Combined effects of climate change and endocrine disruptor compounds on the structure and functioning of coastal ecosystems

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This thesis contains research studies, presented in the form of chapters, which correspond to scientific manuscripts that have been submitted or in preparation for future publication. Considering that all studies were conducted with the collaboration of several authors, namely the supervisor, Patrícia Cardoso (CIIMAR - University of Porto) and co-supervisors Miguel Santos (University of Porto) and Miguel Pardal (University of Coimbra), the student stating that he participated in its design, through the sample collection, data analysis, discussion of results, writing and editing of illustrations.

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Resumo

Os ecossistemas costeiros estão sujeitos a múltiplos impactos de origem antropogénica, sendo as alterações climáticas e a contaminação química alguns dos mais relevantes. No que diz respeito às alterações climáticas, estima-se que, até 2100, a temperatura média dos oceanos aumente cerca de 3 a 4 °C, podendo causar sérios problemas nos ecossistemas aquáticos. Uma das principais causas para esta alteração, deve-se ao aumento dos níveis de CO_2, que acaba por alterar o equilíbrio químico e, assim, desencadear um conjunto de consequências negativas para os ecossistemas aquáticos.

De referir que, nas últimas décadas e com o desenvolvimento industrial, a poluição nos ecossistemas aquáticos aumentou drasticamente, levando à libertação destes compostos de origem antropogénica nos ecossistemas. Há algumas décadas atrás, a libertação desses compostos nos meios aquáticos, obedecia a um menor controlo ou regulamentação capaz de estabelecer limites aceitáveis para os ecossistemas, o que obrigou, ao longo dos anos e com o surgimento de graves problemas ecológicos e de saúde pública, a repensar a forma como eram tratados. Deste modo, foram identificadas diversas classes de compostos disruptores endócrinos (EDCs), que para a comunidade mundial eram e continuam a ser, alguns dos mais problemáticos. Com capacidade de interferir com o normal funcionamento das várias vias metabólicas reguladas por diferentes recetores, os EDCs podem afetar, principalmente, o funcionamento do sistema endócrino, afetando inúmeros processos biológicos (por exemplo, reprodução, sobrevivência e o comportamento). Entre os inúmeros EDCs que se podem encontrar nos ecossistemas aquáticos, as hormonas esteróides, como as progestinas sintéticas são algumas das mais potentes. As progestinas, são micropoluentes de especial preocupação, não só pela sua capacidade como EDC e pelos seus efeitos negativos sobre espécies não alvo, mas também pelo seu uso crescente, em todo o mundo e pelo impacto ambiental que podem ter nos ecossistemas aquáticos.

Por outro lado, o mercúrio (Hg) é um poluente prioritário que pode ser encontrado nos mesmos ecossistemas, e cujo impacto no sistema endócrino, particularmente nas espécies aquáticas, não é bem descrito na literatura. Assim,

atendendo ao facto de todos estes fatores de stresse (isto é, temperatura, Hg e progestinas) agirem no mesmo eixo de interligação (eixo hipotálamo-hipófise-gónada (HPG), é extremamente relevante estudar os efeitos combinados do aumento da temperatura e da exposição às misturas de Hg e progestinas no funcionamento de espécies costeiras relevantes.

Assim, esta Tese inclui, em primeiro lugar uma caracterização ambiental dos níveis de mercúrio e progestinas em ambientes estuarinos ao longo da costa Portuguesa (ou seja, a Ria de Aveiro, o Estuário do Tejo e a Ria Formosa) e depois uma abordagem experimental para estudar os efeitos combinados da temperatura e misturas de Hg e da progestina drospirenona (DRO) na sobrevivência, fitness, dinâmica de maturação das gónadas e respostas de stresse oxidativo do gastrópode *Nucella lapillus*.

Para tal, os principais objetivos dessa primeira fase de estudo foram avaliar as tendências temporais de acumulação de Hg nos três diferentes sistemas e respetivas cadeias tróficas, analisando principalmente componentes abióticos (água) e bióticos (ou seja, produtores primários e espécies macrobentónicas). Posteriormente, avaliamos o risco de consumo de espécies comercialmente importantes (ex., bivalves) para a saúde humana e, finalmente, foi feita uma avaliação do risco ecológico da exposição de Hg.

Os nossos resultados demonstraram que as concentrações totais de Hg nas águas superficiais eram mais elevadas na Ria de Aveiro e no estuário do Tejo do que na Ria Formosa. Em alguns locais da Ria de Aveiro (ou seja, Torreