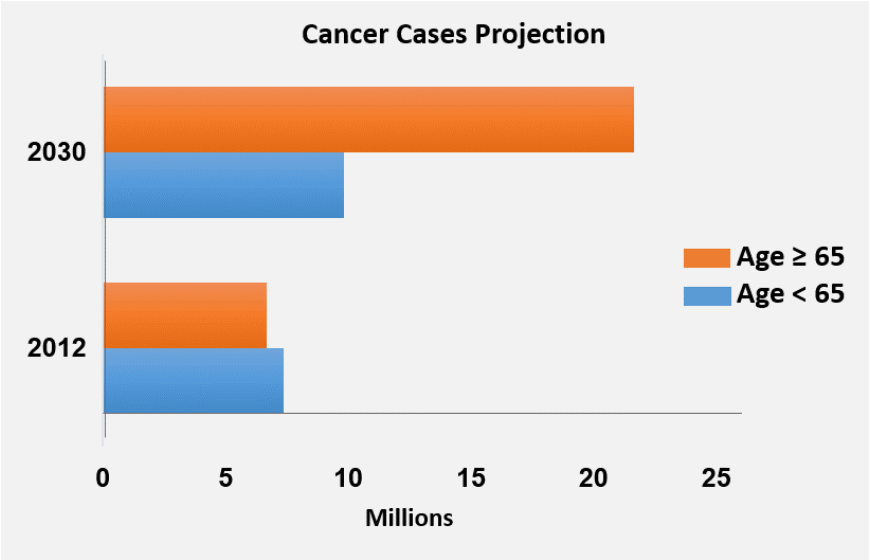


Figure 1.5: Projection of cancer cases between 2012 and 2030 (millions of people). All types of cancer are considered with exception of non-melanoma skin cancer (Ferlay *et al.*, 2015).

As a result, the United Nations has recognized cancer as a priority not only for health but also as a threat to economies and societies, thus placing this disease high on the set of commitments of a global agenda of health coverage (*i.e.* Global Action Plan for the Prevention and Control of NCDs) (WHO, 2014). In this scope, nations aim to monitor the trends of NCDs and evaluate their progress in an attempt to design preventive and control measures. Therefore, substantial increase in availability of affordable basic technologies and essential pharmaceutical compounds are required to treat cancer in both public and private facilities (WHO, 2014). Since cancer consists of a group of heterogeneous diseases that can affect almost any part of the body and has several anatomic and molecular subtypes, specific management of prevention, early diagnosis, treatment, palliative and survivorship care are enforced, regardless the zone of incidence (Torre *et al.*, 2015). Cancer therapy usually involves the alternative of surgery resection, and body´s exposure to agents that kill cancer cells, via radiotherapy and the administration of anticancer medicines through systemic therapies, in a way that these may be applied individually or in combination, depending of the type and stage of the tumour (Helleday *et al.*, 2008; Barczak *et al.*, 2018). Anticancer drugs may correspond to different pharmaceutical modalities, regarding cytotoxic chemotherapy, in addition to hormonal, targeted and immunotherapies (Palumbo *et al.*, 2013).

It is possible to link the increase of cancer incidences in recent years with the rise of drugs production and application, also reflected in global spending on oncology medicine as a leading key therapy area driving pharmaceutical expenditure over the next five years, including treatment and supportive care (Figure 1.6) (Aitken and Kleinrock, 2015). The pipeline of oncology drugs expanded by 45% over the past 10 years (Pineau ad Rink, 2017). By 2021, spending on oncology medicine is expected to grow in a range of 9-12% annually, compared to the 11% seen over 2016, as the demand of new and targeted therapy options raised (Aitken and Kleinrock, 2015) (Figure 1.7). Since 2011, 68 new drugs have been approved for 22 indications, including immune-oncology agents that have considerably changed the paradigm of cancer treatment. Advances in drug discovery have supported the movement towards a more personalized care that relies on the use of validated predictive biomarkers. These novel tools are able to identify genetic attributes of a population presenting a specific cancer case, thus far more likely to respond to therapy and benefits

from a particular drug (Pineau and Rink, 2013). However, conventional treatments, which are less effective for specific drivers of cancer, are still widely applied worldwide. They are represented by cytotoxic drugs which have been prescribed, since the 1960´s, for various solid and hematological malignancies in chemotherapies administered to adults and children (Helleday *et al*., 2008).

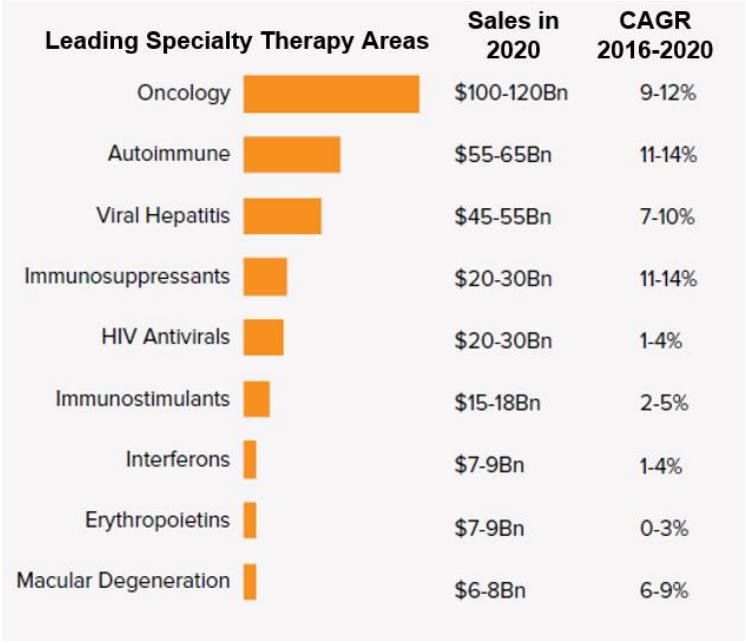


Figure 1.6: Sales in 2020 for leading therapy areas of specialty medicines and compound annual growth rate (CAGR).

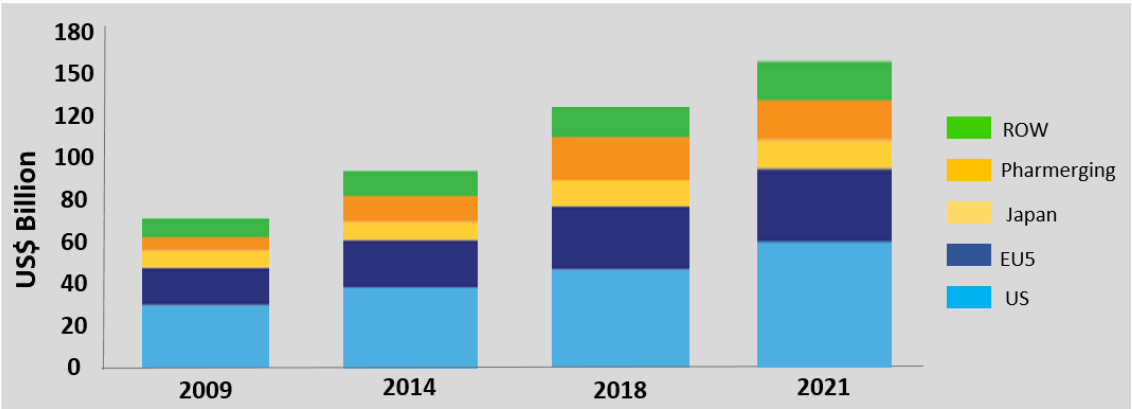


Figure 1.7: Global forecast of spending of oncology pharmaceuticals: EU5 (Germany, Italy, France, Spain, UK); United States; Japan; Pharmerging countries (Brazil, Mexico, Venezuela, Argentina, China, India, Indonesia, Vietnam, etc.); ROW (Rest of the World).

Despite addressed as efficient agents against tumour cells by means of producing high levels of DNA damage, those pharmaceuticals are not selective enough to interact only with highly proliferative cells by providing cell-cycle arrest and cell death in normal and healthy tissues. Such mechanisms often cause undesirable side effects to patients during the chemotherapy and may be potent harmful compounds to all eukaryote organisms (Novak *et al*., 2017).

1.3 Anticancer medicines and the rationale for chemotherapy

Chemotherapy may be administered to cancer patients through usual routes of intravenous, intramuscular or subcutaneous injection. Classification of anticancer drugs is established by the Anatomical Therapeutic Classification (ATC), where they are ranked as Antineoplastic Agents; Endocrine Therapy; Immunostimulants; and Immunosuppressants, as can be seen in Table 1.1 (Booker *et al.,* 2014; Xie *et al*., 2015). Antineoplastic agents are designed to interact directly or indirectly with DNA, by damaging its structure, inhibiting, altering and disrupting mechanisms of its transcription, replication and synthesis. Derivatives of nitrogen mustards were developed, including the DNA alkylating agents cyclophosphamide, chlorambucil and melphalan, widely used in clinical therapeutics (Cheung-Ong *et al*., 2013. Xie *et al.,* 2015). Other examples of DNA-alkylating agents used in cancer treatment include nitrosoureas (e.g., carmustine, lomustine, and semustine) and triazenes (e.g., dacarbazine and temozolomide) (Cheung-Ong *et al.,* 2013).

Since most of these pharmaceuticals do not strictly act over tumour drivers, but also in mechanisms regarding all growing cells (Kosjek and Heath, 2011; Caley and Jones, 2012; Toolaram *et al.,* 2014; Heath *et al.,* 2016), usually leading to detrimental effects in patients owing from adverse toxicity to non-targeted tissues. In contrast, there are compounds accountable for the disruption of biological processes through mechanisms of blocking cells replication factors, or indirectly by recruiting macrophages and monocytes cells (Besse *et al*., 2012; Caley and Jones 2012), thus, not directly involved with DNA. Endocrine therapy manipulates hormone-dependent receptors with tissue-selective interactions that lead to agonist or antagonist activities, represented by the group of selective estrogen receptor modulators (SERMs), like tamoxifen (Paterni *et al.,* 2014; An, 2016). Attachment of these endocrine disrupting compounds (EDC) to respective binding sites acts competitively and subsequently

inhibiting the transcription of estrogen responsive genes responsible for cell replication of estrogen receptor positive cells, such as seen in breast and prostate cancer (Pinto *et al*., 2014).

Currently, the aforementioned groups of drugs are some of the main used in cancer treatments. Most of chemotherapy regimens in clinical practice consist of a combination of several agents from different pharmaceutical groups, in an attempt to provide additive or synergistic effects to achieve maximal tumour cell death and avoid resistance (Caley and Jones, 2012). In spite to their overall application in chemotherapy, there are strong differences in the chemical structure of the several anticancer groups that also have distinct modes of action (MoA) (Booker *et al*., 2014).

Table 1.1: Classification of anticancer drugs

Classification	Subcategory		Compounds	MoA
Antineoplastic agents	Alkylating agents	Nitrogen mustards	Cyclophosphamide, chlorambucil, ifosfamide	Alkylating agents react with nitrogen (N) and extracyclic oxygen (O) atoms of DNA bases to generate a variety of covalent adducts. Induce a range of cytotoxic and mutagenic adducts onto DNA
		Nitrosoureas	Carmustine, lomustine	
		Alkyl sulfonates	Busulfan	
		Other alkylating agents	Temozolomide	
	Antimetabolites	Folic acid analogues	Methotrexate	Structurally similar to endogenous nucleic acids, can be incorporated into the metabolic pathways instead of the endogenous purine and pyrimidines, thereby affecting the enzyme dependent synthesis of DNA and cell reproduction
		Purine analogues	Mercaptopurine, tioguanine, cladribine	
		Pyrimidine analogues	5-fluorouracil, gemcitabine, capecitabine	
	Plant Alkaloids	Vinca alkaloids and analogues	Vinblastine, vincristine	Interacts with microtubules or tubulins leading to inhibition of synthesis of proteins and nucleic acids, disruption of mitotic spindle and eventually cell death
		Podophyllotoxin derivatives	Etoposide	
		Taxanes	Paclitaxel	
	Cytotoxic Antibiotics and related substances	Actinomycines	Dactinomycin	Involve direct toxic action on cellular DNA, interfering with DNA replication and protein synthesis
		Anthracyclines and related substances	Daunorubicin, doxorubicin, epirubicin	
	Other antineoplastic agents	Platinum compounds	Cisplatin, carboplatin	*E.g.* Cisplatin products highly electrophilic. Act towards nucleophilic sites in genomic and mitochondrial DNA, producing DNA inter- and intra-strand adducts that result in DNA distortion, inhibition of DNA replication and interruption of cell division
		Methylhydrazines	Procarbazine	

Endocrine Therapy	Hormones and related agents	Estrogens	Ethinylestradiol, diethylstilbestrol	Antitumor activity involves suppression of luteinizing hormone by inhibition of pituitary function
		Progestogens	Megestrol, medroxyprogesterone	
		Gonadotropin releasing hormone analogues	Leuprorelin, buserelin	
	Hormone antagonists and related agents	Anti-estrogens	Tamoxifen, toremifene	Diverse group of compounds bind to oestrogen α (ERα) and β (ERβ) receptors and produce oestrogen agonist effects in some tissues, but oestrogen antagonist activity in others. Activity determined in part by formation of oestrogen receptor–molecule complexes that vary in their ability to activate genes when bound to ERα or ERβ
		Anti-androgens	Flutamide, nilutamide	
		Aromatase inhibitors	Letrozole, anastrozole	
Immunostimulants	Immunostimulants	Colony stimulating factors	Filgrastim, lenograstim	Immune modulation is based on the stimulation of T-cell function with antibodies that block or activate regulatory receptors is enough to cause the regression of some tumours.
		Interferons	Interferon-α natural, interferon-β natural	
		Interleukins	Aldesleukin	
		Other immunostimulants	Lentinan, roquinimex	
Immunosuppressants	Immunosuppressants	Selective immunosuppressants	Mycophenolic acid, sirolimus	
		Interleukin inhibitors	Daclizumab, basiliximab	
		Calcineurin inhibitors	Ciclosporin, tacrolimus	
		Other immunosuppressants	Methotrexate, azathioprine	

1.4 Drug metabolism and excretion

The major function of drug metabolism in the body is to provide the breakdown of the compound into chemically simpler metabolites for elimination, thereby preventing unwanted accumulation, as well as potentially toxic levels of endogenous molecules (Jančová and Šiller, 2012). In general, the fate of chemical biotransformation includes the generation of metabolites, which may be desired for the therapeutic purpose or not, due to toxicity. Briefly, drug pharmacokinetics occurs in two phases of biotransformation, comprised by the functionalization (Phase I) and conjugation (Phase II) of the compound load, in order to increase its polarity and facilitate its elimination through excretion (King, 2009). Phase I relies on an enzymatic system of defense against most of the xenobiotic compounds, composed by members of the cytochrome 450 gene family (CYP 450), flavin-containing monooxygenases (FMO), monoamine oxidases (MAOs), and xanthine oxidase/aldehyde oxidase (Penner *et al.*, 2012). Those enzymes are differently expressed in tissues, but broadly distributed in liver, kidney and intestine, acting by means of introducing, modifying or unmasking reactive functional groups at the parent drug.

Phase II reactions occur with the introduction of acetyl, sulfate, glucoronid acid, glutathione and amino acids functional groups, either in the parent molecule or in a phase I metabolite structurally changed enabling binding sites for conjugation (Liska, 1998; Jančová and Šiller, 2012). These reactions are mostly catalyzed by the enzyme uridine 5´- diphospho (UDP)-glucoronosyltransferase (UGTs), sulfotransferases (SULTs), glutathione S-transferase (GSTs) and N-acetyltransferases (NATs). Biotransformation mechanisms present low specificity to pharmaceuticals and, in general, enhance polarity of compounds, thus favoring the excretion (King, 2009). Nevertheless, this change is often incomplete, with parent compounds excreted together with the metabolites in a variable proportion (Kümmerer, 2010).

1.5 Anticancer compounds as a threat to the aquatic environment

1.5.1 Pharmaceuticals as Emerging Contaminants (ECs)

Pharmaceuticals are substances intended for the use in diagnosis, cure, mitigation, treatment or prevention of diseases, in humans and animals, also designed to provide therapeutic effects in biological systems by their key molecular structures, the active pharmaceutical ingredients (APIs) (Daughton and Ternes, 1999; Khetan and Collins, 2007). The pharmaceutical compounds also include excipients, additives, inorganic salts and other organic chemicals, such as sugars, scents, pigments and dyes (Kummerer, 2010). A wide range of drugs is displayed by the pharmaceutical industry and are often classified according to their APIs and purpose (e.g. antibiotics, analgesic, lipid regulators, antidepressants, antineoplastics) (Williams, 2005). Classification by similarity in chemical structure and behaviour are also usually applied (e.g. antibiotics: penicillins, cephalosporin, quinolones), which may or not coincide with biological activity. In addition, sorting can be based on the drugs MoA, referring to physiological system to be targeted (i.e. digestive system), or to specific binding receptor to which active ingredient interacts (Kummerer, 2008).

Pharmaceuticals are designed to affect targeted biomolecules and biological critical mechanisms via their specific MoA, triggered even at low doses (Fent *et al*., 2006b; Gunnarsson *et al*., 2008). However, since the end of the last century, new findings regarding pharmaceuticals´ molecular particularities and consumption have emerged questions whether these chemicals are relevant only to human benefits and healthcare (Williams, 2005; Roberts and Bersuder, 2006). According to the societal and health status outlooks, jointly with economic forecasts, it is possible to portray not only the hallmark of the increment usage of anticancer drugs in coastal urban areas, but also outline their continuous loads into aquatic ecosystems worldwide. Considering that, pharmaceuticals have been addressed as ubiquitous compounds detected at trace levels (ng – µg L^{-1}) in aquatic compartments, and their persistent input has brought biota to a long-term exposure (Smital, 2008). These compounds, which profile of distribution, behaviour and biological interactions in the aquatic environment are poorly known (Murnyak *et al*., 2011; Pal *et al*., 2010; Robles-Molina *et al*., 2014) are considered emerging contaminants (ECs), and regulation by legal framework are currently under construction or still absent (Rivera-Utrilla *et al*., 2013). The

term “emerging” does not necessarily concern a new phenomenon, but a recognition of compounds that have been recently identified in the aquatic environment by the improvement of analytical methods, which allowed lowering the detection limit in environmental matrices (Glassmeyer *et al.*, 2008; Nikolaou, 2013).

1.5.2 Environmental Background of anticancer drugs

The first publication regarding the issue of anticancer drugs in the environment dates from the 1980´s and aimed to depict the distribution profile of several classes of pharmaceuticals in different aquatic compartments (*e.g.* sewage influent and effluent, surface water and drinking (Aherne *et al.*, 1985; Richardson and Bowron, 1985). These authors outlined that anticancer drugs are likely to yield environmental risks, particularly to surface waters that receive discharges from sewage treatment plant, since the confirmation of hazards verified to nurses after occupational exposure to pharmaceuticals during chemotherapy manipulation. Following the improvement of analytical technologies, a set of cytotoxic pharmaceuticals were identified in environmental screenings, at a range of ng L^{-1} or below (Kummerer *et al.*, 1997; Buerge *et al.*, 2006; Zuccato *et al.*, 2006; Kovalova *et al.*, 2009;Toolaram *et al.*, 2014). Relevant reviews outlined physico-chemical parameters that determine the fate and behaviour of cytotoxics and predict their pathways in aquatic compartments according to their chemical structure namely, pKa, bioconcentration factor (BCF), octanol/water partition coefficient (Kow), organic carbon partition coefficient (Koc), solubility, Henry´s coefficient (KH) and vapor pressure (Kosjek and Heath, 2011; Xie, 2012; Toolaram *et al.*, 2014).

Transformation products (TPs) are by-products of parent compounds and metabolites formed through physico-chemical (*e.g.* photodegradation, hydrolysis, redox reactions) and biological factors (*e.g.* biodegradation) undergone in WWTPs or over the water bodies (Mompelat *et al.*, 2009). Those chemicals can also be as toxic or even more toxic than their parent compounds (Loffler *et al.*, 2005; Li *et al.*, 2014). Despite their potential importance, the formation and fate of TPs from pharmaceuticals has only been scarcely investigated (Mompelat *et al.*, 2009). Although a significant number of drugs are excreted and released as inactive conjugates, such as glucoronates and sulfates, deconjugation provided by bacterial metabolism in WWTPs and in aquatic environment turn metabolites back to the

biologically active parent compounds (Noppe *et al.*, 2007; Behera *et al.*, 2011). Therefore, mixtures of parental forms, metabolites and TPs started to be also evaluated (Fatta-Kassinos *et al.*, 2011; Toolaram *et al.*, 2014; Haddad *et al.*, 2015; Zhang *et al.*, 2017). The studies of Kovalova *et al. (*2009), Negreira *et al. (*2013, 2014a, 2014b, 2015) and Zhang *et al. (*2013) brought advances in analytical chemistry and processes that underline technologies in WWTPs able to remove anticancer drugs, conform to molecules physico-chemical properties, stability and metabolism.

1.5.3 Sources and routes of anticancer drugs to the marine environment

Cancer treatment is shifting from hospital treatment and medical facilities to home treatment (*i.e.* outpatients) (Johnson *et al.*, 2008; Kosjek and Heath, 2011; Besse *et al.*, 2012). The generalization of this modality and the availability of molecules in pharmacies are growing in order to improve the patient treatment comfort. Consequently, a more diffuse discard of parental and metabolized anticancer agents is prone to succeed during home treatment, compared to discharges from hospital wastewater effluents, which is directly introduced into WWTPs (Kümmerer, 2010; Mater *et al.*, 2014; Zhang *et al.*, 2013) (Figure 1.8). In general, private households are indicated as the most important sources for pharmaceuticals emission into aquatic environment, rather than hospitals (Schuster *et al.*, 2008; Toolaram *et al.*, 2014). In the case of chemotherapy drugs, although the substantial contribution, there is still no clear evidence if household origination is stepping up to or even overtake hospital emissions as their distribution patterns depend on various parameters, such as the size of hospitals, size of catchment area, population density, purchase of pharmaceuticals in town pharmacies, among others (Zhang *et al.*, 2013). WWTPs are primarily designed to serve the purpose of removing pathogens, suspended solids, gross organic and inorganic matter, rather than removing the vast array of modern chemicals like pharmaceuticals, present at trace concentrations of ng to μg L^{-1} in influents (Lenz *et al.*, 2005; Rowney *et al.*, 2009; Besse *et al.*, 2012).

In general, European countries count with conventional sewage treatment processes equipped with screening, degritting, sedimentation, biodegradation and adsorption to biomass as the main elimination mechanisms for various organic contaminants during the passage of conventional activated sludge and confined units (Carballa *et al.*, 2004; Conn *et*

al., 2006; Zhang *et al.*, 2013), although those have shown to be ineffective to remove pharmaceuticals (Yang *et al.*, 2017). Combination of advanced and non-conventional technologies in WWTPs and water reclamation are considered alternatives to improve the elimination performance of elimination of chemotherapy drugs, including electrolysis, UV radiation with H_2O_2, ozonation and membrane bioreactors (MBR) (Česen *et al*., 2015). The cytostatic drug 5-FU was eliminated from hospital wastewaters to levels below the limits of detection by means of biodegradation processes occurring in the MBR system, whereas the major pathway by which anthracyclines (doxorubicin, epirubicin and daunorubicin) were mainly removed was through adsorption to sewage sludge, able to yield an elimination of >90% from the liquid phase (Mahnik *et al.* 2007). The percentage of removal achieved at the end of a batch bioreactor WWTP for tamoxifen, ciprofloxacin and etoposide reached 91, 84 and 100%, respectively (Ferrando-Climent *et al.*, 2015).

In contrast, ifosfamide, cyclophosphamide, vincristine, docetaxel and paclitaxel showed to be recalcitrant due to the inefficiency of conventional biological treatments of WWTPs. Likewise, the alkylating agent cyclophosphamide was also considered resistant to mechanical and conventional biological treatment with suspended biomass, after its feasible detection in WWTP effluents at 17 ng L^{-1} (Česen *et al*., 2015). Alternatively, the use of ozonation technology combined to UV and H_2O_2 (5 g L^{-1}) treatments showed to remove 99% of cyclophosphamide and 94% of ifosfamide from a non-treated wastewater (Česen *et al*., 2015). Other studies demonstrated that cyclophosphamide can withstand degradation processes over the aquatic pathway, until concentrations detected in surface waters reach a range of 0.15 to 17 ng L^{-1} (Buerge *et al*. 2006; Moldovan 2006; Busetti *et al*., 2009).

In fact, literature review demonstrated inconsistencies in the removal performed through biological treatments for the alkylating agents ifosfamide and cyclophosphamide, with reports indicating a range from 0 to 72% (Buerge *et al*., 2006; Kovalova *et al*., 2012). This suggests that the extent to which WWTPs are successful in their removal depends not only on how developed are the selected treatment technologies, but also on the experimental design and on the physico-chemical properties of the effluent (Česen *et al.*, 2015). Moreover, Martín *et al. (*2012) identified a removal rate of cytotoxic drugs in WWTPs, ranging from 0% (*e.g.* doxorubicin, gemcitabine, paclitaxel, cisplatin and vinorelbine) to 100% (*e.g.* etoposide, fluorouracil, methotrexate).

Even though they present wide variations in removal rates for different anticancer drugs, both within and between the same treatment works, combination of advanced technologies promises an alternative solution for preventing cytotoxic drug from continuously entering the environment (Zhang *et al.,* 2013). In any case, the occurrence of these chemical agents at trace concentrations escalates concern about ecotoxicity risks and human health threats. Accordingly, this situation may be even worse in areas without access to sanitation system, specifically in densely-populated urban regions (Zhang *et al.,* 2013). Despite major improvements in sanitation detected in lower income countries over two decades, key differences from developed nations are still depicted by the absence or low sewer connectivity in urban areas, as illustrated by zones that rely on poorly managed septic tanks with direct discharges into surface waters, because some regions are barely equipped with facilities to collect, treat and dispose sewage in an environmentally sustainable manner (Roth *et al*., 2016). Urban centers in Bangladesh, India, Pakistan and Thailand present less than 30% of sewer connectivity, whereas Indonesia and Vietnam indicate 2% and 4%, respectively. In South America, disparities in sewage collection vary from more than 80% in Chile and Peru, 40% in Brazil and to less than 20% in Paraguay and Guyana. These facts have major implications on the distribution and loadings of complex organic mixtures involving pharmaceuticals into estuarine and marine ecosystems (Abessa *et al*., 2005; Pessatti *et al*., 2016) (Figure 1.8). In a worst scenario, those chemicals may also leach into groundwater aquifers and harm drinking water supplies (Zhang *et al.,* 2013). Installation and development of advanced wastewater treatment technologies require significant investment and skilled labor for operation, which was not considered a governmental priority in low-income regions (Rahman *et al*., 2009; Gaw *et al*., 2014).

In this sense, parent molecules and metabolic products of anticancer compounds enter waterways after consumption and metabolism, *via* untreated, partially treated or even treated urban and hospital effluents. Besides that, loads of pharmaceuticals into the marine environment, in general, may also occur via wastewater from manufacturing processes, disposal of unused or expired drug products, and accidental spills during manufacturing or distribution (Díaz-Cruz and Barceló, 2003). Most of the information available about the presence of cytotoxic molecules in aquatic systems are focused on the detection of these compounds at their main sources, such as hospital influents/effluents and sewage wastewater

effluents, where they were detected at levels ranging from 6 to 7973 ng L^{-1}, for methotrexate and ciprofloxacin, respectively (Steger-Hartmann *et al*., 1996, 1997; Ternes, 1998; Castiglioni *et al*., 2005; Lenz *et al*., 2005; Buerge *et al*., 2006; Mahnik *et al*., 2006; Moldovan, 2006; Mahnik *et al*., 2007; Kovalova, 2009; Yin *et al*., 2010; Martín *et al*., 2011; Isidori *et al*., 2016).

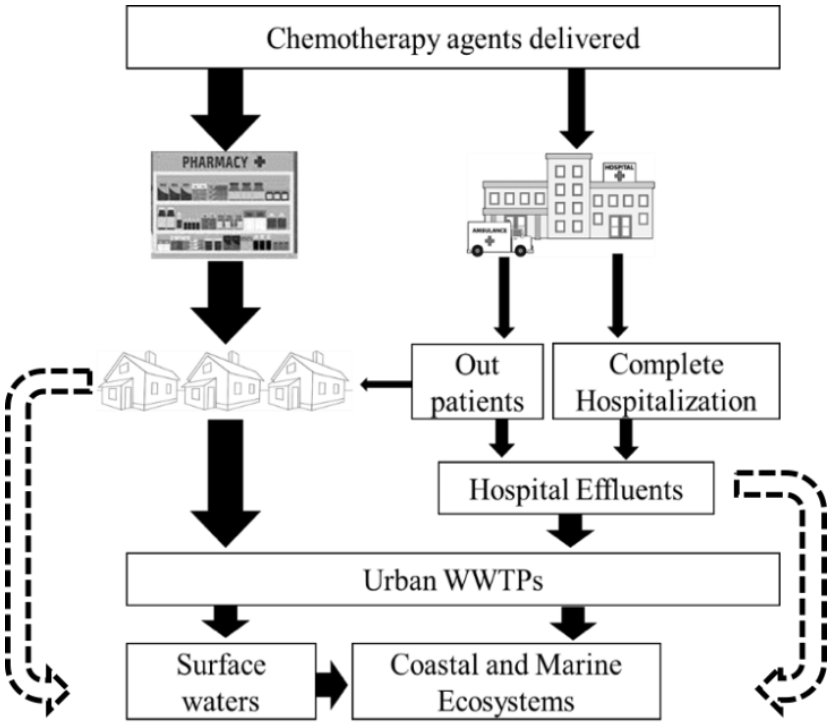


Figure 1.8: Sources and pathways for anticancer drugs into coastal waters. Dashed arrows illustrate the additional course of wastewater in developing regions, where raw domestic and hospital effluents follow straightforward route into aquatic ecosystems.

1.5.4 Potential behaviour and fate of anticancer drugs in marine waters

In the marine environment, physico-chemical and biological conditions are significantly different from limnic waters (*e.g.* salinity, pH, temperature, turbidity, organic compounds, microbial population) and may provide different chemical stability and persistence for pharmaceuticals compounds (Weigel *et al.*, 2002; Lara-Martín *et al.*, 2014). According to Zhang *et al. (*2013), it is important to point out that part of the available information of physico-chemical parameters is estimated by theoretical calculations based

on their chemical structures. Sorption behaviour of pharmaceuticals is often poorly predicted by the estimation of the Kow (Franco *et al.*, 2009; Lara-Martín *et al.*, 2014). Overall, pharmaceuticals consist in polar and highly ionisable molecules, with one or more dissociable functional groups, ranging from acid to alkaline. Electrolytes exist either as neutral or ionic molecules, depending on their dissociation constant (pKa) and pH of the water in which they are dissolved (Rendal *et al*., 2011). In this sense, the application of lipophilicity corrected to pH (log D) is a more reliable strategy that enhances the prediction of environmental behaviour of ionisable species due to the fact that sorption mechanisms can be pH-dependent and change from hydrophobic to ionic interactions (Schaffer *et al*., 2012). Neutral and non-ionised species indicate lower polarity and higher permeability into membranes and fatty acids compared to ionic forms (Fu *et al*., 2009; Rendal *et al*., 2011). The cytotoxic cisplatin is relatively inert, but once in aqueous solutions of low electrolyte concentration (as intracellular medium), chlorine ligands are gradually replaced by water in a stepwise process to form more reactive aqueous species (*i.e.* mono and diaquacisplatin) (Curtis *et al*., 2010; Turner and Mascorda, 2014). Likewise, electrostatic interactions in low salinity environments provide predominance of reactive complexes, whereas in seawater the ionic strength and the presence of chlorine reduces their activity and induce adsorption onto estuarine particles (Curtis *et al*., 2010). This is suggested to decrease levels of pharmaceutical compounds in water and toxicity (Turner and Mascorda, 2015), although bioavailability by deposit feeders may build up the intake.

Therefore, it is crucial to assess whether the levels of these compounds in marine waters become more or less reactive, and what are the processes that drive their bioavailability and toxicity. Bioassays conducted with ionisable pharmaceutical compounds (2,4-dichlorophenol; 2,4,6-triclorophenol; pentachlorophenol; ibuprofen; fluoxetine) at different experimental environmental pH (from 6 to 9) indicate that toxicity depends on the more toxic uncharged fraction (Nakamura *et al*., 2008; Xing *et al*., 2012), but when the ionic fraction is greater, detrimental contributions may be shifted towards the ionic form (Bostrom and Berglund, 2015). Ibuprofen is anionic at a pH 7 (above its pKa), thus hydrophilic, and almost not sorbed onto sediments due to its negative charge, leading to an electrostatic repulsion with each other (Oh *et al*., 2016). In contrast, its sorption was strongly induced by hydrophobicity and neutral forms generated at a pH below pKa (Bui and Choi, 2009). Drugs

distribution is consistent with tidal events and proximity of sewage outfalls, in a way that levels in coastal waters may be orders of magnitude lower compared to the source (*e.g.* wastewater; river water) (Kim *et al*., 2017), reflecting the dilution that these compounds undergo in the marine environment after discharge (Vidal-Dorsch *et al*., 2012). As such, concentrations of pharmaceuticals followed the trend higher during ebb and low tide, and minimum during flood and high tides (Lara-Martín *et al*., 2014). A clear negative correlation between drugs concentration and salinity was demonstrated by the decrease in levels of the beta-blocker metoprolol (16 ng L^{-1} upstream to 8 ng L^{-1} downstream) and the antiepileptic carbamazepine (10 ng L^{-1} upstream to 6 ng L^{-1} downstream) over the course of an estuary, which corroborated with longitudinal and vertical mixing of sewage containing freshwater coming from WWTPs (Lara-Martín *et al*., 2014).

Although detected at low levels (ng L^{-1}), these concentrations may be significant to induce different consequences by virtue of combined effects, that can occur independently of a similar or dissimilar MoA (Cleuvers, 2003). Those can be a simple addition of a toxic effect (*i.e.* additive); less than the sum of the separate constituents (*i.e.* antagonism); and effects exerted by the mixture of the components may be significantly higher than the sum of their individual toxic effects (*i.e.* synergism) (Daughton, 2016; Láng and Kőhidai, 2012) (Figure 1.9). Unlike many organic contaminants historically studied (*e.g.* persistent organic pollutants), partitioning dynamics of the majority of pharmaceuticals are not only due to hydrophobic interactions, but are also influenced by hydrogen bonding, cation exchange, cation bridging and surface complexation, which hinder environmental modelling approaches of exposure, uptake (*i.e.* ingestion, dermal exposure, respiration exchange, larval stage hazard), trophic transfer and bioaccumulation (Du *et al*., 2014; Ribeiro *et al*., 2015; Yamamoto *et al*., 2009). Oh *et al. (*2013) addressed that there is a high possibility that hydrophobic drugs may be trapped in sediments in estuaries and marine waters due to high salinity. Binding to organic matter is recognized as one of the dominant transference processes affecting their environmental fate.

The settling of pharmaceuticals, despite removal from the water column and decrease in drugs levels, represent another route of exposure to biota, particularly to benthos. Fine-grained sediments tend to accumulate high amounts of contaminants due to the high sorptive capacity, and as a reservoir provide sediment dwelling species to be constantly

exposed to complex chemical mixtures, either by bulk sediment dermal contact and ingestion, such as through interstitial water. Physical disturbances of sediments may occur during weather events, incoming tides, sediment transport, rainfall and bioturbation by benthic organisms, in addition to dredging, fishing and shipping (Atkinson *et al*., 2007; Eggleton and Thomas, 2004). Those events are widely known to remobilize pharmaceuticals once settled in benthic compartments and transfer them back to the water column (Ferguson *et al*., 2013). More oxidizing conditions to which contaminants are exposed in overlying water could result in desorption and transformation into more bioavailable or toxic species (Eggleton and Thomas, 2004).

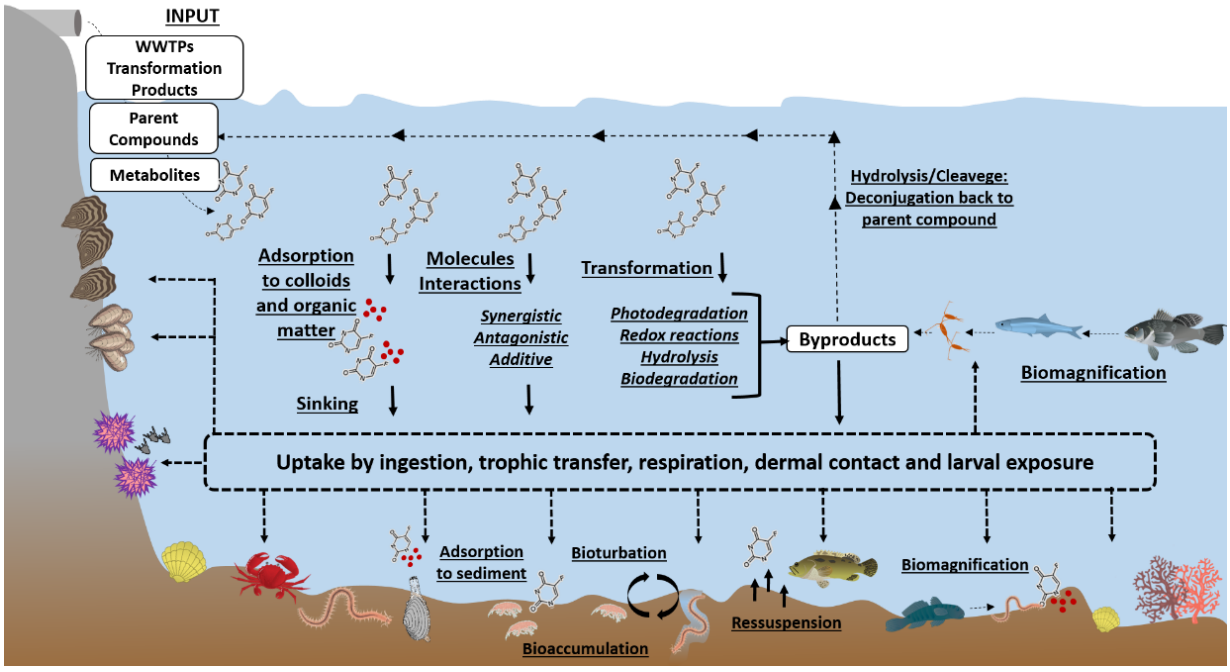


Figure 1.9: Behaviour and fate of anticancer drugs, in conjugation to abiotic and biotic processes, in the marine environment.

1.5.5 Occurrence of anticancer compounds in coastal environments

As an incipient concern, anticancer drugs have been poorly investigated in environmental screenings concerned to pharmaceuticals´ occurrence in coastal and marine zones, with special attention so far destined to the endocrine disruptor applied in breast cancer treatment, tamoxifen. In UK estuaries, tamoxifen was detected at a range between 13

and 212 ng L^{-1} and, due to its high hydrophobicity, it was suggested that its physico-chemical properties may provide its persistence in marine ecosystems (Thomas and Hilton, 2004; Roberts and Thomas, 2006). This compound was also predominant in sediments sampled in the Jamaica Bay (NY, USA) (11.2 ng L^{-1}), where six WWTPs discharge extensive loads of biologically treated sewage (Lara-Martín *et al*., 2015). In the water column, tamoxifen was detected at levels from 141 to 224 ng L^{-1} in sewage-affected sites of the Yangtze River Estuary, and at 120 ng L^{-1} along the shore, whereas in sediment concentrations found were from 30 to 431 ng g^{-1} (Yang *et al*., 2011). Realistic levels of pharmaceuticals were monitored at sampling sites in the Pacific Ocean (from Muir Beach to Monterey Bay – USA), San Francisco Bay (USA), Mediterranean Sea (Israel), Balearic Sea (Ebro estuary and Barcelona), Northern Adriatic Sea (Italy), Northern Aegean Sea (Greece), and tamoxifen was found only at the former location, at a maximum concentration of 93 ng L^{-1} (Nödler *et al*., 2014). Regarding other drugs than tamoxifen, the study of Biel-Maeso *et al. (*2018) showed that the antimetabolite methotrexate end up in the semi-enclosed waters of Cádiz Bay (Spain), at 3.5 ng L^{-1}.

1.5.6 Ecotoxicological effects of anticancer drugs on marine organisms

The occurrence of anticancer drugs in the marine environment triggers concerns about their potential risks to non-target organisms according to the evidence of their unselective MoA towards all eukaryotic cells (Johnson *et al.,* 2013; Heath *et al.,* 2016). Due to the MoA that affect not only highly proliferative cells, but also non-tumour ones, any non-human cells could be vulnerable to biochemical impairments and DNA damage (Zounková *et al.*, 2007; Parrella *et al.*, 2015), with potential effects even at trace concentrations (Bound and Voulvoulis, 2004; Colman *et al*., 2009; Kosjek and Heath, 2011). With regard to hormone therapy, eventual homologies between hormone receptors of humans and non-target organisms may provide binds of drugs to these respective biomolecules and induce antagonist and/or agonist mechanisms that are reflected in alteration of metabolism, homeostatic control or reproduction. Even though non-target aquatic organisms lack specific conserved targets for drug biological effects, interactions via unspecific MoA may operate adversely (Fent *et al*., 2006b; Gunnarsson *et al*., 2008). The application of selected biomarkers to access cytotoxic effects has the potential to offer additional and valuable

information regarding impairments of anticancer drugs on marine non-target organisms. Although biomarkers of exposure and effect may be relatively nonspecific and reflect the influence of multiple environmental stress factors, the evaluation of biochemical and genotoxic alterations are rather consistent findings in biomonitoring of anticancer purposes (Suspiro and Prista, 2011).

Compared with the normal counterparts, most cancer cells have inherently increased amounts of reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), which are crucial to mediate particular signaling pathways in tumour cell growth, proliferation, differentiation, protein synthesis, metabolism and cell survival such as superoxide (Hileman *et al*., 2004; Peng and Gandhi, 2012). The high levels of ROS in cancer cells contributes to cancer-cell proliferation, DNA alterations, apoptosis, metastasis and angiogenesis (Trachootham *et al.,* 2009; Lord and Ashworth, 2012; Gorrini *et al.,* 2013). In contrast, such intensive oxidative stress is counteracted by elevated antioxidant defense mechanisms from tumour cells (Gorrini *et al*., 2013). Therefore, it is important to highlight that chemotherapeutic drugs are designed to exceed cellular ROS levels and overwhelm antioxidant defenses, aiming to induce irreparable damages and subsequently triggering tumour cell apoptosis (Liou and Storz, 2010) (Figure 1.10). Cellular antioxidant mechanisms may be unable to prevent the interference of ROS provided by drugs on critical cellular mechanisms (Conklin, 2004), combined with the inhibition of non-enzymatic molecules (*i.e.* glutathione, flavonoids and vitamins A, C and E) and antioxidant enzymes, as well as the enhancement of lipid peroxidation products (LPO) (Gorrini *et al*., 2013). Cytotoxic therapies are able to block glutathione (GSH) synthesis in order to modulate cancer cell sensitivity to drugs (Cairns *et al*., 2011; Conklin, 2004), since GSH metabolism is actively involved in resistance mechanisms against anticancer compounds (*i.e.* anthracyclines, alkylating agents and platinum-containing drugs). Alterations in the set of the scavenging system (GSH, superoxide dismutase - SOD, and catalase – CAT) and oxidative stress are the first line of action of those drugs, thus, mandatory for assessing further processes that involve cell damage and death. Although anticancer therapies widely aim to prevent cell replication through different cell cycle phases, the MoA based on DNA damage following S phase seems ideal to provide cell cycle arrest and death, since attempts to replicate damaged DNA can cause increased toxicity more directed to replicating cells than to non- replicating ones

(Helleday *et al*., 2008).Platinum-based compounds and alkylating agents are also known to increase ROS to extremely high levels in a way that mitochondrial metabolism is impaired and may lead to caspase activation and cell death, being DNA a critical target for cytotoxicity (Liou and Storz, 2010; Dasari *et al*., 2014). Lesions caused by covalently bindings to DNA bases, such as base alterations, crosslinks and single strand-breaks, may originate chromosomes aberrations and further subsequent malignancies (Lawley and Phillips, 1996, Helleday *et al.,* 2008; Suspiro and Prista, 2011). Cell ability to resist and repair those injuries are widely divergent over the exposed eukaryotic groups (Kondo *et al*., 2010).

Numerous studies were performed with marine species using several classes of pharmaceuticals, by exposing them individually, or as mixtures, prodrugs, metabolites or TPs, *via* water or spiked sediments (Aguirre-Martínez *et al.*, 2013; Gonzalez-Rey and Bebianno, 2014; Aguirre-Martínez *et al.*, 2016a; Pires *et al.*, 2016a, 2016b). Regarding ecotoxicological effects of anticancer agents and its metabolites in marine organisms, studies are still scarce and illustrate a critical discrepancy of more extensive present information in freshwater bioassays (Figure 1.11). Fortunately, even though the quantitative disparity, the quality of acquired data for marine (Table 1.2) and freshwater (Table 1.3) organisms contrast in the analysed endpoints. To date, marine experiments have mainly aimed to observe biochemical analysis, in order to understand the conditions of oxidative stress that occur as responses under chemotherapy administration and corroborate with the prone subtle effects mentioned at relevant levels detected in the environment. The biological responses addressed across different levels of biological organization reinforce the problematic of anticancer agents as emerging contaminants of concern and the need of assessing the risks of priority molecules according to their hazardous impacts (Rowney *et al.*, 2009; Ortiz de García *et al.*, 2014; Lolic *et al.*, 2015).

Table 1.2: Ecotoxicity effects of anticancer drugs in marine species.

Drug	Species	Conc. range ($ng\ L^{-1}$)[a,b]	Time (d)[d]	End-point	Effect[e]	Reference
CisPt	*Mytilus galloprovincialis*	100	4	Oxidative stress, oxidative damage, neurotoxicity, genotoxicity, oxidative damage	(DG, G) AChE, LPO *↑; (G) SOD, T-GPx *↓; (DG) SOD, CAT, T-GPx, GST *↑; LPO *↓	Trombini *et al. (*2016a)
CP	*Anguilla anguilla*	6.5 – 100[a]	2[d]	Sister chromatid exchange	12.5; 25; 50; 100: *↑	Santos and Pacheco (1995)
MET	*Ampelisca brevicornis*	$1 - 10^3$	0	Oxidative stress, oxidative damage, genotoxicity	10^2 and 10^3: EROD, GST, GR, LPO and DNA Damage *↑	Moreira *et al. (*2016)
	Paracentrotus lividus	$10 - 10^9$	[d]	Fertilization	$EC50 = 10^6$	Aguirre-Martínez *et al. (*2016b)
		$10 - 10^9$	2	Larval Development	$EC50 = 1.5 \times 10^6$	
	Isochrysis galbana	$50 - 5\times10^8$	4	Growth inhibition	$EC50 = 8.4 \times 10^7$	Aguirre-Martínez *et al. (*2016b)

	Ruditapes phillipinarum	100 - 5×10^4	14	Oxidative stress, oxidative damage, neurotoxicity, genotoxicity, oxidative damage	10^2: DBF, DNA Damage *↓; 10^3: DBF, DNA Damage *↓, GPx *↑; 10^4: DBF *↓, GPx *↑; 5×10^4: DBF, AChE *↓, GPx *↑	Aguirre-Martínez *et al. (*2016b)
	Paracentrotus lividus	10 - 10^9	1[b]	Fertilization	EC50 = 15×10^6	Aguirre-Martínez *et al. (*2016b)
		10 - 10^9	2	Larval Development	EC50 = 10^9	
		10^{-8} - 10^{-6} M	0.5[d]	Fertilization	10^{-7} M: *↓	Pagano *et al. (*2001)
		10^{-8} - 10^{-6} M	0.5[d]	Offspring Quality	10^{-6} M: *↓	
	Spherechinus granularis	10^{-8} - 10^{-6} M	3	Larval Development	10^{-6} M: Embryotoxicity *↑	Pagano *et al. (*2001)
	Strongylocentrotus purpuratus	Not informed	4	Larval Development	EC50 = 50	Roepke *et al. (*2005)
TAM	*Isochrysis galbana*	1 - 5×10^8	4	Growth inhibition	EC50 = 3.5×10^7	Aguirre-Martínez *et al. (*2016b)
	Ruditapes phillipinarum	100 - 5×10^4	14	Oxidative stress, oxidative damage, genotoxicity, oxidative damage	10^2: EROD, LPO *↑; 10^3 EROD, GR, LPO *↑, GST *↓; 10^4: EROD, LPO *; 5×10^4: EROD, GST, GR, LPO *↑	Aguirre-Martínez *et al. (*2016b)
	Tautogolabrus adspersus	5×10^5 - 5×10^6 [b]	17	Egg production	5×10^5: *↓	Mills *et al. (*2015)

Sparus aurata L.	10^{5} [c]	25	Gene expression	Vitellogenin *↑	García-Hernández *et al.* (2016)
	10^{5} [c]	25	Gene expression	Immune response (ilb1, tnfa, tgfb1,mhc1a, tlr9) *↑	
	10^{5} [c]	25	Sperm concentration	*↑	
	10^{5} [c]	25	Sperm motility	*↑	

- [a] Drug exposure through intraperitoneal injection (mg kg^{-1} body weight);
- [b] Drug exposure through oral gavage (mg kg^{-1} body weight);
- [c] Drug exposure through food intake (mg g^{-1} food);
- [d] Time in hours;
- [e] Unit of concentration effect according to respective concentration range; DG (digestive gland); G (gills)
- CisPt: cisplatin; CP: Cyclophosphamide; MET: methotrexate; TAM: tamoxifen

Table 1.3: Ecotoxicity effects of anticancer drugs in freshwater species.

Drug	Taxon	Species	Concentration Range (mg L^{-1})[a]	Time (d)[b]	Parameter	Endpoint	Concentration of effect (mg L^{-1})	Reference
CisPt	Algae	*Synechococcus leopoliensis*	0.03 - 32	3	Growth Inhibition	EC_{50}	0.7	Brezovsec *et al.* (2014)
		Tetrahymena pyriformis	5 - 70	9[b]	Growth Inhibition	IC_{50}	37.3	Bonnet *et al.* (2003)
		Tetrahymena pyriformis	5 - 70	1[b]	Non-specific esterase activity	IC_{50}	26.5	Bonnet *et al.* (2003)
		Pseudokirchneriella subcapitata	0.1 - 10	4	Growth Inhibition	NOEC	0.1	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.1 - 10	4	Growth Inhibition	LOEC	1	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.1 - 10	4	Growth Inhibition	EC_{50}	2.3	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.05 - 50	3	Growth Inhibition	NOEC	0.5	Brezovsec *et al.* (2014)

	Pseudokirchneriella subcapitata	0.05 - 50	3	Growth Inhibition	LOEC	1	Brezovsec *et al.* (2014)
	Pseudokirchneriella subcapitata	0.05 - 50	3	Growth Inhibition	EC_{50}	1.5	Brezovsec *et al.* (2014)
	Pseudomonas putida	0.016 - 10	16[b]	Growth Inhibition	NOEC	0.03	Zounková *et al.* (2007)
	Pseudomonas putida	0.016 - 10	16[b]	Growth Inhibition	LOEC	0.1	Zounková *et al.* (2007)
	Pseudomonas putida	0.016 - 10	16[b]	Growth Inhibition	EC_{50}	1.2	Zounková *et al.* (2007)
Crustacean	*Daphnia magna*	0.01 - 3.2	2	Immobilization	EC_{50}	≥ 1000	Zounková *et al.* (2007)
	Daphnia magna	0.0001 - 0.03	21	Reproduction Inhibition	NOEC	0.001	Parrella *et al.* (2014a)
	Daphnia magna	0.0001 - 0.03	21	Reproduction Inhibition	LOEC	0.003	Parrella *et al.* (2014a)
	Daphnia magna	0.0006 - 0.1	7	Reproduction Inhibition	NOEC	0.0045	Parrella *et al.* (2014a)
	Daphnia magna	0.0001 - 0.03	21	Reproduction Inhibition	EC_{50}	0.001	Parrella *et al.* (2014a)
	Daphnia magna	0.000001 - 0.1	1	Genotoxicity	LOAEC	0.00001	Parrella *et al.* (2015)
	Daphnia magna	0.000001 - 0.1	1	Genotoxicity	NOAEC	0.000001	Parrella *et al.* (2015)

		Ceriodaphnia dubia	0.0006 - 0.1	7	Reproduction Inhibition	LOEC	0.014	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	0.0006 - 0.1	7	Reproduction Inhibition	EC_{50}	0.016	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	0.00003 - 0.3	1	Genotoxicity	NOAEC	0.00003	Parrella *et al.* (2015)
		Ceriodaphnia dubia	0.00003 - 0.3	1	Genotoxicity	LOAEC	0.0003	Parrella *et al.* (2015)
		Thamnocephalus platyurus	(not informed)	1	Mortality	LC_{50}	8.44	Parrella *et al.* (2014a)
		Brachionus calyciflorus	0.05 - 1	2	Population Growth	EC_{50}	0.4	Parrella *et al.* (2014a)
5-FU	Algae	*Pseudokirchneriella subcapitata*	0.001 - 100	4	Growth Inhibition	NOEC	0.001	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.001 - 100	4	Growth Inhibition	LOEC	0.01	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.001 - 100	4	Growth Inhibition	EC_{50}	0.1	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.004 - 0.13	3	Growth Inhibition	NOEC	0.01	Brezovsec *et al.* (2014)
		Pseudokirchneriella subcapitata	0.004 - 0.13	3	Growth Inhibition	LOEC	0.02	Brezovsec *et al.* (2014)

	Pseudokirchneriella subcapitata	0.004 - 0.13	3	Growth Inhibition	EC_{50}	0.13	Brezovsec *et al.* (2014)
	Pseudomonas putida	0.001 - 10	16[b]	Growth Inhibition	NOEC	0.003	Zounková *et al.* (2007)
	Pseudomonas putida	0.001 - 10	16[b]	Growth Inhibition	LOEC	0.01	Zounková *et al.* (2007)
	Pseudomonas putida	0.001 - 10	16[b]	Growth Inhibition	EC_{50}	0.027	Zounková *et al.* (2007)
	Synechococcus leopoliensis	0.04 - 13	3	Growth Inhibition	EC50	1.2	Brezovsec *et al.* (2014)
Crustacean	*Daphnia magna*	0.01 - 100	2	Immobilization	NOEC	1	Zounková *et al.* (2007)
	Daphnia magna	0.01 - 100	2	Immobilization	LOEC	10	Zounková *et al.* (2007)
	Daphnia magna	0.01 - 100	2	Immobilization	EC_{50}	36	Zounková *et al.* (2007)
	Daphnia magna	0.01 - 2	21	Reproduction Inhibition	NOEC	0.006	Parrella *et al.* (2014a)
	Daphnia magna	0.01 - 2	21	Reproduction Inhibition	LOEC	0.002	Parrella *et al.* (2014a)
	Daphnia magna	0.01 - 2	21	Reproduction Inhibition	EC_{50}	0.02	Parrella *et al.* (2014a)
	Daphnia magna	0.00005 - 5	2	Genotoxicity	NOAEC	0.00005	Parrella *et al.* (2015)
	Daphnia magna	0.00005 - 5	2	Genotoxicity	LOAEC	0.0005	Parrella *et al.* (2015)

		Ceriodaphnia dubia	0.0005 - 0.8	7	Reproduction Inhibition	NOEC	0.002	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	0.0005 - 0.8	7	Reproduction Inhibition	LOEC	0.0067	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	0.0005 - 0.8	7	Reproduction Inhibition	EC_{50}	0.003	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	0.000006 - 0.06	1	Genotoxicity	NOAEC	0.000006	Parrella *et al.* (2015)
		Ceriodaphnia dubia	0.000006 - 0.06	1	Genotoxicity	LOAEC	0.00006	Parrella *et al.* (2015)
	Fish	*Danio rerio*	0.00001 - 0.1	180	Hystopathology		F1 generation: 0.1	Kovács *et al.* (2015)
		Danio rerio	0.00001 - 0.1	180	Genotoxicity		F1 generation: 0.001	Kovács *et al.* (2015)
		Danio rerio	0.00001 - 0.1	180	Gene expression		F1 generation: 0.00001; 0.001	Kovács *et al.* (2015)
		Cyprinus carpio	2.5a	7	Micronucleus frequency		*↑	Grisolia and Cordeiro (2000)
CP	Algae	*Pseudokirchneriella subcapitata*	10 -1000	4	Growth Inhibition	NOEC	250	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	10 -1000	4	Growth Inhibition	LOEC	500	Zounková *et al.* (2007)

		Pseudokirchneriella subcapitata	10 -1000	4	Growth Inhibition	EC_{50}	930	Zounková *et al.* (2007)
		Pseudomonas putida	63 - 10000	4	Growth Inhibition	NOEC	1000	Zounková *et al.* (2007)
		Pseudomonas putida	63 - 10000	4	Growth Inhibition	LOEC	>1000	Zounková *et al.* (2007)
	Crustacean	*Daphnia magna*	6.3 - 1000	2	Immobilization	EC_{50}	>1000	Zounková *et al.* (2007)
	Fish	*Astyanax bimaculatus*	4 - 32[a]	24[b]	Micronucleus frequency		8; 16*↑	Matsumoto and Cólus (2000)
DOX	Algae	*Pseudokirchneriella subcapitata*	0.1 - 63	4	Growth Inhibition	NOEC	1	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.1 - 63	4	Growth Inhibition	LOEC	10	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	0.1 - 63	4	Growth Inhibition	EC_{50}	13	Zounková *et al.* (2007)
		Pseudomonas putida	1 - 1000	16[b]	Growth Inhibition	NOEC	1	Zounková *et al.* (2007)
		Pseudomonas putida	1 - 1000	16[b]	Growth Inhibition	LOEC	10	Zounková *et al.* (2007)
		Pseudomonas putida	1 - 1000	16[b]	Growth Inhibition	EC_{50}	>1000	Zounková *et al.* (2007)
	Crustacean	*Daphnia magna*	0.01 - 10	2	Immobilization	EC_{50}	0.01 - 10	Zounková *et al.* (2007)
		Daphnia magna	0.000002 - 0.02	1	Genotoxicity	NOAEC	0.000002	Parrella *et al.* (2015)
		Daphnia magna	0.000002 - 0.02	1	Genotoxicity	LOAEC	0.00002	Parrella *et al.* (2015)

		Ceriodaphnia dubia	0.000005 - 0.05	1	Genotoxicity	NOAEC	0.000005	Parrella *et al.* (2015)
		Ceriodaphnia dubia	0.000005 - 0.05	1	Genotoxicity	LOAEC	0.00005	Parrella *et al.* (2015)
ETO	Algae	*Pseudokirchneriella subcapitata*	10 - 1000	4	Growth Inhibition	NOEC	<10	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	10 - 1000	4	Growth Inhibition	LOEC	10	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	10 - 1000	4	Growth Inhibition	EC_{50}	250	Zounková *et al.* (2007)
		Pseudokirchneriella subcapitata	3.5 - 350	3	Growth Inhibition	NOEC	10.7	Brezovsec *et al.* (2014)
		Pseudokirchneriella subcapitata	3.5 - 350	3	Growth Inhibition	LOEC	34.3	Brezovsec *et al.* (2014)
		Pseudokirchneriella subcapitata	3.5 - 350	3	Growth Inhibition	EC_{50}	30.4	Brezovsec *et al.* (2014)
		Pseudomonas putida	100 - 1000	16[b]	Growth Inhibition	NOEC	200	Zounková *et al.* (2007)
		Pseudomonas putida	100 - 1000	16[b]	Growth Inhibition	LOEC	250	Zounková *et al.* (2007)
		Pseudomonas putida	100 - 1000	16[b]	Growth Inhibition	EC_{50}	630	Zounková *et al.* (2007)
	Crustacean	*Daphnia magna*	1 - 100	2	Immobilization	EC_{50}	1 - 100	Zounková *et al.* (2007)
		Daphnia magna	1 - 100	1	Mortality	LC50	1.5	Zounková *et al.* (2007)

		Daphnia magna	0.1 - 3	21	Reproduction Inhibition	NOEC	0.1	Parrella *et al.* (2014a)
		Daphnia magna	0.1 - 3	21	Reproduction Inhibition	LOEC	0.3	Parrella *et al.* (2014a)
		Daphnia magna	0.1 - 3	21	Reproduction Inhibition	EC_{50}	0.2	Parrella *et al.* (2014a)
		Daphnia magna	0.00003 - 3	1	Genotoxicity	NOAEC	0.00003	Parrela *et al.* (2015)
		Daphnia magna	0.00003 - 3	1	Genotoxicity	LOAEC	0.0003	Parrela *et al.* (2015)
		Ceriodaphnia dubia	0.03 - 3	7	Reproduction Inhibition	NOEC	0.3	Parrela *et al.* (2014a)
		Ceriodaphnia dubia	0.03 - 3	7	Reproduction Inhibition	LOEC	0.0003	Parrela *et al.* (2014a)
		Ceriodaphnia dubia	0.03 - 3	7	Reproduction Inhibition	EC50	0.1	Parrela *et al.* (2014a)
		Ceriodaphnia dubia	0.00001 - 0.1	1	Genotoxicity	NOAEC	0.00001	Parrela *et al.* (2015)
		Ceriodaphnia dubia	0.00001 - 0.1	1	Genotoxicity	LOAEC	0.0001	Parrela *et al.* (2015)
IMA	Algae	*Pseudokirchneriella subcapitata*	0.120 - 12	3	Growth Inhibition	NOEC	0.38	Brezovsec *et al.* (2014)

	Pseudokirchneriella subcapitata	0.120 - 12	3	Growth Inhibition	LOEC	1.19	Brezovsec *et al.* (2014)
	Pseudokirchneriella subcapitata	0.120 - 12	3	Growth Inhibition	EC_{50}	2.29	Brezovsec *et al.* (2014)
	Synechococcus leopoliensis	1 - 16	3	Growth Inhibition	EC_{50}	5.3	Brezovsec *et al.* (2014)
Crustacean	*Daphnia magna*	0.018 - 3	21	Reproduction Inhibition	NOEC	0.003	Parrela *et al.* (2014a)
	Daphnia magna	0.018 - 3	21	Reproduction Inhibition	LOEC	0.009	Parrela *et al.* (2014a)
	Daphnia magna	0.018 - 3	21	Reproduction Inhibition	EC50	0.3	Parrela *et al.* (2014a)
	Daphnia magna	0.0002 - 0.2	1	Genotoxicity	NOAEC	0.0002	Parrela *et al.* (2015)
	Daphnia magna	0.0002 - 0.2	1	Genotoxicity	LOAEC	0.002	Parrela *et al.* (2015)
	Ceriodaphnia dubia	0.0005 - 10	7	Reproduction Inhibition	NOEC	0.00037	Parrella *et al.* (2014a)
	Ceriodaphnia dubia	0.0005 - 10	7	Reproduction Inhibition	LOEC	0.0009	Parrella *et al.* (2014a)

		Ceriodaphnia dubia	0.0005 - 10	7	Reproduction Inhibition	EC50	0.1	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	0.00003 - 0.03	1	Genotoxicity	NOAEC	0.00002	Parrela *et al.* (2015)
		Ceriodaphnia dubia	0.00003 - 0.03	1	Genotoxicity	LOAEC	0.0002	Parrela *et al.* (2015)
	Fish	*Danio rerio*	0.00029 - 0.15	3	Cell Viability	IC50	0.004	Novak *et al.* (2016)
		Danio rerio	0.000001 - 0.001	1	Genotoxicity	LOEC	1	Novak *et al.* (2016)
CAP	Crustacean	*Daphnia magna*	1 - 400	21	Reproduction Inhibition	NOEC	6.1	Parrella *et al.* (2014a)
		Daphnia magna	1 - 400	21	Reproduction Inhibition	LOEC	1.9	Parrella *et al.* (2014a)
		Daphnia magna	1 - 400	21	Reproduction Inhibition	EC_{50}	0.02	Parrella *et al.* (2014a)
		Daphnia magna	0.0025 - 2.25	1	Genotoxicity	NOAEC	0.002	Parrella *et al.* (2015)
		Daphnia magna	0.0025 - 2.25	1	Genotoxicity	LOAEC	0.02	Parrella *et al.* (2015)
		Ceriodaphnia dubia	2.5 - 200	7	Reproduction Inhibition	NOEC	0.6	Parrella *et al.* (2014)

		Ceriodaphnia dubia	2.5 - 200	7	Reproduction Inhibition	LOEC	1.9	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	2.5 - 200	7	Reproduction Inhibition	EC_{50}	2.4	Parrella *et al.* (2014a)
		Ceriodaphnia dubia	0.012 - 120	1	Genotoxicity	NOAEC	0.012	Parrella *et al.* (2015)
		Ceriodaphnia dubia	0.012 - 120	1	Genotoxicity	LOAEC	0.12	Parrella *et al.* (2015)
TAM	Crustacean	*Daphnia pulex*	0.00012 - 5.2	14	Reproduction Inhibition	NOEC	0.00067	Borgatta *et al.* (2016)
		Daphnia pulex	0.00012 - 5.2	14	Morphological abnormalities	NOEC	0.00012	Borgatta *et al.* (2016)
		Daphnia magna	1 - 10	1	Immobilization	EC_{50}	1.5	DellaGreca *et al.* (2007)
		Ceriodaphnia dubia	1 - 10	7	Population Growth Inhibition	EC_{50}	0.0008	DellaGreca *et al.* (2007)
		Thamnocephalus platyurus	1 - 10	1	Mortality	LC_{50}	0.4	DellaGreca *et al.* (2007)
	Rotifera	*Brachionus calyciflorus*	1 - 10	2	Population Growth Inhibition	EC_{50}	0.25	DellaGreca *et al.* (2007)
		Brachionus calyciflorus	1 - 10	1	Mortality	LC_{50}	1	DellaGreca *et al.* (2007)

	Fish	*Oryzias latipes*	0.001 – 0.625	21	Hatchability		0.125; 0.625: *↓	Sun *et al.* (2007)
		Oryzias latipes	0.001 – 0.625	21 d	Fecundity		0.625: *↓	Sun *et al.* (2007)
		Oryzias latipes	0.001 – 0.625	21 d	Fertility		0.625: *↓	Sun *et al.* (2007)
		Oryzias latipes	0.001 – 0.625	21 d	Vitellogenin		0.025: *↓ ♀	Sun *et al.* (2007)
		Oryzias latipes	0.001 – 0.625	21 d	Vitellogenin		0.001 *↑ ♂	Sun *et al.* (2007)
		Pimephales promelas	0.01 - 8[a]	7	Vitellogenin	LOEC	(♀) 0.1 ↑; 8 ↓	Chikae *et al.* (2004)
		Pimephales promelas	0.01 - 8[a]	7	Vitellogenin	LOEC	(♂) 0.1 ↑	Chikae *et al.* (2004)
		Pimephales promelas	0.00018 - 0.018	42	Vitellogenin	NOEC	0.00018	Williams *et al.* (2007)
VIN	Fish	*Astyanax bimaculatus*	4 - 32	24[b]	Micronucleus frequency		8; 16; 32*↑	Matsumoto and Cólus (2000)
		Cyprinus carpio	20	2, 7, 14, 30	Micronucleus frequency		*↑	Grisolia and Cordeiro (2000)
BLEO	Fish	*Cyprinus carpio*	12.5[a]	48[b]	Micronucleus frequency		*↑	Grisolia and Cordeiro (2000)

- 5-FU: 5-fluorouracil; DOX: doxorubicin; ETO: etoposide; IMA: imatinib; CAP: capecitabine; VIN: vinblastine; BLEO: bleomycin.

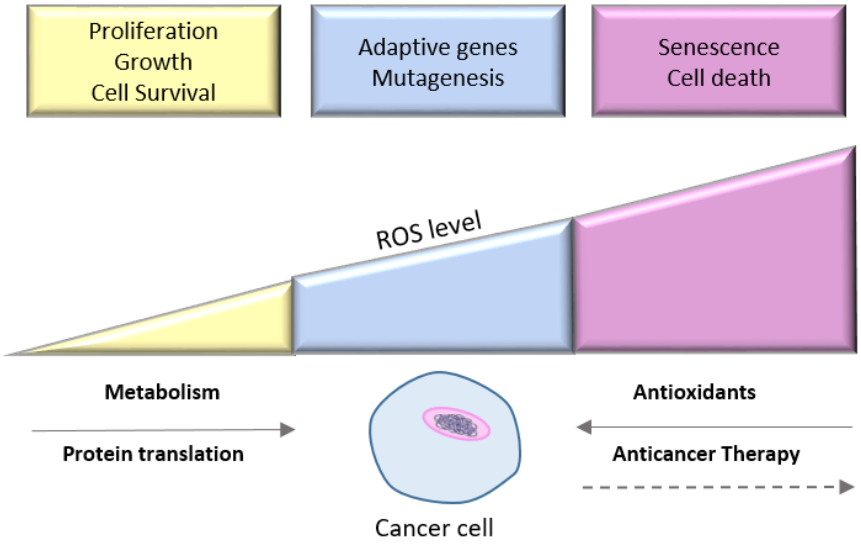


Figure 1.10: Induction and scavenging of ROS levels in cancer cells: Balance between basal (yellow) and excessively high ROS levels (pink), into moderate and ROS-mediated mutagenic events (blue).

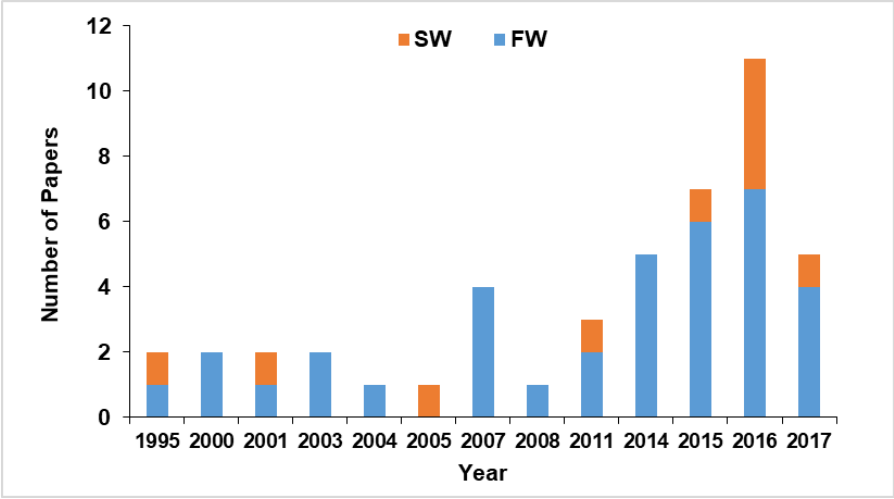


Figure 1.11: Number of papers published per year (Until December 2017) regarding ecotoxicological data of anticancer drugs, considering bioassays with freshwater (FW) and seawater (SW) organisms.

1.6 Environmental Risk Assessment of anticancer drugs

1.6.1 Efforts in the marine environment

An Environmental/Ecological Risk Assessment (ERA) framework is currently considered as the best scientifically approach to evaluate the potential effects of a wide array of chemicals (*e.g.* industrial and pesticides) and stressors on communities and ecosystems (Tarazona *et al.,* 2010). It consists of a set of tools to identify the likelihood for effects and characterize the nature and magnitude of risks to human and ecological health, thus able to help risk managers and decision makers to define protection measures (USEPA, 2001; Tarazona *et al.,* 2010). To date, pharmaceuticals have been evaluated in much the same manner of traditional chemical compounds assessed under a risk spectrum. However, several special aspects must be considered when applying the ERA framework to pharmaceuticals. These include, a range of physical and chemical attributes that influence the behaviour and fate of the compounds in the environment, besides the potential biological activities driven by their respective MoAs.

For this reason, the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency created a guideline for the ERAs of medicinal products for human use, based on a step-wise tiered ERA approach able to reduce the uncertainty of risk to a level deemed acceptable to make a regulatory decision (EMA, 2006; Williams, 2007; Grung *et al*., 2008). Several draft versions of an ERA guidance have been produced by the European Directive, since 2001, with further scientific revisions and public consultation until the release of a final guidelines. Nevertheless, ERA procedures proposed for pharmaceuticals still have some limitations regarding the targeted concerns. Its methodology is exclusively required for pharmaceutical molecules under marketing authorization and excludes compounds released in the market before its publication (Carlsson *et al*., 2006; Besse *et al*., 2012). In addition, while ERA of conventional pharmaceutical compounds covers sewage works and freshwater compartments, the environmental risk in marine ecosystems is still not addressed, with even less attention directed pharmaceuticals bound to sediments (Aguirre-Martínez *et al*., 2016b). The Marine Strategy Framework Directive of the European Union (Directive 2008/56/2008) (European Comission, 2008) aims to protect,

by 2020, the coastal resources based upon marine-related economic and social activities according to the particular features of each European region, that should identify and assess pharmaceuticals as a predominant pressure. The monitoring of prioritized pharmaceuticals and relevant metabolites in coastal aquatic resources is also part of the sustainable development goals (SDGs), established by the Agenda 2030 of the United Nations (Osborne *et al*., 2015). Pharmaceuticals concentrations surveys used to assess risk may be achieved either by mathematical models, compiling hydrological properties of catchments, chemical emissions profiles and underlying assumptions used in fate and distribution calculations, or by monitoring data, reflecting the real environmental complexity, hydrodynamic and chemical processes (Williams *et al.*, 2007).

In the first phase of the two-tiered ERA approach (Figure 1.12), the Predicted Environmental Concentration (PEC) of pharmaceuticals molecules in the aquatic environment is calculated based on its persistence, bioaccumulation and toxicity of the compounds (Koschorreck and Hickmann, 2008). But PEC only provides a rough insight of the overall situation at a national or regional level because it accounts for the following parameters: annual drug consumption ($mg.year^{-1}$), rates of metabolism and excretion from patients, dilution factors from effluents, predicted drug partitioning and susceptibility to biotransformation/degradation (Zhang *et al.*, 2013). Therefore, the array of molecules prone to be present in the final effluent and likely to be released into receiving waters are considered for prioritization of potential risks, prior to implement a monitoring program (Johnson *et al*., 2008; Besse *et al*., 2012; Booker *et al*., 2014; Isidori *et al*., 2016; Santos *et al*., 2017). Although PECs play an important role for decision-making, with acceptable consistency, they may induce a slight overestimation and provide unreal and "worst-case scenarios" (Steger-Hartmann *et al*., 1996; Coetsier *et al*., 2009; Zhang *et al*., 2013). Instead, measured environmental concentrations (MECs) in water bodies (Buerge *et al*., 2006; Coetsier *et al*., 2009) represent a more realistic approach for performing a reliable ERA (Blasco and Delvalls, 2008). For both predicted and measured screening approaches, a wide knowledge regarding pharmaceuticals inputs in the environment is required. It encompasses the form in which they are released, their removal and transformation, as well as their pathways and transport that ultimately determine their concentration in different environmental compartments. If PEC or MEC is below 10 ng L^{-1}, according to EMA

(Europe), or to 1 µg L^{-1} according to FDA (USA), the assessment stops because it is assumed that no environmental risk is expected (USFDA, 1998; Koschorreck and Hickmann, 2008). However, due to the ecotoxicological responses triggered by the environmental levels of anticancer drugs, the threshold criteria set up might be unrealistic to protect the marine environment. Lists of priority anticancer drugs were established based on PEC and MEC and indicate that the following drugs may pose risk to surface waters: the alkylating agents ifosfamide and cyclophosphamide; the antimetabolites capecitabine and methotrexate; the tyrosine kinase inhibitor imatinib; the antiandrogen bicalutamide; methotrexate; and the antiestrogen tamoxifen (Besse *et al.,* 2012; Aguirre-Martínez *et al.,* 2016b).

If levels are equal or higher than the recommended threshold, Phase II of the tiered-approach is required, and should include environment fate and effects of cytotoxic compounds (Toolaram *et al*., 2014). Screening data of active and metabolites of anticancer drugs occurrence are compiled to ecotoxicity data, based on the lowest concentration of the compound accountable for adverse effects on wildlife (*i.e.* Predicted No Effect Concentration, PNEC) (Schowanek *et al*., 2001; Länge and Dietrich, 2002). Calculation of PNEC is often difficult for most of those pharmaceutical compounds once ecotoxicological data is still too scarce on acute and chronic toxicity tests. Extrapolation of acute responses to predict chronic ones are performed, depicting an unrealistic paradigm, because low concentrations and long-term exposures are likely to occur in the environment, thus limiting efficient risk assessment and robust outcomes (Claessens *et al*., 2013). The European Medical Agency proposes that PNEC should be estimated from chronic assays with organisms of each trophic level (algae, aquatic invertebrates and fish), and the lowest value should be used for risk characterization. Despite subtle effects have been registered, together with acute endpoints, most of the studies have been performed with general toxicity responses, rather than a specific endpoint corresponding to the drug´s MoA (Bound and Voulvoulis, 2004; Ferrando-Climent *et al*., 2014).

Further, ERA demands an estimation of the hazard quotient (PEC or MEC/PNEC) for the respective environmental compartment. If the ratio is <1, interpretation relies on an environmental concentration lower than levels resulting in adverse effects for wildlife. Alternatively, if the ratio PEC or MEC/PNEC is >1, levels present in the environment exceed

those prone to harm biota, subsequent approaches must be taken to analyze risk-management options (Grung *et al*., 2008).

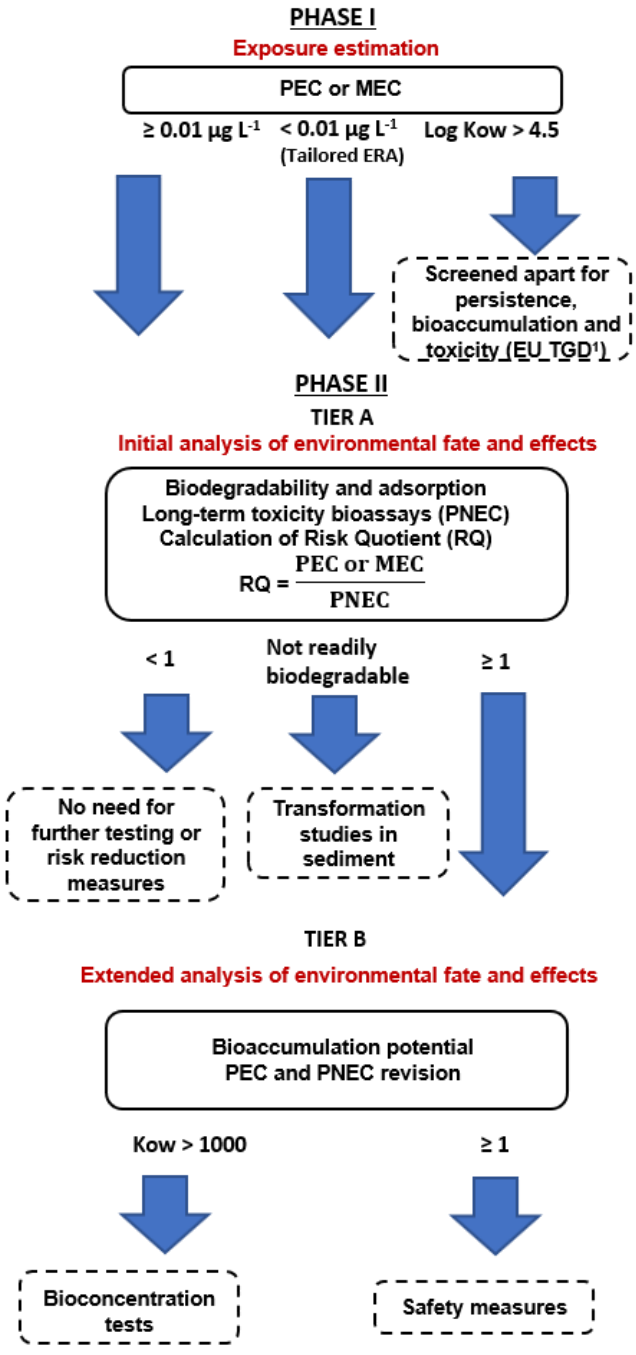


Figure 1.12: Flow chart for the ERA (Environmental Risk Assessment) based on the European Medicines Agency Guideline (EMA, 2006).

[1]European Chemicals Bureau (ECB, 2003) Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances.

1.6.2 The ERA approach involving cytotoxic drugs

Anticancer drugs act by a distinctive MoA, by generating a highly stressful cellular condition via directly or indirectly interaction with DNA, interference on mechanisms and biomolecules of cellular protection and disruption of cell cycle, at last, aiming to interrupt cell proliferation and provide cell death. Moreover, since acting in cell factors and DNA structure and function, anticancer drugs possess mutagenic, cytotoxic, genotoxic, teratogenic and carcinogenic properties that generally ensure the absence of safe doses in laws and environmental regulations, as well as the unfeasibility to estimate threshold levels for lowest effect concentrations (Kosjek and Heath, 2011). In other words, even admitted as a therapeutic class of pharmaceuticals, anticancer agents are, foremost, chemicals and metabolites with carcinogenic or mutagenic potential, which load into the wastewater system are formally banned by the European Community (ECHA, 2008).

Although in most cases genotoxicity of chemicals is a criterion of exclusion for legal market approval (and thus use), pharmaceuticals may be genotoxic and carcinogenic, and are not always completely unavoidable in consumer products. Environmental safety data for anticancer drugs is limited and uncertainties and gaps in the ERA of some anticancer drugs are attributed to the few ecotoxicological data available (Lenz *et al*., 2007a; Besse *et al*., 2012; Toolaram *et al*., 2014). Genotoxicity and cytotoxicity may be triggered by long-term exposure to very low levels of chemicals and have a hereditary and delayed-onset nature that may lead to major consequences at the population level (Llorente *et al*., 2012). Approaches to evaluate the risks associated with this class of compounds to marine species must be developed and incorporated in official protocols for monitoring and determining the maximum permitted levels of anticancer drugs in the environment. A set of genotoxicity bioassays is of particular importance for this kind of assessment but, currently, only mutagenicity tests performed with micronucleus in human lymphocytes, bacterial reverse mutation test conducted with *Salmonella typhimurium* (Ames test) and *Escherichia coli* (SOS chromotest) are standardized (Giuliani *et al*., 1996; Yasunaga *et al*., 2006). It is important to bear in mind that eukaryotic cells cover a broader range of genetic effects than prokaryotic cells, and interpretations are likely to be limited when ERA is based only in tests conducted with bacteria.

1.7 Tropical and temperate marine species

Frameworks and guidelines regarding ERA and monitoring of pharmaceuticals are concentrated in United States and in northern or central European countries, although limited information is available in Mediterranean countries (Aguirre-Martínez *et al*., 2015). In spite of this, there is a marked absence of legislation about this issue in developing regions, as notably Africa, South America and small island nations in Oceania, particularly concerning the marine environment (Bayen *et al*., 2013; Gaw *et al*., 2014). Urbanization pace is accelerating in low-income regions, which are now in the stage of relocation of global pharmaceutical industries (*e.g.* Asian countries). Moreover, pharmaceutical and chemical industries are normally not enforced to comply to environmental regulations, resulting in growing trends of environmental pollution, which include a vast array of pharmaceuticals released in the marine environment through untreated domestic sewage (Rehman *et al*., 2015; Hussain *et al*., 2016; Ashfaq *et al*., 2017). Consequently, concentrations of pharmaceutical compounds are several hundred times higher in aquatic compartments of developing countries than in other regions (Larsson *et al*., 2007; Rehman *et al*., 2015; Ashfaq *et al*., 2017), which may lead to a more severe environmental toxicity (Rehman *et al*., 2015). Such issue is relevant considering that most of those vulnerable nations reside in sub-tropical and tropical climates (Dyer *et al*., 1997; Kwok *et al*., 2007). Despite these regions have shown increasing efforts to detect or estimate environmental concentrations of pharmaceuticals, there is still a paucity of studies describing toxicological endpoints associated with their occurrence (Bayen *et al*., 2013).

In general, ecotoxicological tests conducted with tropical and subtropical species are much less developed than those from temperate regions (Adams and Stauber, 2008). That paucity of tropical dataset has contributed to an environmental quality criteria of developing countries relied on extrapolations from evidences generated in developed countries, based on temperate and coldwater species endemic from Europe and North America (Kwok *et al*., 2007). Therefore, it becomes questionable whether such extrapolation from temperate to tropical counterparts is reliable for regulation standards and decision-making (Peck *et al*., 2002; Wang and Leung, 2015), along with the estimate of toxicity information of freshwater species to protect saltwater species (Leung *et al*., 2014; Wang *et al*., 2014). Differences on relative sensitivity between coastal tropical and temperate species may be attributed to a variety of parameters, major characterized by temperature and salinity-related influences in

chemical bioavailability, uptake and detoxification metabolism (Daam and Van Den Brink, 2010). Those mechanisms may operate as species- and chemical-specific and are not easy to decouple once tropical organisms will always experience higher temperatures and salinities, optimum for their health status (Dyer *et al.*, 1997; Wang *et al.*, 2014). Previous findings with saltwater species indicated that chemical sensitivity is not solely explained by those intrinsic physico-chemical parameters, but also by species composition of the testing taxa (Wang *et al.*, 2014). Temperate saltwater species are more susceptible to un-ionised ammonia, chromium, lead, nickel and tributyltin, whereas tropical species are more sensitive to copper, zinc, mercury, pentachlorophenol and phenol (Kwok *et al.*, 2007; Wang *et al.*, 2014). Hence, temperate data cannot be used to assure suitable environmental protection for tropical or even to polar regions (Chapman *et al*., 2006). The need of proper risk assessments for contaminants under tropical conditions has long been recognized (Daam and Van Den Brink, 2010). Thus, the development of studies regarding the effects induced by pharmaceuticals on tropical marine species is required. This type of study could produce information on the early warning responses caused by pharmaceuticals in the marine environment (Bayen *et al*., 2016). Marine ecotoxicity testing with anticancer drugs is in its infancy and elucidations about sensitivity and biological responses are still lacking in datasets. Knowledge gaps need to be filled for indigenous species from tropical and temperate coastal zones to accomplish future regulatory purposes demanding PNECs.

1.8 Selected biological models

1.8.1 Polychaete *Nereis diversicolor*

Polychaetes constitute the major class of the phylum Annelida, and the family Nereididae is among the most diverse group, comprised by over 43 genera and 540 species (Bakken and Wilson, 2005). These organisms are most common in intertidal soft-sediments of coasts of the North Atlantic of America, North Africa and Northern of Europe (Fidalgo and Costa *et al*., 2006). They are found in shallow marine habitats distributed from the estuaries to the deep sea, and in some freshwater systems. These sediment-dweller organisms exert impact on physical, chemical and biological properties of the marine benthos, through habits of burrowing that contribute to sediment reworking (Gillet *et al*., 2008) and transport

of chemicals from deeper layers of sediments to the water column interface. Hence, they change the organic matter status and metabolism of surrounding microbiota, besides providing absorption of resuspended contaminants *via* uptake by other epibenthic species (Moreira *et al*., 2006; Gillet *et al*., 2008).

The ragworm *Nereis* (*Hediste*) *diversicolor* (O. F. Muller) is recognized as an ecological key-species in soft bottom communities, behaving as a deposit and filter feeder, organic matter and detritus scavenger (Solé *et al*., 2009). Besides of its ecological relevance, *N. diversicolor* comprises an important component of monitoring programs. Due to its low mobility and tolerance to inhabit contaminant-rich sediments, *N. diversicolor* is subjected to chronic exposure of environmental disturbances (Dean, 2008; Lewis and Watson, 2012), and contributes as a pathway of accumulation of contaminants into higher levels of the food chain (Scaps, 2002; Coelho *et al*., 2008). Therefore, increasing research has been conducted with this species aiming to evaluate behavioural, biochemical and genotoxic alterations as part of an environmental quality assessment and field monitoring (Saiz-Salinas and Francés-Zubillaga, 1997; Mouneyrac *et al*., 2003; Moreira *et al*., 2006; Poirier *et al*., 2006; Durou *et al*., 2007b; Solé *et al*., 2009; Gomes *et al*., 2013). Moreover, laboratory-controlled exposures are increasing, and so far they have been used to test the toxicity of trace metals (Bonnard *et al*., 2009; Neave *et al*., 2012), polycyclic aromatic hydrocarbons (PAHs) (Catalano *et al*., 2012), nanoparticles (Cong *et al*., 2011; García-Alonso *et al*., 2011; García-Negrete *et al*., 2013; Buffet *et al*., 2014a, 2014b; Cong *et al*., 2014; Moschino *et al*., 2014; Thit *et al*., 2015 and to a less extent, pharmaceuticals (Maranho *et al.*, 2014, 2015; Pires *et al.*, 2016a, 2016b).

1.8.2 Polychaete *Scolelepis squamata*

The polychaetes *S. squamata* (Muller, 1806) belong to the family Spionidae, which depict significant biomass of macrobenthic fauna in intertidal zones of sandy beaches, as well as in deep sea environment (Amaral *et al.*, 2006). They possess a vast worldwide occurrence from a latitudinal range of 58 °N to 35 °S (Maria *et al.*, 2011). Individuals can reach lengths of about 80 mm (Pardo and Amaral, 2004). This species is considered a deposit feeder, feeding at the sand-water interface using the palps or directly with the mouth, although it can act as a suspension feeder (Dauer, 1983). Like *N. diversicolor*, burrowing presents an ecological relevance in modifying the physico-chemical properties of the marine

benthos (Speybroeck *et al.*, 2007). Previous studies established that *S. squamata* opportunistically changes its feeding behaviour according to environmental conditions, and in the intertidal zone feeds predominantly on suspended matter (Pardo and Amaral, 2004; Dean, 2008). The individuals of *S. squamata* built temporary vertical tubes and occasionally burrow to a depth of 40 cm below the sediment surface (Maria *et al*., 2011). Moreover, those features are likely important to select this species as a bioindicator, particularly along the Southeastern to Southern Brazilian coast, where there is a high abundance, particularly in sandy beaches in São Paulo and Rio de Janeiro areas (Pardo and Amaral, 2004; Amaral *et al.*, 2006).

1.8.3 Sea urchin *Echinometra lucunter*

Echinometra is the most ubiquitous and abundant shallow-water sea urchin in the tropics, from North Carolina to Southern Brazil, inhabiting beds of seagrass, on hard bottoms covered with algae, rock, shell hash or sand (McClanahan and Muthiga, 1998). The species *E. lucunter* has two basic feeding modes, based on algal drift and benthic grazing, although may occasionally consume benthic animals, such as sponges and corals. Besides consuming a large fraction of the benthic primary production, *E. lucunter* can erode reef substratum, reducing its complexity and suitability to other coral reef species. Yet, about half of the food consumed will not be absorbed and becomes detritus. Several marine fishes and invertebrates, as well as shorebirds, feed on *Echinometra* in the intertidal zone (McClanahan and Muthiga, 2007).

Altogether, this species exerts important roles as driver of disturbance and marine community structure. Specimens are commonly reported from shallow waters between the average low tide and depths of 10 m. Temperature and dissecation are the main drivers influencing their distribution and abundance in the upper depth limit, whereas the lower one is represented by predation (Arakaki and Uehara, 1991; Beddingfield and McClintock, 2000). Since embryonic and larval development stages of sea urchin are sensitive to chemical compounds present in seawater, they become suitable organisms for testing developmental, reproductive and cytogenetic effects of marine pollution (Bottger and Mcclintock, 2001; Pusceddu *et al.*, 2007; Maranho *et al.*, 2010; Sousa *et al.*, 2014; Pusceddu *et al.*, 2018).

1.8.4 Amphipod *Tiburonella viscana*

Components of the Amphipoda Order comprise the largest group of peracarid crustaceans, containing more than 9000 species worldwide (Hughes and Ahyong, 2016). Amphipod crustaceans are extremely widespread, occurring from the abyssal depths, throughout the ocean and extending into freshwater and groundwater. Overall, amphipods are relevant in structuring benthic communities, excluding members of other groups by preying upon or disrupting settling larvae, modifying sediment stability and space availability for burrowers (Grant, 1981). Unlike many crustaceans, amphipods do not have a free larval phase, and by brooding their offspring, amphipods can exclude larger and competitive superior species through preying upon settling larvae and newly settled juveniles (Highsmith and Coyle, 1991). Amphipods are trophically diverse and include scavengers, detritivores, filter feeders, predators, and herbivores; whilst they can constitute a large component of the diet of polychaetes, nemerteans, crustaceans, fish, birds, and benthic feeding mammals (Hughes and Ahyong, 2016). Besides their ecological role in community structure, several species of amphipods are standardized for sediment toxicity tests, although sensitivity to pollution may be species dependent (Afli *et al*., 2008). The species *T. viscana* (Barnard, 1964) present important features as bioindicators, such as ease collection, a short life cycle, low dispersion and mobility, direct contact with sediments due to burrowing habit, and a wide sensitivity to pollutants (Abessa *et al.,* 1998; Melo and Nipper, 2007; Maranho *et al.,* 2010; Buruaem *et al.,* 2013; Sousa *et al.,* 2014). *T. viscana* occurs in the Brazilian coast between 23.5° and 25.03° S, inhabiting intertidal zones of sandy beaches, associated with seagrass banks, until 21m depth (Wakabara *et al*., 1991; Melo and Nipper, 2007).

1.9 Selected Drugs

The pharmaceuticals elected for the toxicity assessment in the present thesis were: cisplatin, cyclophosphamide and tamoxifen. They were chosen according to a combination

of criteria regarding their consumption and environmental occurrence. Despite the increase in the number and quality of innovative new drugs currently released in the pharmaceutical market (Aitken and Kleinrock, 2015), these three drugs have been traditionally applied during decades in single and combinatory chemotherapies worldwide, thus with more clinical information available and knowledge about its MoA. Table 1.4 indicates some relevant physico-chemical properties that provide insights regarding the solubility and lipophilicity of compounds (Emadi *et al*., 2009; Dasari and Tchounwou, 2014; Shagufta, 2018). Since occupational exposure to these drugs provide hazards to human health, they were outlined to yield unselective cytotoxic risks once ending up in the aquatic environment (Xie *et al.*, 2012; Toolaram *et al*., 2014). In this sense, environmental screenings including anticancer pharmaceuticals, cisplatin, cyclophosphamide and tamoxifen were targeted as causing environmental risk due to their presence in aquatic compartments (Lenz *et al.*, 2005; Zuccato *et al.*, 2005; Tauxe-Wuersch *et al.*, 2006; Johnson *et al.*, 2008; Orias *et al.*, 2015). However, research gaps in toxicity to coastal and marine organisms, from tropical and temperate zones, are still a caveat in the current knowledge.

1.9.1 Cisplatin

Platinum-based compounds are widely applied in cancer therapy to treat solid tumours, mainly ovarian, colorectal, head and neck and non-small cell lung. The remarkable anticancer property of platinum agents relies on the induction of ICLs (Deans and West, 2011). They are formed by a covalent linkage between nucleotide residues situated in bases of opposite strands, avoiding the separation of DNA double strands during replication (Deans and West, 2011). Although ICLs represent only 5% of bindings generated by platinum agents, their severe genotoxic lesion, usually irreparable, efficiently lead to cell death (Zamble and Lippard, 1995).

Cisplatin (CisPt) (also denominated cis-diamminedichloroplatinum (II)) is the major platinum crosslinking drug, firstly approved by FDA in 1978 (Dasari *et al*., 2014). CisPt is composed of two strong amine ligands interacting with the platinum ion, and two chloride ligands, which are the functional groups that allow platinum ion to bind to DNA bases (Florea and Büsselberg, 2011). Following intravenous administration, 90% of the cisplatin binds with biomolecules and is distributed to tissues, particularly liver, prostate and kidney

(Visacri *et al*., 2017). CisPt is primarily excreted by kidneys, although removal of the drug through urine is incomplete, with approximately 25 to 50 % eliminated after 5 days of intravenous administration (Visacri *et al*., 2017). Overall, administration of cisplatin per course varies from 20 to 200 mg m^{-2} (Vermorken *et al*., 1986). The average values of parent cisplatin excreted in urine in the first 12 h, 12–24 h and 24–48 h were 42 %, 9.4 %, and 9% of dose administered (Gullo *et al*., 1980; Visacri *et al*., 2017). CisPt undergo aquation at greatly lowered concentrations of chloride (4–20 mM), where one or two chloride ligands are displaced by water molecules (Kartalou and Essigmann, 2001; Ma *et al.*, 2015). This mechanism provides cisplatin activation just after its entrance in the cell through the generation of mono and diaquacisplatin reactive metabolites, with high electrophilic potential and able to attack nucleophilic biomolecules as DNA. CisPt is known to increase ROS to extremely high levels in a way that mitochondrial metabolism is impaired and may lead to caspase activation and cell death (Liou and Storz, 2010; Dasari and Tchounwou, 2014).

1.9.2 Cyclophosphamide

The advent of chemotherapy fundamentals emerged at the first four decades of the 20^{th} century. Breakthrough advances undertaken in World War II after an accidental spill of sulfur mustards leading to the markedly depletion of both bone marrow and lymph nodes in men exposed to those chemicals, following discoveries of potential anticancer therapeutic (Huang and Li, 2013). In this course, cyclophosphamide (CP) was developed as a mustard prodrug with cytotoxic and alkylating purposes (Emadi *et al*., 2009).

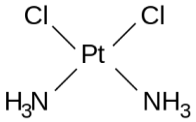
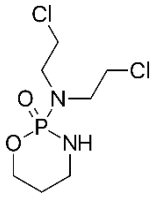
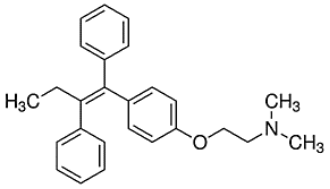
The CP dosage commonly administered in humans varies widely depending on the clinical indication, and can be defined as low (1–3 mg kg^{-1} or 40–120 mg m^{-2}), daily orally administered; intermediate (15–40 mg kg^{-1} or 600–1,500 mg m^{-2}), via intravenous, every 3 to 4 weeks; and high (> 120 mg kg^{-1} or > 5,000 mg m^{-2}), every 2 days. After consumption, CP undergoes subsequent activation and transformation by the CYP 450 enzymes to yield the major cytotoxic species activation, the phosphoramide mustard (PAM) and acrolein. These species form labile covalent DNA adducts and inter-strand crosslinks, accountable for blocking DNA replication and avoiding cell proliferation (Lawley and Phillips, 1996).

According to Bagley *et al. (*1973), not more than 20% of injected CP is excreted intact in urine, at any dose level.

1.9.3 Tamoxifen

To date, the chemotherapeutic drug with more ecotoxicological information available is the selective estrogen-receptor modulator (SERM) tamoxifen (TAM). TAM is a SERM of the triphenylethylene chemical family which possess the ability to antagonize the proliferative action of estrogen through competitive binding to its respective receptor (Goldstein *et al*., 2000). TAM has become the most prescribed drug in hormone therapy of primary and recurrent breast cancer around the world, irrespective of menopausal state (Kiyotani *et al*., 2012). It has shown extensively success on overall survival in clinical trials in women whose tumours are estrogen receptor-positive (HER+) and effective in therapies of metastic breast cancer (Mueller and Korach, 2001; Lim *et al*., 2018). Its MoA is based on stable complex binding with receptors of 17 β-estradiol (Yang *et al*., 2015). The drug has estrogen receptor antagonist properties, with no activation of the receptor to which it is attached, and finally counteracts the proliferative mechanism of oestrogen. Nevertheless, it also has an agonist action in other tissues, with fully activation of the binding receptor (Mater *et al*., 2014; Ma *et al*., 2015). TAM prodrug is metabolized into two more potent molecules, 4-hydroxy-N-desmethyltamoxifen (endoxifen) and 4-hydroxytamoxifen (Besse *et al*., 2012).

Table 1.4: Physico-chemical characteristics of the drugs investigated.

Properties	Cisplatin	Cyclophosphamide	Tamoxifen
Molecular Structure			
CAS Number	15663-27-1	6055-19-2	54965-24-1
pKa	5.3 - 7.2	2.3 - 11.1	8.8 (Basic)
Log Kow	-2	0.6	6.3
Water Solubility	4×10^4 mg L^{-1}	3×10^3 mg L^{-1}	17 mg L^{-1}

1.10 Aim of the thesis

The pseudo-persistence of pharmaceutical compounds in the aquatic environment is an ongoing worldwide problem, requiring extensive awareness on the environmental quality and detrimental responses to non-target biota. Even though anticancer drugs are encompassed in such overall environmental issue, the introductory aspects mentioned above involving their potential MoA in non-target species portray an emerging concern, even less outlined in the environmental context of low/middle income countries. Accordingly, the main objective of this thesis was to assess the MoA and toxicity of anticancer drugs, at environmentally relevant concentrations, on benthic marine species of temperate (Portugal) and subtropical (Brazil) zones.

To achieve this purpose, the thesis is structured in the following chapters: Chapter 1 is a general introduction that reviews the hallmark of environmental problem caused by anticancer drugs, addressing the trends of increase in cancer burden and following outcomes in pharmaceutical consumption that ultimately leads to their introduction in the marine environment. Potential sources and routes into the marine environment are discussed, including their fate and behaviour. Data regarding their occurrence and ecotoxicological

effects are discussed, calling attention for their MoA in the marine environment and in the biota.

In order to determine whether realistic trace concentrations of traditionally prescribed anticancer agents represent a chronic threat to the marine sediment biota, Chapters 2 to 4 describe the effects of the cytotoxic cisplatin (Chapter 2), the alkylating agent cyclophosphamide (Chapter 3) and the endocrine disruptor tamoxifen (Chapter 4) on the sediment dweller *Nereis diversicolor*, by evaluating the behavioural responses, oxidative stress, biotransformation, neurotoxicity, lipid peroxidation and genotoxic outcomes. Furthermore, in Chapter 5, such multi-biomarker approach was applied to respond whether anticancer drugs in mixtures induce distinct responses from the single-drug exposure scenario.

In Chapter 6, chronic and acute endpoints of anticancer agents individually and in mixtures were determined, for the first time, in native benthic organisms (sea urchin, polychaetes and amphipods) from a coastal tropical environment, in Brazil. Lastly, a general discussion of the results from the previous chapters is summarized jointly with the conclusions and future perspectives pertained to environmental risks of this therapeutic class and the study of the benthic compartment (Chapter 7).

Chapter 2

Ecotoxicological assessment of the anticancer drug cisplatin in the polychaete *Nereis diversicolor*

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Abstract

Anticancer drugs are designed to inhibit tumour cell proliferation by interacting with DNA and altering cellular growth factors. When released into the water bodies of municipal and hospital effluents these pharmaceutical compounds may pose a risk to non-target aquatic organisms, due to their mode of action (cytotoxic, genotoxic, mutagenic and teratogenic). The present study aimed to assess the ecotoxicological potential of the alkylating agent cisplatin (CisPt) to the polychaete *Nereis diversicolor,* at a range of relevant environmental concentrations (i.e. 0.1, 10 and 100 ng Pt L^{-1}). Behavioural impairment (burrowing kinetic impairment), ion pump effects (SR Ca^{2+}-ATPase), neurotoxicity (AChE activity), oxidative stress (SOD, CAT and GPXs activities), metal exposure (metallothionein-like proteins - MTLP), biotransformation (GST), oxidative damage (LPO) and genotoxicity (DNA damage), were selected as endpoints to evaluate the sublethal responses of the ragworms after 14-days of exposure in a water-sediment system. Significant burrowing impairment occurred in worms exposed to the highest CisPt concentration (100 ng Pt L^{-1}) along with neurotoxic effects. The activity of antioxidant enzymes (SOD, CAT) and second phase biotransformation enzyme (GST) was inhibited but such effects were compensated by MTLP induction. Furthermore, LPO levels also increased. Results showed that the mode of action of cisplatin may pose a risk to this aquatic species even at the range of ng L^{-1}.

Keywords: Cisplatin, anticancer drugs, polychaetes, oxidative stress, genotoxicity.

2.1 Introduction

The increase of global cancer incidence in the human population led to an increase of development, prescription and combination of drugs, with cytotoxic and cytostatic modes of action (Suspiro and Prista, 2011). They were designed to inhibit cell replication and transcription through direct or indirect interaction with DNA and induce mutagenic, cytotoxic, genotoxic and carcinogenic effects (Johnson *et al*., 2008; Besse *et al*., 2012; Parrella *et al*., 2014a). Excretion of chemotherapeutic drugs, unchanged or metabolized by patients, is considered the primary source by which these pharmaceuticals enter the water bodies, via municipal and/or hospital effluents (Johnson *et al*., 2008; Liu *et al*., 2010; Booker *et al*., 2014). Therefore, concern is now emerging about their fate in aquatic systems and long-term exposure to organisms at environmentally relevant concentrations.

Among the cytotoxic drugs used in chemotherapy, platinum-based drugs (i.e. cisplatin; carboplatin; oxaliplatin) comprise the class L01XA of the anatomical therapeutic chemicals (ATC) which are used individually or in combination to treat various tumours, such as ovarian, testicular, bladder and lung cancers (Deans and West, 2011; Parrella *et al.*, 2014a). Cisplatin (CisPt), or cis-diammine-dichloroplatinum II, was approved for clinical uses in 1978 and is considered one of the most promising and widely used platinum-based therapeutic anti-cancer drugs (Arnesano and Natile, 2009; Gómez-Ruiz *et al*., 2012). Within 24 h of intravenous infusion (i.e. 50-100 mg/m^2 body surface), patients excrete 28 ± 4% of platinum complexes via urine (Dwyer *et al*., 2000; Vermorken *et al*., 1986), 40% in the form of monoaquacisplatin (Hann *et al*., 2003). In aqueous solutions of low chloride levels (i.e. intracellular fluids), CisPt chloro ligands are gradually hydrolysed according to the ionic strength of receiving waters in a stepwise process to generate cis-$[PtCl(H_2O)(NH_3)_2]^+$ (monoaquacisplatin) and cis-$[PtCl(H_2O)(NH_3)_2]^+$ (diaquacisplatin), the former being associated with antitumour activity and toxicity (Hann *et al.*, 2003; Lenz *et al.*, 2005; Curtis *et al.*, 2010). More than 75% of CisPt enters sewage treatment plants and surface waters as the reactive monoaquacisplatin (Hann *et al.*, 2003), with a propensity to interact with biotic and abiotic matrix (Curtis *et al*., 2010; Vyas *et al*., 2014).

The contribution of platinum (Pt) from Pt-based drugs to the environment is smaller compared to other Pt sources such as car catalytic converters (Lenz *et al*.2007a; Curtis *et al.*,

2010). Pt from chemoterapeutic drugs was detected in sewage treatment plants influents (1 ng.L^{-1} to 250 µg L^{-1}) and effluents (i.e. 2 µg L^{-1} to 150 µg L^{-1}), from hospitals (< 10 to 145 µg L^{-1}) and surface waters (0.01 to 0.54 ng L^{-1}) (Kümmerer *et al*., 1999; Lenz *et al*., 2005, 2007a, 2007b; Vyas *et al*., 2014) but in the form of CisPt was only detected in hospital effluents (1.7 ng Pt L^{-1}) (Hann *et al*.2005) and surface waters (0.0028 to 0.0125 ng Pt L^{-1}) (Vyas *et al.,* 2014) (Table 2.1). However, the behaviour and fate of CisPt in the marine environment remains unclear (Turner and Mascorda, 2015). Although pharmaceuticals are mainly hydrophilic and tend to be present in the dissolved form of the water column, the highly soluble CisPt molecule (log Kow = -2.19) has a high potential to be adsorbed to suspended solids and sewage sludge (Lenz *et al*.2005; 2007a; 2007b). The interaction with the solid fraction induces the formation of complexes with organic matter and partitions with the sediments (Moreno-González *et al.*, 2015), where CisPt settles. Sediments can then act as a reservoir of these compounds and provide their continual remobilization to the water column via resuspension or trophic transfer (Buruaem *et al*., 2012; Araujo *et al*., 2013; Rodrigues *et al*., 2013).

Environmentally relevant concentrations of CisPt in aquatic sediments is unknown. Only Pt levels were detected in coastal and estuarine waters, ranging from 0.02 to 25 ng g^{-1} (Tuit and Ravizza, 2000; Pratt and Lottermoser, 2007; Prichard *et al.,* 2008; Cobelo-García *et al.,* 2011). However, CisPt adsorption to sediments decrease from river to estuarine waters which is consistent with the reduction of reactivity of the CisPt degradation (Curtis *et al*., 2010; Turner and Mascorda, 2015) and indicates that dispersal of CisPt is favored towards the marine environments, where physico-chemical conditions inhibit the hydroxo species formation (Turner and Mascorda, 2015).

Table 2.1: Concentrations of total Pt from Pt-based anticancer drugs (e.g. cisplatin; carboplatin; oxaliplatin) and Pt detected from cisplatin drug (ng L^{-1}), in aquatic compartments.

Hospital Effluent		Wastewater Treatment Plants (WWTPs)		Surface Water		Detection Method	Reference
Total Pt	Pt CisPt	WWTPs influent (Total Pt)	WWTPs influent (Total Pt)	Total Pt	Pt CisPt		
-	-	3,000 - 250,000 (a)	2,000 - 150,000 (a)	-	-	ICP-MS; HPLC-ICP-MS	Lenz *et al. (*2007a, 2007b)
4,700 - 145,000	-	-	-	-	-	ICP-MS	Lenz *et al. (*2005)
30 - 84,590	-	-	-	0.01 - 0.540	0.0028 - 0.0125 (b)	ICP-MS	Vyas *et al. (*2014)
<10 – 601	-	-	-	-	-	Adsorptive Voltammetry	Kummerer *et al. (*1999)
38.2 - 110.6	-	38.2 - 110.6	-	-	-	Adsorptive Voltammetry	Steger-Hartmann *et al. (*1997)
-	1.7 (a)	-	-	-	-	HPLC-ICP/MS	Hann *et al. (*2005)

(a) 24-hour composite samples; (b) Estimated concentration according to effluent rate discharge.

Although the MoA and uptake of CisPt to aquatic organisms remains poorly understood, the uptake in cancer cells occurs by passive diffusion or via copper transporter proteins, such as copper transporter 1 (CTR 1) (Holzer *et al*., 2006). CisPt reactive products are highly electrophilic and act towards nucleophilic sites in genomic and mitochondrial DNA, producing DNA inter- and intra-strand adducts that result in DNA distortion, inhibition of DNA replication and interruption of cell division (Gonzalez *et al*., 2001; Fuertes *et al*., 2003; Dasari and Tchounwou, 2014). Furthermore, CisPt has DNA-unrelated effects and can bind to nitrogen and sulphur nucleophilic sites of proteins, phospholipid membranes and cytoskeleton, while enzyme activities, receptor and protein functions are highly affected, with consequent metabolic impairment that can lead to cellular apoptosis or necrosis (Gonzalez *et al*., 2001; Fuertes *et al*., 2003; Dasari and Tchounwou, 2014; Gatti *et al*., 2015). The resistance to counteract CisPt activity is mainly due to the reduction of CisPt accumulation in cells; the increase of DNA repair; Pt inactivation through conjugation with glutathione; and binding to methalothioneins (Arnesano and Natile, 2009; Tadini-Buoninsegni *et al*., 2014).

The ecotoxicological impact of CisPt in aquatic organisms is scarce and was mainly reported for freshwater species, such as protozoans (*Tetrahymena pyriformis*), rotifers (*Brachionus calyciflorus*), microalgae (*Pseudokirchneriella subcapitata*), crustaceans (*Daphnia magna*; *Ceriodaphnia dubia*) and fish (*Danio rerio),* which showed effects on growth and reproduction inhibition (Bonnet *et al.,* 2003; Zounková *et al.,* 2007; Brezovsek *et al.,* 2014), acute immobilization (Zounková *et al.,* 2007), embryotoxicity (Kovács *et al.,* 2015), cytotoxicity (Gajski *et al*., 2016) and mortality (Parrella *et al.,* 2014a; Kovács *et al.,* 2015). CisPt causes genotoxic effects in aquatic organisms, such as the induction of DNA strand breaks as observed in the microcrustaceans *Daphnia magna* and *Ceriodaphnia dubia* after 24 hours of exposure (10 and 300 ng Pt L^{-1}, respectively) (Parrella *et al.,* 2015), and in the marine mussel *Mytillus galloprovinciallis* after exposure to 100 ng Pt L^{-1} (Trombini *et al.,* 2016a).

Benthic and epibenthic species accumulate contaminants by dermal contact and dietary strategies, such as filtration of suspended solids and/or intake of sediment particles and fractions associated to organic matter (Simpson *et al.,* 2005), but little is known about the fate of pharmaceuticals in these species. The polychaete *Nereis* (*Hediste*) *diversicolor* is

a deposit feeder that also scavenges for organic matter and detritus on the sediments surface where it plays a relevant ecological role due to bioturbation, particle mixing and irrigation of benthic galleries (Solé *et al.,* 2009). Therefore, *N. diversicolor* was considered a suitable model for biomonitoring purposes and application in ecotoxicology assays with emergent contaminants (Cong *et al.,* 2011; Buffet *et al.,* 2014a), including pharmaceutical compounds (Maranho *et al.,* 2015).

Adverse sublethal effects regarding physiological and biochemical responses after exposure of benthic species to different classes of pharmaceuticals (e.g. anti-inflammatory; oral contraceptive; anticonvulsant; antidepressant) were reported (Maranho *et al.,* 2014; 2015). Biomarkers of behaviour impairment, neurotoxicity (acetylcholinesterase – AChE), antioxidant activity (superoxide dismutase – SOD; catalase – CAT; glutathione-peroxidases – GPx), biotransformation (glutathione-S-transferases – GST), oxidative damage (lipid peroxidation - LPO) and genotoxicity (DNA strand breaks) were considered reliable endpoints to assess pharmaceuticals toxicity (Aguirre-Martínez *et al.,* 2013; Gonzalez-Rey and Bebianno, 2013; Maranho *et al.,* 2014; 2015). In this sense, the present study aims to assess the adverse effects of the CisPt anticancer drug to the ragworm *N. diversicolor*, at a range of relevant environmental scenarios (i.e. 0.1, 10 and 100 ng Pt L^{-1}). The approach involves the assessment of behavioural impairment (burrowing kinetic impairment), ion pump effects (SR Ca^{2+}-ATPase), neurotoxicity (AChE activity), oxidative stress (SOD, CAT, GPXs activities), biotransformation (GST activities), metal exposure (metallothionein-like protein- MTLP), oxidative damage (LPO) and genotoxicity (DNA damage), after 14 days of exposure to a contaminated water-sediment system. To the best of our knowledge, this is one of the first multibiomarker approaches encompassing anticancer drugs and marine organisms, with relevance to understand their risk in coastal and benthic habitats.

2.2 Materials and Methods

2.2.1 Chemicals

Analytical standard of cis-platinum (II) diamine dichloride (CisPt) (CAS 15663-27-1) was obtained from Sigma-Aldrich (Portugal). CisPt stock solution (123.07 mg L^{-1}) was

prepared in ultrapure Milli-Q water. For safety handling of cytotoxic drug, experimental work was performed using class II biological safety cabinet, with appropriate clothing (open-back, impervious chemotherapy protection gown, double powder-free latex gloves and safety goggles). Test solution concentrations were selected according to a range of Pt-based anticancer drugs levels screened in water bodies and effluents (Table 2.1) and were 0.1, 10 and 100 ng Pt L^{-1} (all nominal concentrations).

2.2.2 Sediment characterization

Sediments were sampled in the Ria Formosa lagoon (Tavira, Portugal), and sieved through a 2-mm mesh to remove large debris. Sediments were dried at 80°C (adapted from Thain and Bifield, 2001; ASTM, 2009) to remove volatile compounds and water. Grain size distribution was assessed on dry aliquots of sediments according to the method proposed by Royse (1970). Organic matter content was determined by loss on ignition (550°C, for 5 h), by the method described by Gross (1971). Results obtained were 22.3% of sand, 77.7% of fines and 0.55% or organic matter content.

2.2.3 Experimental design

Specimens of *N. diversicolor* (5-7 cm length; 0.21 g ± 0.06) were supplied from an aquaculture of the Portuguese Institute of the Sea and Atmosphere (IPMA) (Tavira, Portugal) and transported alive to the laboratory in tanks with natural seawater and sediments and maintained at constant aeration for one week. Twenty animals were randomly transferred to plastic aquaria of 10 L (each animal/30 cm^2), in a triplicate design, with a proportion of 1:4 sediments/water. Systems were kept under constant aeration and a light period of 12:12 hours. Natural seawater was renewed every 48 hours followed by redosing of CisPt to maintain nominal concentrations in the water phase during the experiment. Physico-chemical parameters: salinity, temperature and pH were determined in seawater (35.45 ± 1.2; 18.3 ± 0.8 °C; 7.99 ± 0.07, respectively). For each biochemical analysis (*i.e.* antioxidant enzymes; AChE; LPO/MT), measurements were carried out individually (n = 6 per treatment). Animals were collected at the beginning of the experiment (Control CT 0) and after 14 days of exposure (Control CT 14; 0.1, 10 and 100 ng Pt L^{-1}). Organisms used for behavioural (n = 15 per treatment) and comet assays (n = 15 per treatment) were immediately

handled, whereas those for biochemical endpoints were rinsed with clean seawater, frozen in liquid nitrogen and stored at -80 °C until further use.

2.2.4 Sarcoplasmic reticulum Ca^{2+} - ATPase

Preparation of sarcoplasmic reticulum vesicles

Isolated sarcoplasmic reticulum vesicles (SRV), prepared from freshly obtained skeletal rabbit muscles described elsewhere (Fraqueza *et al.*, 2012) were suspended in 0.1 M KCl, 10 mM HEPES (pH 7.0), diluted 1:1 with 2.0 M sucrose and frozen in liquid nitrogen prior to storage at -80 °C. Protein concentrations were determined spectrophotometrically at 595 nm, by the Bradford method, using bovine serum albumin as a standard. The percentage of each protein present in the SRV preparations was determined through densitometry analysis of SDS-polyacrylamide gel electrophoresis (7.5% acrylamide). The SR Ca^{2+}-ATPase analysed by SDS polyacrylamide gel electrophoresis comprised at least 70% of the total protein in the SR-vesicles. The SERCA-1, (sarcoplasmic, or endoplasmic reticulum Ca^{2+}-ATPase-1) was the predominant isoform in the SR preparations (Fraqueza *et al*., 2012).

ATP Hydrolysis by Calcium Pump

Steady-state assays of the sarcoplasmic reticulum Ca^{2+}-ATPase were measured spectrophotometrically at 25˚C using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay (Aureliano *et al*., 2008), under the following conditions: 25 mM HEPES (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, 2.5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, 18 IU lactate dehydrogenase and 7.5 IU pyruvate kinase, with or without CisPt. The experiments were initiated by the addition of 10 μg/ml calcium ATPase, in the presence or absence of 4% (w/w) of calcium ionophore A23187. CisPt concentrations (up to 4 and 8 mM) were added to the medium immediately prior to protein addition. Furthermore, within the concentration range studied, CisPt solutions did not affect the coupled enzyme method used in the assays, as observed upon addition of 40 μM ADP. Briefly, after the addition of the enzymes to the medium, NADH was added followed by the vesicles from skeletal muscle sarcoplasmic reticulum. Then, after the addition of ATP the absorbance was recorded during about 1 minute (basal activity) and

after the ionophore was added the decreased of the absorbance was measured during about 2 minutes (uncoupled ATPase activity). The ATPase activity and the inhibition of CisPt was measured taken into consideration the decrease of OD per minute in the absence (100%) and in the presence of CisPt (Fraqueza *et al*., 2012).

2.2.5 Behaviour assay

Unexposed and exposed worms were submitted to a burrowing test according to Bonnard *et al. (*2009). Ten animals were carefully placed individually in 150 mL-plastic containers, filled with natural seawater and 5 cm of sediments. Over a period of 30-minutes, the position of each worm was recorded every two minutes to establish the time that each worm was fully buried. The results are expressed as the percentage (%) of unburied specimens over time (min).

2.2.6 Biochemical analysis

Neurotoxicity

For AChE activity determination, whole tissues were individually homogenized in 100 mM Tris-HCl buffer (pH 8) containing Triton 0.1 % and centrifuged at 12,000 *g* (30 min, 4 °C). The supernatant was split into two 500 µL aliquots for total protein determination and AChE activity. The assay was conducted by inserting the reaction mixture in each microplate well, containing 50 µL of the sample, 0.75 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in 0.1 M Tris HCl, and 3 mM acetylcholine (ATC). The method measures the absorbance of 5-mercapto-2-nitrobenzoato (yellow) (coefficient of extinction of ε = 13.6 mM^{-1} cm^{-1}) formed by the reaction of thiocholine, a product of ATC cleavage by AChE with DTNB, at 405 nm (λ_{max} for DTNB is 412 nm) (Ellman *et al.*, 1961; Colovic *et al.*, 2013). AChE activity is expressed as $ATC.min^{-1}$ mg^{-1} protein.

Antioxidant enzymes

Tissues for the measurement of antioxidant enzymes were individually homogenized in 20 mM Tris-HCl buffer (0.5 M sucrose, 0,075 M KCl, 1 mM DTT, 1 mM EDTA), adjusted

to pH 7.6 and centrifuged at 500 g (15 min, 4 °C) to separate the cytosolic fraction. The supernatants were further re-centrifuged at 12,000 g (45 min, 4 °C), to separate the mitochondrial fraction. Antioxidant enzymes activities were determined in the cytosolic fraction. SOD activity was analysed according to the method of McCord and Fridovich (1969), by measuring the decrease of absorbance of substrate cytochrome-c through xanthine oxidase/hypoxanthine system, at 550 nm. Results are expressed as U mg^{-1} protein. CAT activity was determined by spectrophotometry using a microplate reader by measuring the decrease of absorbance at 240 nm due to the presence of hydrogen peroxide concentration (Greenwald, 1985). CAT is expressed in µmol min^{-1} mg^{-1} protein. Total GPX activity was measured in a microplate reader, at 340 nm, based on the method adapted from Lawrence and Burk (1976). The reaction consists in the reduction of oxidized glutathione linked to the oxidation of NADPH in the presence of excess glutathione reductase. Cumene hydroperoxide and H_2O_2 were used as substrates for T-GPx and Se-GPx, respectively. Results are expressed in nmol min^{-1} mg^{-1} protein.

Glutathione-S-transferases (GST)

The activity of GST was measured in the spectrophotometric assay according to the method described by Habig *et al. (*1974). The conjugation of the worm samples with 0.2 mM reduced GSH with 0.2 mM 1-chloro-2,4-dinitrobenzene (CDNB) in a mixture of 0.2 M KH_2PO_4/K_2PO_4 buffer (pH 7.9) was measured, with a molar extinction coefficient (ε) of 0.6 mM^{-1} cm^{-1}. The change in absorbance was recorded at 340 nm. GST activity is expressed in nmol min^{-1} mg^{-1} protein.

Metallothionein-like proteins (MTLPs)

Whole tissues of *N. diversicolor* were individually homogenized in Tris-HCl buffer (0.02 M, pH 8.6) and butylated hydroxy toluene (BHT) (10 µl BHT per mL Tris–HCl buffer). The homogenate was centrifuged at 30,000g for 45 min (4°C). Aliquots of the supernatant were separated for further use in LPO and total proteins quantification (Cravo *et al*., 2012). The remaining supernatant was heat-treated at 80 °C for 10 min to precipitate the high molecular weight proteins, and re-centrifuged at 30,000 g for 45 min at 4 °C. The obtained cytosolic fraction was stored at −80 °C for MTLP quantification by differential pulse

polarography according to the method described by Bebianno and Langston (1989). Levels of MTLPs are expressed as mg mg^{-1} protein.

Oxidative damage

LPO was assessed by determining the absorbance of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations, at 540 nm, following the method described by Erdelmeier *et al. (*1998), adapted for microplate reader. LPO is expressed as nmol MDA + 4-HNE mg^{-1} protein.

Total proteins concentration

Total proteins concentration was determined following the method described by Bradford (1976), adapted for microplate reader using bovine serum albumin (BSA) as a standard.

Genotoxicity assay

Genotoxicity endpoint was assessed by the Comet assay, based on the slightly modified protocol of Singh *et al. (*1988), and described in Gomes *et al. (*2013). Extraction of the polychaetes coelomocytes was performed at the beginning and end of the exposure period, according to Lewis and Galloway (2008). Briefly, coelomic fluid of *N. diversicolor* (n=15) was extracted from the posterior region of the polychaete body into 20 µL of PBS buffer with a 0.5 mL-syringe fitted with hypodermic needle. The mixture was centrifuged at 835 *g* (3 min, 4 °C), and the pellets were used for the comet assay. Slides were previously cleaned in alcohol/ether and coated with 0.65 % normal melting point agarose (NMA) in Tris-acetate EDTA. Isolated cells from centrifugation were suspended in 0.65 % low melting point agarose (LMA, in Kenny's salt solution; 0.4 M NaCl, 9 mM KCl, 0.7 mM KH_2PO_4, 2Mm $NaHCO_3$, 1000 ml Milli-Q water) and casted on the microscope slides. Then slides with the embedded cells were immersed for 1 hour, in a lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, 10% Dimethylsulfoxide,1% Sarcosil, pH 10, 4 °C) for the diffusion of cellular components and DNA immobilization in agarose. Following the lysis step, slides were gently placed in an electrophoresis chamber containing electrophoresis

buffer (300 mM NaOH, 1 mM EDTA, adjusted at pH 13, 4 °C) and left for 15 min to permit DNA unwinding. The electrophoresis was performed at 25 V and 300 mA, over 5 min.

Afterwards, microscope slides were soaked with neutralizing solution (0.4 mM Tris, pH 7.5), for 15 min, and finally rinsed with distilled water and dried overnight. For further comets assessment, slides were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 mg ml^{-1}) and analysis made through optical fluorescence microscope (Axiovert S100) coupled with a camera (Sony). The image analysis system Komet 5.5 (Kinetic Imaging Ltd) was used to score 50 randomly chosen cells for each slide, at a total magnification of ×400. The amount of DNA in the comet tail (tail DNA %) was used and results are expressed as mean ± standard deviation.

Statistical analysis

Data was tested for normality and homogeneity of variance by Kolmogorov-Smirnov and Brown-Forsyth tests, respectively. Once these assumptions were tested, one-way analysis of variance (ANOVA or non-parametric Kruskal-Wallis test was performed. As a result of these tests, all data, except GPx, was treated as parametric. The post-hoc Tukey test for multiple data comparisons was applied to discriminate significant differences among treatments ($p < 0.05$). Behavioural status of organisms was analysed by linear regression, considering the interval of continuous burrowing in the sediments. Principal component analysis (PCA) was used to evaluate the influence CisPt concentrations on biochemical biomarkers and DNA damage, in control and exposed polychaetes. Statistical analysis was conducted with GraphPad Prism® software (v. 6, 2014) and PAST® software (v. 3.10, 2015).

2.3 Results

2.3.1 Effects of CisPt in Ca^{2+} - ATPase activity

Sarcoplasmic reticulum (SR) plays a crucial role in calcium homeostasis and in regulating muscle contraction. Sensitivity of SR Ca^{2+}-ATPase activity has been documented by the increase of basal cytosolic Ca^{2+} concentration after incubation with toxic and/or

contaminant metals solutions (Aureliano and Crans, 2009). In the present study, the inhibition of the SR Ca^{2+}-ATPase activity by CisPt was analysed to relative higher concentrations (8 mM) of CisPt. As observed in Figure 2.1, even for concentrations up to 8 mM of CisPt, the activity of the calcium pump was only inhibited about 40%. For this ion pump activity, it was estimated an IC_{50} of about 13.7 mM for CisPt solutions.

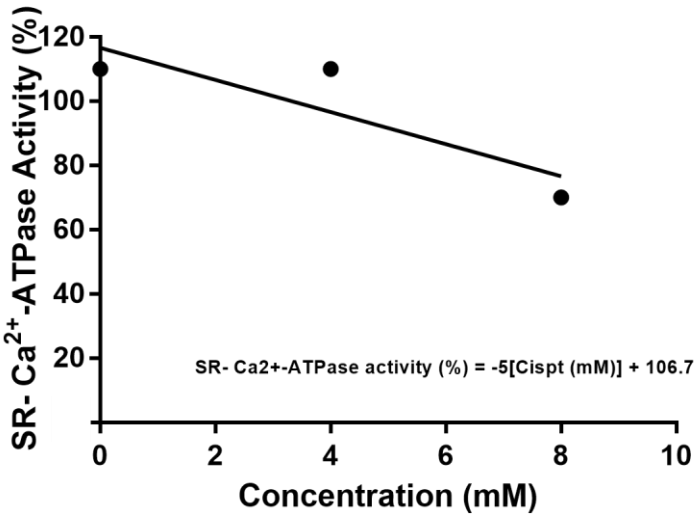

Figure 2.1: Inhibition of sarcoplasmic reticulum calcium pump by CisPt.

2.3.2 Behavioural Assay

Burrowing behaviour of polychaetes over time is presented in Figure 2.2. Linear regression showed no significant differences among slopes in the burrowing of worms exposed to the different treatments ($p > 0.05$). Nevertheless, a similar trend of burrowing kinetic was observed between unexposed organisms (Y = -12X + 106; r = 0.97; $p < 0.05$) and those exposed to 0.1 ng Pt L^{-1} (Y = -14X +114; r = 0.9615; $p < 0.05$), where all polychaetes were fully buried after 8 minutes. Meanwhile, specimens exposed to 10 (Y = -10X + 106; r = 0.96; $p < 0.05$) and 100 ng Pt L^{-1} (Y = -7.1X + 97.14; r = 0.93; $p < 0.05$)

showed a slower burrowing rate, with organisms unable to burrow the sediment by the end of the assay.

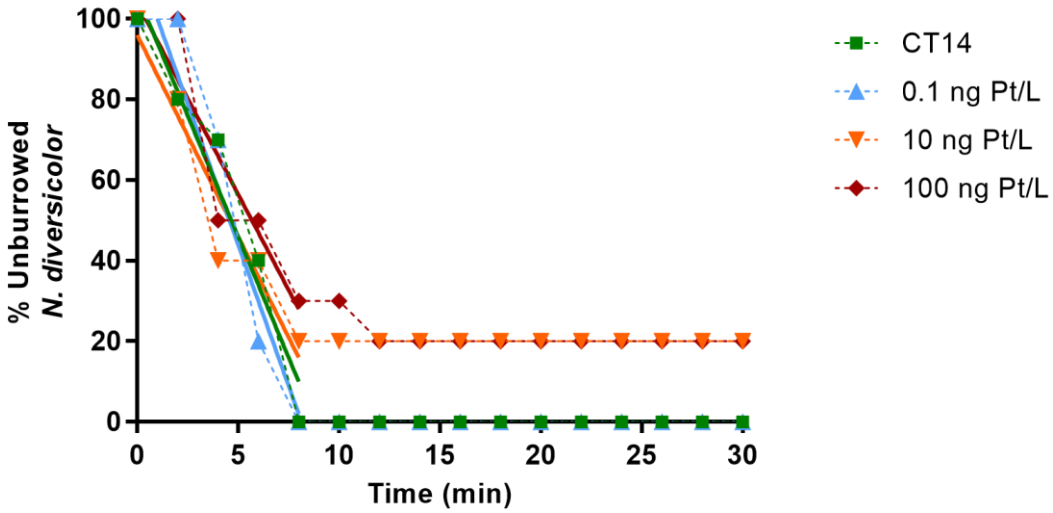

Figure 2.2: Burrowing behaviour of *N. diversicolor* unexposed (CT14 – control at day 14) and exposed to CisPt (0.1, 10 and 100 ng Pt L^{-1}), expressed as percentage of unburied organisms over time (minutes).

2.3.3 Biochemical analysis

Neurotoxicity

Results of AChE activity are expressed in Figure 2.3. AChE levels were similar between controls over time ($p > 0.05$). An increasing trend was observed for polychaetes exposed to 0.1 ng Pt L^{-1} and 10 ng Pt L^{-1} although not significantly different from controls ($p > 0.05$). Polychaetes exposed to 100 ng Pt L^{-1} showed a significant inhibition of AChE activity compared to controls ($p > 0.05$).

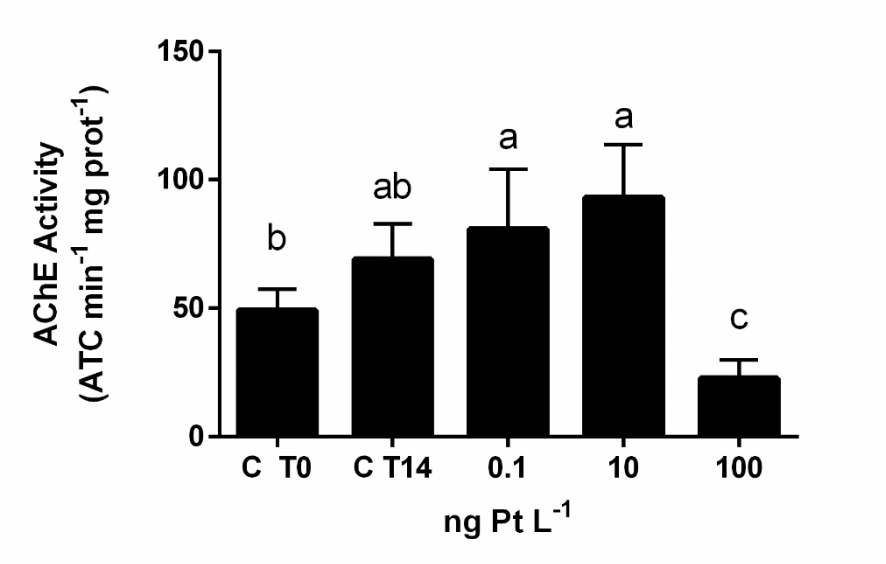

Figure 2.3: AChE activity (mean ± standard deviation) (ATC.min-1.mg^{-1} protein) in *N. diversicolor* unexposed (CT0 and CT14) and exposed to CisPt (0.1, 10 and 100 ng Pt L^{-1}) for 14 days. Different letters indicate statistically significant differences among treatments (One-way ANOVA; $p < 0.05$).

Antioxidant enzymes

In the present study, antioxidant enzymes of control polychaetes did not change over time ($p > 0.05$). SOD activity showed a significant inhibition (*i.e* 2.3-fold) in worms exposed to 100 ng Pt L^{-1} ($p < 0.05$) (Fig. 2.4A). Similarly, exposure to the highest CisPt concentration resulted in a clear inhibition (i.e. 2.3-fold) of CAT activity ($p < 0.05$) (Fig. 2,4B). In contrast, CisPt yielded a significant increase in GPx Se-dependent activity and levels ranged from 0.05 ± 0.01 nmol min^{-1} mg^{-1} protein to 0.22 ± 0.07 nmol min^{-1} mg^{-1} protein for worms exposed to 100 ng Pt L^{-1} ($p < 0.05$) (Fig. 2.4C). Regarding T-GPx, the activity indicated a bell-shape behaviour, where the activity of this enzyme was significantly inhibited after 0.1 ng Pt L^{-1} exposure and induced after 100 ng Pt L^{-1} exposure (Fig. 2.4D) ($p < 0.05$). Furthermore, GST activity was only inhibited at the highest CisPt concentration ($p < 0.05$) (Fig. 2.4E).

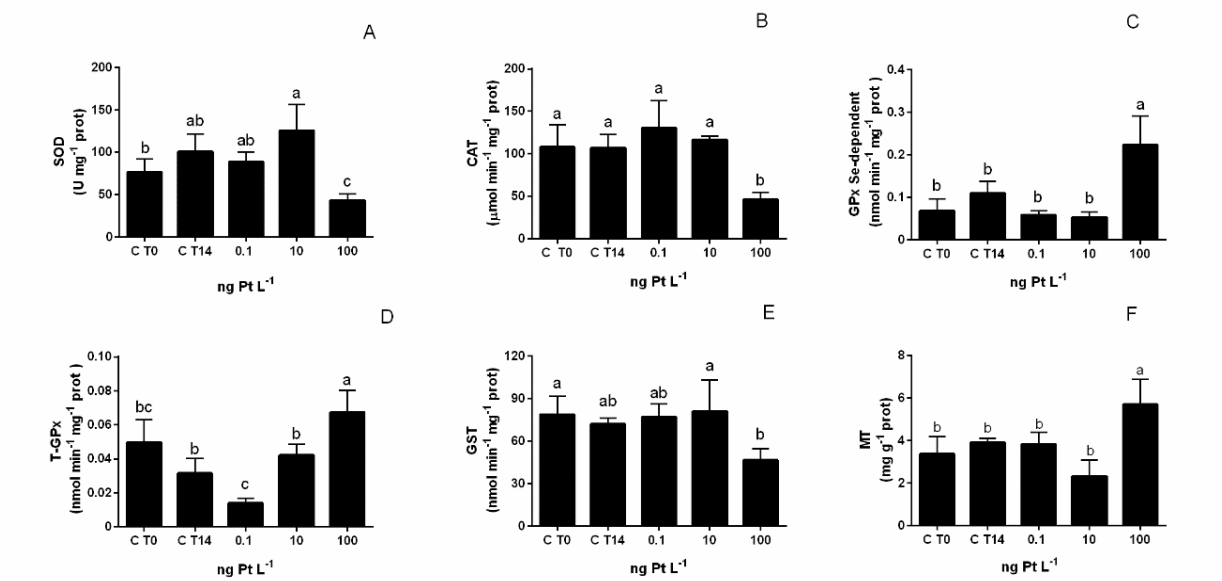

Figure 2.4: Mean ± standard deviation of (A) SOD, (B) CAT, (C) GPx Se-dependent, (D) Total GPx, (E) GST and (F) MT in *N. diversicolor* unexposed and exposed to CisPt (0.1, 10 and 100 ng Pt L^{-1}) for 14 days. Different letters indicate statistically significant differences among treatments (One-way ANOVA (A, B, C, D, F) and Kruskal Wallis (E); $p < 0.05$).

Metallothionein-like Proteins (MTLPs)

Results showed a significant increase in MTLPs levels ($p < 0.05$) only at the highest CisPt concentration (100 ng Pt L^{-1}), accounting for a 2-fold increase when compared to controls (Fig. 2,4F).

Oxidative damage

LPO products over the exposure conditions presented an increasing trend but only significant at the highest drug concentration compared to controls ($p < 0.05$) (Fig. 2,5).

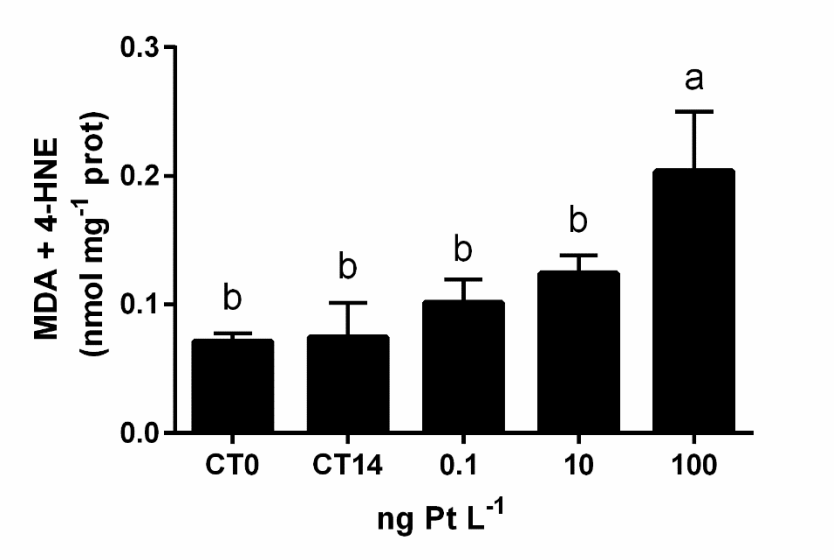

Figure 2.5: LPO activity (mean ± standard deviation) (MDA+ 4-HNE nmol.mg^{-1} protein) in *N. diversicolor* unexposed (CT0 and CT14) and exposed to CisPt (0.1, 10 and 100 ng Pt L^{-1}) for 14 days. Different letters indicate statistically significant difference among treatments (One-way ANOVA; $p < 0.05$).

2.3.4 Genotoxicity

Genotoxicity data, expressed as percentage of DNA tail, are presented in Figure 2,6. No changes in DNA damage were observed for any treatment ($p > 0.05$).

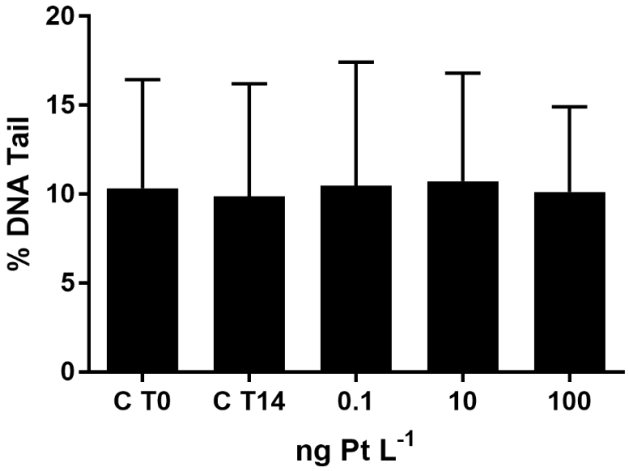

Figure 2.6: DNA (mean ± standard deviation) in tail, in *N. diversicolor* unexposed (CT0 and CT14) and exposed to CisPt (0.1, 10 and 100 ng Pt L-1) for 14 days. Different letters indicate statistically significant differences among treatments (One-way ANOVA; $p < 0.05$).

2.3.5 Principal Component Analysis (PCA)

Biochemical biomarkers and genotoxicity data were integrated in a multivariate analysis in order to detect patterns of variation (Figure 2.7). PCA results indicated that the two principal components represent 89.8% of total variance (PC1 = 75.6 %; PC2= 14.2%). High correlation loadings in the first component indicated that antioxidant enzymes SOD, CAT, GST and AChE activity were inhibited at the highest CisPt level (i.e. 100 ng Pt L^{-1}). SOD and CAT neutralize ROS, and their decrease together with GST reduction favor the depletion of cell defenses while Se-GPx, MT and LPO were the opposite. The second component indicates a relationship between LPO and genotoxicity endpoints. The overall PCA clear separates the worms exposed to 100 ng Pt L^{-1} from controls and from those exposed to the lower CisPt concentrations. Moreover, worms exposed to 0.1 ng Pt L^{-1} were closely associated to those for controls, reflecting the effectiveness of antioxidant systems and absence of harmful effects at this level of CisPt concentration.

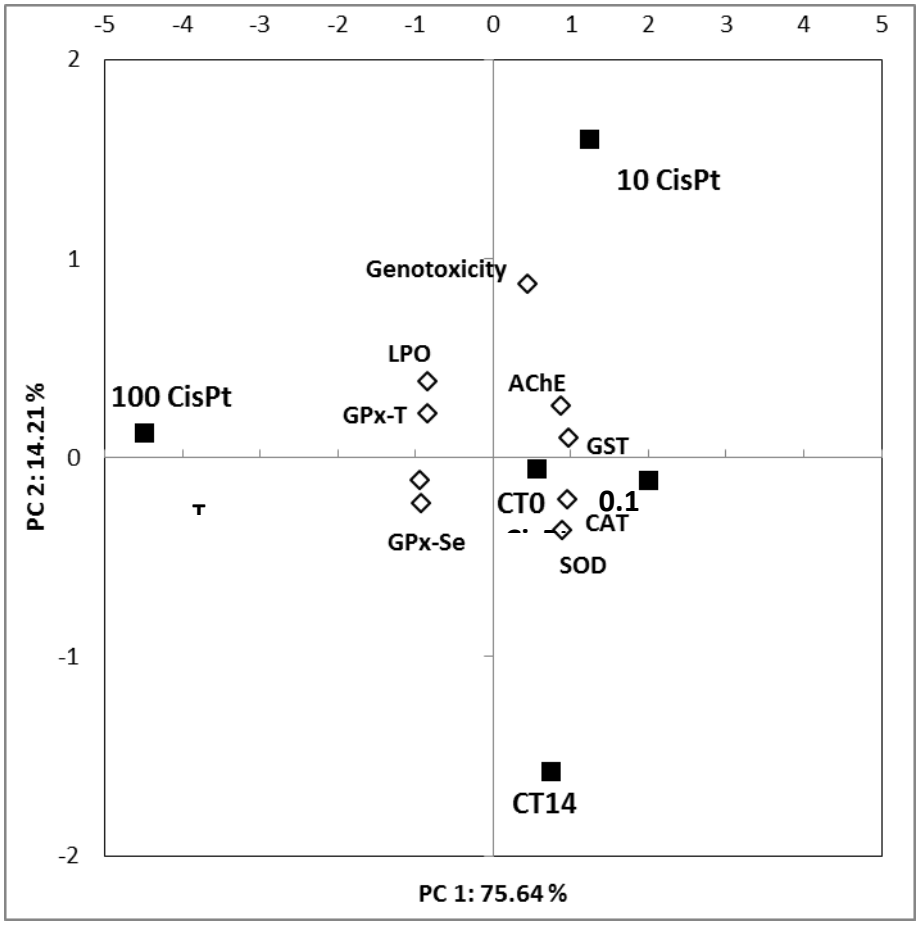

Figure 2.7: Principal Component Analysis (PCA) of biochemical biomarkers and DNA damage analysed in *N. diversicolor* at controls (CT0 and CT14) and CisPt-contaminated conditions, for 14 days.

2.4 Discussion

CisPt is widely used for treatment of different types of tumours including head and neck, lung, ovarian, leukaemia, breast, brain, kidney and testicular cancers (Dasari and Tchounwou, 2014). CisPt acts in mammals over the N7 centres of purine residues, especially guanine, mainly generating 1,2-intrastrand *d* (GpG) adducts and 1,2-intrastrand d (ApG), besides the enhancement of oxidative stress and damage in mitochondrial proteins, which result in an overall failure of cellular function and apoptosis (Chu, 1994; Dasari and Bernard Tchounwou, 2014; Reedijk, 1999). The drug´s MoA and toxicity was extensively studied in mammals, where ototoxicity, nephrotoxicity and gastro toxicity are the main side effects

(Karasawa and Steyger, 2015). The diffuse, worldwide and frequent discharge of effluents containing cytotoxic drugs in coastal water bodies brings ecological and environmental concern regarding the chronic exposure of these chemicals to biota, even at low concentrations. Ecotoxicological assays with anticancer drugs in aquatic organisms are now emerging, although approaches with marine benthic species are lacking.

In the present study, the effects of CisPt on the function of the sarcoplasmic reticulum Ca^{2+}-ATPase were investigated to evaluate putative effects on calcium homeostasis. It was assumed that the onset of contraction in polychaetes muscle is triggered by similar mechanisms as in vertebrates, once previous studies have shown that energy transduction catalysed by Ca^{2+}-ATPase is highly conserved through evolution, as observed in marine invertebrates (Garcia *et al.,* 1975; Cario *et al.,* 1995; Landeira-Fernandez *et al.,* 2001). CisPt had no effect on Ca^{2+}-ATPase activity, in particular for the CisPt concentrations normally found in the environment (Figure 2.1). Therefore, inhibition of Ca^{2+}-ATPase activity by cisplatin seems unlikely as a cause of CisPt toxicity, although CisPt can decrease ATPase activity *in vitro*. Other studies estimated IC_{50} values in the order of 2 to 5 mM for similar ion pumps, but for longer incubation periods such as 2 hours (Uozumi and Litterst, 1985). However, the lower ATPase activity inhibition by CisPt can, at least in part, be due to the solvent (DMSO) used. It can react with CisPt and therefore and in this situation is not a good solvent. Recently it was reported that IC_{50} values increased up to about 10- and 60-fold, respectively, for carboplatin and cisplatin prepared in DMSO whereas no effects were observed for others Pt complexes such as oxaliplatin and satraplatin (Hall *et al.,* 2014). Therefore, certain precautions should be taken when using solvents in *in vitro* and *in vivo* Pt drugs studies. The multibiomarker approach linking behavioural, biochemical and genotoxicity endpoints provide important information about the impact caused by pollutants in aquatic organisms (Durou *et al.,* 2007; Gusso-Choueri *et al.,* 2016). Burrowing impairment comprises an ecological valuable biomarker of stress reflecting disturbances in organisms´ fitness, such as food seeking and predator avoidance (Bonnard *et al.,* 2009).

The suitability of burrowing behaviour is a reliable and sensitive biomarker when defence mechanisms are not enough to prevent impairments (Amiard-Triquet, 2009; Buffet *et al*., 2012). *N. diversicolor* plays an important ecological role due to bioturbation, that provides irrigation and particle mixing via burrowing and feeding (Thit *et al.,* 2015).

Alteration of such behaviour provide harmful ecological outcomes (Bonnard *et al.,* 2009). In addition, increase of burrowing time may facilitate worm predation on the sediment surface (Amiard-Triquet, 2009). Results on behaviour (Figure 2.2) clearly indicate a perturbation on burrowing of organisms exposed to the highest CisPt concentration (100 ng Pt L^{-1}) that were not fully buried by the end of the assay (Fig. 2.2). To date, burrowing responses of endobenthic organisms after exposure to pharmaceuticals are lacking. Meanwhile, Cong *et al.* (2014) addressed an increase in burrowing time in specimens of *N. diversicolor* exposed to Ag nanoparticles, at concentrations of 50 and 100 µg Ag mg^{-1} d.w. in the sediments, corroborating with high cytotoxic and genotoxic potential of Ag nanoparticles on burrowing behaviour. Decrease burrowing of *N. diversicolor*, compared to controls, was also observed after exposure to copper (Buffet *et al.,* 2011; Thit *et al.,* 2015) and sediment-spiked copper oxide nanoparticles (Thit *et al.,* 2015).

Alteration of behaviour and locomotion may be a response to neurotransmitters inhibition, which play a key role in the nervous system (Boyd *et al.,* 2002; Bonnard *et al.,* 2009). AChE activity is vital for normal muscular function and behaviour of most species, because it is involved in the nervous transmission by a rapid hydrolysis of the neurotransmitter acetylcholine in acetate and choline at the nervous systems (Payne *et al.,* 1996; Carajaville *et al.,* 2000). Inhibition of AChE is well-recognized as a biomarker of exposure to neurotoxic compounds (Aguirre-Martínez *et al.,* 2016a; Damásio *et al.*, 2011). Herein, AChE levels increased with increasing Pt concentrations until 10 ng Pt L^{-1}, following a strongly inhibition at the highest CisPt concentration (100 ng Pt L^{-1}) (Fig. 2.3). This behaviour depicts a hormesis phenomenon, comprised by the stimulation of a response (AChE activity) at a dose below the pharmacological/toxicological threshold (Calabrese and Blain, 2005; Aguirre-Martínez *et al.,* 2016a) that is in an attempt to mitigate the neurotoxic effects against the transitory drug effect (Milan *et al.*, 2013; Aguirre-Martinez *et al.*, 2016a).

According to Calabrese and Baldwin (2001) it is an adaptive response that results in an improved physiological fitness, for a finite and generally short period. In this sense, behavioural interferences at 10 ng Pt L^{-1} may be associated to such mitigation effort, whereas at 100 ng Pt L^{-1} it may be due to AChE inhibition, thus neurotoxicity (Milan *et al.,* 2013; Aguirre-Martinez *et al.,* 2016a). A similar upregulation followed by an inhibition of AChE activity was observed in the clam *Ruditapes phillipinarum* after exposure to caffeine (5 to

50 µgL^{-1}) and carbamazepine (0.1 to 50 µg L^{-1}) for 14 days (Aguirre-Martinez *et al.*, 2016a). This trend was also addressed at fluoxetine exposure using the clam *Venerupis philippinarum* (1 and 5 µg L^{-1}; Munari *et al.*, 2014) and the mussel *Mytillus galloprovincialis* (75 ng L^{-1}; Gonzalez-Rey and Bebianno, 2013).

Levels of AChE activity in *N. diversicolor* also increase significantly after 14 day-exposure to sediment spiked with ibuprofen, carbamazepine, fluoxetine and EE2, showing a situation where hormesis did not occur, due to a combination of the test organisms, dose and pharmaceutical compounds selected (Cossu *et al.*, 2000; Gonzalez-Rey and Bebianno, 2013; Maranho *et al.*, 2015; Aguirre-Martinez *et al.*, 2016a). CisPt is an organo-metallic compound designed to perform neurological side-effects in human erythrocytes, arising neurotoxicity as an effect of interaction of the molecule with AChE (Aljafari, 1995). The electrophilic reactive products of hydrolysis, containing Pt may be the key factor for the inhibition of AChE activity and this may be the case in the worms exposed to the highest CisPt concentration.

CisPt hydrolysed species are strongly electrophilic and can react with nucleophile binding sites, including the thiol groups SH- of biomolecules. In this sense, the nucleophile reduced glutathione (GSH) is one of the main target of CisPt drug (Gonzalez *et al*., 2001) and acts as a protection ligand against drug toxicity and side effects without interfering in the antitumour activity (Reedjik, 1999). It has an important role in regulation of inner mitochondrial permeability and enzyme function by keeping SH- in the reduced state, thus acting on protection against oxidative stress (Reedjik, 1999). GSH reacts with CisPt and other electrophilic compounds to form deactivated conjugates that are readily excreted by a GS-conjugated export pump. This reaction may occur spontaneously or with the enzymatic help of GST (Fuertes *et al.*, 2003; Penner *et al.*, 2012). GSH depletion in cells may lead to the reduction of GST activity, as observed in worms exposed to 100 ng Pt L^{-1} (Fig. 2.4E). In addition, CisPt-GSH conjugation generates thiyl radicals (RS$^{\bullet}$) able to produce reactive oxygen species (ROS) (Slater, 1984; Desoize, 2002; Aquilano *et al.*, 2014).

In order to prevent ROS interaction with critical cellular macromolecules, SOD plays the primary defense role against oxygen toxicity by catalyzing the conversion of O_2^-, to H_2O_2 through dismutation reaction, whilst CAT degrades H_2O_2 to H_2O (Van den Berg *et al.*, 2005).

In the present study, the significant decrease of both enzyme activities in worms exposed to the highest CisPt concentration (Fig. 2.4) indicate an impairment of their activity that could be attributed to a possible excess of reactive oxygen intermediate species. Hence, the increase of Se-GPx in worms exposed to 100 ng Pt L^{-1} may be a response to counteract the impairment of hydrogen peroxide metabolism, usually provided by SOD and further generation of pro-oxidant products required for neutralization (Catalano *et al.,* 2012; Gomes *et al.,* 2014). Se-GPx acts by reducing organic peroxides and is known as a critical tool to mitigate oxidative stress caused by a wide diversity of electrophilic species (Prahbu *et al.,* 2004).

Previous biochemical approaches using *N. diversicolor* indicated a noteworthy responsiveness of antioxidant system to contaminated sediments (Durou *et al*., 2007; Kalman *et al*., 2009; Maranho *et al*., 2014). The trend of detoxification process encompasses the increment of GST and of CAT levels, which might suggest a relationship between those enzymes playing their role against oxidative stress, due to an improvement of neuromuscular status and the absence of LPO products (Solé *et al.,* 2009; Buffet *et al.,* 2011; Buffet *et al.,* 2014a). In the present study, the first axis of PCA (75.6%) supports the connected mechanisms of biochemical impairments but does not occur in worms exposed to the higher concentration where a clear inhibition of SOD, CAT and GST indicate a lack of enough protection against ROS, neurotoxicity and membrane damage, as supported by the PCA analysis (Fig. 2.7). The deficiency on enzymatic activities in the worms exposed to the highest CisPt concentration suggest a precarious state of biochemical conditions, promoting a high susceptibility for biological systems (Cossu *et al*., 2000), as observed in burrowing behaviour, and propensity to lipid peroxide generation (Buffet *et al.,* 2012).

The induction of MTLP was recognized as a potential biomarker of metal exposure in aquatic organisms (Amiard *et al.,* 2006; Won *et al.,* 2008). These cysteine-rich metal-binding proteins act on metal detoxification and homeostasis by forming inactive complexes with metal in the cytosol or excreted through their thiol groups (Gagné *et al.,* 2008; Won *et al.,* 2008; Huska *et al.,* 2009). The presence of MTLPs in annelids was addressed by several authors (Geracitano *et al.,* 2004; Perez *et al.,* 2004; Poirier *et al.,* 2006; Won *et al.,* 2008). However, previous studies reported no significant relationship between MTLP induction and the overall metal concentrations in *N. diversicolor*, suggesting that this biomarker does not

reflect metal bioavailability on this biological model (Poirier *et al.*, 2006; Gomes *et al.*, 2013). Comprehension of functional genomic and transcriptomic responses of MTLPs in ragworms was first described by McQuillan *et al. (*2014), which identify two putative MTLP genes encoding cysteine-rich proteins (i.e. Cd/Se MT and atypical MTLP), after Cu long-term exposure. In the present study, MTLP levels significantly increased in worms exposed to the highest CisPt concentration (i.e. 100 ng CisPt L^{-1}) (Fig. 2.4). On the route to DNA attack, CisPt species interact with biomolecules containing methionine and cysteine residues (Reedjik, 1999), followed by an increase of the thiol-containing protein levels (Jamieson and Lippard, 1999; Siddik *et al.*, 2003; Supalkova *et al.*, 2008).

MTLP overexpression seems to be an useful cellular defence against CisPt and is determinant in drug resistance during chemotherapy, by decreasing the level of the antitumour agent available for interaction with the target DNA (Doz *et al.*, 1993; Hagrman *et al.*, 2003; Smith *et al.*, 2006). Besides evidence on modulation of drug responses, MTLPs also mediate a decrease in toxicity of CisPt (Jamiesen and Lippard, 1999). Despite the remarkable binding strength of GSH to CisPt and outcomes in drug resistance, MTLPs trap on the drug molecule is significantly higher and limits the amount of the drug available for binding to DNA (Doz *et al.*, 1993; Fuertes *et al.*, 2003; Hagrman *et al.*, 2003). In this sense, MTLPs indicate a potential antioxidant performance regarding electrophilic cytotoxic agents (Doz *et al.*, 1993; Geret *et al.*, 2003; Gagné *et al.*, 2008) for the highest CisPt concentration, induced by the decrease of the other antioxidant enzymes activity. Likewise, levels of LPO increased with the increasing CisPt concentration (Fig. 2.5) but were only significant at 100 ng Pt L^{-1} confirming a decrease in the antioxidant lines of defense, leading to alterations such as protein degradation and membrane damage (Viarengo *et al.*, 1990; Viarengo *et al.*, 2007; Gomes *et al.*, 2014). Consequently, unpaired electrons in the lipid molecules give rise to hydroperoxide radicals, able to trigger chain reactions based on assemblage of electrons from other lipid structures. The LPO pathway may also arise from the depletion of GSH and NADH resulting in dehydrogenase inhibition and uncoupling of oxidative phosphorylation (Dasari and Tchounwou, 2014). This process leads to hydroxyl radical formation and more oxidative stress. The decrease of the activity of GST enzymes implies the need to protect cells against peroxides (Hurst *et al.*, 1998). In this sense, the action of Se-GPx is an important alternative to counteract harmful electrophiles produced during oxidative damage (Fig. 2.7).

Despite the increase in Se-GPx activity, the defence pathway is not enough in particular for the worms exposed to the highest concentration, as accounted in PCA by the positive relationship between both Se-GPx and LPO products (Fig. 2.7). The detection of genotoxic alterations caused by exposure to cytotoxic drugs at environmental conditions could be of great relevance to address aquatic ecotoxicological impacts.

Polychaetes are highly sensitive to genotoxic damage (Lewis and Galloway, 2008; Catalano *et al.*, 2012; Buffet *et al.*, 2013; Maranho *et al.*, 2014). Agents such as CisPt binds covalently to base residues on DNA strands producing DNA-DNA intra- and inter-strand cross-links (ICLs) (Siddik, 2003) that, respectively, comprise the link of two nucleotides in the same and opposite strands (Deans and West, 2011). Intra and inter-strand cross-links binds strongly to prevent DNA strands separation, thus blocking DNA replication and transcription (Jamisien and Lippard, 1999; Noll *et al.*, 2006; Deans and West, 2013). The absence of detection of the double and single strand breaks in CisPt contaminated worms, assessed by alkaline comet assay, may be attributed to the presence of ICLs and DNA-DNA intra-strand crosslinks, leading to the closure of DNA strands and less DNA migration in electrophoresis (Merk and Speit, 1999). Contrarily, genotoxicity outcome by strand breaks formation occurred in freshwater daphnids *Daphnia magna* and *Ceriodaphnia dubia* exposed to CisPt (100 ng L^{-1} and 300 ng L^{-1}, respectively) over 24 h (Parrella *et al.*, 2015). DNA damage was also present throughout the 14 days of exposure of *M. galloprovincialis* to 100 ng Pt L^{-1}, suggesting that CisPt exerts its toxic effects probably by direct breaking of DNA strands and ROS production (Trombini *et al.*, 2016a). In general, CisPt induces DNA repair mechanisms to remove adducts and promote cell survival, by stalling the helix distorting occasioned by chemotherapeutic drugs and UV radiation (Siddik, 2003). Through the SOS chromotest, CisPt drug was recognized as a potent inducer of SOS reparation system in *Escherichia coli* PQ37, where DNA repair mechanism and recombinant enzymes were expressed (Lantzsch and Gebel, 1997; Nowosielska *et al.*, 2004; Parrella *et al.*, 2015).

Thus, certain mutations of genes responsible for repair pathways inherent to each species may be prone to generate differences in sensitivity to cytotoxic drugs (Huang and Li, 2013) that need to be confirmed in this case. Still, the absence of genotoxicity, in the present study, may also stems from the remarkable induction of antioxidant potential at the highest drug concentration, acting to supply the overwhelmed enzymes activity against CisPt

toxicity. In addition, the nucleophilic trapping capacity of MTLP and GSH to CisPt are widely known to contribute to resistance to the drug, once its availability to nucleus is reduced (Doz *et al.,* 1993). According to the European Medicine Agency (EMA) guidelines, ecotoxicological assays with representative aquatic species of each trophic level must be performed to screen and assess environmental risk posed by drugs (EMA, 2006). Nevertheless, these bioassays are only required for pharmaceuticals under processes for production authorization and market implementation, which discard anticancer drugs for the submission to such a procedure (Besse *et al.,* 2012). Besides, very few studies exist that apply the biochemical, metabolic and physiological responses in aquatic organisms exposed to chronic environmental realistic concentrations (Fent *et al*., 2006b; Maranho *et al*., 2014).

This study clearly demonstrates the response of multibiomarker approach involved in behaviour assessment, oxidative stress and genotoxic responses that are consistent to the MoA of this drug in this species. Despite that the responses addressed are mostly depicted at the highest CisPt concentration, Pt concentration at ng L^{-1} range reflects that very low concentrations combined with chronic exposure may represent a hazard for aquatic species. In addition, the bioassay was conducted with only one pharmaceutical compound, whereas a mixture of several other compounds with similar and different MoA towards anticancer activity, also at low concentrations, have already been detected in coastal waters (Besse *et al.,* 2012). The present approach and exposure stand out of a standardized and classic biotest applied for regulatory considerations, and holds significance based on the reality and complexity of the benthic aquatic environment, including the environmentally realistic concentrations of the chosen drug.

2.5 Conclusions

To the best of our knowledge, this is the first (eco)toxicity assessment dealing with cytotoxic drugs between water and sediment compartments. The present study indicate that CisPt has the potential to induce neurotoxic effects that may alter the burrowing profile of polychaetes. Inhibition of antioxidant enzymes activity (SOD, CAT) and biotransformation (GST) and potential accumulation of pro-oxidant radicals led to stress able to further increase membrane susceptibility to lipid peroxidation. These biological alterations were only observed at highest CisPt levels, which if detected in the environment may depict a high

biological risk. Nevertheless, the lack of enough biochemical protection to prevent impairment was verified at ng L^{-1} range highlighting concern regarding the drug potential to non-target aquatic species. Putting all biomarkers data together, confirm, once again, the reliability of this species for ecotoxicology assays, as well as the sediment compartment as an important target, especially regarding emergent contaminants such as anticancer pharmaceuticals.

Chapter 3

Environmental relevant levels of the cytotoxic drug cyclophosphamide produce harmful effects in the polychaete *Nereis diversicolor*

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Abstract

Cytotoxic drugs applied in chemotherapy enter the aquatic environment after patients's metabolism and excretion, in both main compounds and their respective metabolites. The increased consumption and discharge of these drugs raise concern on the genotoxic burden to non-target aquatic species, due to their unselective action on DNA. Settlement and adsorption of cytotoxic drugs to aquatic sediments pose risks to benthic species through chronic exposure. The aim of the present study was to assess the effects induced by the anticancer drug cyclophosphamide (CP) on the polychaete *Nereis diversicolor*, after 14 days of exposure to environmental relevant concentrations (10, 100, 500 and 1000 ng L^{-1}). Burrowing impairment, neurotoxicity (Acetylcholinesterase - AChE activity), oxidative stress (superoxide dismutase – SOD; catalase – CAT; glutathione peroxidases - GPXs activities), biotransformation (glutathione-S-transferases - GST), oxidative damage (lipid peroxidation - LPO) and genotoxicity (DNA damage) were assessed. Burrowing impairments were higher at the lowest CP concentrations tested. The higher CP levels tested (500 and 1000 ng L^{-1}) induced a significant inhibition on the enzymatic antioxidant system (SOD, GPx) and on GST activity. DNA damage was also significant at these concentrations as an outcome of CP metabolism, and high levels of oxidative damage occurred. The results showed that the prodrug CP was metabolically activated in the benthic biological model *N. diversicolor*. In addition to the potential cytotoxic impact likely to be caused in aquatic species with similar metabolism, *N. diversicolor* proved to be reliable and vulnerable to the cytotoxic mode of action of CP, even at the lower doses.

Keywords: Anticancer drugs, cyclophosphamide, sediments, polychaetes, oxidative stress, genotoxicity.

3.1 Introduction

Anticancer drugs (or antineoplastic drugs) are a group of pharmaceuticals routinely and widely administered in chemotherapy all over the world, whether individually or in combination with other cytotoxic agents. In 2012, 14.1 million new cancer cases were reported around the world, and the World Health Organization estimates an enhancement of 57% of new cases until 2030 (WHO, 2014). Besides the subsequent increase of cytotoxic prescriptions, approximately 80% of oncology patients receive treatment at oncology wards and go home after drug administration (i.e. out-patients). Additionally to conventional elimination into hospital sewage systems, metabolites and unchanged fractions of the administered drug are excreted through urban effluents, following to municipal WWTPs, where elimination of these substances is incomplete (Johnson *et al.,* 2008; Kosjek and Heath, 2011). Furthermore, these chemicals may also be released directly to the water bodies, in regions not properly attended by sewage collection and treatment systems (Abessa *et al.,* 2005; Pessati *et al.,* 2016).

Consequently, anticancer drugs are detected at low levels (ng to $\mu g.L^{-1}$ range) in aquatic systems (Crane *et al.,* 2006; Johnson *et al.,* 2008; Kosjek and Heath, 2011; Booker *et al.,* 2014; Mater *et al.,* 2014) and long-term exposure to non-target species has led to the concern of genotoxic burden of cytotoxic drugs to aquatic species (Steger-Hartmann *et al.,* 1996, 1997; Buerge *et al.,* 2006; Ferrando-Climent *et al.,* 2014). In general, the conventional chemotherapy drugs act directly on the DNA double strand, in order to avoid the synthesis and proliferation of tumour cells (Farber, 1973). Such covalent interaction primarily alters the DNA helical structure, influences the binding of chromatin protein and induces DNA strand breaks, subsequently producing genotoxic effects, followed by the death of the tumour cells (Harris, 1976; Ou and Lien, 1985). However, once these "targets" are not specific drivers of cancer cells, normal growing cells are also affected through the same MoA by which they may be killed or left unrepaired, followed by genomic instability with neoplastic transformation and eventual carcinogenic mutations (Johnson *et al.,* 2008; Kosjek and Heath 2011; Ferrando-Climent *et al.,* 2014). Acting unselectively on DNA, it has been hypothesized that anticancer drugs may harm all eukaryotic organisms by cytotoxic, genotoxic, mutagenic and carcinogenic effects (Johnson *et al.,* 2008; Vyas *et al.,* 2014). The

biological risk posed by these anticancer molecules and their metabolites in aquatic species is not well elucidated (Mater *et al.,* 2014), with few data on acute and chronic ecotoxicological assessments in algae *Tetrahymena pyriformis* (Bonnet *et al*., 2003), *Pseudomonas putida* (Zounková *et al.,* 2007) and *Pseudokirchneriella subcapitata* (Zounková *et al.,* 2007; Brezovsec *et al.,* 2014; Česen *et al.,* 2016); polychaete *Nereis diversicolor* (Fonseca *et al.,* 2017); bivalve mollusc *Mytilus galloprovincialis* (Trombini *et al.,* 2016a); cladocera crustaceans *Daphnia pulex* (DellaGreca *et al.,* 2007; Borgatta *et al.,* 2015, 2016), *D. magna* (Zounková *et al.,* 2007; Parrella *et al.,* 2014a, 2014b, 2015) and *Ceriodaphnia dubia* (DellaGreca *et al.,* 2007; Parrella *et al.,* 2014a, 2014b, 2015); crustacean amphipod *Ampelisca brevicornis* (Moreira *et al*., 2016)*;* and the fishes *Danio rerio* (Kovács *et al.,* 2015), *Pimephales promelas* (Winter *et al*., 2007) and *Oryzias latipes* (Sun *et al.,* 2011). Limited information exists regarding Environmental risk assessment (ERA) for anticancer drugs. ERA guidelines are assigned only for newly authorized pharmaceuticals (since 2006) and does not include molecules prone to cause genotoxicity impairments in aquatic ecosystems (Johnson *et al.,* 2008; Aguirre-Martínez *et al.,* 2016b).

Cyclophosphamide (CP) is one of the oldest and most frequently prescribed cytotoxic and alkylating agents used in cancer treatment, approved since 1960´s (Gilard *et al.,* 1994; Buerge *et al.,* 2006; Česen *et al.,* 2015). It consists of a phosphoramide ring linked to a bifunctional moiety containing two chloro-ethyl groups. CP is a prodrug and its activation consists in the oxidation by cytochrome P450 mixed function oxidase to form 4-hydroxy-CP (4-OHCP), undergoing subsequent transformations to yield the major cytotoxic species, the phosphoramide mustard (PAM) and acrolein (Figure 3.1).

After administration, around 80% of the dose is excreted as metabolites, which accounts for substantial amounts of metabolites disposed into sewage systems (Bagley *et al.,* 1973; Steger-Hartmann *et al.,* 1996). In the absence of metabolic activating systems, CP fails to bind to DNA and its genotoxic activity is prevented. Even without metabolic activation by S9-rat extracted fraction, the parental drug CP caused mutagenic effects in the bacteria *Escherichia coli* and *Salmonella typhimurium*, and in the cyanobacteria *Synecococcus leopoliensis* (320 mg L^{-1}) (Česen *et al.,* 2016), indicating that not only the metabolites are involved in cellular impairments (Mohn and Ellenberger, 1976; Benedict *et al.,* 1977; Balbinder *et al.,* 1981; Winckler *et al.,* 1984). Research concerning CP and its

ecotoxicological effects in aquatic organisms is scarce and do not comprise exposure routes by which species would uptake the drug. Data available addresses the drug as a toxic positive control in *in vivo* short-term bioassays assessing mortality, immobilization, growth and reproduction inhibition, larval development and genotoxicity as end-points with CP levels far from environmental relevant concentrations (Matsumoto and Cólus, 2000).

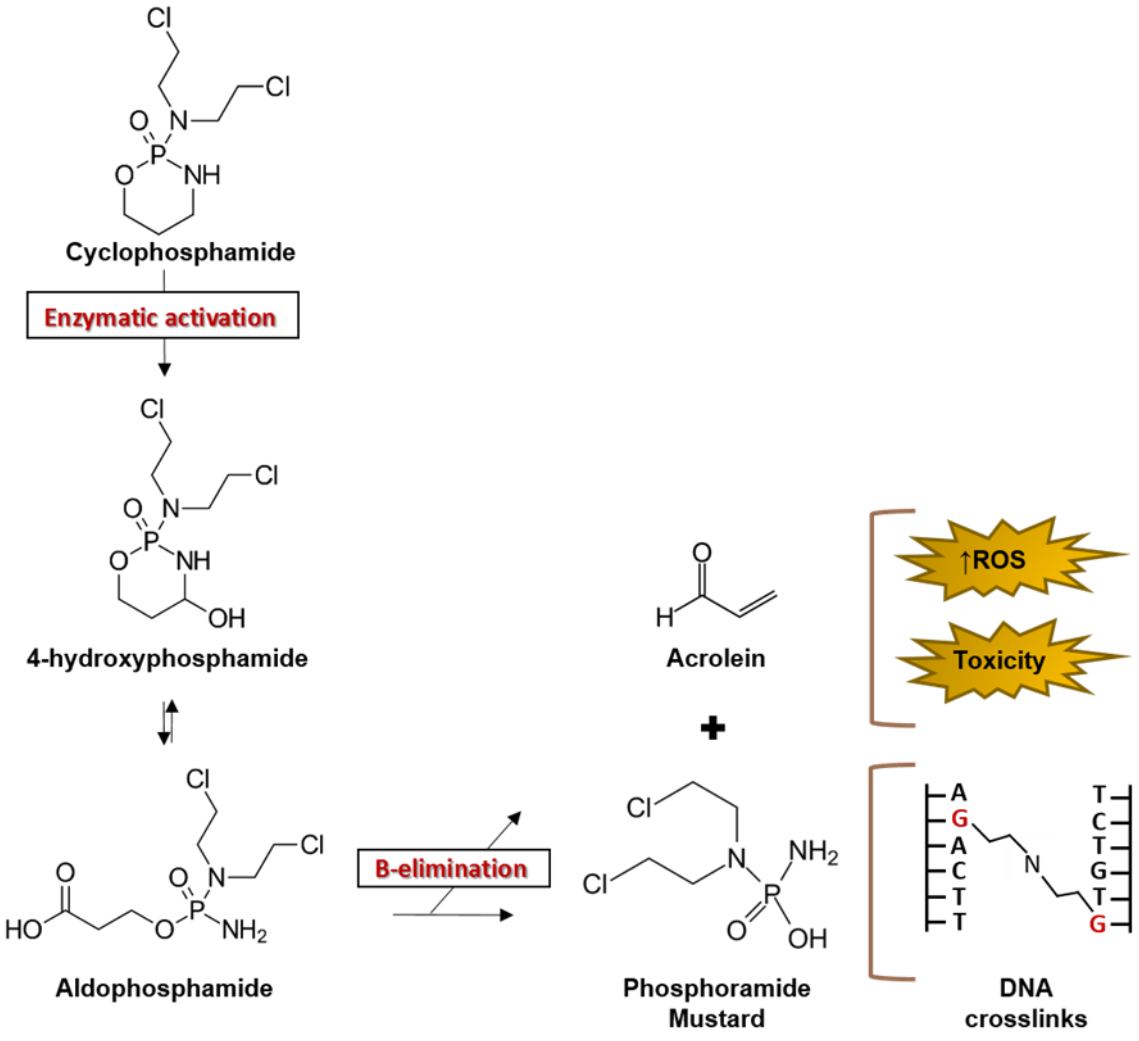


Figure 3.1: CP metabolism pathway. Enzymatic activation by cytochrome P-450 enzyme system generates 4-hydroxycyclophosphamide, coexisting with the non-cytotoxic aldophosphamide metabolite. It decomposes into acrolein, that is toxic, and the DNA-binding phosphoramide mustard.

Considering that most of cytotoxic drugs are highly polar, with low Kow, the fraction of drugs adsorbed into sewage sludge could be neglected and distribution is assumed to be mainly in the water column (Kosjek and Heath, 2011; Seira *et al*., 2016). Negligible biodegradability, volatilization and adsorption of CP to biosolids confirms this assumption (Steger-Hartmann *et al.*, 1996, 1997; Buerge *et al.*, 2006). However, pharmaceuticals are multi-functional ionisable compounds and conventional partitioning models are not suited for a straightforward prediction of their environmental fate (Kwon and Armbrust, 2008). Once in the aquatic environment, hydrophobic interactions providing adsorbance of polar compounds to particulates may not be disregarded, especially for organic cations, which are formed during intra-molecular cyclizations for cytotoxic drugs activation (*e.g.* CP aziridinium cation) (Emadi *et al.*, 2009). Therefore, specific transformations in coastal environments may allow their adsorption, transference and persistence to the sediments (Kosjek and Heath, 2011; Maranho *et al.*, 2014; Moreno-González *et al.*, 2015).

Sediments are the ultimate sink for contaminants in the marine environment, posing benthic species at risk through chronic exposure and bioaccumulation (Araujo *et al*., 2013; Buruaem *et al*., 2012; Rodrigues *et al*., 2013; Vethaak *et al*., 2017). Nevertheless, aquatic ecotoxicological assessment of anticancer drugs involving sediments is lacking (Fonseca *et al.*, 2017). In addition, to the best of our knowledge, only two studies exist on the occurrence of anticancer drugs in river sediments, where levels ranged below the detection limit (Zuccato *et al*., 2000) and 391 ng kg^{-1} (bicalutamide), 392 ng kg^{-1} (doxifluridine), and 250 ng kg^{-1} (tamoxifen) (Azuma *et al*., 2017). So far, no screening approach was directed to the coastal environment. The levels of CP detected in hospital effluents, WWTPs influents and effluents, surface waters and sediments are indicated in Table 3.1. Data suggests that this drug and its metabolites are likely to contribute to overall toxicity in receiving waters (Kiffmeyer *et al.*, 1998; Yasunaga *et al.*, 2006; Kosjek and Heath, 2011; Česen *et al.*, 2016).

Polychaetes are widely used in marine environmental health assessments due to their ecological key role in benthic dynamics (Solé *et al.*, 2009), and their rapid and easy response to disturbances induced by different stressors (Sivadas *et al.*, 2010; Cong *et al.*, 2011; Buffet *et al.*, 2014; Maranho *et al.*, 2015). The species *Nereis* (*Hediste*) *diversicolor* is highly recommended for ecotoxicological assessment regarding their physiological and biochemical responses after exposure to different classes of pharmaceuticals (e.g. anti-

inflammatory; oral contraceptive; anticonvulsant; antidepressant drugs) (Aguirre-Martínez *et al.*, 2013; Maranho *et al.*, 2014, 2015; Pires *et al.*, 2016a; Fonseca *et al.*, 2017). Exposure to the cytotoxic drug cisplatin, at trace concentrations (0.1; 10 and 100 ng Pt L^{-1}) in seawater, caused behavioural and biochemical impairments in this species, after 14 days of exposure (Fonseca *et al.*, 2017).

The aim of the present study is to assess the effects induced by the anticancer drug CP in the polychaete *N. diversicolor*, exposed to a range of environmental relevant concentrations of CP detected in water compartments. For this purpose, the following biomarkers were assessed (1) behavioural impairment (burrowing kinetic impairment), (2) neurotoxicity (acetylcholinesterase - AChE activity), (3) oxidative stress (superoxide dismutase – SOD; catalase – CAT; glutathione peroxidases - GPXs), (4) Phase II conjugation reaction (glutathione-S-tranferases - GST activity), (5) oxidative damage (Lipid peroxidation LPO) and (6) genotoxicity (DNA damage), after exposure for 14 days to a contaminated water-sediment system.

Table 3.1: Concentrations of CP in water (ng L^{-1}) and sediments (ng g^{-1}).

Study area	Hospital Effluent	STP Influent	STP Effluent	Surface Water		River Sediment	Detection Method	Reference
				Upstream	Downstream			
Australia	-	-	125	-	< 100	-	SPE; LC-MS/MS (ESI)	Busetti *et al. (*2009)
Canada		< 4 - 22	4 - 21			-	SPE; LC-MS/MS (ESI)	Rabii *et al. (*2014)
China	6 - 2,000	-		-	-	-	SPE; UPLC-MS/MS (ESI)	Yin *et al. (*2010)
Thailand	-	-			1,907	-	SPE; HPLC-MS/MS	Usawanuwat *et al. (*2014)
England	-	-	19 - 0.37	-	-	-	SPE; LC-MS/MS (ESI)	Llewellyn *et al. (*2011)
Germany	19 - 4,500	< 6 - 143	< 6 - 17	-	-	-	SPE; GC-MS (EI)	Steger-Hartman *et al. (*1997)
Germany	146	-		-	-	-	SPE; GC-MS (EI)	Steger-Hartman *et al. (*1996)
Germany	-	-	nd	-	nd	-	LC-MS/MS (ESI)	Ternes (1998)
Italy	-	-		-	nd (8 rivers)	-	SPE; HPLC-MS/MS (ESI)	Calamari *et al. (*2003)

Italy	-	-	nd - 9	-	-	-	SPE; HPLC-MS/MS (ESI)	Castiglione *et al. (*2005)
Italy	-	-	0.6	-	nd	-	SPE; HPLC-MS/MS	Zuccato *et al. (*2005)
Italy	-	-		-	2.2 - 10.1	< 12	SPE; HPLC-MS/MS	Zuccato *et al. (*2000)
Japan	-	-		-	nd	nd	SPE; UPLC-MS/MS	Azuma *et al. (*2017)
Norway	< 2 - 21	< 2	2	-	-	-	SPE; LC-TOF/MS (ESI)	Thomas *et al. (*2007)
Romania	-	-		-	< 30 - 64.8	-	SPE; GC-MS (EI)	Moldovan (2006)
Spain	< 1.1 - 43	8 - 26	7 - 25	< 0.9	< 0.9 - 20	-	SPE; UPLC QqLiT	Ferrando-Climent *et al. (*2014)
Spain	-	-		-	< 3	-	SPE; HPLC-MS/MS	Valcárcel *et al. (*2011)
Spain					7.8 - 13.7	-	SPE; LC-MS (ESI)	Franquet-Griell *et al. (*2017)
Spain	5.73	< 3.1 - 13,100	3.1	-	-	-	SPE; LC-MS (ESI)	Gomez-Canela *et al. (*2012)
Spain	-	< 2.1	<2.3	-	< 1.7	-	SPE; HPLC-MS/MS (ESI)	Martín *et al. (*2011)
Spain	-	nd	nd	-	-	-	SPE; HPLC-MS/MS (ESI)	Martín *et al. (*2014)

Spain		nd - 43.8	nd - 25	-	-	-	SPE; LC-MS/MS	Negreira *et al. (*2014a)
Slovenia	14 - 22,000	19-27	17	-	-	-	SPE; GC-MS (EI)	Česen *et al. (*2015)
Switzerland	-	2 - 11	2 - 10	-	0.15 - 0.17	-	SPE; LC-MS/MS (ESI)	Buerge *et al. (*2006)
Switzerland	0.161	-		-	-	-	SPE; HPLC-MS/MS (ESI)	Kovalova *et al. (*2012)

- STP (Sewage treatment plant).

3.2 Materials and Methods

3.2.1 Chemicals

Cyclophosphamide monohydrate (Cytoxan) (CAS 0768) was purchased from Sigma-Aldrich (Portugal). For safety handling of the cytotoxic drug, experimental work was performed using class II biological safety cabinet, with appropriate clothing (open-back, impervious chemotherapy protection gown, double powder-free latex gloves and safety goggles). CP stock solution (40 mg L^{-1}) was diluted in ultrapure Milli-Q water to prepare test solutions.

3.2.2 Experimental setup

Sediments and specimens of *N. diversicolor* were handpicked during summer, at low tide at the intertidal estuarine mudflat in Mira River estuary (Vila Nova de Milfontes), located in the Southwest coast of Portugal (37.729031 N, -8.751585 W). The site is considered undisturbed and was used as a reference site for sediment quality assessment (Ferreira *et al.*, 2003; Moreira *et al.*, 2006; Fonseca *et al.*, 2017). Sediments were mainly composed of silt and clay (73% of particles <63 µm), with a organic matter content of 7.8%. Animals were transported alive to the laboratory with sediments and water from the site of origin. The organisms were acclimated for 5 days in aerated aquaria filled with natural filtered seawater (salinity 35) from the Ria Formosa lagoon (Faro, Portugal) and sediments from the sampling site. The sediments were wet-sieved through a 2-mm mesh for removal of large debris and other living organisms, followed by drying at 80°C (Thain and Bifield, 2001; ASTM, 2009; Maranho *et al.*, 2014), to remove volatile compounds and water. Sediments were then re-hydrated with the same amount of water (w.w./d.w.). Dried aliquots of the sediments were used to determine grain size distribution by the method proposed by Royse (1970). Organic matter content was determined by loss on ignition (550°C, for 5 h), as described by Gross (1971).

The 14-day bioassay was carried in 20-L glass aquaria, with a proportion of 1:4 sediment/seawater (Faro, Ria Formosa), under constant aeration, controlled temperature (19 ± 2°C), salinity (35 ± 1.8) and light period (12:12 hours). Each treatment was performed in

triplicate, including seawater controls (day 0: CT0; day 14: CT14), solvent control (0.001% DMSO) and CP at a range of concentrations based on reported levels in the environment (Table 3.1). Polychaetes were divided into groups of 75 per treatment (25 per aquarium). Over the 14 days of exposure, water was renewed every 48 hours avoiding sediment resuspension, with redosing of the drug in the water phase (0, 10; 100; 500 and 1000 ng L^{-1}) in order to simulate the input of pharmaceuticals in aquatic system. Animals used in the burrowing and comet assays were immediately handled for respective analysis, while those regarding biochemical end-points were rinsed with clean seawater and stored at -80 °C until further use.

3.2.3 Burrowing Assay

Worms of control conditions (day 0: CT0; day 14: CT14) and those exposed to the different CP concentrations were submitted to a burrowing test according to Bonnard *et al. (*2009). Fifteen animals of each treatment were carefully placed individually in 150 mL-plastic containers, filled with natural seawater and 5 cm of sediments. Over a period of 30-minutes, the position of the polychaetes was recorded every two minutes, to assess the time for fully burrowing. The results are expressed as the percentage (%) of unburied specimens, over time (min).

3.2.4 Biochemical analysis

Neurotoxicity

Animals (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment) were individually homogenized in 100 mM Tris-HCl buffer (pH 8.0) and 0.1% Triton. The homogenates were centrifuged at 12,000 *g*, for 30 min, at 4 °C, and further separated in aliquots for total protein determination (Bradford, 1976) and AChE activity analysis (Ellman *et al.,* 1961). AChE activity was measured through the increase of the absorbance of the yellow compound resulting from the production of 5-mercapto-2-nitrobenzoate (ε = 13.6 mM^{-1} cm^{-1}) formed by the reaction of thiocholine, a product of acetylcholine cleavage by AChE with DTNB, at 405 nm (Ellman *et al.,* 1961; Colovic *et al.,* 2013). AChE activity is expressed as $ATC.min^{-1}$ mg^{-1} protein.

Tissue preparation for enzyme activities analysis

Whole organisms (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment) were homogenized in 20 mM Tris-HCl buffer (0.5 M sucrose, 0,075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6), according to the protocol described by Geret *et al.* (2002). The homogenates were centrifuged at 500 g, for 15 min, at 4 °C, and the supernatants obtained centrifuged again (12,000 g, 45 min, 4 °C). Aliquots (150 µL) of the cytosolic fraction were separated for determination of each antioxidant enzyme activity (SOD, CAT, GPX) and biotransformation (GST). In addition, total proteins concentrations (mg protein g^{-1} tissue) were determined following the method described by Bradford (1976), adapted for microplate reader using bovine serum albumin (BSA) as a standard.

Antioxidant enzymes activities

SOD activity was assessed by measuring the decrease of absorbance of the substrate cytochrome-c by xanthine oxidase/hypoxanthine system, at 550 nm (McCord and Fridovich, 1969), and the results are expressed as U mg^{-1} protein. CAT activity was determined spectrophotometrically by measuring the decrease of absorbance at 240 nm (Greenwald, 1985). GPX activity was measured at 340 nm by using cumene hydroperoxide and H_2O_2 as substrates for T-GPx and Se-GPx, respectively (adapted from Lawrence and Burk, 1976). The activity of CAT, Se- and T-GPx are expressed in nmol min^{-1} mg^{-1} protein.

Biotransformation

GST activity was measured in the cytosolic fraction by the conjugation of 0.2 mM reduced glutathione (GSH) with 0.2 mM CDNB (molar coefficient of extinction = 0.6 mM^{-1} cm^{-1}) in a reaction mixture of 0.2 M KH_2PO_4/K_2PO_4 buffer (pH 7.9), at 340 nm (adapted from Habig *et al.*, 1974). The results are expressed in nmol CDNB min^{-1} mg^{-1} protein.

Oxidative damage

LPO was determined in whole organisms (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment), by the absorbance of malondialdehyde (MDA) and 4-

hydroxyalkenals (4-HNE) concentrations, at 540 nm (adapted from Erdelmeier *et al.*, 1998). LPO is expressed in nmol MDA + 4-HNE mg^{-1} protein.

Genotoxicity assay

DNA damage was assessed by the alkaline Comet assay, adapted from Singh *et al.* (1988) and described by Gomes *et al.* (2013). Slides were previously cleaned in alcohol/ether and coated with 0.65 % normal melting point agarose (NMA) in Tris-acetate EDTA. Coelomocytes present in the coelomic fluid of *N. diversicolor* (5 specimens per aquaria, in triplicate; total of 15 organisms per treatment) were extracted from the posterior region of the polychaete body into 20 µL of PBS buffer in a 0.5 mL-syringe fitted with hypodermic needle, based on the procedure described by Fonseca *et al.* (2017). The mixture was centrifuged at 835 g (3 min, 4 °C) and the pellet suspended in 0.65 % low melting point agarose (LMA, in Kenny's salt solution; 0.4 M NaCl, 9 mM KCl, 0.7 mM KH_2PO_4, 2Mm $NaHCO_3$, 1000 ml Milli-Q water) and casted on the microscope slides. Subsequently, slides were immersed in a lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, 10% Dimethylsulfoxide,1% Sarcosil, pH 10, 4 °C), over one hour, for the diffusion of cellular components and DNA immobilization in agarose. Slides were then placed in electrophoresis chamber, embedded with buffer (300 mM NaOH, 1 mM EDTA, adjusted at pH 13, 4 °C) and left for 15 min to permit DNA unwinding. The electrophoresis was performed at 25 V and 300 mA, over 5 min. Afterwards, slides were neutralized with appropriate buffer (0.4 mM Tris, pH 7.5), rinsed with distilled water and left to dry overnight. Slides were analysed with an optical fluorescence microscope Axiovert S100 (total magnification of ×400), coupled with a camera, with an aid of 4,6-diamidino-2-phenylindole (DAPI, 1 mg ml^{-1}). Image analysis was made with the software Komet 5.5 (Kinetic Imaging Ltd) by classifying 50 randomly chosen cells from each slide. The amount of DNA in the comet tail (DNA tail %) was used as an end-point and results are expressed as mean ± standard deviation.

3.2.5 Statistical analysis

Obtained data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene´s test) in order to determine whether they satisfy the assumptions

associated with parametric tests, and one-way analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis were applied. Tukey´s or Dunn´s post-hoc tests were performed to compare treatment effects. Results were significant when $p < 0.05$. Burrowing behavioural data was analysed by linear regression. Principal Component Analysis (PCA) were applied to evaluate the relationship among biomarkers responses and between pharmaceuticals levels. Statistical analysis was carried out using the Statistica 8.0 software (Statsoft Inc., 2007, USA).

3.3 Results

3.3.1 Behavioural Assay

Results of burrowing assessment are indicated in Figure 3.2. Slopes of trend lines depicting by linear regression analysis showed significant statistical differences between all treatments ($p < 0.05$). Both control conditions (CT0 and CT14) indicate similar trend, as well as for animals exposed to the concentration of 100 ng L^{-1}, that was entirely overlapped by CT0. Perturbation in burrowing, however, were higher with the decrease of cyclophosphamide levels. Polychaetes exposed to 1000 ng L^{-1} were fully buried after the first 5 minutes, whereas at 500 ng L^{-1}, animals lasted 20 minutes at surface until complete burrowing while among those exposed to 10 ng L^{-1} not all the animals were buried.

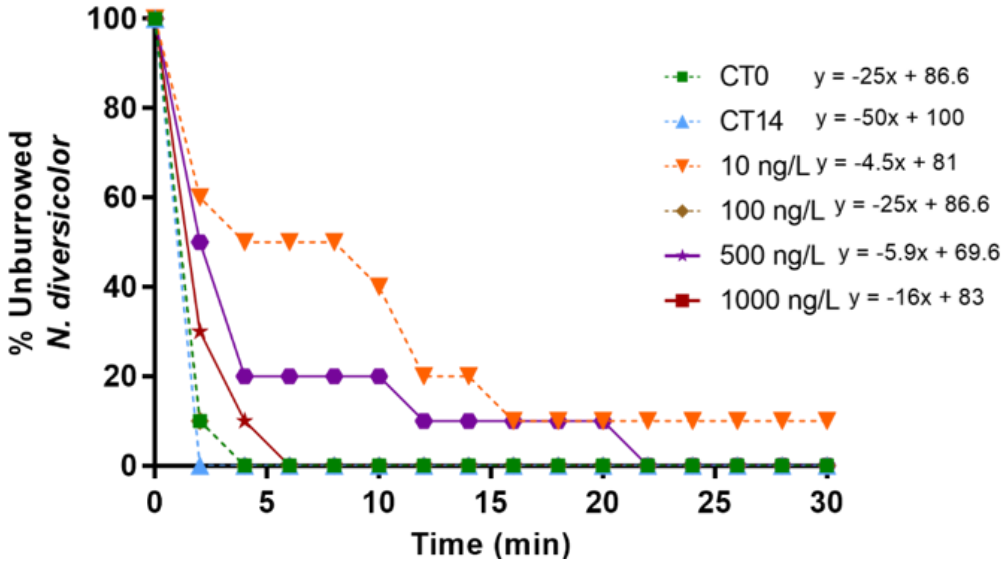


Figure 3.2: Burrowing behaviour of *N. diversicolor* from control conditions (day 0: CT0; day 14: CT14) and those exposed to CP-contaminated systems (10, 100, 500 and 1000 ng L^{-1}), expressed as percentage of unburied organisms over time (lines with symbols). Continuous lines represent least-square best-fit regression lines, with respective equations.

3.3.2 Biochemical analysis

Neurotoxicity

AChE activity did not change between controls over time ($p > 0.05$). A slight decreasing in AChE activity was observed in polychaetes exposed to the lowest CP concentrations (10 ng L^{-1}), although not significant ($p > 0.05$). With the increase of CP concentrations, AChE activity was similar to the controls ($p > 0.05$) (Figure 3.3).

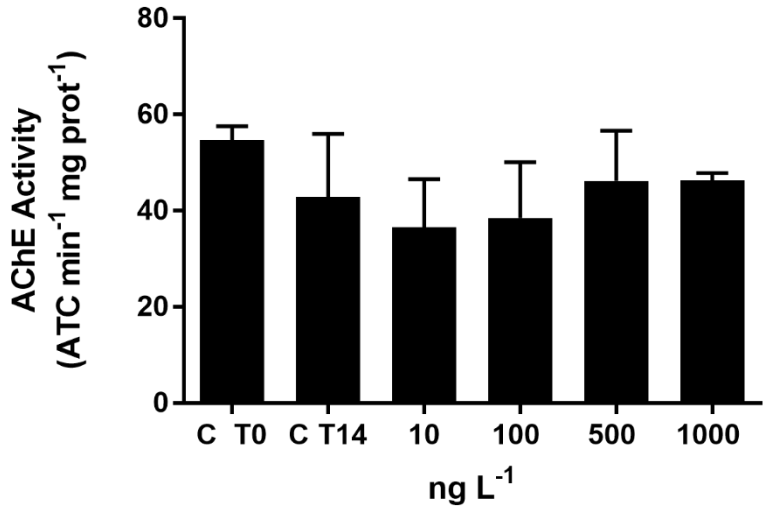


Figure 3.3: AChE activity (mean ± S.D.) (ATC.min^{-1}.mg^{-1} protein) in *N. diversicolor* unexposed (CT 0 and CT 14) and exposed to CP (10, 100, 500 and 1000 ng L^{-1}) for 14 days. Absence of letters indicates no significant differences among treatments (Kruskal-Wallis, $p > 0.05$).

Antioxidant and biotransformation enzymes

Antioxidant enzymes activities were similar between controls over time ($p > 0.05$). Despite no significant statistical differences between CP-treatments and controls, it was observed an increase in SOD activity in ragworms exposed to 10 and 100 ng L^{-1} of CP, followed by an inhibition in organisms exposed at the higher CP levels (i.e. 500 and 1000 ng L^{-1}) (Figure 3.4A). A significant 2-fold increase in CAT activity was detected in worms exposed to concentrations of 10 and 500 ng L^{-1} (Figure 3.4B), whereas no significant changes were observed for GPx Se-dependent activity with CP increasing levels ($p > 0.05$). On the other hand, a clear decreasing trend in T-GPx activity was detected with increasing CP concentrations, significantly different from controls only in polychaetes exposed to 500 and 1000 ng L^{-1} ($p < 0.05$). Likewise, GST activity decreased with the increasing CP concentrations, significant at 100, 500 and 1000 ng L^{-1} ($p < 0.05$) (Figure 3.5).

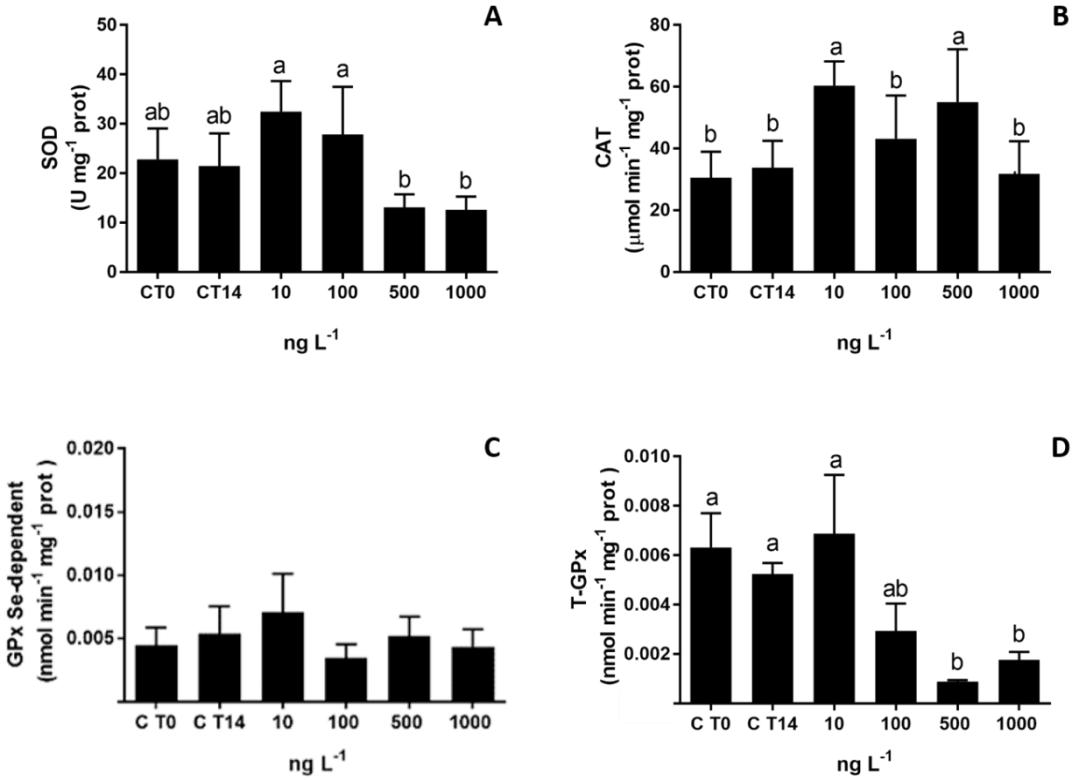


Figure 3.4: Antioxidant enzymes activities (mean ± S.D.) of (A) SOD, (B) CAT, (C) GPx Se-dependent, (D) T-GPx in *N. diversicolor* of control conditions (CT0 and CT14) and exposed to CP (10, 100, 500 and 1000 ng L^{-1}) for 14 days. Different letters indicate significant difference among treatments (ANOVA: SOD, CAT; Kruskal-Wallis: GPx Se-dependent, T-GPx; $p < 0.05$). Absence of letters indicates no significant differences among treatments ($p > 0.05$).

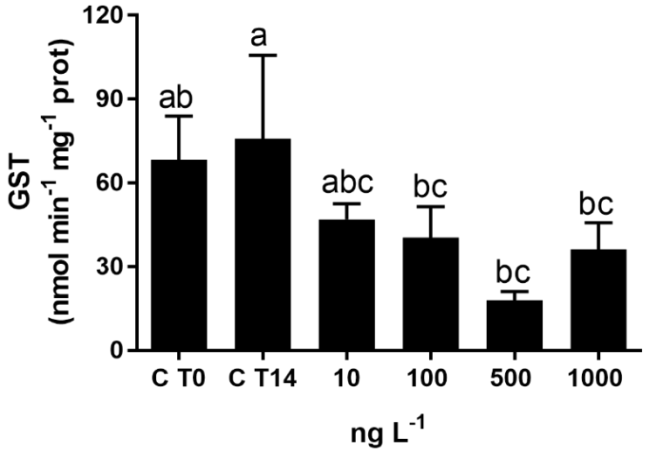


Figure 3.5: Biotransformation enzyme activity (mean ± S.D.) in *N. diversicolor* of control conditions (CT0 and CT14) and exposed to CP (10, 100, 500 and 1000 ng L^{-1}) for 14 days. Different letters indicate significant differences among treatments (Kruskal-Wallis, $p < 0.05$).

Oxidative damage

An increase of oxidative damage was clear in polychaetes with the increase of CP concentrations with significant differences at 500 and 1000 ng L^{-1} compared to controls ($p < 0.05$) (Figure 3.6).

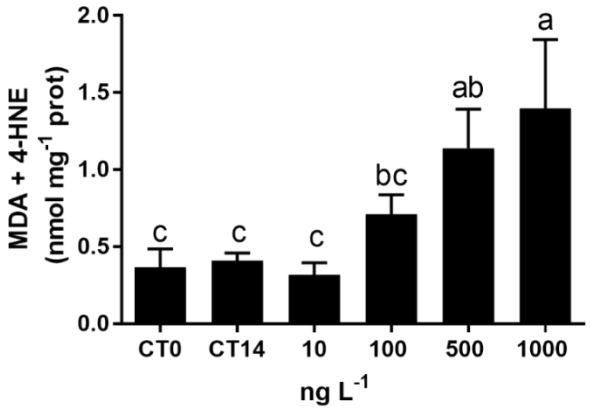


Figure 3.6: LPO activity (mean ± standard deviation) (MDA+ 4-HNE nmol.mg^{-1} protein) in animals of control conditions (CT0 and CT14) and exposed to CP at 10, 100, 500 and 1000 ng L^{-1} for 14 days. Different letters indicate significant differences among treatments (ANOVA, $p < 0.05$).

3.3.3 Genotoxicity

Data of DNA tail (%) and DNA damage grade after 14 days of exposure are presented in Table 3.2. Despite all polychaetes exposed to CP treatments indicated DNA damage compared to its respective control ($p<0.05$), the grade of impairment was always between minimal to mid damage.

Table 3.2: Genotoxic effects in polychaetes *N. diversicolor* unexposed and after 14 days of exposure to CP. DNA damage (average ± SEM) is expressed as tail DNA %. Frequency of coelomocytes distributed by grade of DNA damage (%). Different letters represent statistical differences between treatments, during the exposure period. Percentage of coelomocytes distributed through damage criteria (i.e. Minimal to Extreme).

Treatment	DNA Tail (%)	DNA Damage grade (%)				
		Minimal	Low	Mid	High	Extreme
Control	7.5^{a} (± 0.4)	69.2	30.8	0	0	0
10 ng CP L^{-1}	10.26^{b} (± 0.57)	59	30.1	10.9	0	0
100 ng CP L^{-1}	10.59^{b} (± 0.36)	51.5	46.2	2.3	0	0
500 ng CP L^{-1}	9.97^{b} (± 0.42)	57.2	40.0	2.8	0	0
1000 ng CP L^{-1}	11.83^{b} (± 0.55)	50.8	41.2	8.0	0	0

3.3.4 Principal Component Analysis (PCA)

PCA results indicate a two-dimensional pattern explaining 81.4% of the total variance (PC1= 46.3 %; PC2 = 36.2 %) (Figure 3.7). Overall, the plot score indicated a clear separation between organisms from control conditions and contaminated treatments, in which CT0 and CT14 organisms are close-related in both axis projections. The first axis mainly explains the decline in SOD, T-GPx, GST, in addition to the induction of oxidative and significant DNA damage as detrimental effects of suppression of the lines of defense, in animals exposed to the two highest CP levels. In the second component it is highlighted the divergence of biomarkers profile between control groups and the lowest CP concentration (*i.e.* 10 ng L^{-1}). The non-monotonic behaviour of burrowing in such exposed group elicited a reduced number of animals excavating. Likewise, at the lower CP level, SOD, CAT and Se-GPX are triggered, although no significant differences were detected ($p > 0.05$).

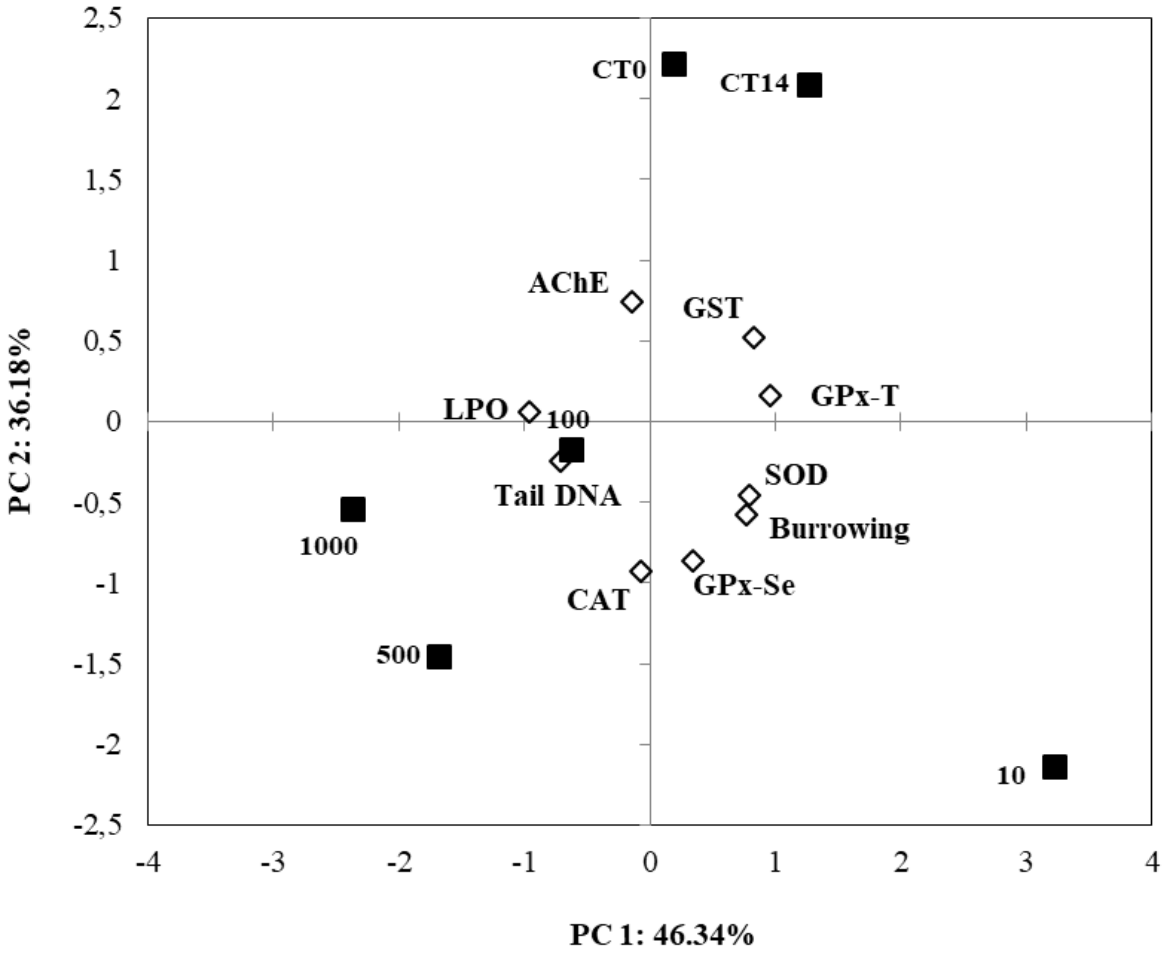


Figure 3.6: Principal Component Analysis (PCA) integrating responses of antioxidant enzymes (SOD, CAT, Se-GPx, T-GPx, GST), AChE, DNA Tail, oxidative damage (LPO) and burrowing behaviour of *N. diversicolor*, exposed to control conditions (CT0: day 0; CT14: day 14) and to different CP concentrations (10, 100, 500 and 1000 ng L^{-1}).

3.4 Discussion

CP is a widely used chemotherapy agent applied worldwide, with diffuse release in aquatic systems through hospital and domestic wastewater discharges. Despite that its partition is preferential through the water phase, geochemical and hydrodynamic data indicate that CP may settle in the sediments (Xie 2012; Kosjek and Heath, 2011). Coastal polychaetes have been increasingly applied as bioindicators in ecotoxicological assessments evaluating biochemical alterations caused by pharmaceuticals (Maranho *et al.,* 2014; Pires *et al.,* 2016a, 2016b; Fonseca *et al.*, 2017). The species *Nereis diversicolor* indicates, as

addressed in previous studies, high sensitivity to low levels (ng L^{-1} and g^{-1}) of pharmaceuticals containing different physico-chemical properties and MoA, translated by disturbances in behaviour, energy status, oxidative stress, neurotoxicity, oxidative damage, and genotoxicity (Maranho *et al.*, 2014, 2015; Pires *et al.*, 2016a; Fonseca *et al.*, 2017).

In the present study, burrowing activity seemed to be non-monotonic over CP levels. In other words, such behaviour was most impaired at lower concentrations, whereas at the highest concentrations (*i.e.* 1000 ng L^{-1}) all polychaetes were fully buried by the end of the assay (Figure 3.2). As stated by Doyotte *et al.* (1997), it is not a general rule that an increase in contaminant concentrations induces the expected harmful effect, as proposed by the dose-response curve. Lagarde *et al.* (2015) reviewed different profiles of non-monotonic dose responses for various physiological and behavioural effects, in several organs or systems. Accordingly, the present burrowing profile corroborated with a bell-shaped dose-effect relationship in which minimal effects are induced at the two extreme doses (control and 1000 ng L^{-1}), although the mechanisms involved in the burrowing at the highest CP concentration may be different to the observed in controls. Hence, at very high doses, unspecific effects will, for instance, occur because the organism would be completely overwhelmed by the substance, through non-specific biological mechanisms (Lagarde *et al.*, 2015). Many components of signaling pathways and their cross-talk could contribute to understand the non-linearity and dynamical monotonicity in response to stimuli (Wijk *et al.*, 2015).

Contrarily to the present results, animals remained emerged at the highest concentration of the cytotoxic drug cisplatin (100 ng Pt L^{-1}), together with neurotoxic effects associated to AChE inhibition (Fonseca *et al.*, 2017). CP has been widely reported to inhibit brain and retinal AChE activity in different biological models (Al-Jafari *et al.*, 1993, 1995; Kamal *et al.*, 2010) with hydrophobic interactions providing CP positioning within the acyl pockets and catalytic site of the enzyme (Shakil *et al.*, 2011). However, herein, no effects of neurotoxicity were observed (Figure 3.3), as a potential result of docking between the enzyme and CP that may not be well accomplished in the present biological model (Valasani *et al.*, 2013). Besides, CP levels applied herein are too low to observe neurotoxic effects, compared to concentration range (mg – g L^{-1}) used in experiments with vertebrates (Santos and Pacheco, 1995).

In general, cancer cells are characterized by increased aerobic glycolysis, followed by high levels of reactive oxygen species (ROS), which need to be counteracted by expression of ROS-scavenging systems for the success of the first stage of tumour formation (Gorrini *et al.,* 2013). The administration of anticancer drugs relies on the enhancement of ROS levels to trigger cancer cells death through signaling pathways and cellular damage (Conklin *et al.,* 2004; Gorrini *et al.,* 2013). CP produces additional oxidative stress through the acrolein metabolite (Arumugam *et al.,* 1999; Dumontet *et al.,* 2001; Oboh and Ogunruku, 2010). In this sense, its cytotoxic MoA combined with the suppression of cellular defenses are effective to impair normal growing cells, including marine non-target species (Fonseca *et al.,* 2017). In the present study, although significant differences were not statistically detected in SOD activity when compared to controls ($p > 0.05$), data showed a slight induction of SOD activity at the two lowest concentrations (Figure 3.4A), triggered as a potential consequence of polychaetes adaptation to overcome the stress from the drug (Sun and Zhou, 2008). At the two highest levels of CP, SOD activity was inhibited (Figure 3.4A), as observed at 14-day of exposure to the anticancer drug CisPt, at 100 ng Pt L^{-1} (Fonseca *et al.,* 2017). In rats, the inhibition of SOD activity was also observed in animals treated with intraperitoneal injection, either with 5 or 100 mg CP kg^{-1} (Patel and Block, 1985).

Conversely, in the marine mussel *Mytilus galloprovincialis*, over the 2-week exposure to CisPt (100 ng Pt L^{-1}), a higher induction and detoxification of SOD activity was observed in the digestive gland compared to the gills, denoting the primary role of the former in recovery processes in this species (Faria *et al.,* 2009; Trombini *et al.,* 2016). Giving the predominant role of SOD as the first line of defense in ROS scavenging, by converting the highly reactive superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2), its depletion yields cell redox unbalance by $O_2^{\bullet-}$ accumulation, thus elevating the oxidizing potential (Djordjevic *et al.*, 2011; Ben Ameur *et al.*, 2012; Cozzari *et al.*, 2015). Regarding the second line of defense against ROS, CAT activity acts as a catalyst of the SOD activity by-product H_2O_2 for its detoxification into water (Faria *et al.*, 2009; Gonzalez-Rey and Bebianno, 2012). Even though it is expected a coping behaviour among enzymes in the metabolic pathway, CAT activity responses depicted a non-monotonic profile over CP levels not in accordance to SOD (Figure 3.4B), which may be attributed to the interference of other ROS detoxifying process, as stated by Aguirre-Martínez *et al.*, (2013) and Gonzalez-Rey and Bebianno

(2011). In the case of CP metabolism, the CYP450 activation for PAM and acrolein detoxification combined with the uncoupled catalytic cycle may be accountable to form extra amounts of oxyradicals that need to be neutralized (Dumontet *et al.*, 2001; Harskamp *et al.*, 2012; Oboh and Ogunruku, 2010). The burst of superoxide induced by CP that cannot be efficiently eliminated through SOD activity is promptly buffered by the GSH (Aquilano *et al.*, 2014). The electrophilic acrolein binds to GSH and reduces their availability inside the cells, hence impairing the antioxidant glutathione dependent-system and also increasing free radical generation (Peña-Llopis *et al*., 2002; Singh *et al*., 2014).

The activity of Se-GPx is an important alternative to counteract harmful electrophiles produced during oxidative damage (Djordjevic *et al.*, 2011) and is a key enzyme that detoxifies H_2O_2 and converts lipid hydroperoxydes to non-toxic alcohols (Charushila and Subodhini, 2015). The present results demonstrate that Se-GPx was unaffected at any of the CP levels to which worms were exposed to (Figure 3.4C). In contrast, *N. diversicolor* exhibited a clear induction of that enzyme when specimens were exposed to CisPt, as an offset of CAT activity decrease (Fonseca *et al.*, 2017), once both enzymes are associated to enzymatic hydrogen peroxide scavenging (Cozzari *et al.*, 2015). As in the present study CAT activity was not inhibited, it may be hypothesized that the activation of Se-GPx was not particularly relevant to counteract severe oxidative challenge. Notwithstanding, T-GPx is also recognized as the most important enzymatic reductor agent of lipid peroxides and emerges as a compensatory response in cases where SOD activity is inhibited (Aquilano *et al.*, 2014). In contrast, the excess of non-neutralized oxyradicals, such as superoxide, may lead to exhaustion or inhibition of the defense system culminating in T-GPx inactivation, as observed in worms exposed to 500 and 1000 ng L^{-1} (Figure 3.4D). Thus, once T-GPx activity decreased, more hydrogen peroxide is accumulated in the cell to the point of causing oxidative damage and activation of inflammatory pathways (Yu *et al.*, 2006). T-GPx activity inhibition also occurred in the digestive gland of the clam *Ruditapes phillipinarum* exposed to the antiepileptic carbamazepine and the antibiotic novobiocin, as a reflection of pro-oxidant forces overcoming antioxidant defenses (Aguirre-Martínez *et al*., 2013).

GST is involved in the Phase II biotransformation metabolism by catalyzing the nucleophilic attack of GSH onto highly electrophilic compounds, thus denoting an important role in homeostasis as well as in detoxification and clearance of the drugs (Hayes *et al.*,

2005; Faria *et al.*, 2009; Aguirre-Martínez *et al.*, 2013). Such enzyme can reduce lipid hydroperoxides together with GPx, and is related to protection against apoptosis (Prabhu *et al.*, 2004). Several studies have pointed out its induction in aquatic invertebrates after pharmaceuticals exposure as an outcome of its activation and antioxidant role (Martín-Diaz *et al.*, 2009; Aguirre-Martínez *et al.*, 2013; Buffet *et al.*, 2014; Maranho *et al.*, 2014; Aguirre-Martínez *et al.*, 2016a). The mismatch between our results and those reported may be related to different patterns of response associated to the drug´s MoA and disparities across species regarding their defensive systems to metabolize xenobiotics (Sun and Zhou, 2008; Faria *et al.*, 2009; Cozzari *et al.*, 2015). No CP immunotoxicity was observed in the aquatic gastropod *Lymnaea stagnalis* even at therapeutic exposure levels, which was suggested to be related to the lack of appropriate converting enzymes of the pro-drug (Boisseaux *et al.*, 2017). Contrarily, polychaetes *N. diversicolor* exhibited a sharp decline in the biotransformation system in a 14-day bioassay conducted with the electrophilic CisPt, which resembles the highly electrophilic binding to the drug (Fonseca *et al.*, 2017). Nevertheless, Gonzalez-Rey and Bebianno (2011) observed a significant decrease of GST activity in mussels *Mytilus galloprovincialis* exposed to ibuprofen (250 ng L^{-1}), corroborating the data regarding the polychaete *Diopatra neapolitana*, in systems containing carbamazepine (3 µg L^{-1}) (Pires *et al.*, 2016b).

Lipid peroxidation is initiated by the attack on fatty acids of biomolecules that possess sufficient reactivity to abstract a hydrogen atom from methylene carbon in the side chain (Halliwell and Chirico, 1993). Cellular lipids are the primary targets of generated ROS and subsequently LPO occurs, as in human cells exposed to hydroxyl-cyclophosphamide (Dumontet *et al.*, 2001). Morphological changes were observed in type II-alveolar rats´ epithelial cells as a result of oxidative stress (e.g. decrease in SOD and glutathione reductase activities) and an enhancement of LPO occurs after 7 days of exposure to 150 mg CP kg^{-1} body weight (Sulkowska *et al.*, 1998). LPO is one of the main toxic effects caused by CP and responsible for several side effects during chemotherapy (Sulkowska *et al*., 1998) due to the production of the reactive aldehyde acrolein formed by β-elimination of aldophosphamide. Besides an important component produced by CP for cytotoxicity in cancer cells, this metabolite is also known as an overall secondary reactive species-product of LPO that triggers further chain reaction (Dumontet *et al.*, 2001; Conklin 2004; Singh *et*

al., 2014). The increased levels of LPO by-products at the higher CP levels (500 and 1000 ng L^{-1}) (Figure 3.6) may be a result of an inefficient biochemical antioxidant defense system (Gonzalez-Rey and Bebianno 2011; Freitas *et al.,* 2016; Pires *et al.,* 2016a; Fonseca *et al.,* 2017), depicted by the depletion of SOD, T-GPx and GST activities, as indicated in the first axis of the PCA (Figure 3.7). Besides enzymes activity suppression, chemotherapy treatment produce additional ROS generation (Conklin, 2004), which may lead to DNA damage, mutations and apoptosis to normal growing cells, such as observed in marine non-target species exposed to cisplatin and methotrexate (Moreira *et al.*, 2016; Fonseca *et al.,* 2017).

CP has bifunctional and S_N1 molecular properties, with two reactive moieties towards bases of opposite DNA double strands (Anderson *et al*., 1995). The major alkylating agent following CP metabolism is PAM, which undergoes spontaneous hydrolysis to form reactive aziridinium intermediate species, that binds to nucleophilic N7-position of guanine in DNA. Alkylation to oxygen atoms of DNA, especially at the O6-position of guanine, are also generated at lower frequency than N-alkyl adducts, however its biological relevance is even greater considering the readily mispair of bases during DNA replication (Fu *et al.,* 2012). The array of lesions caused by CP alkylation impair genome integrity by inducing mutagenesis, besides DNA replication and eventual responsive signaling for cell death if DNA repair mechanisms are not efficient (Deans and West, 2011; Fu *et al*., 2012). DNA damage caused by CP is widely described in vertebrates cells, in which administration by therapeutically treated-patients and rats exposed to this drug showed gene mutations, sister chromatid exchange, DNA adducts, DNA-DNA and DNA-protein crosslinks in somatic cells, especially by the ultimate crosslinking metabolite PAM (Crook *et al.,* 1986; Codrington *et al.,* 2004). Few studies were so far conducted to access genotoxicity responses to anticancer drugs considering *in vivo* uptake by marine organisms (Parrella *et al.,* 2015; Trombini *et al.,* 2016a; Fonseca *et al.,* 2017). In the present study, results showed a significant increase in DNA damage in CP-treated animals compared to controls (Table 3.2). Animals exposed to CP, particularly to 1000 ng L^{-1}, had a significantly higher density of DNA in the tail compared to controls (CT14) ($p < 0.05$). The grade of DNA damage was similar among CP treatments, although the lowest and highest concentrations indicated more burden of mid damage.

DNA damage caused by anticancer drugs were addressed in other aquatic biological models, at levels ranging from low ng L^{-1} to µg L^{-1}: the bivalve mollusc *Mytilus galloprovincialis*, exposed to cisplatin (Trombini *et al.*, 2016a); the microcrustaceans *Ceriodaphnia dubia* and *Daphnia magna*, exposed to 5-FU, capecitabine, cisplatin, doxorubicin, etoposide and imatinib (Parrela *et al.*, 2014a, 2015); and ZFL cell lines of the fish *Danio rerio* to the tyrosine kinase inhibitor imatinib (Novak *et al*., 2016). However, the 14-day exposure of *N. diversicolor* to the alkylating agent cisplatin (Fonseca *et al.*, 2017) yielded no genotoxicity effect at a similar range of concentrations. Cytotoxic metabolites of CP are transported and diffused into cells interacting with DNA sites after prodrug enzymatic activation, certified with the increasing DNA damage over increasing CP levels, however, this needs to be confirmed with CP levels accumulated in the worms.

3.5 Conclusions

The present study highlights the cytotoxic effects of the antineoplasic drug CP on normal proliferative cells of polychates, by binding to DNA and interfering in antioxidant status. Chronic exposure of polychaetes *N. diversicolor* to CP levels of 500 and 1000 ng L^{-1} in seawater led to an inhibition of the antioxidant enzymes activity, stimulating LPO by-products and DNA damage. The analysed biomarkers suggest that the prodrug CP is probably metabolically activated by *N. diversicolor* into PAM and acrolein, respectively responsible for the antineoplastic properties and toxic effects observed, nonetheless, this needs to be confirmed.

Chapter 4

Impacts of tamoxifen exposure to *Nereis diversicolor*

Prepared for submission to:

∝ Fonseca, T.G., Carriço, T., Fernandes, E., Mestre, N.C., Abessa, D. M. S., Tavares, A., Bebianno, M.J. Impacts of *in vivo* and *in vitro* exposures to tamoxifen: comparative effects on human cells and marine organisms

Abstract

Tamoxifen (TAM) is a first generation-SERM administered for hormone receptor-positive (HER+) breast cancer in both pre- and post-menopausal patients and may undergo metabolic activation in organisms that share similar receptors and thus face comparable mechanisms of response. The present study aimed to assess whether environmental trace concentrations of TAM are bioavailable to the deposit feeder *N. diversicolor* (0.5, 10, 25 and 100 ng L^{-1}) after 14 days of exposure. Behavioural impairment (burrowing kinetic), neurotoxicity (AChE activity), oxidative stress (SOD, CAT, GPXs activities), biotransformation (GST activities), oxidative damage (LPO) and genotoxicity (DNA damage) were assessed. TAM exerted a remarkable oxidative stress and damage at the lowest concentration (0.5 ng L^{-1}), whereas significant genotoxicity was reported at the highest exposure level (100 ng L^{-1}).

4.1 Introduction

In recent years, the occurrence of pharmaceuticals in the aquatic environment is recognized as one of the worldwide emerging issues regarding environmental quality and human health (Hereber. 2002). Increasing consumption of medicines leads to the continuous load of pharmaceuticals´ parent compounds and its metabolites into sewage and WWTPs, where low removal efficiencies have been reported (Hernando *et al.*, 2006; Furuhagen *et al.*, 2014; Daughton, 2016; Pereira *et al.*, 2017). Therefore, it is well established that several hundreds of pharmaceutical compounds are ubiquitous in WWTPs effluents, ground and surface waters, ultimately reaching estuarine and marine ecosystems (Frédéric and Yves, 2014; Verlicchi and Zambello, 2014).

Among the variety of pharmaceuticals classes released into the aquatic environment, anticancer drugs are one of the critical groups that raise concern regarding the ecotoxicological potential risk of long-term exposure to non-target organisms (Rowney *et al.*, 2009; Besse *et al.*, 2012; Booker *et al.*, 2014). Overall, the MoA of these molecules rely on the interaction with DNA, cellular growth factors, proteins and signaling pathways that lead to apoptosis, thus promoting the tumour cell death (Gačić *et al.*, 2014). Nevertheless, detrimental effects of anticancer drugs are not restricted to the highly proliferative cells or

onto cancer drivers, thus presenting cytotoxic, mutagenic, carcinogenic and teratogenic potential to all normal growing cells (Mater *et al.,* 2014; Parrella *et al.,* 2014a).

Endocrine therapy is an alternative anticancer treatment that uses selective mechanisms of interaction with cancer targets, applied individually or in combination with cytotoxic molecules (Hoskins *et al*., 2009). It consists of the administration of a structurally diverse group of compounds that binds to the estrogen receptors α (ERα) and β (ERβ), and may produce estrogen agonist or antagonist effects, depending of the targeted tissue (Goldstein *et al*., 2000; Paterni *et al*., 2014). These molecules are referred as selective estrogen receptor modulators (SERM) (Goldstein *et al.,* 2000; Rodenas *et al.,* 2015; An, 2016). The antagonist effect following the formation of estrogen receptor-SERM complexes is crucial for the management of hormone-dependent breast cancer and tumour cell survival (Goldstein *et al.,* 2000; Zheng *et al.,* 2007; Criscitiello *et al.,* 2011).

Tamoxifen (TAM) is a first generation-SERM administered for hormone receptor-positive (HER+) breast cancer in both pre- and post-menopausal patients (Goetz *et al*., 2007). Its antitumour ability derives from the antagonism towards the proliferative action of estrogen through competitive binding to ERs (Goldstein *et al*., 2000). As a pro-drug, TAM requires a complex metabolic activation to elicit its designed pharmacological activity (Kisanga *et al*., 2005). The 4-hydroxytamoxifen and 4-hydroxy-N-desmethyltamoxifen (endoxifen) are the main products of hepatic oxidation, elicited by CYP2D6 and CY3A4, with high anti-estrogen potential in humans (Johnson *et al.,* 2008; Murdter *et al.,* 2011) (Figure 4.1). Since pharmaceuticals active compounds are designed to have a specific MoA, targeting a specific metabolic and molecular pathway in humans, their biological activity may occur in organisms that share similar receptors and face comparable mechanisms of responses (Fent *et al*., 2006a). Conservation of biomolecules during evolution may trigger similar responses of chemicals in different non-targeted biological systems (Franzellitti *et al.,* 2013; Zhang *et al.,* 2017). Although organisms may lack homologues of estrogen receptors, they may contain genes for estrogen-related receptors (ERRs) that, according to Borgatta *et al.* (2015) are structurally close to human ERα and ERβ.

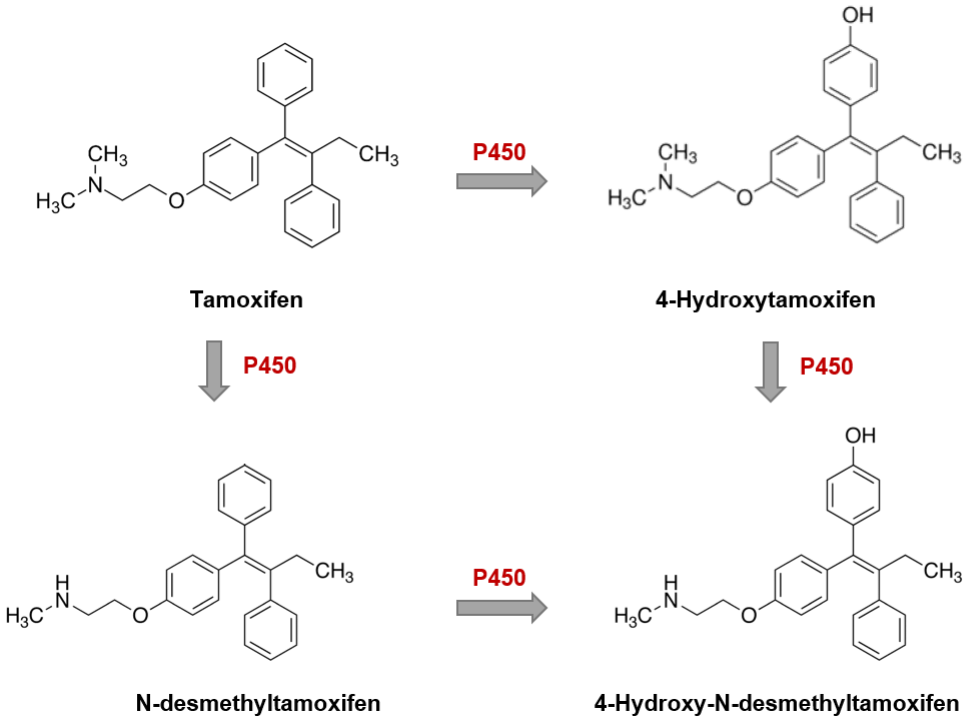


Figure 4.1: Metabolic pathways of tamoxifen in humans, following activation by cytochrome P450.

Assumptions based on physico-chemical properties indicate that TAM has a high hydrophobic potential (Kow = 6.3), thus prone to adsorb to organic particles (Orias *et al.*, 2015) and settle onto bottom sediments. However, once in coastal areas, TAM as other pharmaceuticals molecules, can be subject to physico-chemical and biological conditions different from those found in freshwater environments (*e.g.* salinity, pH, temperature, turbidity, organic compounds, microbial population) and thus TAM may present different chemical behaviour and unexpected partition over water, suspended particles and sediments, therefore a distinct persistence (Weigel *et al.*, 2002; Lara-Martín *et al.*, 2014).

Ecotoxicity assays conducted with TAM, in freshwater and marine species, showed inhibition in the larval development of the sea urchin *Spherechinus granularis* (Pagano *et al.*, 2001), a decrease in egg production of the fish *Tautogolabrus adspersus* (Mills *et al.*, 2015), and an alteration of vitellogenin gene expression in the fish *Sparus aurata* L. (García-Hernández *et al.*, 2016) (See details in Table 4.1). These effects were detected at a concentration range from ng L^{-1} to µg L^{-1}, encompassing TAM concentrations detected in aquatic compartments (Table 4.1). Despite the increasing efforts devoted to comprehend the

risks of TAM occurrence in aquatic compartments, there is still a lack of environmental concentrations screening in coastal and marine ecosystems, as well as information around its bioavailability and potential routes of exposures that may derive toxicological effects to non-target species (Thomas and Hilton, 2004; Roberts and Thomas, 2006; Lara-Martín *et al.,* 2014).

In light of the trend of increasing concentrations of anticancer drugs in the aquatic environment, even though below human therapeutic doses, it is important to understand whether pharmaceuticals are likely to pose a risk to the biota *via* similar metabolic pathways detected in humans (Furuhagen *et al*., 2014; Mater *et al*., 2014). Estrogen receptor expression varies across species and can be differentially expressed according to tissues (McGinnis and Crivello, 2011). Human cell lines such as the retinal pigment epithelium (RPE) cell line have shown to express ER subtypes (Wang *et al.,* 2015; Koulisis *et al.,* 2016), in contrast to cervical carcinoma HeLa cells, in which ER does not mediateestrogen responsive reporter expression (Maminta *et al.,* 1991; Shanle and Xu, 2010; Klinge, 2015;).

Polychaetes are sediment dwelling organisms from estuarine and marine benthic zones which possess high ecological relevance, besides being an important component of an ecotoxicological toolbox to assess the impact of contaminants in sediments due to their life habits that provide a constant contact with chemicals adsorbed onto particles and interstitial water (Freitas *et al*., 2015). Putative detrimental effects of anticancer drugs in sediments have been depicted through biochemical impairments in the polychaete *N. diversicolor*, exposed to the cytotoxic drug cisplatin (0.1 to 100 ng Pt L^{-1}) (Fonseca *et al.,* 2017) and cyclophosphamide (10 to 1000 ng L^{-1}) (Fonseca *et al.,* 2018), and the amphipod *Corophium volutator* exposed to the drug methotrexate (1 to 1000 ng L^{-1}) (Moreira *et al.,* 2016). Due the paucity of research to effects of TAM to marine organisms, particularly considering their routes of uptake, one of the aims of the present study was to investigate whether environmental trace concentrations of TAM are bioavailable to the deposit feeder *N. diversicolor.* The approach consisted of a 14-day assessment of behavioural impairment (burrowing kinetic impairment), neurotoxicity (AChE activity), oxidative stress (SOD, CAT, GPXs activities), biotransformation (GST activities), oxidative damage (LPO) and genotoxicity (DNA damage). Organisms were exposed to a range of concentrations that encompass environmental relevant levels and those considered a worst-case scenario

Table 4.1: TAM Concentrations in water (ng L^{-1}), suspended solids and sediments (ng g^{-1}) from ground, riverine and marine waters.

Study Area	Groundwater	River			Marine			Reference
		Surface Water	Suspended Solids	Sediment	Seawater	Suspended Solids	Sediment	
France	-	<5.8 - 25	-	-	-	-	-	Coetsier *et al.* (2009)
Japan	-	-	658	0.042	-	-	-	Azuma *et al.* (2017)
Germany	6 - 16.5	-	-	-	-	-	-	Reh *et al.* (2013)
England	-	27 - 212	-	-	-	-	-	Roberts and Thomas (2006)
England	-	<10	-	-	-	-	-	Ashton *et al.* (2004)
England	-	13 - 71	-	-	-	-	-	Hilton and Thomas (2004)
England	-	<10 - 23	-	-	-	-	-	Hilton *et al.* (2003)
Spain	-	12.4 - 26.8	-	-	-	-	-	López-Serna *et al.* (2012)
Spain	11.2 - 223	-	-	-	-	-	-	López-Serna *et al.* (2013)
Spain	-	12 - 38	-	-	-	-	-	Ferrando-Climent *et al.* (2014)
Spain	-	0.5 - 25	-	-	-	-	-	Franquet-Griell *et al.* (2017)
USA	-	-	-	-	-	8 - 44	7	Lara-Martín *et al.* (2014)
USA	-	n.d. - 11.2	-	-	-	-	-	Lara-Martín *et al.* (2015)
USA	-	-	-	-	93	-	-	Nödler *et al.* (2014)
China	-	0.01 - 1.23	-	-	-	-	-	Zhao *et al.* (2015)

4.2 Materials and Methods

4.2.1 Chemicals

Tamoxifen analytical standard (CAS 10540-29-01) was purchased from Sigma-Aldrich (Portugal). For safety handling of the drug, the experimental work was performed using class II biological safety cabinet, with appropriate clothing (open-back, impervious chemotherapy protection gown, double powder-free latex gloves and safety goggles). Since TAM is not readily soluble in water, the stock solution was prepared in the carrier solvent dimethyl sulfoxide (DMSO). Experimental solutions were prepared from sequential dilutions in ultrapure Milli-Q water, with a final concentration of 0.001% (v/v) DMSO in order to avoid a solvent toxic effect.

4.2.2 Experimental design

Polychaetes *N. diversicolor* and sediment samples were collected in an intertidal mudflat in Mira River Estuary (SW, Portugal), during low tide, and transported alive to the laboratory. Sediment grain size distribution and organic matter content were determined according to methods described by Royse (1970) and Gross (1971) (see Fonseca *et al.*, 2018). Animals were maintained in glass aquaria filled with aerated natural seawater (salinity 35) and sediments from the site of origin, during 5 days before testing. A total of 75 polychaetes was randomly selected and added to each treatment, in a triplicate design (25 animals per aquaria), in 10-L glass aquaria containing sediments and seawater (1:4 ratio, respectively). Specimens were unexposed: controls (CT0 - day 0; and CT14 - day 14) and exposed to DMSO (0.001%), and to TAM (0.5, 10, 25 and 100 ng L^{-1}) for 14 days. Experimental set-up was kept under constant aeration and light period (12:12 hours), and physico-chemical parameters registered (pH 7.92 ± 0.2; temperature 19 ± 1°C; salinity 35 ± 1). During the experiment, water was carefully renewed every 48 hours avoiding sediment resuspension, with addition of DMSO (0.001%) and TAM concentrations into water column aftermath. Animals were not fed during the bioassay.

For burrowing and comet assays, specimens were immediately handled and managed for respective analysis, while those regarding biochemical end-points (AChE, SOD, CAT,

GST, GPx, LPO) were rinsed with clean seawater and stored at -80 °C until further use. For the DNA damage analysis coelomic cells of *N. diversicolor* (5 specimens per aquaria, in triplicate; total of 15 organisms per treatment) were extracted from the posterior region of the polychaete body into 20 µL of PBS buffer with a 0.5 mL-syringe fitted with hypodermic needle (adapted from Lewis and Galloway 2008).

4.2.3 Burrowing assay

The behavioural endpoint of the burrowing assay was verified only over the exposure of *N. diversicolor*. Polychaetes from control conditions, DMSO and TAM-exposed were submitted to a burrowing test after the 14-days bioassay, according to Fonseca *et al.* (2017). Polychaetes (5 specimens per aquaria, in triplicate; total of 15 organisms per treatment) were carefully and individually displaced from their test containers and transferred to 150 mL-plastic flasks, filled with natural seawater and 5 cm of sediments. The vertical position of polychaetes in sediment column was recorded every two minutes, to assess the time for fully burrowing in the 30 min-assay. The results are expressed as the percentage (%) of un-burrowed specimens, over time (min).

4.2.4 Biochemical Analysis

Total proteins

Total proteins concentrations (mg protein g^{-1} tissue) were determined according to the method described by Bradford (1976) adapted for microplate reader, using bovine serum albumin (BSA) as a standard.

Neurotoxicity

AChE activity was determined in whole tissues of *N. diversicolor* (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment). Samples were individually homogenized in 100 mM Tris-HCl buffer (pH 8.0) with addition of 0.1% Triton, following centrifugation at 12,000 *g*, for 30 min, at 4 °C. Supernatants were split into aliquots for total protein determination (Bradford, 1976) and AChE activity was analysed using the method of Ellman *et al.* (1961) adapted to microplate reader. Briefly, 50 µl of non-diluted samples,

or blank solution, were incubated with 200 µl of 0.75 mM DTNB solution (Sigma) in 0.1 M Tris-HCl buffer, pH 7.5. The reaction started by the addition of 50 µl of 3 mM ATC (Sigma), following incubation for 10 min at room temperature. Changes in absorbance were recorded over 5 min at 405 nm, and results are expressed as ATC.min^{-1} mg^{-1} protein.

Antioxidant enzymes

Antioxidant enzymes activities were determined in whole tissues of *N. diversicolor* (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment). Samples were individually homogenized in 20 mM Tris-HCl buffer (0.5 M sucrose; 0.075 M KCl; 1 mM DTT; 1 mM EDTA; adjusted to pH 7.6 with HCl), according to the protocol described by Geret *et al.* (2002). Homogenates were centrifuged at 500 *g*, for 15 min, at 4 °C, and the supernatants obtained centrifuged again (12,000 *g*, 45 min, 4 °C). Enzymatic activities were analysed in the cytosolic fraction. SOD activity was assessed by measuring the decrease of absorbance of the substrate cytochrome-c by xanthine oxidase/hypoxanthine system, at 550 nm (McCord and Fridovich 1969), and the results are expressed as U mg^{-1} protein. CAT activity was evaluated through the decrease of absorbance originated by hydrogen peroxide (H_2O_2) consumption at 240 nm, according to Greenwald (1985). GPX activity was measured at 340 nm by using cumene hydroperoxide and H_2O_2 as substrates for T-GPx and Se-GPx, respectively (adapted from Lawrence and Burk, 1976). The activity of CAT, Se- and T-GPx are expressed in nmol min^{-1} mg^{-1} protein.

Biotransformation enzyme activity

The cytosolic fraction for the determination of GST activity was obtained according the protocol described in 2.3.2. Measurement of this biotransformation enzyme activity was conducted in whole specimens of *N. diversicolor* (6 organisms per treatment). Reaction mixture undergoes by the conjugation of 0.2 mM reduced glutathione (GSH) with 0.2 mM CDNB (molar coefficient of extinction = 0.6 mM^{-1} cm^{-1}) in a reaction mixture of 0.2 M KH_2PO_4/K_2HPO_4 buffer (pH 7.9), at 340 nm (adapted from Habig *et al.*, 1974). GST activity is determined in nmol min^{-1} mg^{-1}.

Oxidative damage

Polychaetes *N. diversicolor* (6 organisms per treatment) were individually homogenized in a Tris–HCl buffer (20 mM, pH 8.6) and BHT (100:1 µL, respectively). The resulting homogenates were centrifuged, for 45 min at 30,000 *g* at 4 °C, in order to obtain the cytosolic fraction. Aliquots of supernatants were used for the quantification of total proteins (Bradford, 1976) and determination of oxidative damage (Erdelmeier *et al*., 1998), adapted for microplate reading. The absorbance of LPO by-products, the malondialdehyde (MDA) and (2E)-4-hydroxy-2-nonenal (4-HNE), was measured at 586 nm and expressed as nmol MDA + 4-HNE mg^{-1} protein.

4.2.5 Genotoxicity

Genotoxicity was estimated by the alkaline Comet assay, as described in Fonseca *et al.* (2018). Briefly, cells collected from polychaetes and mussels were centrifuged at 3000 rpm (3 min, 4 °C), and resulting pellets suspended in 0.65 % low melting point agarose (LMA, in Kenny's salt solution; 0.4 M NaCl, 9 mM KCl, 0.7 mM KH_2PO_4, 2Mm $NaHCO_3$, 1000 ml Milli-Q water). Subsequently, cells were smeared in duplicate on microscope slides, coated with 0.65 % normal melting point agarose (NMA) in Tris-acetate EDTA. To allow membrane permeability and release of DNA, slides were immersed in lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, 10% Dimethylsulfoxide,1% Sarcosil, pH 10, 4 °C), for at least 1 h, and placed in a horizontal electrophoresis box. Cells were exposed to alkali buffer (300 mM NaOH, 1 mM EDTA, adjusted at pH 13, 4 °C) over 15 min to allow DNA unwinding. Electrophoresis current was set up at 25 V and 300 mA, over 5 min, following slides neutralization (0.4 mM Tris, pH 7.5). Cells were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 mg ml^{-1}) and analysed with an optical fluorescence microscope Axiovert S100 (total magnification of ×400). The amount of DNA in the comet tail (DNA tail %) was determined by the software Komet 5.5 (Kinetic Imaging Ltd) in 50 cells per sample (25 in each duplicate). Results are expressed as mean ± standard deviation (S.D.).

4.2.6 Statistical analysis

Statistical analysis was carried out using the Statistica 8.0 software (Statsoft Inc., 2007, USA). The results were compared using parametric tests (ANOVA, followed by the Tukey's test), or non-parametric equivalent test (Kruskal-Wallis), according to data distribution and variances homogeneity (Shapiro-Wilk and Levene's tests, respectively). Results were significant when $p < 0.05$.

4.3 Results

4.3.1 Burrowing behaviour

The percentage of polychaetes from CT0, CT14 and exposed to DMSO buried were 100%, 90% and 90%, respectively, within 6 minutes of bioassay. In contrast, organisms exposed to the different TAM concentrations showed a general increase in burrowing time with the increase of TAM concentrations exposure. Polychaetes treated with 25 ng L^{-1} and 100 ng L^{-1} failed to burrow after 4 minutes and 26% and 53% of polychaetes, respectively, remained unburied (Figure 4.2).

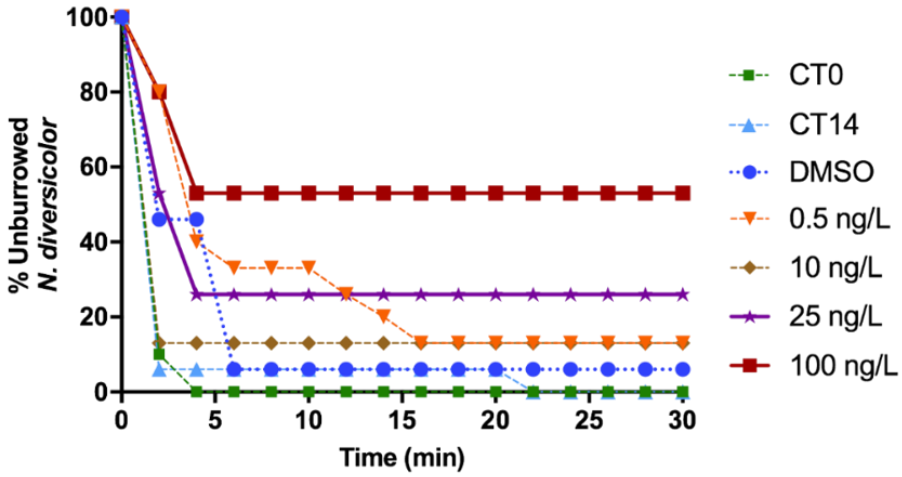


Figure 4.2: Percentage of unburied *N. diversicolor* over time (minutes) in controls (CT0, CT14) and exposed to DMSO and to TAM concentrations (0.5, 10, 25, 100 ng L^{-1}). Lines with symbols indicate the behaviour during the 30-min experiment.

4.3.2 AChE activity

No differences in AChE activity were detected among controls (*i.e.* CT0, CT14) and DMSO ($p > 0.05$) (Figure 4.3). Polychaetes exposed to TAM showed an induction of AChE activity, except for those exposed to 10 ng L^{-1}.

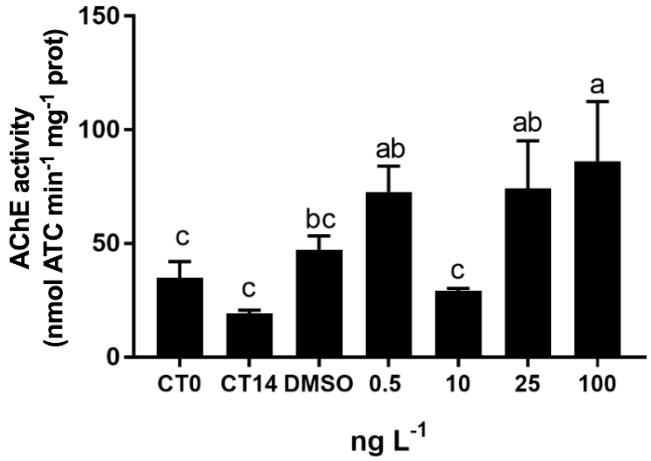


Figure 4.3: *N. diversicolor* AChE activity (mean ± S.D.) ($ATC.min^{-1}$ mg^{-1} protein) in unexposed (CT0, CT14) and exposed to DMSO and to TAM concentrations (0.5, 10, 25 and 100 ng L^{-1}). Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

4.3.3 Antioxidant enzymes responses

Overall, enzymatic activities of unexposed specimens (*i.e.* CT0, CT14) and exposed to DMSO were similar ($p > 0.05$), with the exception of T-GPx that indicated an increase in DMSO exposed group. In polychaetes exposed to the lowest TAM concentration (*i.e.* 0.5 ng L^{-1}), SOD activity increased compared to all other treatments ($p < 0.05$) (Figure4.4-A). Although not significant ($p > 0.05$), there was an increasing trend of SOD activity in the other TAM concentrations. Regarding CAT activity, a significant inhibition occurred at the highest TAM concentration compared to control conditions ($p < 0.05$) (Figure 4.4-B). The activity of Se-GPx and T-GPx, like for SOD, also increased in polychates exposed to 0.5 ng L^{-1} of TAM ($p < 0.05$) (Figure 4.4-C and 4.4-D) (2-fold compared to controls).

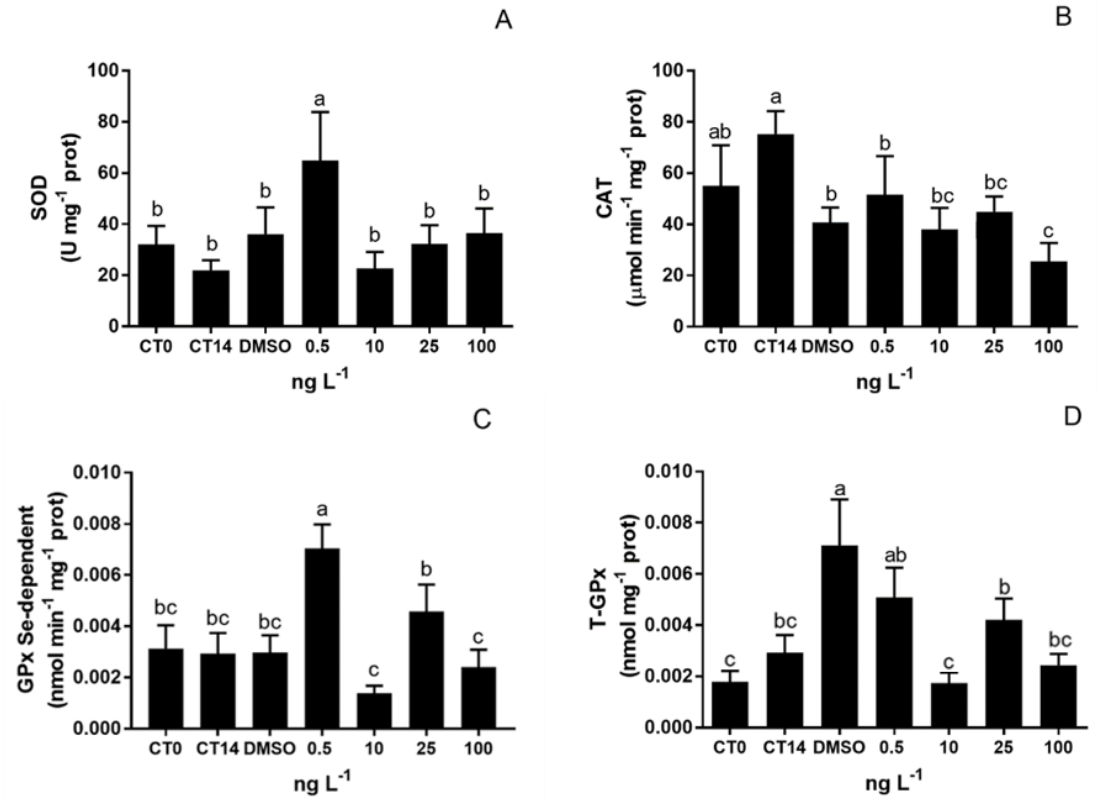


Figure 4.4: *N. diversicolor* antioxidant enzymes activities (mean ± S.D.) expressed in unexposed polychaetes (CT0, CT14) and exposed to DMSO and to a range of TAM concentrations (0.5, 10, 25 and 100 ng L^{-1}): (A) SOD, (B) CAT, (C) GPx Se-dependent, (D) T-GPx. Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

4.3.4 Biotransformation enzyme activity

Relatively to GST activity, no significant alteration was observed among treatments ($p > 0.05$) (Figure 4.5).

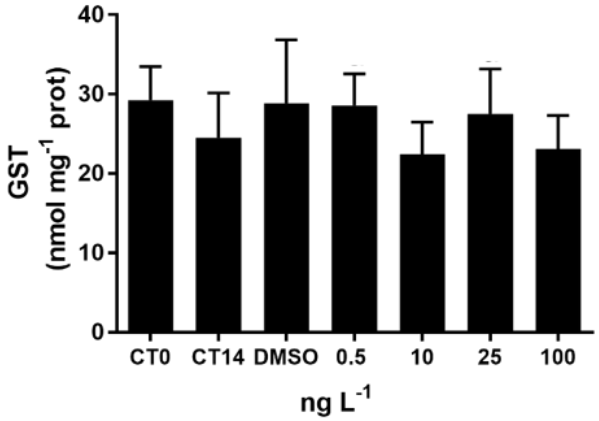


Figure 4.5: *N. diversicolor* GST activity (mean ± S.D.) in unexposed (CT0, CT14) and exposed to DMSO and to TAM concentrations (0.5, 10, 25 and 100 ng L^{-1}) Absence of letters indicate no differences among treatments (One-way ANOVA, Kruskal-Wallis; p > 0.05).

4.3.5 Oxidative damage

LPO levels in polychaetes controls and exposed to DMSO were similar ($p > 0.05$). In contrast, the exposure of polychaetes to TAM induced the generation of LPO by-products since there was a similar increase of MDA + 4HNE concentrations in all of exposed animals compared to controls ($p < 0.05$; Figure 4.6).

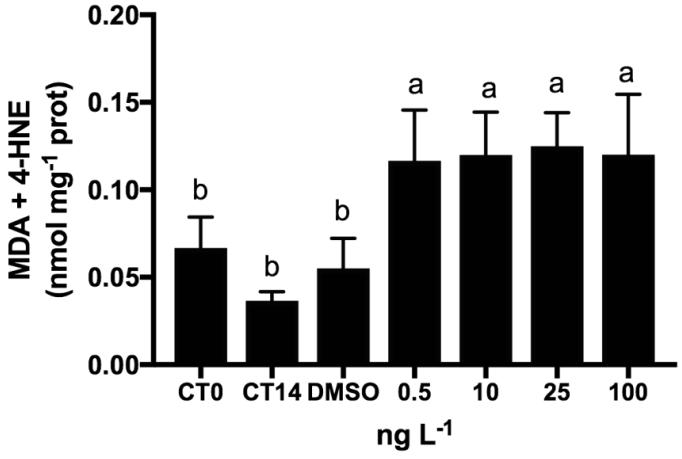


Figure 4.6: *N. diversicolor* LPO levels (mean ± STD) (MDA+ 4-HNE nmol.mg^{-1} protein) in unexposed (CT0, CT14) and exposed to DMSO and to different TAM concentrations (0.5, 10, 25 and 100 ng L^{-1}). Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

4.3.6 Genotoxicity

No significant changes in % of DNA tail were detected on coelomocytes from control conditions (CT0, CT14 and DMSO) ($p > 0.05$; Figure 4.7). In TAM-treated polychaetes, DNA damage was only addressed in specimens exposed to the highest drug concentration (100 ng L^{-1}) ($p < 0.05$; Figure 4.7).

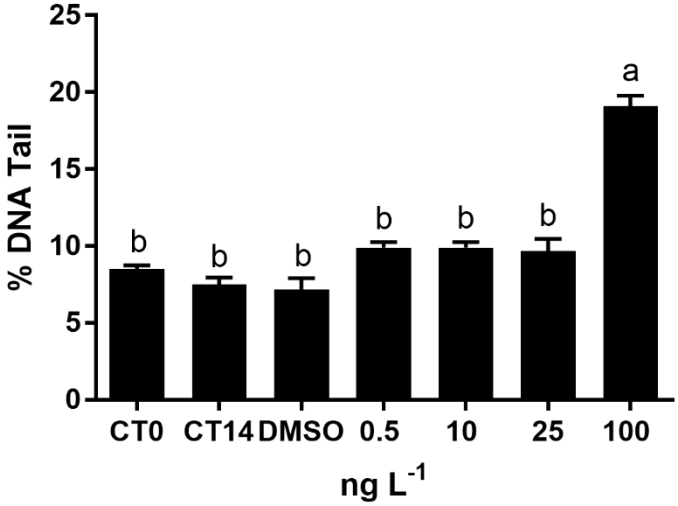


Figure 4.7: DNA damage (mean ± standard deviation) in coelomocytes of polychaetes *N. diversicolor* unexposed (CT0, CT14) and exposed to DMSO and to different TAM concentrations (0.5, 10, 25 and 100 ng L^{-1}). Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

4.4 Discussion

Specific physico-chemical properties of pharmaceuticals are known to provide predictable behaviour and fate in aquatic compartments. The high log Kow and low water solubility of TAM suggest a potential removal from wastewater through drug adsorption onto sewage sludge and sediments. However, detection of TAM in WWTPs effluents (concentrations up to 369 ng L^{-1}) (Roberts and Thomas, 2006) confirm its low removal efficiency (*i.e.* 17 to 43%) and drug recalcitrance over conventional technologies that use sorption processes (Fernando-Climent *et al.*, 2014). Once released in the aquatic

environment, besides its residence in water column, pharmaceuticals compounds also sorb onto suspended organic matter, clay, sediments and microorganisms, that eventually settle in the bottom areas (Silva *et al.,* 2011; Zenker *et al.,* 2014; Rocha *et al.,* 2015). Therefore, differential uptake, trophic transfer and bioaccumulation are experienced by aquatic species subjected to different ways of exposure, via water or sediment contact, although there is still a lack of information around TAM´s bioavailability, that may derive toxicological effects to non-target species (Table 4.2) (Chapman and Wang, 2001; Torres *et al.,* 2009; Yamamoto *et al.,* 2009; Du *et al.,* 2014; Ribeiro *et al.,* 2015).

The present findings indicated that SOD and Se-GPx activities significantly increased in polychaetes exposed to the lowest TAM concentration (0.5 ng L^{-1}) ($p < 0.05$) (Figure 4.2). SOD upregulation illustrates the prompt of the antioxidant system to overcome those toxic by-products, by means of converting superoxide radical into less toxic hydrogen peroxide (H_2O_2), following detoxification into water and oxygen by GPx´s (Van der Oost *et al.,* 2003; Gonzalez-Rey and Bebianno, 2014). At the lowest TAM concentration, GPx may have exerted a critical compensatory mechanism over the unchanged CAT activity (Cozzari *et al*., 2015; Marchi *et al*., 2017). The non-linear dose response characterized by the lower dose stimulation (0.5 ng L^{-1}) observed in SOD and Se-GPx activities (Figure 4.4), is referred as hormesis and may include mechanisms to overcompensate alterations in homeostasis and adaptive response based on inducible repair processes (Chapman, 2002; Hoffmann, 2009). In other words, a small exposure to a stressful stimulus increases the resistance of the cell or organism to a moderate or severe level of stress (Arumugam *et al.,* 2006; Hoffmann, 2009). Besides repairing any damage, organisms also reduce background damage more effectively (Chapman, 2002). The hormetic curve is described as non-monotonic and biphasic shape, in which effects at low doses are opposite to those at high doses (Hoffmann, 2009). This kind of relationship have been described from bacteria to vertebrates, as an outcome at 1000 different environmental stressors (Chapman, 2002; Mater *et al.,* 2014), and is also attributed to the response of *N. diversicolor* exposed to the lowest CP concentration (10 ng L^{-1}) (Fonseca *et al.,* 2018).

In the case of TAM administration to human patients, an increase in enzymatic activity was registered for choline acetyltransferase, accountable for the synthesis of the acetylcholine neurotransmitter critical for synapses (Silva *et al*., 2000). In mammalian brain,

TAM acts as an ER agonist and exerts estrogen-like effects associated to modulation of neuroprotection and increase of human cognition, attention and verbal skills (Dicko *et al.,* 1999; McEwen and Alves, 1999; Simpkins *et al.,* 2009; Newhouse *et al.,* 2013). Besides the beneficial effects of TAM in human neuroactivity, there are still confounding factors in the mammalian group itself related to features of hormonal status, age, tissue-specific activation of ERs, and whether ERs possess ability to bind estrogens or anti-estrogens at low concentrations (Newhouse *et al.,* 2013; Lv *et al.*, 2017). At a concentration range from 0.1 to 50 µg L^{-1}, TAM was unable to alter AChE activity in the freshwater clam *Corbicula fluminea* and disagree with the present observations, reinforcing that its role as a receptor-estrogen modulator in invertebrates is far from clear (Aguirre-Martínez *et al*., 2018)

The biotransformation enzyme, GST, conjugates reactions of active electrophilic metabolites or their parental compounds with reduced glutathione (GSH), enabling its transformation to more extractable hydrophilic metabolites (Van der Oost *et al.,* 2003; Luis *et al.,* 2016). In addition, GST provides protection against ROS, playing a critical role in the defence against oxidative damage and peroxidative products of DNA and lipids (Nuwaysir *et al*., 1996). However, in human liver, TAM is extensively metabolized by phase I cytochrome P450 and phase II sulfotransferases (SULTs) and UDP-glucoronosyltransferases (UGTs) (Kiyotani *et al.,* 2012), whereas the mechanism by which TAM affects GST expression is poorly studied. According to Nuwaysin *et al. (*1996), TAM administration at relevant clinically therapeutic doses produce significant suppression in GST mRNA expression in rat liver, resembling the outcomes associated to cisplatin MoA (Fuertes *et al.,* 2003). Herein, the activity of GST did not change in polychaetes exposed to any TAM-concentration, in contrast to the significant inhibition in *N. diversicolor* specimens exposed to cisplatin at 100 ng Pt L^{-1} (Fonseca *et al.,* 2017 - Chapter 2). In the present case, either TAM metabolism may not be involved in GST phase II enzyme, or the concentration range applied in the bioassay was not enough to trigger this detoxification pathway.

In this study, TAM induced LPO in polychaetes exposed to all concentrations (Figure 4.6). TAM embeds itself in the lipid membranes and generates superoxide, which causes LPO and subsequent 4-HNE formation, following activation of caspase-3 cascade and cell death (Bekele *et al*., 2016). Such mechanism supports the contribution of oxidative damage on killing cancer cells during TAM therapy. Interferences in the oxidative damage may be

attributed either to the metabolic activation of TAM in *N. diversicolor,* following production of ROS, and/or by oxidative stress generated by TAM molecule intrusion in the lipid bilayer, which does not depend on the prodrug metabolism neither ERα expression and its activation (Trachootham *et al*., 2009; Lushchak, 2011; Bekele *et al*., 2016).

Accordingly, the noteworthy DNA damage herein revealed in specimens exposed to the highest TAM concentration may be particularly linked to activation of prodrug at such great drug levels (Figure 4.7), since the conversion of TAM into its intermediary putative active metabolite α-hydroxytamoxifen is a pre-requisite for DNA covalent binding and adducts formation (Boocock *et al*., 2002), as reported in human peripheral blood lymphocytes and MCF-7 cells using alkaline Comet Assay (Wozniak *et al*., 2007). Such explanation is plausible considering that the antioxidant disruption elicited in polychaetes from 100 ng L^{-1} treatment only occurred through inhibition of CAT activity in addition to levels of LPO by-products, at the same grade of oxidative damage generated in all tested TAM concentrations. In other words, different mechanisms are involved in DNA damage at the highest TAM concentration.

Table 4.2: Ecotoxicological effects of TAM reported in coastal and marine species.

Taxon	Species	Concentration range (ng L^{-1})[a]	Time (d)[b]	Parameter	Effect	Reference
Microalgae	*Isochrysis galbana*	1 - 5 × 10^8	4	Growth inhibition	EC50 = 3.5 × 10^7	Aguirre-Martínez *et al. (*2016a)
Bivalve Mollusc	*Ruditapes phillipinarum*	100 - 50000	14	Oxidative stress, oxidative damage, genotoxicity, oxidative damage	**100**: *↑ EROD, LPO; *↓GPx, DBF; **1000**: *↑ EROD, GPx, GR, LPO, DNA; *↓ DBF, GST; **10000**: *↑ EROD, GPx, LPO**;** *↓DBF **50000**: *↑ EROD, GPx, GR, LPO**;** *↓ AChE, GST, DBF	Aguirre-Martínez *et al. (*2016a)
Echinorderm	*Paracentrotus lividus*	10 - 10^9	1[b]	Fertilization	EC_{50} = 1.5 × 10^7	Aguirre-Martínez *et al. (*2016a)
		10 - 10^9	2	Larval Development	EC_{50} = 1 × 10^9	
		10^{-8} - 10^{-6} M	0.5[b]	Fertilization	1 × 10^{-7} M: *↓	Pagano *et al. (*2001)
		10^{-8} - 10^{-6} M	0.5[b]	Offspring Quality	1 × 10^{-6} M: *↓	
	Spherechinus granularis	10^{-8} - 10^{-6} M	3	Larval Development	1 × 10^{-6} M: Embryotoxicity *↑	
	Strongylocentrotus purpuratus	-	4	Larval Development	EC_{50} = 50	Roepke *et al. (*2005)

	Tautogolabrus adspersus	5×10^5 - 5×10^{6a}	17	Egg production	5×10^5: *↓	Mills *et al. (*2015)
	Sparus aurata L.	100000[a]	25	Gene expression	Vitellogenin *↑	García-Hernández *et al. (*2016)
Fish		100000[a]	25	Gene expression	Immune response (ilb1, tnfa, tgfb1,mhc1a, tlr9) *↑	
		100000[a]	25	Sperm concentration	*↑	
		100000[a]	25	Sperm motility	*↑	

4.5 Conclusions

Our present findings confirmed that the prodrug TAM elicits detrimental impacts in *N. diversicolor*, at environmental realistic concentrations, by means of a hormetic trend of responses. These results highlight the risks to which coastal species are chronically subjected. Although the lack of evidence regarding nuclear ERs-like expression in *N. diversicolor*, which would enable drug metabolic activation and apoptotic outcomes, the generation of oxidative stress, membrane damage, and genotoxicity were registered, and could have been attributed to the direct contact of prodrug to cytoplasmic membrane. In this sense, it is relevant to determine whether TAM acts as an agonist and antagonist in the tissue of analysis, and to which extent the presence of ERs is crucial for cell growth inhibition in non-target organisms.

Chapter 5

Effects of mixtures of anticancer drugs in the benthic polychaete *Nereis diversicolor*

Prepared for submission to:

∝ Fonseca, T.G., Abessa, D. M. S., Bebianno, M.J. Effects of mixtures of anticancer drugs in the benthic polychaete *Nereis diversicolor*

Abstract

The increasing consumption of anticancer drugs through single and/or combinatory chemotherapy administration worldwide raised concern regarding their toxicity burden towards non-target species, particularly in the coastal zone. The toxicity of tertiary mixtures involving the drugs cisplatin (CisPt), cyclophosphamide (CP) and tamoxifen (TAM), at an increasing range of concentrations (Mixtures A, B, C, and D), was determined in the marine polychaete *Nereis diversicolor* by analyzing different endpoints like the effects on burrowing behaviour, neurotoxicity (acetylcholinesterase – AChE - activity), antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), selenium dependent glutathione peroxidase (Se-GPx) and total glutathione peroxidases (T-GPx) activities), biotransformation metabolism (Glutationne-S-transferases (GST), lipid peroxidation (LPO) and genotoxicity (DNA damage). The effects obtained from the exposure to mixtures were compared to those of the same concentration of the single-drugs. Regarding SOD activity, TAM showed an antagonist effect over CisPt and CP in mixtures C and D. However, in Mix D, there was a synergistic effect of TAM and CisPt that resulted in the sharp inhibition of CAT activity, and an additive interaction of CisPt and CP on the Phase II biotransformation enzyme. Even at low concentrations, the drugs present in Mix A also suppressed polychaetes´ GST activity, although different from the respective single-drug responses, in addition to a protective mechanism of hormesis detected by T-GPx activity induction not sufficient to avoid oxidative damage and mid-grade DNA damage. Due to the absence of burrowing impairment in Mix A, mechanisms involved in neurotoxicity were other than the one driven by AChE alterations. At the intermediary concentrations (Mix B and C), only LPO occurred, as a potential effect of TAM interaction over cellular membranes. Despite the range of concentrations tested, the sediment dweller *N. diversicolor* showed to uptake and metabolize the anticancer compounds in combination indicating that mixtures may act differently according to the dose of the individual drugs. These findings show that single compound toxicity data are not enough to predict aquatic toxicity such as anticancer drug mixtures.

5.1 Introduction

It is well established that aquatic organisms are exposed to multiple stressors, containing complex mixtures of chemicals, among them pharmaceutical residues and their respective metabolites (González-Ortegón *et al*., 2016; Thrupp *et al*., 2018). Despite the fact that pharmaceuticals are jointly discharged into water bodies via effluents from hospitals and household WWTPs, with limited efficiency to remove complex molecules, or directly from the sources, as is the case of developing countries, environmental risk assessment still relies on the impact of individual substances (Altenburger *et al*., 2000; Fent *et al*., 2006a; Santos *et al*., 2010). The assessment of ecotoxicological impacts elicited by combined effects of pharmaceuticals in mixtures is still an incipient topic of research, particularly regarding coastal and marine organisms.

Evidence suggests that some drugs interact at environmentally relevant levels in a given mixture, conferring unexpected toxicity effects not predicted based on individual concentrations, by virtue of interactions among their modes of action (MoAs) (Cleuvers, 2004; Pomati *et al.*, 2008; Trombini *et al.*, 2016b). Almeida *et al. (*2018) showed that the antiepileptic carbamazepine (CBZ) or the anti-histaminic cetirizine (CTZ) combined with Cd elicited different biochemical responses in marine clams *Ruditapes philippinarum* compared to the drugs alone, in a 28-day bioassay. GST activity and levels of LPO by-products were similar between individuals from controls and those exposed to the mixture CBZ + Cd, whereas the single exposure to CBZ caused oxidative stress and damage. On the other hand, CBZ combined with Cd inhibited GST activity, while individual exposure to this drug did not cause biochemical alterations. A 6 h-exposure of the Pacific oyster *Cassostrea gigas* to a mixture containing ibuprofen (5 µg L^{-1}) and the herbicides diuron (5 µg L^{-1}) and isoproturon (5 µg L^{-1}) caused a significant inhibition of phagocytosis (50%) and a 20% reduction in the catecholase phenoloxidase activity, in contrast to the absence of effects on exposure only to diuron (Luna-Acosta *et al.*, 2012).

The therapeutic purpose and MoA of mixtures of drugs are important parameters of concern, such as their respective individual concentrations (Trombini *et al*., 2016b). It is crucial to consider the potential toxic mechanisms underlying drugs´ MoA and the complexity of responses in aquatic species, particularly those produced by hazardous therapeutic classes (Pomati *et al.,* 2008; Toolaram *et al.,* 2014). In this context, the anticancer drugs applied in conventional chemotherapies arise as an incipient environmental concern given their overall design to ultimately interrupt tumour cell proliferation and provoke cells' death (Johnson *et al.,* 2008; Xie, 2012; Toolaram *et al.,* 2014). Such therapeutic class comprise a wide array of subgroups, in which molecules exert cytotoxic activity through a range of mechanisms, including disruption of DNA transcription, replication and synthesis; significant generation of reactive oxygen species (ROS) following biochemical alterations and cellular damage; modulation of kinases and inactivation of vital proteins; interaction with microtubules or tubulins in order to disrupt mitotic spindle, among others (Conklin, 2004; Gorrini *et al.,* 2013; Toolaram *et al.,* 2014; He *et al.,* 2016). Since these "targets" are not specific drivers of tumour proliferation, normal growing cells are also affected, with cytotoxic, genotoxic and mutagenic outcomes (Zounková *et al.*, 2007; Caley and Jones, 2012; Heath *et al.,* 2016). Particularly regarding the administration of these pharmaceuticals, chemotherapy regimens combine multiple anticancer drugs with distinct MoAs, in order to provide an additive or synergistic activity, thus achieving clear rationale cytotoxic effects and overcome patients´ resistance (Tkaczuk, 2009; Shi *et al.,* 2012; He *et al.,* 2016). To mitigate potential increases in toxicity, the combination approach to treatment frequently involves using lower doses of chemotherapeutic agents than would typically be considered optimal (Tkaczuk, 2009). Consequently, excreted residues consist of mixtures of these therapeutic compounds and their by-products (Parrella *et al*., 2014a).

A vast array of pharmaceuticals with anticancer purposes were detected in freshwater and marine compartments at concentrations from sub-ng to ng L^{-1} (Thomas and Hilton, 2004; Buerge *et al.,* 2006; Zuccato *et al.,* 2006; Ortiz de García *et al.,* 2013; Česen *et al.,* 2015; Franquet-Griell *et al.*, 2017). Regardless of being present at

very low levels, anticancer agents cause subtle effects in non-target aquatic organisms under chronic exposure, via similar toxic mechanisms reported in targeted biological models (Moreira *et al.,* 2016; Trombini *et al.,* 2016b; Fonseca *et al.,* 2017, 2018), thus highlighting that ecotoxicological data of aquatic species should not be merely based on acute toxicity determinations (Brezovsek *et al*., 2014; Elersek *et al*., 2016). Although knowledge regarding toxicity elicited by mixtures of anticancer drugs is growing, there is still a caveat around the potential responses in marine species.

Once in the aquatic environment, pharmaceuticals undergo hydrodynamic, biological and chemical processes over the water course until they reach the marine environment, where the gradient of salinity and pH, microbial population, among others, may provide differential partitioning of pharmaceuticals compounds and contribute to their aggregation and settling onto sediments (Yamamoto *et al.,* 2009; Du *et al.,* 2014; Ribeiro *et al.,* 2015). Sediment dwelling species are then constantly exposed to complex pharmaceuticals mixtures, either by bulk sediments dermal contact and ingestion, such as through interstitial water, depicting important routes of exposure to benthic biota. Altogether, factors that must not be neglected in these combinatory approaches are the range of concentrations present in mixtures, differences in bioavailability and routes of drug uptake, presence of biologically relevant targets of the drug and reasonable interactions between them (Escher and Hermens, 2002; Parrella *et al.,* 2014a; Brezovsek *et al.,* 2014) .

Cisplatin (CisPt) is a platinum-based anticancer agent mainly applied to treat ovarian, colorectal, head and lung cancers, by inducing covalent linkages with DNA and generation of interstrand crosslinks (see Chapter 2). Although also acting on DNA, cyclophosphamide (CP) consists of an alkylating mustard that requires metabolic activation to exert its cytotoxic purpose by forming covalent adducts (Emadi *et al.,* 2009) (see also Chapter 3). Tamoxifen is a selective estrogen-receptor modulator (SERM) prescribed for therapy of breast cancer with the purpose to antagonize the proliferative action of estrogen through competitive binding to its respective receptor (ER) (Goldstein *et al.,* 2000; Besse *et al.,* 2012) (see also Chapter 4).

Because these anticancer drugs have been traditionally applied worldwide in single and combinatory chemotherapy during decades, they were chosen for the present mixture toxicity assessment aimed to determine the effects exerted in an ecological representative species from marine benthos, the polychaete *Nereis diversicolor*. Mixtures consisted of an increasing range of concentrations and exposure took over 14 days. The objective of the present chapter was to assess the effect of mixtures of CisPt, CP and TAM on a multibiomarker approach, including behavioural impairment in burrowing activity; neurotoxicity (AChE activity); antioxidant enzymes, namely the superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidases (Se-GPx and T-GPx); the biotransformation phase II enzyme, glutathione-S-transferase (GST); lipid peroxidation (LPO); and genotoxicity (DNA damage). Secondly, results on the effects obtained of the mixtures exposures were compared to their individual effects previously determined in *N. diversicolor* (Fonseca *et al.*2017; 2018).

5.2 Materials and Methods

5.2.1 Chemicals

The analytical standards of *cis*-platinum (II) diamine dichloride (CisPt) (CAS 15663-27-1), cyclophosphamide monohydrate (Cytoxan) (CAS 0768) and tamoxifen (CAS 10540-29-01) were purchased from Sigma-Aldrich (Portugal). Stock solutions of each chemical were prepared in ultrapure Milli-Q water, except for that of TAM that due to its low water solubility was first dissolved in the carrier solvent dimethyl sulfoxide (DMSO). Experimental solutions of pharmaceuticals were individually prepared from serial dilutions of respective stock solutions (CisPt 123.07 mg Pt L^{-1}; CP 40 mg L^{-1}; TAM 100 mg L^{-1}) in ultrapure Milli-Q water. Aliquots of 100 µL of each pharmaceutical solution, at respective concentrations, were added and mixed in microtubes, following subsequent seed into respective treatment tanks. In order to avoid toxic effects derived from solvent, a final concentration of 0.001% (v/v) DMSO was set up in test aquaria (Fonseca *et al.,* 2017, Chapter 4). Since dermal exposure is

considered to have the predominant role in the uptake of anticancer agents by health care workers, drugs herein studied were safety handled using class II biological safety cabinet, with appropriate clothing (open-back, impervious chemotherapy protection gown, double powder-free latex gloves and safety goggles).

5.2.2 Experimental setup

Polychaetes *N. diversicolor* were handpicked during summer, at the intertidal estuarine mudflat in Mira River estuary (Vila Nova de Milfontes, Portugal), following their transport to the laboratory with sediments and water from the site of origin. Sediments treatment and characterization were performed according to Fonseca *et al. (*2017, 2018, See Chapters 2 and 3). Grain size distribution was composed predominantly by silt and clay (73% of particles <63 µm), following fine sand (23%), and organic matter content of 7.8%. Polychaetes were acclimated for five days in aerated aquaria filled with natural filtered seawater (salinity 35) from the Ria Formosa lagoon (Faro, Portugal) and sediments from the sampling site. Specimens of *N. diversicolor* were placed in 10-L glass aquaria, in triplicate, containing sediment and water (1:4), under constant aeration, controlled temperature (19 ± 2°C), salinity (35 ± 1.8) and light period (12:12 hours).

Seawater was carefully renewed every 48 hours, avoiding sediment resuspension, following addition of pharmaceutical mixtures into water column. Polychaetes were exposed for 14 days to four tertiary drug combinations, including CisPt + CP + TAM, at increasing concentrations (ng L^{-1}), respectively: Mix A: 0.1 + 10 + 0.1; Mix B: 10 + 100 + 10; Mix C: 100 + 500 + 25; Mix D: 100 + 1000 + 100 ng L^{-1}. Concentrations studied were selected according to environmental levels reported in water bodies and worst-case scenarios of pharmaceutical pollution. Along with drug-exposed treatments, controls of seawater (day 0, before systems contamination: CT0; day 14: CT14) and solvent (0.001% DMSO) were set up. Seventy-five polychaetes were distributed per treatment (25 per aquarium). Animals used in the burrowing and comet assays were immediately handled for respective analysis, while

those regarding biochemical end-points were rinsed with clean seawater and stored at -80 °C until further use.

5.2.3 Burrowing Assay

At the first (CT0) and 14th days (CT14; DMSO; MIX A, B, C and D) of the bioassay, fifteen animals were removed from each exposure treatment (5 per aquarium) and individually placed in 150 mL-plastic containers, filled with natural seawater and 5 cm of clean sediments. Worms were submitted to a burrowing test according to Fonseca *et al. (*2017) (Chapter 2), over a period of 30-minutes. The position of the polychaetes was recorded every two minutes, to assess the time for fully burrowing. The results are expressed as the percentage (%) of unburied specimens, over time (min).

5.2.4 Biochemical analysis

Neurotoxicity

For determination of AChE activity, polychaetes were individually homogenized (2 specimens per replicate; total of 6 organisms per treatment) in 100 mM Tris-HCl buffer (pH 8.0) and 0.1% Triton. The homogenates were centrifuged at 12,000 *g*, for 30 min, at 4 °C, and further separated in aliquots for total protein determination (Bradford, 1976) and AChE activity analysis (Ellman *et al.*, 1961). The product of acetylcholine (ATC) cleavage by AChE was determined at 405 nm (Ellman *et al.*, 1961; Colovic *et al.*, 2013), and enzymatic activity expressed as ATC.min^{-1} mg^{-1} protein.

Antioxidant and biotransformation enzymes activities

Procedures regarding tissue preparation and enzymatic analysis were previously detailed in Fonseca *et al.* (2018). Whole organisms (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment), were homogenized in 20 mM

Tris-HCl buffer (0.5 M sucrose, 0,075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6), following cytosolic fractioning obtained by serial centrifugations, according to Fonseca *et al. (*2018) (Chapter 3). Supernatant aliquots (150 µL) were separated for further determination of antioxidant enzymes (SOD and CAT) and biotransformation (GST) activities, normalized by total proteins concentration (mg protein g^{-1} tissue) by using bovine serum albumin (BSA) as standard (Bradford, 1976). Absorbance of SOD activity was measured at 550 nm and results expressed as U mg^{-1} protein (McCord and Fridovich, 1969). Absorbance regarding CAT activity was measured at 240 nm (Greenwald, 1985) and results expressed as nmol min^{-1} mg^{-1} protein.

GST activity was measured at 340 nm, by the conjugation of 0.2 mM reduced glutathione (GSH) with 0.2 mM CDNB in a reaction mixture of 0.2 M KH_2PO_4/K_2PO_4 buffer (pH 7.9) (adapted from Habig *et al.*, 1974), and results expressed in nmol CDNB min^{-1} mg^{-1} protein. Total GPX activity was measured in a microplate reader, at 340 nm, based on the method adapted from Lawrence and Burk (1976). The reaction consists in the reduction of oxidized glutathione linked to the oxidation of NADPH in the presence of excess glutathione reductase. Cumene hydroperoxide and H_2O_2 were used as substrates for T-GPx and Se-GPx, respectively. Results are expressed in nmol min^{-1} mg^{-1} protein.

Oxidative damage

Levels of LPO by-products were measured in whole organisms (2 specimens per aquaria, in triplicate; total of 6 organisms per treatment), through malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) absorbances measured at 540 nm adapted from the method of Erdelmeier *et al. (*1998). Results are expressed in nmol MDA + 4-HNE mg^{-1} protein.

5.2.5 Genotoxicity assay

Genotoxicity assessment conducted with alkaline Comet assay in *N. diversicolor* coelomocytes was previously detailed in Fonseca *et al. (*2018) (Chapter

3). Briefly, collected cells were centrifuged at 3000 rpm (3 min, 4 °C), and resulting pellets suspended in 0.65 % low melting point agarose (LMA). Cells were smeared on microscope slides, following immersion in lysis buffer, for at least 1 h. Slides were placed in a horizontal electrophoresis box aftermath, and immersed in alkali buffer allowing DNA to unwind. Electrophoresis current was set up at 25 V and 300 mA, over 5 min, following slides neutralization (0.4 mM Tris, pH 7.5). Cells were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 mg ml^{-1}) and analysed with an optical fluorescence microscope Axiovert S100 (total magnification of ×400). DNA in the comet tail (DNA tail %) was determined in 50 cells per sample (25 in each duplicate) (Komet 5.5 - Kinetic Imaging Ltd). Results are expressed as mean ± standard deviation (S.D.). In addition, cells were also categorized for the frequency of DNA damage grade, according to the criteria used by Jo (2014). Damage is determined zero or minimal when it is DNA in tail is lower than 10%; low damage from 10-25%; mid damage from 25-50%; high damage from 50-75%; and extreme damage >75%.

5.2.6 Statistical analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene´s test) in order to determine whether they satisfy the assumptions associated with parametric tests, then the one-way analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis were applied. Tukey´s or Dunn´s post-hoc tests were performed to compare treatment effects. Results were significant when $p < 0.05$. Burrowing behavioural data was analysed by linear regression. Principal Component Analysis (PCA) was applied to evaluate the relationship among biomarkers responses and between pharmaceuticals levels. Statistical analysis was carried out using the Statistica 8.0 software (Statsoft Inc., 2007, USA).

5.3 Results

5.3.1 Burrowing behaviour

Results of burrowing bioassay are shown in Figure 5.1. A similar behavioural profile was addressed for polychaetes collected from control, DMSO and those from the highest-concentration mixture (Mix D), in a way that 100% of individuals were buried within 2 (CT14, DMSO, Mix D) and 4 minutes (CT0). On the other hand, no trends of behaviour could be depicted for the remaining treatments. Polychaetes exposed to the Mixture A and B failed to burrow after 2 minutes of the bioassay, thus resulting in 33% and 13% of organisms left unburied until the end of analysis. Regarding polychaetes exposed to Mixture C, 26% of individuals remained at the sediment surface.

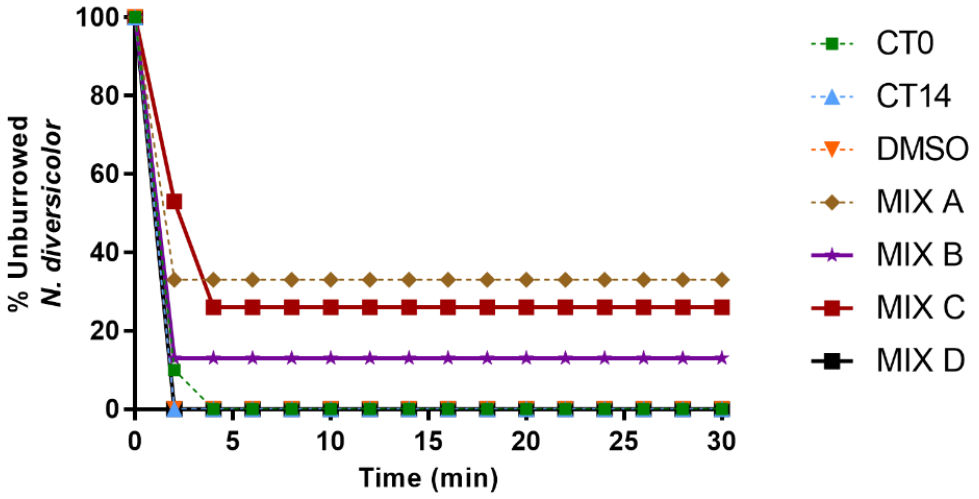


Figure 5.1: Burrowing behaviour of *N. diversicolor* from control conditions (CT0; CT14; 0.01% DMSO) and exposed to a combination of anticancer drugs, at increasing concentrations (Mix A, B, C and D). Results were expressed as percentage of unburied organisms over time (lines with symbols).

5.3.2 Biochemical analysis

Neurotoxicity

AChE activity did not change among controls ($p > 0.05$). Polychaetes exposed to Mixture A and C exhibited similar AChE activity compared to the unexposed organisms. Conversely, neurotoxicity was detected in mixtures B and C, by means of a 2- and 3-fold inhibition of AChE activity ($p < 0.05$) (Figure 5.2).

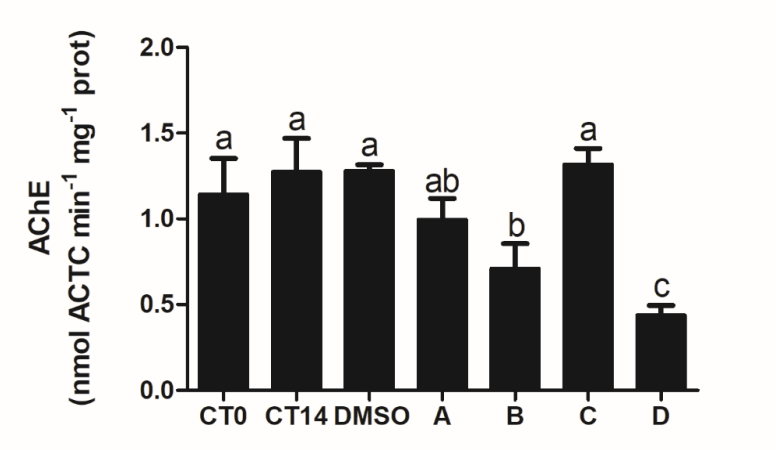


Figure 5.2: AChE activity (mean ± S.D.) (ATC.min^{-1}.mg^{-1} protein) (mean ± S.D.) in *N. diversicolor* unexposed (CT0; CT14; 0.01% DMSO) and exposed to a combination of anticancer drugs, at increasing concentrations (Mix A, B, C and D), over 14 days. Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

Antioxidant and biotransformation enzymes

Activity of antioxidant enzymes were similar between controls and indicated that the percentage of solvent concentration applied to prepare the mixtures of pharmaceuticals solutions provided no significant effects in *N. diversicolor*. Although a slight trend of increase was depicted in SOD activity, over the increasing concentrations of the mixtures, no significant alterations were detected ($p > 0.05$) (Figure 5.3-A). In contrast to SOD, there was a decreasing trend of the CAT activity in the polychaetes exposed to the mixture containing the highest drugs concentrations

(*i.e.* Mix D), CAT activity was significantly inhibited 12-fold when compared to the other mixtures ($p < 0.05$) (Figure 5.3-B). No significant impairments in Se-GPx were addressed in control conditions neither in drug-treated polychaetes, in contrast to T-GPx, induced in individuals exposed to Mix A ($p < 0.05$).

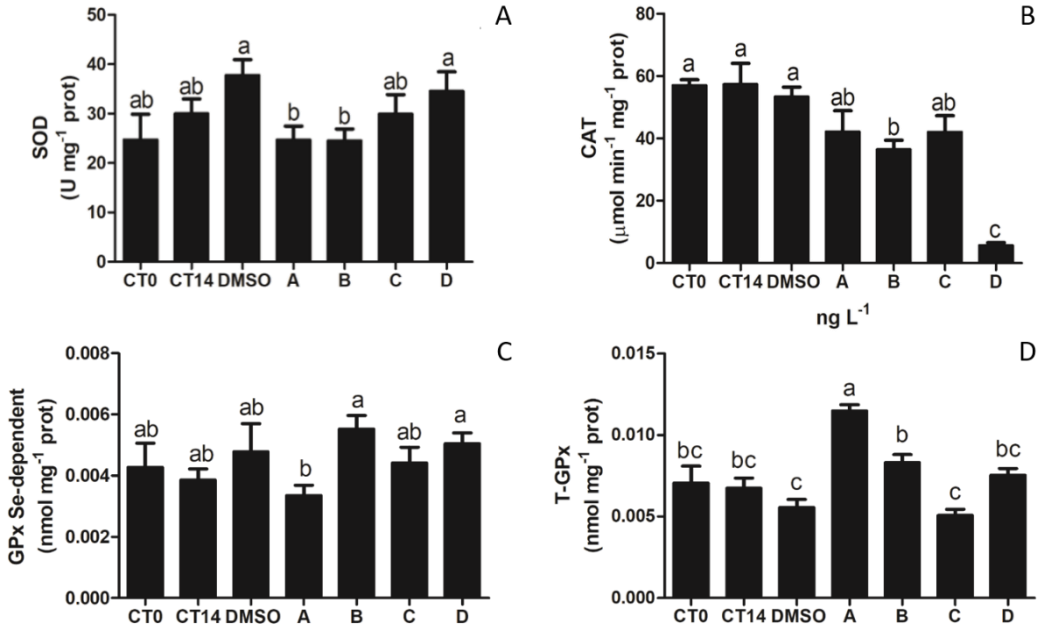


Figure 5.3: Antioxidant enzymes activities (mean ± S.D.) of (A) SOD, (B) CAT, (C) Se-GPx and (D) T-GPx (mean ± S.D.) in *N. diversicolor* unexposed (CT0; CT14; 0.01% DMSO) and exposed to increasing concentrations of mixtures of anticancer drugs (Mix A- D), over 14 days. Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

Regarding GST activity, a bell-shaped response profile was displayed (Figure 5.4). A significant 2-fold inhibition of GST activity was detected in polychaetes exposed to the lowest mixture concentration (*i.e.* Mix A), followed by an increase until Mix C and ultimately decreasing at the highest mixture concentration (Mix D), similar to Mix A ($p < 0.05$).

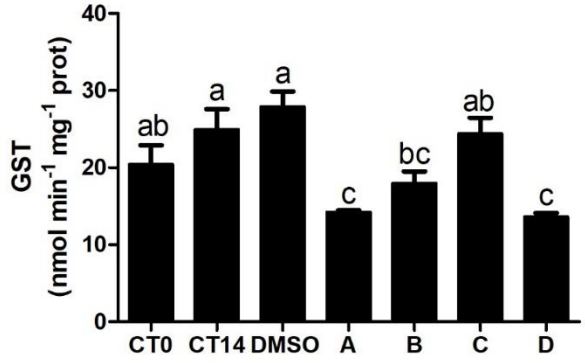


Figure 5.4: GST activity (mean ± S.D.) in *N. diversicolor* unexposed (CT0; CT14; 0.01% DMSO) and exposed to increasing concentrations of mixtures of anticancer drugs (Mix A-D), over 14 days. Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

Oxidative damage

Levels of LPO by-products were similar among controls ($p > 0.05$) (Figure 4.5). Conversely, in polychaetes exposed to the mixtures, LPO significantly increase in the presence of mixtures with Mixtures A and C having LPO levels 2-fold higher than controls. The highest oxidative damage was detected in polychaetes exposed to Mixtures B and D (3-fold higher than controls) ($p < 0.05$).

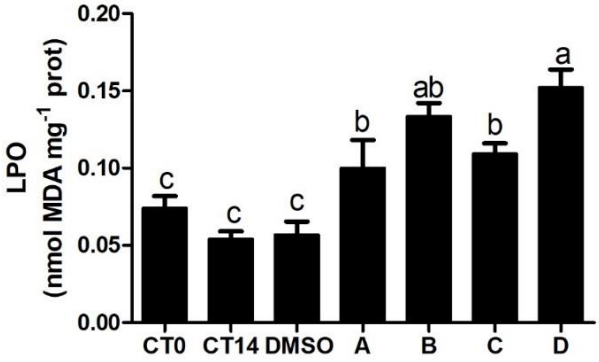


Figure 5.4: Lipid Peroxidation (LPO) levels (mean ± STD) (MDA+ 4-HNE nmol.mg^{-1} protein) in *N. diversicolor* unexposed (CT0; CT14; 0.01% DMSO) and exposed to increasing concentrations of mixtures of anticancer drugs (Mix A-D), over 14 days. Different letters indicate significant differences among treatments (One-way ANOVA; $p < 0.05$).

5.3.3 Genotoxicity

No significant differences were reported in DNA tail (%) among control conditions and groups exposed to pharmaceutical mixtures (Table 5.1). However, according to the grading of DNA damage in coelomocytes, unexposed polychaetes showed minimal or low damage along the 14-day exposure period (95.6 to 98.8%), whereas those exposed to Mix A indicated the highest level of mid damage (57.1%), followed by Mix C, B and D (Table 5.1). In the present study, no extreme DNA damage was reported.

Table 5.1: Genotoxicity in polychaetes *N. diversicolor* unexposed (CT0; CT14; 0.01% DMSO) and after 14 days of exposure to mixtures (Mix A-D), expressed as percentage of DNA in tail (mean ± SEM). Frequency of coelomocytes distributed by grade of DNA damage (%) (i.e. minimal to mid).

Treatment		DNA Tail (%)	DNA Damage grade		
			Minimal	Low	Mid
Control	CT0	9.49 (± 3.3)	60.1	36.7	3.2
	CT14	7.6 (± 1.8)	67.2	31.6	1.2
	DMSO	10.8 (± 1.78)	53.8	41.8	4.4
Mixtures	A	14.2 (± 3.3)	7.9	35	57.1
	B	12.85 (± 2.9)	7.9	45.1	47
	C	12.3 (± 6.6)	7.3	52	40.7
	D	11.3 (± 3)	5.9	52.6	41.5

5.3.4 Principal Component Analysis

Integration of biomarkers responses regarding organisms exposed to pharmaceuticals in mixtures is depicted in Figure 5.6. PCA of the data explained 81%

of variance, in which PC1 and PC2 corresponded to 53.3% and 27.7%, respectively. According to Durou *et al. (*2007), correlation coefficients are significant when they are higher than $\sqrt{d/n}$ (*i.e. d* is the number of principal components; *n* the number of variables), thus equal to 0.35 for this data analysis. In PC1, positive correlations were registered for CAT, GST and AChE activities, jointly with loading contributions of control conditions (CT0, CT14 and DMSO) and Mix C, which indicates the high expression range of these enzymes. In contrast, PC1 exhibited a negative correlation regarding Se-GPx, T-GPx, LPO and DNA damage explaining the effects on these endpoints in individuals exposed to Mix A, B and D.

Regarding PC2, the variables of significant positive distribution are represented by SOD, Se-GPx and GST enzymes, grouping individuals exposed to DMSO control condition and Mix B, C and D. Interpretations of this axis suggest higher levels of these enzymatic activities compared to the other groups present in the negative axis, although no significative differences were observed and no clear elucidations could be concluded. On the other hand, the induction of GPx-T activity in individuals exposed to Mix A and the clear inhibition of CAT activity in those exposed to Mix supported the separation of both treatment groups in PC2, and negative correlations attributed to both variables.

Integrating biomarkers responses from mixtures and individual exposure data of anticancer drugs (Chapter 2, 3 and 4) on a PCA analysis results were distributed in three main axes (PC1, PC2 and PC3), which accounted for 74.3% of variance. Figure 4.7-A shows the plot of PC1 and PC2 that respectively represent 41% and 18.5% of the total variance. The correlation coefficient for this dataset was 0.61. Therefore, PC1 illustrated a significant positive correlation between the antioxidant status, defined by SOD, CAT, Se-GPx and T-GPx, jointly with the biotransformation enzyme GST. Loadings concerning polychaetes exposed to pharmaceuticals in mixtures are distributed on higher scores of the negative PC1, particularly regarding the group D (Figure 5.7-A) in comparison to control groups closer to the origin, thus indicating a general trend of inhibition in these biochemical markers in mixture-exposed

individuals. Due to the high scores in the positive axis of PC1, biomarkers effects from the singular exposure to CisPt (Chapter 2) showed substantial distance from those registered for the other anticancer drugs, particularly attributed to the higher baseline values addressed in enzymatic activities. Interestingly, PC1 also illustrated a relationship between the effects of TAM- and CP-treated groups with those under the mixtures. In addition, exposure of pharmaceuticals mixtures A, B and D showed a similar contribution in PC1 regarding enzymes activities, which corroborates to responses observed at the highest CP concentration (1000 ng L^{-1}).

According to biomarkers´ loadings in drug-treated groups, animals exposed to CisPt seemed to fail to contribute in mixtures´ toxicity, at any of the concentrations tested. Likewise, the lowest level of TAM (0.5 ng L^{-1}), which individually induced higher activity of SOD, CAT and GST during its single exposure (Chapter 4), was not able to elicit enzymatic alterations within Mix A. Moreover, PC2 exhibited a significant positive correlation between Se-GPx and T-GPx, activities related to effects from individuals exposed to Mix A, particularly attributed to the sharp induction reported in T-GPx activity (Figure 3-D). Once again, biochemical responses of Mix A show a disagreement with the effects reported for pharmaceuticals individually exposed, as can be seen by the opposite distribution of treatments. PC3 explained 14.8% of the total variance (Figure 5.7-B) in which a significant positive loading was attributed to LPO. In this biplot, individuals exposed to CP showed a range of MDA products higher than that registered in the other singular and mixture exposures.

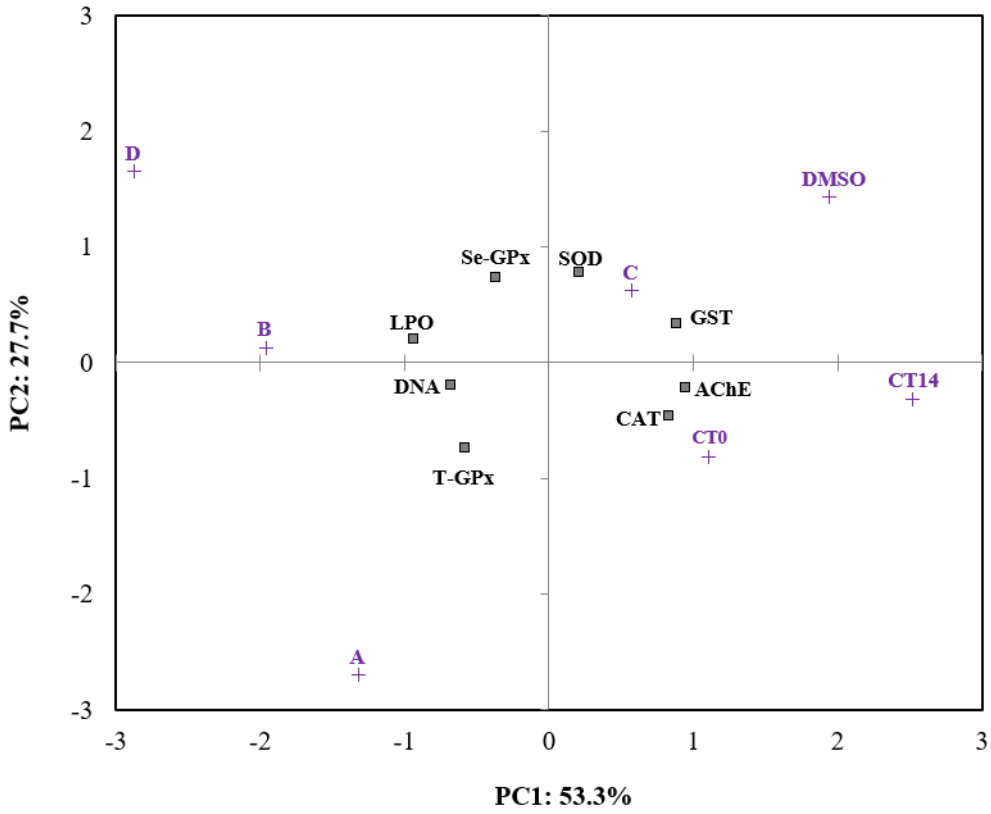


Figure 5.5: PCA biplot of PC1 vs. PC2 integrating biomarkers responses (■ AChE, SOD, CAT, Se-GPx, T-GPx, GST, LPO, DNA damage) from polychaetes *N. diversicolor* unexposed and exposed to different mixtures concentrations of anticancer drugs (✚ Mix).

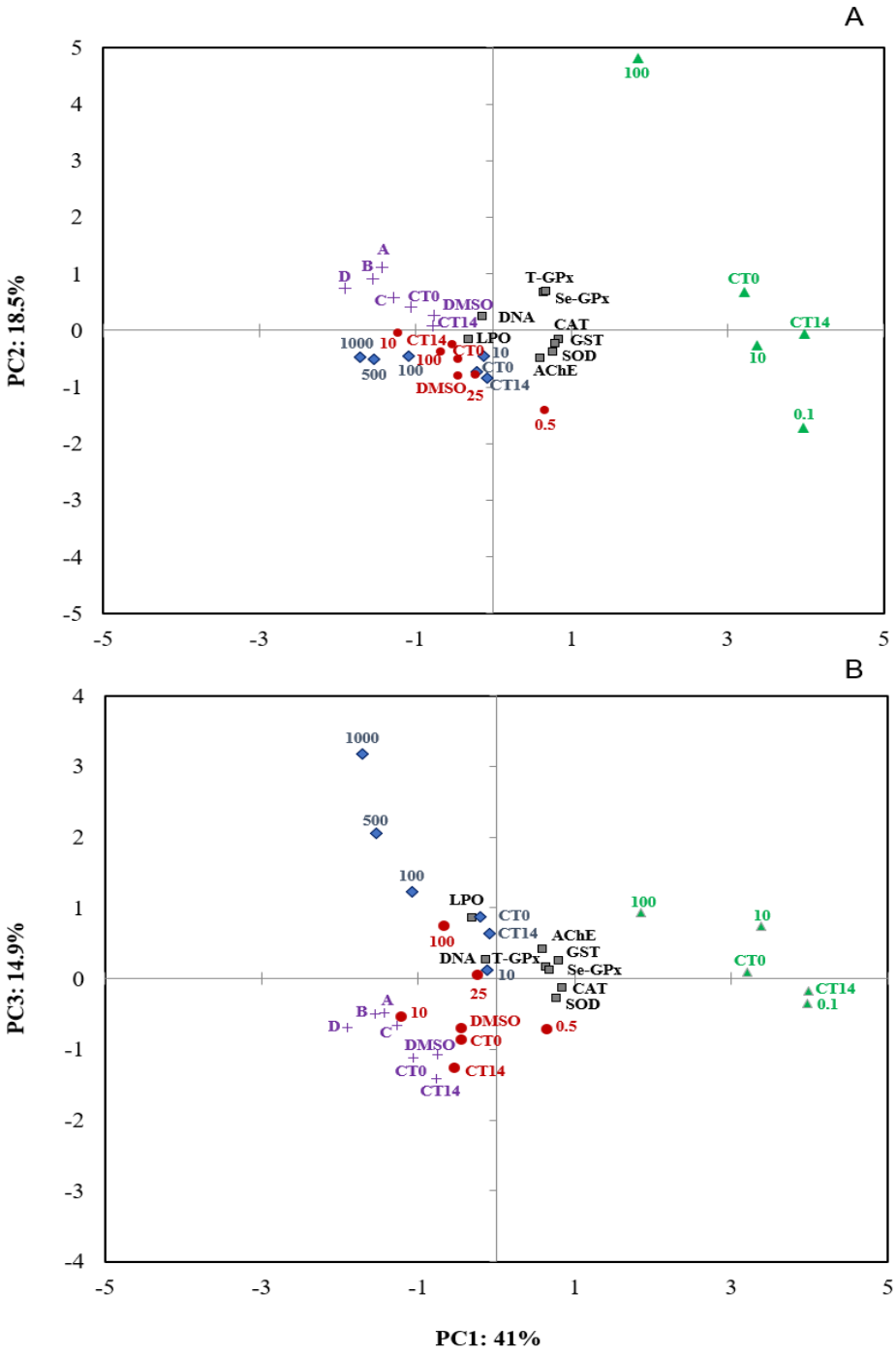


Figure 5.6: PCA biplot from (A) PC1 vs. PC2 and (B) PC1 vs. PC3 integrating biomarkers responses (■ AChE, SOD, CAT, Se-GPx, T-GPx, GST, LPO, DNA damage) in polychaetes *N. diversicolor* from bioassays conducted with singular exposure of anticancer drugs in (▲ CisPt; ◆ CP; ● TAM) and in combination (✚ Mix), at different concentrations jointly with control conditions.

5.4 Discussion

Anticancer drugs have been applied for about sixty years in chemotherapy treatments worldwide, but since then the outlook of non-communicable diseases indicates an increase in cancer incidence for the next decades, trends of consumption of these specialized medicines are also estimated to rise, as do their excretion on waterways and the consequent impact to water resources (López-Gómez *et al*., 2013; Toolaram *et al*., 2014; Torre *et al*., 2015). Data regarding marine ecotoxicological effects of pharmaceutical mixtures have been mainly conducted with non-steroidal anti-inflammatories, analgesics, fungicides, antiepileptics, beta-blockers, anti-depressants and blood lipid lowering drugs, whereas concerns directed to the issue of anticancer drugs has shown to be incipient (Canesi *et al.,* 2007; Luna-Acosta *et al.,* 2012; Gonzalez-Rey *et al.,* 2014; González-Ortegón *et al.,* 2016; Pires *et al.,* 2016a; Trombini *et al.,* 2016b).

Cancer cells have an aberrant metabolism (Che *et al*., 2016) characterized by a highly oxidant intracellular environment in which critical ROS-sensitive signaling pathways act as drivers and regulators for tumour proliferation (Trachootham *et al.,* 2009; Liou and Storz, 2010). Therefore, the pharmacologic rationale of anticancer drugs relies on the induction of irreparable damages, cell cycle arrest or death, either through the ability to exceed threshold levels of intracellular ROS and/or by suppressing antioxidant defenses (Liou and Storz, 2010; Gorrini *et al.,* 2013). SOD activity plays an important role by the dismutation of superoxide anion ($O_2^{\cdot -}$) into hydrogen peroxide (H_2O_2), a critical messenger of antiapoptotic mechanisms and tumour proliferation (Sarsour *et al*., 2008). Although assumed as a target for cytotoxic purpose, no alterations in SOD activity were addressed in polychaetes tested in mixtures. In Mix C and D, the absence of changes in SOD could be associated to the antagonistic potential of TAM over CisPt and CP, once both drugs were accountable for its inhibition in polychaetes under single-drug exposure (Figure 5.8). According to the PCA (Figure 5.7) and the overall effects illustrated in Figure 5.8, the profile of antioxidant system (*i.e.* SOD, CAT, Se-GPx and T-GPx activities) of polychaetes

exposed to Mix C and D were mainly driven by the responses reported in the bioassay conducted only with TAM.

When CisPt and CP were exposed individually, both were able to induce and inhibit T-GPx, respectively, while in combination their effects were cancelled (Figure 5.8). Antagonism is generally defined by the interaction between two or more drugs resulting in an overall effect intensity lower than any of the drugs alone (Becker, 2011); a toxicity in mixture lower than the sum of the individual effects (Láng and Kőhidai, 2012), although Chou *et al. (*2010) infer that drugs interactions should not be limited to a simple arithmetic sum and the shape of dose-effect curve must be considered. Antagonistic effects on growth inhibition were addressed in the freshwater ciliate *Tetrahymena pyriformis* exposed to binary mixtures involving diclofenac, ibuprofen, metoprolol and propranolol and predominate at the highest concentrations due to the potential competitive inhibition between the two pharmaceuticals acting on the same molecular target (Láng and Kőhidai, 2012). Although the designed pharmacological MoA of TAM diverges from the cytotoxicity of CisPt and CP, studies have shown that TAM may also elicit toxic effects in ER-negative cells, thus via a strategy disregarding the anti-estrogenic pathway (Scripture and Figg, 2006). According to Becker (2011), pharmacokinetic interactions between administered drugs alter one´s delivery to its respective target, due to changes in absorption, distribution, and elimination, which may change the interaction toward specific pathways and receptors, thus its ultimate efficacy (Scripture and Figg, 2006; Becker, 2011). Interactive outcomes provided by the tertiary mixtures may have altered the uptake of the cytotoxic agents by the cells via changes in structure or function of proteins involved in drug transport delivery. In the case of CisPt, the copper transporter proteins CTR1 are accountable for its accumulation, and impairments in this influx is linked to resistance (Dasari and Tchounwou, 2014; Ma *et al.*, 2015). However, to the best of our knowledge, no studies have clarified an interaction of TAM or CP with this membrane protein. In fact, studies have highlighted that the combinatory administration of CisPt with the alkylating CP is more effective in clinical responses

than when managed individually, particularly in ovarian carcinoma (Neijit *et al.*, 1984; Mcguire *et al.*, 1996).

Likewise, the adjuvant administration of CisPt with TAM led to an enhancement of cytotoxic and apoptotic effect in the ER-positive OSCC (oral squamous cell carcinoma) cells and concluded that the binary treatment could be a more effective alternative (Kim *et al.*, 2007). In addition, TAM and CisPt have demonstrated synergistic action in the human melanoma cell line T-289 (Mcclay *et al.*, 1994; Nakata *et al.*, 1995), similar to the results obtained in combinatory treatment of CP with TAM, showing a significant benefit in breast cancer patients (Abram *et al.*, 1988). Although the divergence in drug-combination outcomes, it is important to be aware that the range of concentrations applied herein are nearly 10^{-6} lower than those used in the chemotherapy, which may have triggered responses of multidrug resistance phenotype, drug efflux metabolism, or metabolism into less active metabolites and activation of kinases pathways according to the targeted tissue (Tan *et al.*, 2014; Zhu *et al.*, 2017). In the study of Goldenberg and Froese (1985), an antagonistic effect was registered at the co-administration of TAM with melphalan, suggesting that the former either inhibited the other´s influx and stimulated its efflux.

In addition, the combination of TAM with the cytotoxic docetaxel, able to inhibit microtubule depolymerization, produces antagonist outcomes in ER-positive MCF-7 human cell lines, while in the ER-negative breast cancer cell lines the response is synergistic, thus indicating that mechanisms leading to drug resistance may diverge according to targeted tissues (Zhu *et al.*, 2017). Toxicity predictions of binary mixtures failed to estimate growth inhibition in the green algae *Pseudomonas subcapitata*, underestimating the detrimental effects caused by 5-FU + CisPt and 5-FU + IM, and overestimating those of CisPt + ET (Bresovzec *et al.*, 2014). Also, growth inhibition caused by 5-FU + IM on the cyanobacteria *S. leopoliensis* was lower than that modeled (Bresovzec *et al.*, 2014). Therefore, besides the particular physico-chemical features of mixtures under study, some species may be more affected than others, which generates particular orders of susceptibility (Ortiz de García *et al.*, 2014). Although

resistance to anticancer drugs is an issue of high interest for chemotherapy effectiveness, the mechanisms involved in the drug failure are also relevant for ecotoxicological approaches over non-target species.

Drugs (ng L^{-1})		SOD	CAT	Se-GPx	T-GPx	GST	AChE	LPO	DNA Damage
MIX A	CisPt 0.1				↓				
	CP 10								↑
	TAM 0.5	↑		↑				↑	
MIX B	CisPt 10		↓						
	CP 100		↑						↑
	TAM 10		↓					↑	
MIX C	CisPt 100	↓	↓	↑	↑	↓	↓	↑	
	CP 500	↓	↑		↓	↓		↑	↑
	TAM 25						↑	↑	
MIX D	CisPt 100	↓	↓	↑	↑	↓	↓	↑	
	CP 1000	↓			↓	↓		↑	↑
	TAM 100		↓				↑	↑	↑

Figure 5.7: Biomarkers profile in *N. diversicolor* exposed to tertiary mixtures (A, B, C and D), according to the single-drug responses. Arrows ↓ indicate inhibition; arrows ↑ induction; absence of arrows indicates effects similar to controls, in each single-drug bioassay. Green and red boxes indicate, respectively, similar and distinct effects in relation to the corresponding mixture.

In contrast, the single-exposure of CisPt and TAM, conducted with the same Mix D concentrations, inhibited CAT activity by 57% and 51%, respectively, whereas in combination enzymatic decrease accounted for 90%, indicating synergistic effects on this enzyme. In addition, polychaetes exposed to this high set of concentrations experience a significant disruption of the first line of ROS scavengers by a severe

inhibition of CAT activity (Figure 5.3). The significant inhibition of GST activity was similar to the response addressed in *N. diversicolor* under single exposure to 100 ng Pt L^{-1} CisPt and to 1000 ng L^{-1} CP (Fonseca *et al.*, 2017, 2018), showing their additive interaction (Figure 5.8). In addition, Becker (2011) addressed that the most frequent and significant pharmacokinetic interaction between drugs is associated to the biotransformation metabolism. Even at low concentrations (Mix A) suppressed polychaetes´ biotransformation metabolism, although with no similarity to the respective single-drug responses (Figure 5.8). Indeed, PCA indicated in PC1 that biochemical responses addressed in specimens exposed to Mix A correspond to those reported for Mix D as well as those from single CP exposure (Figure 5.7). Although interferences in Mix D toxicity may also derive from CisPt´s MoA, PC1 loadings separate this group from the others in the positive side of axis, since the overall range of biomarkers responses are higher than those reported in any other experiment. It is well-known that the thiol groups in the cytoplasm, especially consisting of glutathione (GSH) may conjugate to electrophilic pharmaceuticals like CisPt and CP, forming adducts that provide their inactivation and detoxification (Vertuani *et al.*, 2004; Galadari *et al.*, 2017). Since GST catalyzes the conjugation of GSH with the reactive species acrolein and mono-aquacisplatin from CP and CisPt metabolism, its inhibition leads to hydroxyl radical formation and oxidative stress, able to trigger LPO (Basu *et al*., 2015; Siddik, 2003). Therefore, it seems that, once combined at low concentrations (Mix A), CisPt and CP may impair GST detoxification to the same extent that was observed at high concentrations (Mix D) and turn it insufficient to prevent membrane damage (Gonzalez-Rey *et al*., 2014) (Figure 5.5), as illustrated by the PCA dispersal pattern (Figure 5.6).

Drugs at the low concentrations (Mix A) may be accountable for inducing significant levels of T-GPx activity (Figure 5.3-D) as a potential protective effect of hormesis promoted by responsive mechanisms facilitated at low concentrations, such as drug permeability (Alfarouk *et al*., 2015). In other words, although the balance of antioxidant status and mechanisms involved in toxicity vary among concentrations, the stress performed by Mix A could have prompted physiological protective

mechanisms to compensate a disruption in homeostasis (Calabrese and Baldwin, 2001). A hormetic trend response was also depicted by the induction of SOD and Se-GPx activities in the TAM single-exposure at 0.5 ng L^{-1} (Chapter 4), although such biochemical profile was offset in Mix A and not associated to the herein experienced hormesis. Likewise, CisPt at 0.1 ng Pt L^{-1} showed to disrupt T-GPx activity in polychaetes, by a significant decrease of the activity (Figure 5.8), (Chapter 2). T-GPx acts in the intracellular space together with CAT on the role of H_2O_2 breakdown by reducing it to water. Accumulation of this ROS contribute to the generation of hydroxyl radicals (OH·) by Fenton reaction and have damaging effects on proteins function, DNA and lipids (Liou and Storz, 2010; Basu *et al.*, 2015). In this sense, it is hypothesized that despite the activation of the antioxidant system in polychaetes submitted to Mix A, ROS were not sufficiently scavenged and detoxification was not enough to overcome injuries provided by LPO by-products (Pires *et al.*, 2016a), culminating in mid-grade DNA damage at levels higher than those of the other mixtures (Table 5.1), as indicated by the positive correlation in PC1 joining T-GPx, LPO and DNA damage responses (Figure 5.6). In addition, the single exposure to 0.5 ng L^{-1} of TAM induced high levels of oxidative damage (Chapter 4), suggested to be responsible for the levels of LPO by-products in this mixture (Figure 5.8).

Despite the favorable anti-tumour properties, CisPt are also known to cause severe neurotoxicity as side effects in humans (Aljafari, 1995; Dasari and Bernard Tchounwou, 2014). The accumulation of platinum compounds and respective metabolites in the dorsal root ganglion of rats generates platinum-DNA adducts, known as key-steps to neurotoxicity (Kanat *et al*., 2017). According to Baig *et al. (*2014), CisPt molecular docking within the acyl pocket of AChE is permitted by the hydrophobic interactions and hydrogen bonds that play an equally important role in the drug´s correct positioning, thus allowing the inhibition of AChE. This also occur in the exposure of CisPt (100 ng Pt L^{-1}) to *N. diversicolor*, along with the impairment to burrow (Fonseca *et al.*, 2017 – See Chapter 2). However, even though organisms submitted to Mix D indicated the same neurotoxic profile, they showed an efficient burrowing capacity, in contrast to the expected results (Figure 5.1). Specimens

exposed to CP (1000 ng L^{-1}) were fully buried by the end of the bioassay and indicated no alterations of AChE activity profile (Fonseca *et al.,* 2018 – See Chapter 3). In this sense, according to the overall results, it is suggested that the mechanism involved in the burrowing impairment in Mix A was different from the one driven by AChE alterations. Therefore, further assessments around energy expenditures related to mitochondrial electron transport and lipid reserves are needed to better understand the mechanisms involved in burrowing interferences (Maranho *et al.,* 2015).

Progressively, findings around the toxicity or estrogenic assessment of mixtures have shown that combination of endpoints cannot be calculated by simply adding the effects of the individual components present in the mixture, especially if they have different dose-response curves (Fent *et al*., 2006a; Payne *et al*., 2000). Binary mixtures involving the drugs 5-fluorouracil (5-FU), cisplatin (CisPt), etoposide (ET) and imatinib (IM), at concentrations in a µg L^{-1} range, were accountable for the offspring reduction of the crustacean *Daphnia magna*, similar to single-exposures (Parrella *et al.*, 2014a). Hence, drugs exerted detrimental effects at concentrations independent of the other anticancer agents present in the mixtures, confirming the predictive power of independent action (IA) model at lower concentrations (Cleuvers, 2003; González-Ortegón *et al*., 2016), which roles out when the combined compounds have different MoAs. However, at higher concentrations, antagonist interactions occurred in mixtures containing IM as a potential sign of interference in the activity of ET and CisPt (Parrella *et al.*, 2014c), revealing a clear demonstration that interactions with targeted receptors, influx and efflux transporters and expression of resistance pathways may vary according to the concentration and nature of chemicals present in the mixture.

At the intermediary concentrations of Mix B and C, the anticancer compounds also triggered a significant oxidative damage in polychaetes, although no pronounced effects on neuroactivity, antioxidant and biotransformation metabolisms were detected. In these two mixtures, biochemical effects in polychaetes were similar to those reported in single-TAM exposures (10 and 25 ng L^{-1}) (Chapter 4): no significant

changes in antioxidant outcomes were registered, although drugs elicited an ultimate response of LPO, potentially triggered by drugs interaction directly over cellular membrane. In fact, TAM is known to impair the lipid bilayer integrity that ultimately influence down-stream nuclear events (Cabot *et al.,* 1997; Engelk *et al.,* 2002; Bilge *et al.,* 2013), disregarding the presence of ERs to trigger oxidative mechanisms recognized as important pathways for cell death (Bekele *et al*., 2016). Furthermore, besides the potential mechanisms associated to resistance against anticancer drugs in mixture, it is important to bear in mind that even that the target tissue under study express ERs, the mechanisms that mediates TAM over them may vary, culminating in estrogen agonist or antagonist, according to cell type (Sun *et al.,* 2011; García-Hernández *et al.,* 2016). The effects of TAM and its MoA in aquatic species have been investigated in fish, although results showed that estrogenic or anti-estrogenic outcomes depend on the gender, concentration and tissue analysed (Sun *et al.,* 2011).

5.5 Conclusions

In summary, some of the biological effects exerted by mixtures were in accordance to those previously reported for single-drug exposures, although corroborating only in specific endpoints, rather than similar to the whole set of biomarkers. Responses varied according to the range of concentrations assessed, but not in a strict dose-response manner. Although high levels of LPO by-products were reported in polychaetes exposed to all mixtures, mechanisms involved in the oxidative damage exerted at intermediary-drug levels (Mix B and C) seemed to stray from the oxidative stress pathway as reported in low-drug levels (Mix A). In addition, with the increase of drug concentrations in the mixture, the trend of effects was more similar to that observed in TAM single-exposure, suggesting an antagonist interaction of the cytotoxic CisPt and CP at their respective high doses and the dominance of TAM MoA over their cytotoxic potential in Mix C and D. Despite the range of low concentrations tested, the sediment dweller *N. diversicolor* showed to uptake and metabolize the mixture of anticancer compounds, indicating that the combined compounds may interact differently according to their doses. Therefore, our findings showed that single

compound toxicity data are not sufficient to predict the environmental risks offered by anticancer drugs in combination, although it is relevant to comprehend whether the divergences registered in the oxidative status of organisms exposed to the mixtures of anticancer drugs and individually are dominated by the mechanisms involved in chemotherapy resistance.

Chapter 6

Toxicity of anticancer drugs: a first insight to tropical species

Prepared for submission to:

T. G. Fonseca; G. Daniel; G. Eufrasio; A. C. Feitosa; L. Mello; Maria João Bebianno; Denis M. S. Abessa. Toxicity of anticancer drugs: a first insight to tropical species.

6.1 Introduction

Over the last twenty years, the occurrence of pharmaceuticals in the aquatic environment, particularly in surface waters, became a critical issue due to demographic projections that prompts an ever-increasing demand for drug consumption worldwide. Once these imminent trends prevail in the upcoming global scenario, the input of pharmaceuticals as parent molecules, their metabolites and transformation products in water bodies are recognized as a great environmental challenge because traditional technology of WWTPs are poorly efficient to remove these complex molecules (Lenz *et al.,* 2005; Rowney *et al.,* 2009; Besse *et al.,* 2012; Parrella *et al.,* 2014b), and also because many regions, especially in developing countries, do not have suitable systems to collect and treat sewage. In this sense, household and hospital effluents containing pharmaceutical compounds ultimately reach marine ecosystems acting as pseudo-persistent stressors, to which organisms undergo continuous exposure. With the advance of analytical methods, pharmaceutical compounds, including anti-depressants, analgesics, antibiotics, anti-inflammatories, beta-blockers, among others, have been detected at very low concentrations in estuarine and marine waters (ng L^{-1} to µg L^{-1}) (Buser *et al.,* 1999; Weigel *et al.,* 2002; Ashton *et al.,* 2004; Togola and Budzinski, 2008; Vidal-Dorsch *et al.,* 2012; Pereira *et al.,* 2016a) and sediments (ng g^{-1}) (Silva *et al.,* 2011; Long *et al.,* 2013; Beretta *et al.,* 2014; Lara-Martín *et al.,* 2014; Moreno-González *et al.,* 2015). In addition, ecotoxicological responses associated to these drugs´ MoA have been assessed in representative species living in the water column nd sediments, at environmentally relevant concentrations (Escher *et al*., 2011; Madureira *et al*., 2012; Aguirre-Martínez *et al*., 2013, 2016a, 2016b).

A watch list of priority pharmaceuticals including the sex hormones 17α-ethinylestradiol, 17β-estradiol and the anti-inflammatory diclofenac was established by European Union in order to regulate the assessment of these compounds in the freshwater environment (Hughes *et al.,* 2013; Lolic *et al*., 2015). Increasing efforts have been dedicated on the identification of priority pharmaceutical compounds either by the application of accurate monitoring or modelling approaches. In the policy

perspective of pharmaceuticals´ occurrence in marine waters, the European Marine Strategy Framework Directive (European Comission, 2008) establishes strategies on the methodological criteria to assess the status of environmental quality and anthropic environmental pressures, although they seem unclear and elusive, tailored to each Member State´s concerns and priority substances compatible to the demand of its respective and specific marine conditions and pressures. Since this set of measures are devised on the basis of a sound knowledge of the state of marine environment, scientific evidence of pharmaceuticals´ fate, bioavailability, and potential early warning impacts to biota have asserted the relevance of pharmaceuticals on an environmental risk assessment (ERA) in the marine context. In a worldwide outlook, regulatory actions with respect to pharmaceuticals in the environment are well documented in northern and central European countries, as well as in the United States and Canada, where frameworks and guidelines withstand deliberation, and represent a progress towards the protection of the marine environment (EMA, 2006; Environmental Canada, 2011; Hughes *et al.,* 2013).

In contrast, scientific knowledge and environmental legislation in most developing regions still face significant challenges, as traditionally experienced for other aquatic pollutants (Rahman *et al.,* 2009; Fang *et al.,* 2012). Low- and middle-income countries challenge economical limitations revealed by issues in quality of sanitation, inefficient water quality programs and simple technologies (Rahman *et al.*, 2009; Mohapatra *et al.,* 2016). When existing, sewage treatment alternatives rely on household septic systems using filtration and biodegradation performed by naturally occurring microorganisms (Singh *et al*. 2010), systems of pre-conditioning plants set up with degritting units following disinfection through chlorination (Abessa *et al.,* 2006); and primary and/or secondary treatments of wastewater including sedimentation, fermentation, photolysis and biodegradation. According to Mohapatra *et al. (*2016), these techniques are less costly than innovative treatment technologies and are serviceable for communities with limited resources and infrastructures. These processes culminate in the disposal of untreated or partially treated wastewater directly into the marine environment, or into inland waters that reach the coast, with substantial

loads of chemical mixtures including pharmaceuticals. It is assumed that dilution elicited by the receiving water bodies decreases the harmful potential of these hazardous substances; however, their continuous release may make the concentrations to remain relatively high, thus these chemicals can be sometimes considered pseudopersistent. In this sense, the extent of pharmaceutical impact in coastal waters is presumably higher in developing countries, where the capacity for wastewater treatment is far below the quantity of sewage generated by population (Fang *et al.*, 2012; Subedi *et al.*, 2015; Balakrishna *et al.*, 2017).

Accordingly, there is a pressing need to expand the research around the occurrence and impacts of pharmaceuticals in aquatic ecosystems in developing countries (Rahman *et al.*, 2007; Hughes *et al.*, 2013; Gaw *et al.*, 2014) as there are currently far fewer data for Africa, Asia and South America compared to the Europe and North America. In general, such reality occurs in the tropical and subtropical scenario, where around 75% of global biodiversity is found, and where the number of species potentially affected by exposure to pollutants is substantially higher, in contrast to the inherent low resilience following disturbing events (Kwok et al., 2007; Hubner et al., 2009). Ecotoxicological data used in risk assessment conducted in tropical regions are often generated in North America or Europe, based on bioassays with temperate test organisms (Kwok *et al.*, 2007; French *et al.*, 2015) and in particular, considering the levels of environmental contamination found in regions where sewage is generally collected and properly treated. To date, the information on the occurrence and distribution of pharmaceuticals in tropical ecosystems is scarce, and even less ecotoxicological knowledge was produced to the respective native organisms. Such extrapolation may lead to erroneous inferences due to the different sensitivity of selected organisms to a chemical on comparable tests from both regions (Garcia *et al.*, 2008).

In Todos os Santos Bay (Bahia, Brazil), ibuprofen, atenolol, carbamazepine, erythromycin, diclofenac and diazepam were detected in sediments from sub ng g^{-1} to ng g^{-1} (Beretta *et al.*, 2014). In the Danshui Estuary, at the northern coast of Taiwan,

concentrations of pharmaceuticals in seawater ranged as follows: clofibric acid (< 1.4 to 55.1 ng L^{-1}), diclofenac (< 2.5 to 53.6 ng L^{-1}), ibuprofen (< 2.5 to 57.1 ng L^{-1}) and ketoprofen (< 1.7 to 6.59 ng L^{-1}) (Fang *et al*., 2012). In Bangladesh, the antibiotics metronidazole (13.51 ng L), tylosin (16.68 ng L^{-1}) and trimethoprim (17.2 ng L^{-1}) were some of the pharmaceutical compounds detected at a high detection frequency in the river surface waters (Old Brahmaputra river), as a result of the wide consumption in livestock manure, poultry rearing and human treatment (Hossain *et al*., 2018). Recently, a wide array of therapeutic classes and illicit drugs were detected at ng L^{-1} in seawater of Santos Bay (São Paulo, Brazil), including ibuprofen, diclofenac, acetaminophen, losartan and cocaine (Pereira et al., 2016a; Cortez *et* al., 2018; Fontes et al., 2018; Pusceddu et al., 2018). Ecotoxicological approaches conducted in this region revealed that the anti-inflammatory diclofenac (DCF) (Fontes *et al*., 2018) and the anti-hypertensive losartan (LOS) (Cortez *et al*., 2018) can trigger subtle toxicity responses on the sentinel brown mussel *Perna perna* even via waterborne exposures at trace ng L^{-1}, with effects linked to the designed drugs´ MoA, namely the alteration of the antioxidant status, lysosomal membrane destabilization and COX inhibition. Sulfonamide antibiotics and the antidepressant norfluoxetin showed to bioaccumulate in the liver of wild armored catfishes *Hypostomus commersoni*, from the Acaraguá river (Argentina), representing up to 5.6 and 9.1 µg kg^{-1} wwt, respectively.

Altogether, the environmental and economic state of low- to middle-income regions corroborate to the establishment of an urgent framework for assessment and management of ecological risk caused by pharmaceuticals, reinforced by growing research with the focus on the impacts to tropical aquatic ecosystems. However, the attention to the therapeutic class of anticancer agents has not been yet considered. The situation of developing countries can become more complex, because the present decade is referenced as an important period in the global health history, since the disease burden profile changed toward more non-communicable diseases, particularly in these countries, which are expected to experience a significant increase in cancer incidence according to the milestone demographic shift promoted by the population growth and longevity improvement (Torre *et al*., 2015). According to the World Health

Organization (WHO, 2014), by 2030, less developed regions will experience an increase of 44% and 97% in new cancer cases among population aged < 65 and > 65 years old, respectively, against to 2.8% and 48% increase in more developed regions. Therefore, it is possible to link such increase in cancer incidences with the enhancement of anticancer drugs production and application in treatment and supportive care (WHO, 2014); and their increasing concentrations in the environment. Anticancer agents are administered in cancer treatments, individually or in combination, by means of provoking cell death through cellular and DNA damage and the increase of reactive oxygen species (ROS) levels that modulates apoptotic signaling pathways (Conklin *et al.*, 2004; Gorrini *et al.*, 2013). Despite efficiency against tumour proliferation, these pharmaceuticals are not selective to cancer drivers and interact with common biomolecules of normal cells, resulting in cytotoxicity to healthy tissues (Heath *et al*. 2016; Novak *et al*. 2017). Thus the presence of anticancer substances in the aquatic environment may represent substantial risk to non-target biota at both freshwater (Grisolia and Cordeiro, 2000; Zounková et al., 2007; Parrella et al., 2014b, 2014c; Kovács et al., 2015) and marine ecosystems (Aguirre-Martínez *et al.*, 2016; Moreira *et al.*, 2016; Trombini *et al.*, 2016a; Fonseca *et al.*, 2017; Aguirre-Martínez *et al.*, 2018; Fonseca *et al.*, 2018).

According to local hydrodynamic and physical disturbances, pharmaceuticals may be trapped in sediments, due to their capacity to bind to organic matter, being transferred from water column to sediments. Thus, sediments become another route of exposure to biota, particularly the benthic and epibenthic species. Considering the forecast of anticancer drugs consumption and the inexistence and/or inefficient technologies of WWTPs in tropical countries, allied to the current status of absent awareness and data around the potential toxicity of these compounds to tropical native organisms, knowledge contributions are essential regarding screening of environmental concentrations and marine species responses towards chronic exposures. The present study aimed to evaluate the effects of the widely administered anticancer agents namely cisplatin (CisPt), cyclophosphamide (CP) and tamoxifen (TAM), individually and in tertiary mixtures on the representative tropical marine

invertebrates, namely the sea urchin *Echinometra lucunter*, the amphipod *Tiburonella viscana* and the polychaete *Scolelepis squamata*. The selection of CisPt, CP and TAM for the present toxicity assessment was made according to a combination of criteria regarding their consumption and environmental occurrence in water bodies worldwide, due to their traditional administration in single and combinatory chemotherapies, thus with more clinical information available and knowledge about their physico-chemical properties and MoA (Emadi *et al*., 2009; Dasari and Tchounwou, 2014; Shagufta, 2018).

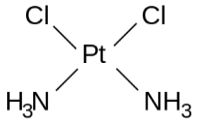
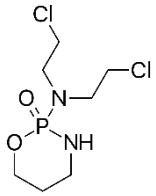
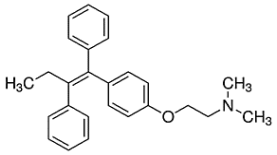
6.2 Materials and Methods

6.2.1 Chemicals

The analytical standards of *cis*-platinum (II) diamine dichloride (CAS 15663-27-1), cyclophosphamide monohydrate (Cytoxan) (CAS 0768) and tamoxifen (CAS 10540-29-01) were purchased from Sigma-Aldrich (Portugal) (Table 6.1). Individual stock solutions of CisPt and CP were prepared in ultrapure Milli-Q water, whilst TAM was firstly dissolved in the solvent dimethyl sulfoxide (DMSO), following serial dilutions in Milli-Q water, with a final concentration of 0.001% (v/v) DMSO to avoid solvent toxicity effect during experimental use. Since dermal route of exposure is considered to have the predominant role in the uptake of anticancer agents in humans, the chemicals under study were handled under safety criteria using class II biological safety cabinet, with appropriate clothing (*i.e.* open-back, impervious chemotherapy protection gown, double powder-free latex gloves and safety goggles).

Table 6.1: Physico-chemical characteristics, structure and metabolism of the selected anticancer drugs.

Properties	**Cisplatin**	**Cyclophosphamide**	**Tamoxifen**

Molecular Structure			
MoA	Causes oxidative stress; Binds to nucleophilic sites in genomic and mitochondrial DNA, producing DNA adducts and damage that inhibits its replication and cell division	Causes oxidative stress; Reacts with N and O atoms of DNA bases generating cytotoxic and mutagenic DNA adducts	Causes oxidative stress; Binds to estrogen α (ERα) and β (ERβ) receptors and interrupts cell proliferation
CAS Number	15663-27-1	6055-19-2	54965-24-1
pKa	5.3 - 7.2	2.3 - 11.1	8.8 (Basic)
Log Kow	-2	0.6	6.3
Water Solubility	4×10^4 mg L^{-1}	3×10^3 mg L^{-1}	17 mg L^{-1}

6.2.2 Toxicity of single-drug exposures

Embryo-larval development of *E. lucunter*

Adult specimens of the sea urchin *E. lucunter* were collected at low tide, in the Palmas Island (Santos, São Paulo - Brazil), rapidly transported alive to the laboratory, where animals were maintained under constant aeration and temperature (25 ± 2°C), according to the protocol NBR 15350 established by the Brazilian Association for Technical Standards (ABNT, 2012). Spawning was induced by the injection of 3 mL KCl 0.5 M into the coelomic cavity of sexually mature individuals. Females were individually placed on 400 mL beakers containing filtered seawater (salinity 35) for oocytes collection, which were observed under microscope to confirm viability. The selected batches of oocytes were pooled and allowed to decant, following serial washings by supernatant removal and solution filtration through a 350 µm-mesh, with

the addition of 600 mL of dilution water. Spermatic fluid was collected directly from the gonopore with the aid of a glass Pasteur pipette and placed into a dry 30-mL glass beaker kept on ice. In order to prompt sperm activation, 0.5 mL of spermatic fluid was added into 24.5 mL of seawater. Afterwards, 2 mL of active sperm solution was introduced into the oocytes solution and stirred with a glass stick rod, over 10 minutes, to elicit fertilization. An aliquot of eggs solution was seeded in a Sedgwick–Rafter and observed under microscope to ensure that 80–100% of the eggs were fertilized. Four 10-mL test tube replicates were set up for each treatment, comprised by pharmaceuticals individually tested at five test concentrations (CisPt: 0.1, 1, 10, 50 and 100 ng L^{-1}; CP:10, 50, 100, 500 and 1000 ng L^{-1}; TAM: 0.5, 10, 25, 100 and 500 ng L^{-1}), jointly with control conditions consisting of seawater and 0.001% DMSO solvent. In each replicate, 300-500 embryos were seeded and exposed over 36 ± 4 h, at constant temperature (25 ± 2°C) and photoperiod (16 h light:8 h dark), fixed with 0.5 mL of 40% buffered formaldehyde-borax (pH 7.0) aftermath. Individuals from the control groups were analysed under microscope to confirm normal pluteus larvae development, by establishing a criterion of minimum 80% to indicate suitability of the test.

6.2.3 Toxicity of pharmaceuticals in mixtures

Sediment spiking

Reference sediments were collected in Engenho d´Água beach, at the north coast of São Paulo State (Brazil), during low tide. This spot has been often used as a control site, as reported in previous ecotoxicological studies (Abessa *et al.*, 1998; Sousa *et al.*, 2007; Maranho *et al.*, 2010; Araujo *et al.*, 2013; Souza *et al.*, 2016). Sediments were transported to the laboratory, where they were wet-sieved through a 2-mm mesh for removal of large debris, seaweed and macrofauna, following drying at

80 °C (Thain and Bifield, 2001; ASTM, 2009; Maranho *et al.*, 2014). Sediments were disaggregated and stored at 4°C over seven days until the spiking procedure. Dried aliquots of the sediments were used to determine grain size distribution by the method proposed by Royse (1970). Organic matter content was determined by loss on ignition (550 °C, for 5 h), as described by Gross (1971). Afterwards, sediments were re-hydrated with the same amount of water loss (w.w./d.w.). Procedures of sediment spiking were adapted from the sediment suspension technique, described by USEPA (2001).

Briefly, the total volume of sediments correspondent to each treatment, conducted in triplicate (*i.e.* 200 mL of each replicate × 3 = 600 mL of sediment), was introduced into 2 L-glass jars, plus inserting aliquots of pharmaceutical solutions to compose the tertiary pharmaceutical mixtures with water collected from the reference site of sediment, filling a total aqueous volume of 330 mL. Nominal concentrations of tertiary pharmaceutical mixtures ranged as follows: 0.1 CisPt + 10 CP + 0.5 ng L^{-1} (Mixture A); 10 CisPt + 100 CP + 10 ng L^{-1} (Mixture B); 100 CisPt + 500 CP + 25 ng L^{-1} (Mixture C); 100 CisPt + 1000 CP + 100 ng L^{-1} (Mixture D). Each pharmaceutical mixture was stirred at a moderate speed with a bench homogenizer, over 30 min. Control conditions consisted of unspiked sediments, with DMSO (final concentration 0.001%) and without. For partitioning and equilibration of pharmaceuticals between sediments solid-phase and interstitial water, spiked sediments were kept in the dark, at 4°C, over seven days. Afterwards, sediment samples with the pharmaceutical mixtures were stirred with a spatula and distributed in triplicate in 2 L-glass test beakers.

Survival of the amphipod *T. viscana*

Amphipods were collected in subtidal zone of the Engenho d´Água beach, at low tide, and transported alive to the laboratory where they were acclimated over three

days at room temperature (25 ± 2°C), constant aeration and light exposure. The whole-sediment toxicity bioassay was adapted from the method described by Melo and Abessa (2002) and ABNT (2008). The experimental system was set up in a triplicate design, depicted by thirty adult individuals transferred to each glass beaker, filled with 200 mL of spiked sediment and 800 mL of filtered seawater from the site of amphipods´ origin. The exposure of amphipods to mixtures A, B, C and D, jointly with seawater and DMSO solvent controls, was carried over 10 days under constant aeration and lightning, at room temperature (25 ± 2°C). Animals were not fed and overlying water was not renewed during the bioassay. At the end of the exposure, the content of each test vessel was individually sieved through a 0.5 mm-mesh, and surviving organisms registered for acute endpoint. Physico-chemical experimental conditions were measured in the beginning (salinity 35.2 ± 0.7; pH 7.65 ± 0.05; air saturation 78.7 ± 5%) and end (salinity 35.6 ± 0.5; pH 8.22 ± 0.1; air saturation 71.5 ± 6.4 %) of the bioassay.

Survival of the polychaete *S. squamata*

Specimens of the spionid *S. squamata* (Muller, 1806) were handpicked in the intertidal zone of the sandy beach of Itaguaré, close to the Restinga de Bertioga State Park and within the Environmental Protection Area of the Central Coast (Bertioga, São Paulo – Brazil), far from urbanized areas. After collection, polychaetes were transported alive to the laboratory with sediment and seawater from the site of origin. Animals were acclimated over three days until the beginning of the bioassay, kept at room temperature (25 ± 2°C), constant aeration and light exposure. Clean control and spiked sediments with pharmaceutical mixtures (200 mL) were inserted in 2 L- glass jars filed with 800 mL of filtered overlying seawater, in a triplicate design. Twenty-five individuals were placed in each replicate chamber and exposed over 14 days, with static renewal every 48 h necessary to maintain the pharmaceuticals exposure levels, in accordance to the experimental set-up conducted with the polychaete *N. diversicolor*, detailed in the previous Chapters 2-4 (Fonseca *et al.*, 2017; 2018). Physico–chemical parameters were monitored in the beginning (salinity 35.1 ± 0.7;

pH 8.1 ± 0.06; air saturation 114.5 ± 6.2 %) and at the end (salinity 35.3 ± 0.5; pH 8.03 ± 0.05; air saturation 115.2 ± 6.4 %) of the bioassay. At the end of the exposure period, the number of living organisms in each treatment replicate was recorded.

6.2.4 Statistical Analysis

Data of toxicity bioassays were first checked for normality and homocedasticity, respectively by Shapiro Wilk's and Bartlett tests. Once these premises were confirmed, Student´s T-test was applied on the embryotoxicity dataset of *E. lucunter*, by comparing each pharmaceutical treatment to the respective control, whereas the analysis of variance (One-way ANOVA) was applied for the acute toxicity tests conducted with *T. viscana* and *S. squamata* in spiked sediment, followed by Tukey's test of multiple comparisons to detect significant differences among treatments ($\alpha = 0.05$).

6.3 Results and Discussion

To the best of our knowledge, the present study comprises the first ecotoxicological assessment of anticancer drugs on tropical marine organisms, considering the potential responses provoked by these pharmaceuticals. According to the obtained results, single-drug exposures of *E. lucunter* embryos demonstrated the reliability of the embryo-larval development as a chronic endpoint for assessing the toxicity of anticancer agents present in seawater at trace concentrations. Surprisingly, malformation of embryos did not follow the usual dose-response pattern, in which effects increase with the dose. Instead, a U-shaped effect was depicted with a non-linear significant reduction of viable pluteus larvae at CisPt and CP treatments, respectively at the range from 0.1 to 10 ng Pt L^{-1}, and from 50 to 500 ng CP L^{-1} (Figure 6.1). However, even though the highest levels of these anticancer agents indicated no toxicity in relation to control, these samples would be considered toxic according to the acceptability criteria proposed by both USEPA (2002) and CETESB (1999), which establishes a minimum of 80% of successful embryo-larval development.

The observed effects exhibited a non-monotonic dose-response relationship, in which the very low doses exerted significant teratogenicity, defined by birth defects, congenital disorders and structural anomalies, in accordance to their designed cytotoxic MoAs. In humans, teratogenicity caused by chemotherapy administration generates fetal changes over mothers' pregnancy, deficient growth and a small-for-gestational-age newborn (Paskulin *et al*., 2005). Likewise, external abnormalities responses in mice fetus were also addressed (Ghaseminezhad and Hejazi, 2015). In contrast to the present results, a significant decrease in larval development occurred in fertilized eggs of the sea urchin *Paracentrotus lividus* exposed to the anti-metabolite methotrexate, from the concentration of 5 µg L^{-1} to 1 g L^{-1}, in a dose-response manner (Aguirre-Martínez *et al*., 2016b), whereas the lower concentrations on the range of ng L^{-1} did not depict effects over the development endpoint. In this comparative scenario between studies, it is important to bear in mind that not only the drugs´ MoAs are distinct, but species´sensitivity are, ever so, crucial to consider. Under an exposure to ibuprofen, *P. lividus* exhibited an EC_{50} of 10 ng L^{-1} for the embryotoxicity endpoint (Aguirre-Martínez *et al*., 2015), whilst the same drug at the concentration of 15 ng L^{-1} was enough to provide a null larval viability in the tropical counterpart *Lytechinus variegatus* (Pusceddu *et al.*, 2018).

In fact, according to a review on clinical trials data regarding chemotherapy administration, the application of cytotoxic agents at low-doses achieves a more satisfactory efficacy in anti-proliferative responses compared to high conventional-dose regimen (Xie *et al*., 2017). Findings obtained from the exposure of embryos of *Xenopus laevis* to CisPt at a range of high doses (10 µg L^{-1} to 10 mg L^{-1}) did not elicit any effect in their development or survival, which reinforce the variation of resistance across taxa (Van der Grinten *et al.*, 2010; Bakopoulou *et al.*, 2011; Isidori *et al.*, 2016). However, the concentrations of anticancer agents herein tested were chosen according to their respective levels detected in the aquatic compartment, particularly in freshwater systems of temperate zones, once coastal zones have not been foreseen under the screening spectra of anticancer drugs, which includes, in particular, the coastal zones of tropical developing regions.

Considering both the non-monotonic profile of response herein addressed and the trace realistic concentrations at which detrimental effects were elicited, experimental set-ups conducted with pharmaceuticals should include a wide range of concentrations corresponding to environmental analytical disclosures. This is particularly stressed in the case of anticancer drugs, not only because of their high cytotoxic, genotoxic and mutagenic potential (Novak *et al*., 2016; Toolaram *et al*., 2014), but also due to their feasible biphasic trend of dose-response, depicted as a challenge to the use of a traditional threshold model, which proposes that there is a dose below which no effects of a chemical are observed (Chapman, 2002; Vandenberg *et al*., 2012, 2014; Agathokleous, 2018).

Thus, the comprehension of these trends of responses has crucial implications for the risk assessment of anticancer drugs and the establishment of safety levels for these chemicals in the nature. Nevertheless, regarding the effects from the exposure to environmental concentrations of TAM, a monotonic dose-response relationship was evidenced, with increasing teratogenic effects observed from the lowest level (Figure 6.2). Similarly, embryotoxicity was reported for *P. lividus* exposed to TAM, with a significant decrease in larval viability at the lowest concentration of 1 ng L^{-1} (Aguirre-Martínez *et al.*, 2016b). Besides a higher sensitivity compared to adult life stages, the selection of embryolarval development represent a quick, relatively easy and sensitive toxicity test, with the added advantage of having a low cost and test duration (Hutchinson *et al.*, 1998; Aguirre-Martínez *et al.*, 2016b).

In both experiments conducted with the sediment-dwellers amphipods and polychaetes, no significant differences were detected between survival in organisms from control conditions with and without DMSO solvent. In contrast, results indicated in Figure 6.1 and 6.2 show the detrimental effects exerted by anticancer mixtures in amphipods and polychaetes, respectively. Comparatively, *T. viscana* presented a higher sensitivity to pharmaceuticals than *S. squamata*. Like the non-monotonic dose relationship herein reported for *E. lucunter*, survival response in amphipods also illustrated a non-linear reduction of survival in Mix B and D, both represented by

mortality rates of 60%. According to Lagarde *et al.* (2015), such non-monotonic profile has been typically linked to endocrine disrupting compounds, with reports of similar ecotoxicological effects in experiments conducted with aquatic organisms (Morales *et al*., 2018; Teng *et al*., 2018) and on pharmacological studies developed with human cell lines (Mater *et al.*, 2014; Vandenberg *et al.*, 2014, 2012; Lagarde *et al.*, 2015). Therefore, this biphasic profile could be associated to the predominance of TAM´s MoA in mixture, able to interact differently with estrogen receptors and signalling proteins, depending of their affinity across the range of tested doses and the alternance of antagonist and agonist effects according to the targeted tissue.

According to the Mixed-Ligand hypothesis, described by Maness *et al.* (1998), at low concentrations of the drug, dimers are more likely to form and induce a response, whereas with a subtle increase in concentration (Mix C) there is a block in the molecular receptor activity and no interaction with the drug occurs. In contrast, at a higher drug concentration (Mix D), dimers are more likely to form and induce a response, herein expressed as mortality. Moreover, modulation of TAM MoA may disregard ERs´presence (Gundimeda *et al.*, 1996; Bekele *et al.*, 2016), because its anti-proliferative and cytotoxic effects may be also derived from the prodrug metabolic activation and generation of the oxidative metabolites endoxifen and 4-hydroxytamoxifen. Such metabolites are accountable for the oxidative stress and the cascade of signal transduction mediated by protein kinases that culminate in both interruption of cell proliferation and apoptosis. It appears credible that the extremes of the biphasic response curve are prone to be driven by complementary mechanisms conjugated by the cytotoxic potential of CisPt and CP, together with the oxidative outcomes of TAM´s metabolic activation, following modulation of ERs, whether existent. To date, no evidence exists regarding the expression of ERs in *T. viscana* that could validate the antagonist role of TAM over specific estrogenic targets. However, present non-monotonic dose relationship may suggest the presence of a nuclear estrogen-like receptor, thus requiring further refined characterization of nuclear ERs and its ligand-induced transcriptional activation.

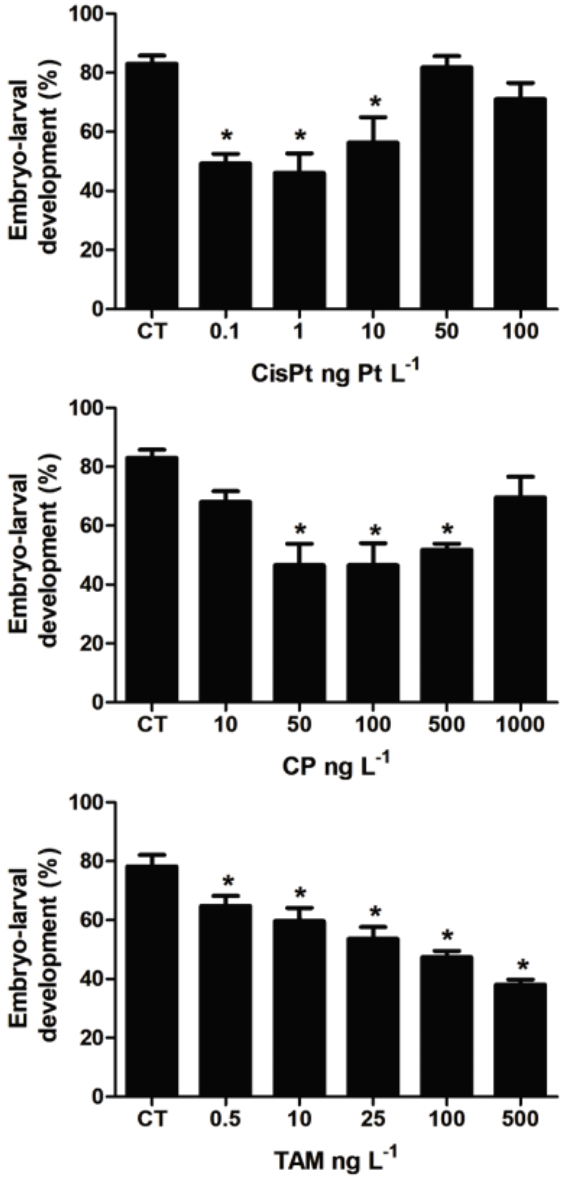


Figure 6.1: Percentage of normal pluteus of *E. lucunter* after exposure of fertilized eggs to single anticancer drugs. Asterisks indicate significant differences between drug treatments and control (T-test, $p < 0.05$).

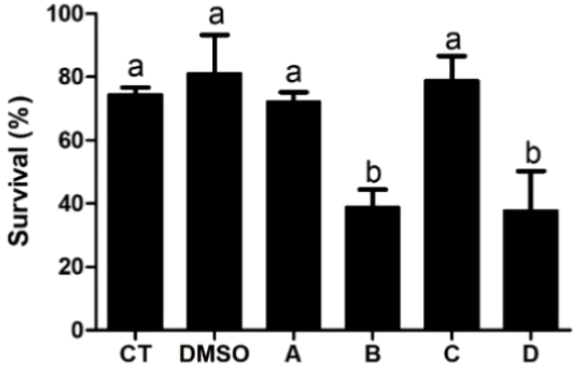


Figure 6.2: Survival rates of the amphipod *T. viscana* (mean and S.D) exposed over 10 days to control conditions (CT and DMSO), and tertiary mixtures of the anticancer drugs. Different letters indicate significant differences ($p < 0.05$).

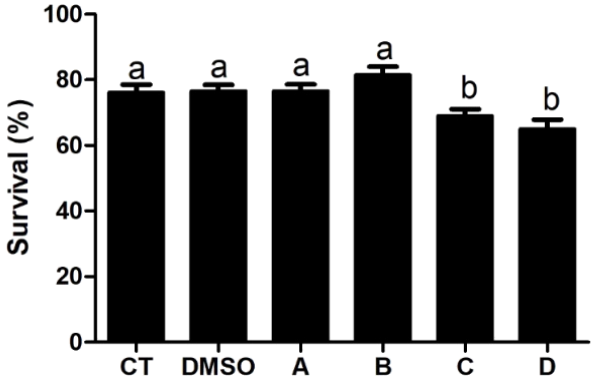


Figure 6.3: Survival rates of the polychaete *S. squamata* (mean and S.D) exposed over 10 days to control conditions (CT and DMSO), and tertiary mixtures of the anticancer drugs. Different letters indicate significant differences ($p < 0.05$).

The native tropical species *T. viscana* has been so far applied in several environmental quality assessments of Brazilian coastal and marine zones under the anthropic burden of harbour pollution, as well as untreated industrial and domestic effluents, containing complex mixtures of chemical at environmental realistic concentrations (Abessa *et al*., 1998; Melo and Nipper, 2007; Maranho *et al*., 2010; Araujo *et al*., 2013; Rodrigues *et al*., 2013; Cesar *et al*., 2014). This species was sensitive to anti-cancer drugs, as observed by the mortality rates. However further

studies are necessary to provide the comprehension of biochemical and molecular pathways linked to mechanisms that underpin the ultimate response of mortality, which are decisive to insert tropical indigenous species in the scenario of comparison to temperate biological models. Regarding polychaetes, although the significant acute toxicity exerted in organisms from the higher pharmaceutical doses (Mix C and D), the survival rate in these drug-treated organisms was reduced less than 10% compared to unexposed groups.

6.4 Conclusions

Considering the growing need for data concerning the biological impacts of pharmaceuticals in the marine environment, this study presents the first data based on the toxicity effects of anticancer drugs with biological models of Latin America. Larval development of the sea urchin *E. lucunter* demonstrated to be a sensitive endpoint indicating the adverse effects posed by CisPt, CP and TAM at an environmental range of concentrations, with respective profiles of response including the non-monotonic shape. The sediment dweller organisms herein applied in the sediment spiked bioassays with pharmaceuticals in mixtures showed consistent sensitivity to anti-cancer drugs in the acute toxicity tests, considering the trace concentrations of the chemicals applied. Further analysis of chronic and subchronic endpoints are required in order to elucidate and/or confirm the molecular and biochemical mechanisms underlying the lethal responses observed to the amphipod *T. viscana* and the polychaete *S. squamata,* which may be designed as suitable bioindicators for studies on the environmental quality of tropical and subtropical regions regarding pharmaceuticals.

Chapter 7

GENERAL DISCUSSION

7.1 The issue of anticancer drugs in the marine environment

Global demographic trends have led to a record in population´s growth, which today comprises 7.7 billion people, on a pace to represent 8.9 billion by 2050, mainly derived from low- and medium-resource countries. Allied to that, attributes of an ever-increasing urban society have prompted a decline in fertility rates and an increase in life expectancy, accountable for the demographic milestone shift towards structural aging of population (Ferlay *et al*., 2015). These trends, together with modern life-style habits (*i.e.* diet, smoking, infectious agents and environmental pollutants) have been crucial to the prominence of chronic and non-communicable diseases, which are displacing infection-related ones as major causes of morbidity and mortality in many parts of the world (Anand *et al*., 2008; Stewart and Wild, 2014). In this sense, cancer arises as one of the leading causes of death, and remains striking, in a way that, in 2012, 14.1 million new cases of cancer were diagnosed worldwide, likely to turn into 27.5 million incidences in 2040 (GLOBOCAN, 2012). In light of the implications that cancer disease burden brings to public health and economy, United Nations Resolution on noncommunicable diseases have joined efforts with governments in a global monitoring framework, aimed at tackling this malignancy through strategies of early detection, preventive and control actions (WHO, 2002). Such scenario contributes to the increment in production and consumption of anticancer agents applied in traditional systemic therapy, managed individually or in mixtures according to the type and stage of tumour (Caley and Jones, 2012; Aitken, 2016), under an increasing trend of home-based drug administration.

As every other pharmaceutical consumed, intravenously or via oral ingestion, parent molecules of anticancer compounds are metabolized and excreted, in their native form or as metabolites, into waterways following to WWTPs. According to analytical assessments with particular focus on pharmaceuticals´ detection in effluents and surface waters, a large number of anticancer molecules have shown considerable recalcitrance either by biological conventional treatments or by highly advanced technologies, thus ending up in the aquatic ecosystems at range of sub ng to ng L^{-1},

confirming the inefficiency of their removal. Although anticancer drugs represent lower consumption rates compared to other classes of over-the-counter drugs, leading to represent a smaller fraction of pharmaceuticals in the environment, they act upon essential biomolecules and cell proliferation signaling pathways, such as generate signal transduction cascades of cell death, potentially conserved in non-target species (Mater *et al*., 2014). In spite of the increasing number of studies devoted to comprehend their environmental toxicity, research has been mainly focused on freshwater species in the spotlight of ecotoxicological knowledge, whereas studies conducted with coastal counterparts are still scarce and reveal a critical disparity in current datasets (Figure 1.11). Therefore, the vulnerability to which marine ecosystems undergo in face of the increasing inputs of complex cytotoxic mixtures of pharmaceuticals has been neglected (Claessens *et al*., 2013a; Gaw *et al*., 2014; Moreno-González *et al*., 2016). Moreover, there is a general paucity of information in the scientific literature concerning the multitude of exposure routes to drugs in the environment, which clearly disregards the sediment compartment, where higher concentrations of contaminants are present in comparison with the overlying water (Burton *et al*, 2003; Simpson *et al*, 2005). Sediments may constitute not only a sink but also a secondary source of pollution, to which special attention should be given (Buruaem *et al*., 2012).

7.2 Ecotoxicological impacts of anticancer drugs

When this thesis started, efforts on analytical detection and ecotoxicological investigation of pharmaceuticals, mainly elicited in freshwater environments, were focused in therapeutic classes including antibiotics, non-steroidal anti-inflammatory drugs, antidepressants, antihypertensives, antiepileptic and contraceptives (Weigel *et al.*, 2004; Roberts and Thomas, 2006; Togola and Budzinski, 2008; Paíga and Delerue-Matos, 2013; Nödler *et al.*, 2014). Even though the occurrence of anticancer drugs has already been measured in effluents of WWTPs and riverine compartments (Aherne *et al*., 1990, 1985; Richardson and Bowron, 1985), the interest on their fate in marine waters and sediments has been reflected only in the detection of TAM (Hilton and

Thomas, 2003), and more recently, the survey for methotrexate occurrence (Biel-Maeso *et al.,* 2018). Until the present thesis starts, only few marine species were used as biological models for the investigation of anticancer´s toxicity, under exposure to high concentrations and route of exposure not in accordance to a realistic scenario (Santos and Pacheco, 1995; Pagano *et al.*, 2001; Roepke *et al.*, 2005).

Cancer cells, to which these drugs are designed, have a high demand for ATP as a "fuel" for aberrant proliferation, resulting in high accumulation of ROS that needs to be counteracted by scavenging mechanisms to avoid the death of tumour cells. In fact, ROS levels are regulated to a range able to activate cell transduction pathways critical for cancer growth and progression (Gorrini *et al*., 2013; Qiu *et al*., 2015). In this sense, the MoA of cytotoxic drugs relies on the overwhelming tumour cells´ defense through suppression of ROS scavengers and induction of oxidative stress, following physiological dysfunction, cell damage and apoptosis (Chu, 1994; Dasari and Bernard Tchounwou, 2014; Fukai and Ushio-Fukai, 2011; Qiu *et al*., 2015; Reedijk, 1999).

7.2.1 Effects of cisplatin in *N. diversicolor*

The platinum-based drug CisPt remains as one of the most widely utilized antineoplastic pharmaceuticals worldwide. The present findings clearly showed that the presence of CisPt at 100 ng Pt L^{-1} in the seawater elicit effects linked to the drug´s MoA, by means of the sharp inhibition of SOD and CAT, accountable for the dismutation of the superoxide anion to H_2O_2, following transformation to H_2O, respectively (Fukai and Ushio-Fukai, 2011). In this sense, the burst of oxidizing superoxide and hydrogen peroxide, that could not be eliminated due to the suppression of this first line of defense against ROS, seemed to be buffered by the protective reduced GSH, due to the significant induction of Se-GPx activity (Ben Ameur *et al.*, 2012; Cozzari *et al.*, 2015; Djordjevic *et al.*, 2011). This enzyme has a critical role in the detoxification of products deriving from ROS-promoted oxidation of lipids, like malondialdehyde and 4-hydroxyalkenals (Aquilano *et al*., 2014). However, the

considerable high levels of LPO by-products addressed in polychaetes submitted to the highest Pt level clearly indicated that such scavenging mechanisms were not sufficient to avoid membrane damage (Gomes *et al*., 2014; Viarengo *et al*., 2007).

Besides, GSH maintain the essential thiol status of cysteine residues on proteins, and conjugates with a great variety of electrophilic compounds like CisPt, more often through the nucleophilic attack catalysed by GST, which aims to increase drug solubility and favour its excretion (Aquilano *et al*., 2014; Fuertes *et al*., 2003; Penner *et al*., 2012; Prabhu *et al*., 2004).The significant inhibition of GST activity registered at 100 ng Pt L^{-1} could be attributed to the GSH depletion in cells in face of a high conjugation rate to the drug (Doz *et al.*1993; Fuertes *et al.*2003; Hagrman *et al.*2003). Stornetta *et al. (*2017) addressed that elevated GSH levels in KB-3-1 epidermoid carcinoma cells were associated with a 3-fold reduction in drug-DNA adducts, in the same way that the treatment of cancer cells with buthionine sulfoximine, a thiol inhibitor, induced the formation of DNA crosslinks and cell death (David-Cordonnier *et al*., 2003). Similarly, metallothionein (MT) comprises another endogenous cysteine-rich molecule that limit the amount of CisPt available for interaction with DNA (Doz *et al*., 1993; Huska *et al*., 2009; Wang, 1998). The significant increase in intracellular concentration of MT in *N. diversicolor,* at the highest CisPt concentration (*i.e.* 100 ng CisPt L^{-1}) (Fig. 2.4), is in agreement with several oncological studies reporting its induction in human cell lines treated with cytotoxic platinum-based drugs (Doz *et al*., 1993; Florea and Büsselberg, 2011; Fuertes *et al*., 2003; Huska *et al*., 2009; Kelley *et al*., 1988; Komiya *et al*., 1991; Smith *et al*., 2006; Wang, 1998). Huska *et al.* (2009) demonstrated that the electrochemical signal corresponding to the MT-cisPt complex, detected via voltammetry in rat-blood treated intraperitoneally with the drugs, enhanced over time. On the other hand, it was addressed that the Cat2 signal, representative to presence of free –SH moieties, decreased, correlating well with the fact that free thiol moieties of MT are saturated by CisPt.

It has been found that resistance undergo through the mechanism in which the aquated CisPt lose one or two chloride ligands inside the cell, followed by the replacement by cysteine thiolates of MTs (Zhang *et al*., 2011). Altogether, intracytoplasmic covalent binding of thiol to platinum compounds reduces the proportion of drug available to interact with nuclear DNA, widely known to modulate resistance mechanisms during chemotherapy (Dabrowiak *et al*., 2002; Gagné *et al*., 2008; Huska *et al*., 2009; Reedijk, 1999; Won *et al*., 2008). In this sense, it is conceivable that the absence of genotoxicity, herein assessed by the Comet assay, was attributed to mechanisms of drug modulation and resistance described in mammalians. Another possible explanation for that is based on the different types of crosslinks between DNA, that increase structural alterations of crosslinked DNA fragments and contributed to less DNA migration in electrophoresis (Merk and Speit, 1999). Detrimental responses leading to an ultimate cytotoxicity are not solely attributed to genotoxicity, since only a small fraction of CisPt actually binds to DNA. Thus, the above-mentioned imbalance in antioxidant potential play a consistent role in the toxicity of CisPt (Sadzuka *et al*., 1992).

Biochemical mechanisms of defence seemed to be inefficient to fight against oxidative stress and may have contributed to alterations in the energy expenditure (Pires *et al.,* 2016a) as indicated by the decrease of burrowing rates among those polychaetes exposed to the highest concentration. This behavioural outcome may ultimately resound in ecological impacts, since organisms become more susceptible to predators when lying on the sediment surface (Amiard-Triquet, 2009; Bonnard *et al*., 2009). Moreover, an impaired burrowing is also reflected in the bioturbation event, a relevant habit displayed by *N. diversicolor* for the irrigation and particle mixing that ultimately afford changes in the oxi-reduction state of chemicals and provide displacement of organic matter to another infauna (Thit *et al.,* 2015). Few studies have so far linked this behavioural alteration to neurotransmitters´ role, vital for normal muscular function, particularly in the investigation of pharmaceuticals (Boyd *et al.*2002; Bonnard *et al.*2009). As such, it was hypothesized that the reduction in the number of buried organisms at the highest CisPt concentration was attributed to the

neurotoxic event of AChE activity inhibition (Figure 2.3), similar to findings described in previous studies conducted with human erythrocytes, where the electrophilic reactive products of CisPt are accountable for the neurological side-effects (Aljafari, 1995). An interesting aspect to highlight is that despite the neurotoxic response of AChE inhibition was observed only at 100 ng Pt L^{-1}, which represents an environmental worst-case concentration, modulation of AChE activity also occurred at 10 ng Pt L^{-1} by its significant induction, suggested to be linked to an adaptation of physiological fitness over the hormetic phenomena (Calabrese and Blain, 2005). Therefore, it is important to remark that besides a profile of toxicity consistent with the platinum-based MoA was addressed in *N. diversicolor*, at 100 ng Pt L^{-1}, the sediment dweller biological model showed to respond, in a non-monotonic way, to the drug uptake at sub-toxic level.

7.2.2 Effects of cyclophosphamide in *N. diversicolor*

Along with CisPt, CP is one of the oldest and most frequently prescribed cytotoxic drug in cancer treatment, but the first one to draw attention in an aquatic ecotoxicological perspective (Matsumoto and Cólus, 2000; Zounková *et al*., 2007). Nevertheless, the present results fulfill gaps of risks under an environmental realistic way including the benthic environment. The antioxidant responses depicted by *N. diversicolor* exposed to CP showed to disregard the cytotoxic profile caused by CisPt, due to significant enzymatic disruptions at the lowest CP level tested (10 ng L^{-1}), in which burrowing performance of individuals seemed to be more impaired, in contrast to the fully-buried organisms at the highest CP level (Figure 3.2). In face to the lack of neurotoxic responses, the changes determined in the behaviour of polychaetes are assumed to have no relationship with AChE activity.

The metabolism of CP includes the production of the reactive acrolein via β-elimination of aldophosphamide. This electrophilic and highly toxic reactive species is readily bound to GSH in the cytosol and interferes in the glutathione-related detoxification pathway (Peña-Llopis *et al.,* 2002; Singh *et al.,* 2014). Since CAT

showed to exert its role in clearance of hydrogen peroxide, there was no significant alteration of Se-GPx activity, in contrast to the induction addressed in CisPt exposure at the highest concentration. In the present case, it may be hypothesized that the increment of CP concentration, at 500 and 1000 ng L^{-1}, led to an exhaustion of radicals´ neutralization and accumulation of ROS, able to cause T-GPx inactivation. Because of such defense failure, the accumulation of hydrogen peroxide levels is potentially involved in oxidative damage to cellular components, liable for the side effects reported over chemotherapeutic treatment with this drug (Dumontet *et al*., 2001; Yu *et al*., 2006).

Increased levels of GST activity are directly associated with resistance to alkylating drugs in model tumour systems (Dumontet *et al*., 2001). Furthermore, herein, the 14-day exposure to the highest CP concentration led to a marked depletion of GST, as observed at 100 ng Pt L^{-1}, providing basis to the rationale of glutathione-depleting compounds as sensitizing agents in alkylating-resistant tumour cells (Dumontet *et al*., 2001; Singh *et al*., 2014). The present findings on the profile of GST activity confirm the suppression of this metabolic route during the cytotoxic MoA of CP, in resemblance to CisPt (Singh *et al*., 2014). Such effect diverges from other pharmaceuticals´ detoxification which are usually associated with increased GST activity (Martín-Diaz *et al.,* 2009; Aguirre-Martinez *et al.,* 2013; Buffet *et al.,* 2014; Maranho *et al.,* 2014; Aguirre-Martinez *et al.,* 2016a) and emphasizes the inclination to the activation of specific pathways of cellular defense according to the chemical (Sun and Zhou, 2008; Faria *et al.*, 2009; Cozzari *et al.*, 2015). Again, the production of cytotoxic and reactive metabolites indicates the failure in the antioxidant defense and the elevation of products from lipid peroxidation. Following their generation, these primary by-products undergo further reactions to form secondary products of lipid peroxidation, including the malondialdehyde and 4-HNE, herein determined, in addition to acrolein. In other words, along with the production of acrolein by means of CP metabolism, this reactive species is a general product of lipid peroxidation able to trigger further oxidative chain reaction in membranes (Conklin 2004; Singh *et al.*, 2014). This may be the explanation to the increase of 1.8-fold levels of LPO by-

products determined in *N. diversicolor* exposed to CP in comparison to those of cisplatin, besides the reasonable effects resultant from the depletion of the antioxidant status. Collectively, these impairments could have been responsible for the increased levels of DNA damage. However, the actual MoA relative to genotoxicity request the metabolic activation of the CP prodrug by CYP450 and ultimately generate the aziridinium intermediate species, able to bind to nucleophilic N7-position of guanine in DNA (Crook *et al.*, 1986; Codrington *et al.*, 2004), although it needs to be confirmed.

7.2.3 Effects of tamoxifen in *N. diversicolor*

The findings regarding the biochemical outcomes of TAM exposure in the benthic polychaete confirmed its potential to cause critical effects at an environmental realistic concentration (0.5 ng L^{-1} in seawater). Although there is little research pertaining the quantification of anticancer drugs in coastal zones, such concentration may be prone to be encountered at a distant location from land, where currents play an important role in the dilution of the target analytes (Moreno-González *et al*., 2015; Alygizaks *et al*., 2016). Again, as reported for the above-mentioned cytotoxic drugs, a very low concentration can stimulate and trigger antioxidant mechanisms, as herein indicated by the significant increase of SOD and Se-GPx activities (Figure 4.2), in a non-monotonic dose-response. Although not extensively explored in ecotoxicology, due to the pursuit to determine chemicals´ threshold levels, this kind of relationship was described from bacteria to vertebrates under a wide array of environmental stressors (Chapman, 2002; Mater *et al.*, 2014).

In contrast to the biotransformation metabolism described for *N. diversicolor* exposed to cisplatin and cyclophosphamide, the activity of GST did not change in polychaetes exposed to any TAM-concentration, in accordance to the gathered knowledge of TAM metabolism in mammalians, that does not encompass GST phase II enzyme but is extensively metabolized by phase I cytochrome P450. It may be then suggested that the unaltered GST activity is derived from a pharmacokinetic pathway

rather than the fact that the concentration range herein applied was not enough to trigger this detoxification via. Surprisingly, TAM evoked oxidative damage in the whole range of concentrations and, as documented in human cells under drug therapy, LPO increase may disregard prodrug metabolism neither ERα expression and its activation for the simple fact that TAM prodrug can embeds itself in the lipid membranes and generates superoxide and oxidative stress, with further activation of apoptotic cascade (Trachootham *et al.*, 2009; Lushchak, 2011; Bekele *et al.*, 2016). However, only under the highest TAM concentration, polychaetes presented an expressive DNA damage which may be attributed to the conversion of TAM into its intermediary putative active metabolite α-hydroxytamoxifen, which is able to bind covalently to DNA and damage the double strands (Figure 4.7), as reported in human peripheral blood lymphocytes and MCF-7 cells (Wozniak *et al*., 2007). In other words, even though there is a lack of disclosure on estrogen receptors´ expression in *N. diversicolor,* it is plausible that the metabolic activation has only occurred at 100 ng L^{-1}, and that the prodrug oxidative potential provided detrimental effects all over the concentration range tested. It is important to bear in mind that TAM metabolic activity is described as complex in pharmacology, considering its capacity to display agonist and antagonist potential depending of the target line of cells. Likewise, herein, AChE activity increased in a dose-response manner, also related to the increment of impairments in burrowing ability, which is dissonant to the previous results obtained with *N. diversicolor* exposed to CisPt and CP.

7.2.4 Effects of mixtures of anticancer drugs on *N. diversicolor*

Knowledge concerning the presence of anticancer drugs in the marine environment is very scant in detriment to the broad conventional classes of pharmaceuticals, and even less investigation deals with the intricate reality of chemical interactions involving different anticancer agents. Joining the facts that the selected drugs have a highly toxic potential to ultimately elicit cell death, and their administration in combination during cancer treatment, it was hypothesized that the toxic effects caused by tertiary mixtures would be increasingly toxic to the benthic

model *N. diversicolor*, in a dose-response manner. Nevertheless, the results indicated that at the intermediary concentrations (Mixtures C and D), the overall profile of oxidative stress seemed to be driven by TAM, when compared with the effects derived in the bioassay conducted individually. It is suggested that over the single-drug exposure, at these respective concentrations, TAM activate oxidative stress by prodrug membrane contact. This pathway avoided (or inhibited) the influx of the cytotoxic CisPt and CP, a required step for the activation of the drugs in the cytosol, thus silencing endpoints observed in the respective single-drugs bioassays. Changes could have occurred in structure or function of proteins involved in drug transport delivery, namely in the copper transporter proteins CTR1, accountable for the intake of CisPt and drug resistance (Dasari and Tchounwou, 2014; Ma *et al.,* 2015).

Yet, at the highest mixture concentration (Mix D), the sharp inhibition of CAT activity (90%) is a potential synergistic effect, whereas the inhibition of GST activity indicates an additive interaction (Figure 5.8). At the lowest mixture concentration (MIX A), the suppression of GST activity is dissonant to the respective single-drug responses (Figure 5.8). Indeed, PCA indicate that biochemical responses in specimens exposed to Mix A correspond to those reported for Mix D as well as to those from single CP exposure (Figure 5.7). Drugs at the low concentrations (Mix A) may be accountable for inducing significant levels of T-GPx activity (Figure 5.3-D) as a potential protective effect promoted by responsive mechanisms facilitated at low concentrations, such as drug permeability (Alfarouk *et al*., 2015). It is hypothesized that despite the activation of the antioxidant system in polychaetes submitted to Mix A, ROS were not sufficiently scavenged and detoxification was not enough to overcome injuries provided by LPO by-products (Pires *et al.,* 2016a), culminating in mid-grade DNA damage at levels higher than those of the other mixtures (Table 5.1), as the effect reported in the single exposure to 0.5 ng L^{-1} of TAM, potentially prone to generate LPO by-products in this mixture (Figure 5.8).

Regarding neurotoxicity, AChE activity was inhibited in organisms exposed to Mix D, although burrowing capacity was similar to unexposed animals (Figure 5.1),

which is in contrast with those exposed to Mix A, where burrowing was impaired but no changes is AChE activity were observed. According to these results, it seems that the mechanism involved in the burrowing impairment is different from those driven by AChE activity alterations. At the intermediary concentrations (Mix B and C), the anticancer compounds also triggered oxidative damage in polychaetes, although no pronounced effects on neuroactivity, antioxidant and biotransformation metabolisms. In these two mixtures, biochemical effects of polychaetes were similar to those reported in single-TAM exposures (Chapter 4), suggesting an antagonist interaction of the cytotoxic CisPt and CP at their respective doses and the dominance of TAM MoA over their cytotoxic potential in Mix C and D. These results confirm that the combination of endpoints cannot be calculated by simply adding the effects of the individual components in the mixture.

7.2.5 Effects of anticancer agents on tropical/subtropical biological models

Considering that pharmaeuticals have been left behind in governmental frameworks of low- to middle-income nations, it is essential that the scientific community addresses current gaps in ecotoxicological knowledge by taking into account the vulnerability of aquatic ecosystems in tropics, where the biodiversity is greater and more sensitive to contaminants (Freitas and Rocha, 2012; Moreira et al., 2014). Concomitantly, the incidence of cancer is growing at a fast pace and anticancer drugs´discharge and impacts to the coastal ecosystems have been never questioned in these zones. An ongoing debate is to what extent toxicological benchmarks and environmental criteria developed for species living in temperate regions are valid and applicable for ecological risk assessment of species in other geographical areas such as in tropical regions, which demands more ecotoxicological data under these conditions, using native indigenous test organisms (Gunnarsson and Castillo, 2018).

According to the obtained results, single-drug exposures to *E. lucunter* embryos demonstrate the reliability of such early-life as a chronic endpoint for toxicity assessment of anticancer drugs present at trace concentrations in seawater. As

illustrated by some of the effects displayed by *N. diversicolor* under single-drug exposures, the development of the pluteus larvae also revealed a significant decrease in its viability through a U-shaped dose-response in embryos submitted (Figure 6.1) to CisPt and CP. In other words, the lowest concentration of CisPt (0.1 ng Pt L^{-1}), able to produce the herein reported teratogenic effects, was predicted to occur in surface waters from a tidal river in UK (Vyas *et al*., 2014) receiving hospital wastewaters with oncology ward burden. However, it is important to stress that sources of Pt from platinum-based drugs coexist with, typically much larger, inputs from vehicle emissions in the urban setting. In the case of CP, levels far superior to 50 ng L^{-1} of CP, able to cause embryotoxicity in *E. lucunter*, were already measured in surface waters from Australia, Romania and Thailand (Busetti *et al.,* 2009; Moldovan, 2006; Usawanuwat *et al.,* 2014). To date, environmental screening of anticancer molecules was not yet executed in the Brazilian coastal zone, neither in any other region from Latin America. In contrast to the effects elicited by both cytotoxic drugs, a non-monotonic dose-response relationship was observed in embryos exposed to TAM, at which the lowest concentration 0.5 ng L^{-1} caused significant teratogenicity (Figure 6.2). In reference to the criteria proposed by the regional environmental guidelines established in São Paulo (Brazil) (CETESB, 1999), it is noteworthy that a significant sensitivity of this biological model was registered in the present study, which confirms its reliability as a tropical biological model counterpart in the toxicity assessment of anticancer drugs, such as its feasible performance by features as low cost and test duration (Hutchinson *et al.,* 1998; Aguirre-Martínez *et al.,* 2016).

In the bioassays conducted with the sediment-dwellers biological models, in spiked sediment with mixtures, *T. viscana* demonstrated a higher sensitivity to pharmaceuticals compared to *S. squamata*, in a way that the survival response was also characterized by a non-linear reduction of survival in Mix B and D. According to Lagarde *et al. (*2015), such non-monotonic profile has been typically linked to endocrine disrupting compounds, with reports of similar ecotoxicological effects in experiments conducted with aquatic organisms (Morales *et al*., 2018; Teng *et al*., 2018) and on pharmacological studies developed with human cell lines (Mater *et al.*,

2014; Vandenberg *et al.*, 2014, 2012; Lagarde *et al.*, 2015). The present non-monotonic dose relationship suggests the presence of a nuclear estrogen-like receptor, thus requiring further refined characterization of nuclear ERs and its ligand-induced transcriptional activation. Sequencing of cDNA encoding for ERs, analysis of its expression and homologies with vertebrate counterparts are further steps for a more comprehensive application of tropical indigenous species, not only regarding endocrine disruptive ability of receptor modulators at environmental trace levels, but also to understand the key role that ERs play in cell growth or maintenance (Lv *et al*., 2017). The present findings from sediment spiking procedure conducted with both tropical species of amphipods and polychaetes pinpoint the relevance of the sediment compartment as an essential target of study on environmental impacts of anticancer drugs, even as if an acute endpoint, which actually depicts a reasonable concern in view of the detrimental effects reported over survival at low ng L^{-1}.

7.2.6 Anticancer drugs in an Environmental Risk Perspective

In Europe, the environmental legislation framework involved in the definition of protective measures for aquatic pollutants is represented by a broad set of policies that directly or indirectly encompass the issue of conventional pharmaceuticals. The Marine Strategy Framework Directive of the European Union (Directive 2008/56/2008) establishes that each country member is responsible for conducting its monitoring in accordance to particularities of coastal and marine regions, also connected with other transversal policies. In addition, as part of the strategy implemented by the Directive 2013/39/EU, all the member states shall monitor the emerging risks prone by each pharmaceutical compound (Pereira *et al.,* 2016b). This is also an issue that must be gauged and circumscribed by action plans under the global agenda of Sustainable Development Goals, established by United Nations. In contrast, anticancer drugs still face several limitations and gaps in the set of environmental assessment, since many of them were formulated before the new criteria for authorization and release in the pharmaceutical market (EMA, 2006). In this sense,

CisPt, CP and TAM are just some of the anticancer molecules not assisted in terms of risk control.

The European Medicine Agency guideline proposed a trigger value of PEC of 10 ng L^{-1}, as a limit that separates the exposure estimation in the first phase of the environmental risk assessment from the test requirements in the subsequent second phase (Kummerer *et al.,* 2016). With regard to the review of literature, both CP and TAM needed to be examined with toxicity tests. According to the present toxicological outcomes, harmful biochemical effects occurred even at sub ng L^{-1}, as verified in the single drug exposure of TAM to *N. diversicolor*. Regardless of the value established by ERA, the scientific validity for determining an "action limit" approach may be questioned, particularly for this endocrine disruptive drug. This compound is fitted together with other endocrine disruptor chemicals that trigger an overall trend of non-monotonic dose-response and, therefore, does not conceive a safe dose and must be addressed irrespective of the quantity released into the environment (EMA, 2006).

The non-monotonicity represents a challenge to fundamental concepts in toxicology and standardized approaches for investigating this aspect within the risk assessment context are still missing (Vandenberg *et al.,* 2014; Lagarde *et al.,* 2015). Another parameter entangled in the TAM assessment is that even though this compound remain in Phase I because of a PEC of water column below the action limit, its log Kow is > 4.5, thus the assessment is recommended through another technical guidance, in line with the European approach for risk assessment of general industrial chemicals in the context of REACH regulation (Registration, Evaluation, Authorization and Restriction of Chemicals) (ECHA, 2008).

In accordance to the thesis´ findings, it can be assumed that a traditional dose-response toxicity profile, to which a threshold level is associated, is not appropriate to set up protection measures also for the cytotoxic agents CisPt and CP. These results challenge risk assessment dogma, as an issue stemming primarily from the scarcity of relevant ecotoxicity data on aquatic non-target organisms. Such compounds interact directly with DNA and are therefore of special concern to be considered in-depth in

Phase II of the EMA guideline (EMA, 2006). However, at that point, the purpose of the analysis is to predict the concentration of the substance for which adverse effects were not expected to occur, by the use of standardized bioassays conducted with algae growth inhibition endpoint, *Daphnia sp*. reproduction test and the early life stage test with fish. In addition to the urgency of a commitment with the marine species counterparts and data generation, ecotoxicological approaches from now on developed with anticancer drugs should include discussions whether the analysed endpoints applied in a risk assessment are in fact consistent to their designed biological effects (Booker *et al.,* 2014; Kummerer *et al.,* 2016). Besides, the use of more sensitive species, including those sediment-dwelling ones, is highly required, with application right on the first step of an ERA in the marine context. Herein, the use of a battery of biochemical biomarkers with the polychaete *N. diversicolor* reinforced the application of this biological model in the assessment of sublethal changes triggered by pharmaceuticals, particularly in regard to this potent therapeutical class of drugs.

Another restraint of this tiered approach regards to the expression of a degree of uncertainty through the extrapolation from the test data to the real environment, since pharmaceuticals do not occur isolated. It relies on covering the additional risk of unknown components in a non-defined mixture, by applying an additional assessment factor of 10 to account for 'mixture uncertainty', in every single-substance assessment (Backhaus and Karlsson, 2014). On the other hand, in accordance to the Brazilian environmental reality (probably similar to other developing countries), sources of oncological waste are most likely to feed into municipal waste systems and discharged directly into the surface/coastal waters via straight-piping of raw sewage. The national concern regarding pharmaceuticals in the environment is currently hampered only by tools to provide the proper discard of waste from hospital and health care services, according to the Brazilian Constitutional text (Art. 200) that attributes the role of control and monitoring of substances and medical procedures to the National Health System, jointly to the Federal Resolution CONAMA 358/2005 (Brazil, 2005). This set of laws acts in order to avoid illicit practices that would generate harmful discards to the environment, besides affording the duty to the responsible parties to execute

actions for the appropriate and responsible waste management, from its generation until the destination. According to the law, the antineoplastic drugs must be treated by means of "reuse, recuperation or recycling" or "specific treatment and disposal (Brazil, 2005). It is important to point out that according to this legislation, effluents containing a cytotoxic burden are jointly inserted in the group of chemicals able to be "discharged into water bodies or public sewage system, as long as complying the guidelines from environmental agencies and water resources managers", with the absence of requirements for an environmental screening and monitoring of molecules in the aquatic compartment. At a federal aegis of decision-making, the scenario of dialogue with scientific production and background about the risk of pharmaceutical in Brazilian coastal waters is still incipient or missing. Developing nations, as Brazil, should ground their environmental legislation to practices developed and successfully experienced by Northern countries.

It is of high priority that these regions generate a wide background of information regarding the sensitivity of tropical biological models towards anticancer exposures, in accordance to a range of environmental concentrations recommended to soon get discovered in coastal zones. As showed in this investigation, tropical/subtropical species seem to be more sensitive to anticancer drugs than temperate organisms. Furthermore, knowledge of possible overseen risks associated with the introduction of such substances into the aquatic environment can help to propose a proper environmental framework that include the assessment of these molecules and that can also underline the demand for suitable technologies of wastewater treatment from the municipal burden, and also hospital/oncology wards. Such measures must be taken before critical ecologic and human health problems take place due to long-term exposure, assuming the perception that invest in pollution prevention is preferable than remediation and restoration actions, with the pertinent logic to minimize both risks and public cost. The overall environmental issue of this particular class of anticancer agents diverges substantially from those over-the-counters´groups, which introduction into waterways is able to be minimized through a

variety of individual actions by population, such as the proper disposal of the traditional medicines employed.

7.3 Conclusions

Altogether, the multibiomarker approach herein applied revealed that the occurrence of anticancer agents, at a realistic range of concentrations in the aquatic environment, are able to disrupt the burrowing behaviour, biochemical and antioxidant status, cause neurotoxicity, oxidative damage and genotoxicity in *N. diversicolor*, right in agreement to drug´s designed MoAs over cancer treatment, which then confirms their unspecific toxicity not only to normal growing cells, as already reported to humans, but also to non-target organisms. Besides, results collectively indicated that CisPt and CP loads into water column exert hazardous effects in the sediment compartment even though the physico-chemical properties of these hydrophilic drugs do not lead to estimates of risks towards subtropical and temperate sediment dwelling species, thus highlighting the sediment compartment as an important repository of these harmful molecules in the marine environment. It is relevant to remark that TAM, assumed to comprise a targeted therapy on nuclear estrogen receptors, also altered significantly the biochemical parameters and depicted a genotoxic potential. The present thesis also confirmed that research and regulatory frameworks shall adjust the environmental pollution perspective to the scenario of anticancer drugs´pollution, which indicated non-monotonic profiles of toxicity responses at trace concentrations of single-drug and combined exposures, both in tropical and temperate biological models. In addition, it is noteworthy to reinforce that the sublethal responses obtained through drug´s exposures, individually, are not enough to predict the effects offered by these pharmaceuticals when combined.

There is a scarcity of knowledge about the environmental impacts posed by these anticancer agents in the aquatic ecosystems, therefore, a monumental gap between decision making gathered to this issue in developed and developing regions. Although contrasting, challenges currently faced represent a typical shortage related

to political and economical realities of each country, which must be assumed as a priority to the progress in the sanitary and ecological perspectives. Dialogues across disciplines connected to new drug design and manufacturing, WWTPs engineering, environmental chemistry and ecotoxicology must be conducted to gather new solutions to control and minimize pollution by anticancer agents, and therefore risks to environmental and health, that cannot be dissociated from any other aspects of sustainable living.

7.4 Future Perspectives

- In light of the evidence of the multiple toxicity effects of anticancer identified throughout this thesis and the strength of their presence in the marine environment, it is crucial that further research are committed not only to investigate the several biological mechanisms that still need to be clarified in marine non-target organisms, but also to ultimately engender reasonable attention to their input in water bodies worldwide, under the spectra of priority and hazardous substances. To that end, the following key points represent relevant aspects to be considered for future approaches, from environmental features to pharmacokinetic particularities of drugs in exposed species:

  - Perform environmental assessments and monitoring of the current realistic concentrations of anticancer agents and their active metabolites present in coastal waters and sediments worldwide, also quantifying their respective bioaccumulation in tissues of representative species, both in developed and under-development regions, in order to set up a panorama of drug distribution and exposure scenarios across the global coastal zones.

  - Assess whether bioavailability and reactivity of CisPt, CP and TAM vary according to environmental changes of ionic strength (or changes in physical-chemical properties, such as pH, Eh and temperature), depicting the relevance of estuarine water course.

- Evaluate drug-resistance mechanisms activated in representative marine species during exposure to the cytotoxic CisPt, individually and in mixture with other anticancer compounds.

- Target the signaling pathways and caspase activity in representative marine species, in order to confirm the efficacy of the ultimate MoA of anticancer drugs at environmentally relevant levels.

- Investigate the MoA involved in the metabolic activation of the prodrugs CP and TAM in marine species.

- Identify and characterize of estrogen-like receptors in marine organisms, and the interference of TAM in endocrine metabolism and cell proliferation.

- Estimate species sensitivity distributions (SSDs) on the basis of laboratory toxicity data for a wide array of marine organisms, in order to demonstrate differences in sensitivities derived in tropical and temperate zones, as well as determine potential non-monotonic profiles of responses.

- Determine shifts in protein and gene profile expressions (based on genomics, metabolomics, transcriptomics and proteomics), thus subsidizing the identification of precise metabolism pathways of anticancer agents and the selection of profitable biomarkers.

- Considering the genotoxic and mutagenic potential of anticancer agents, investigate their potential to trigger mechanisms of cancer generation in non-target organisms.

- Propose dialogues across environmental health scientists, toxicologists, and risk assessors to determine how the low-dose effects and non-monotonic dose responses influence a specific risk assessment to anticancer agents.

- Foster fellowships across medical research in an attempt to forewarn this science branch about drug´s cradle-to-grave and encourage its active involvement on environmental stewardship, including ecological and human health consequences, with an emerging discussion of the overall issue in the root of medical literature.

- Engage society in scientific debates as part of the environmental responsibility in aquatic pollution regarding the consumption of pharmaceuticals in general, highlighting the individual conduct as a measure to prevent and control impacts.

- Expand sewage connection and implement efficient WWTPs´ technologies able to reduce the pharmaceuticals burden, particularly in regions reliant on septic tanks or small decentralized systems for sewage treatment disposal.

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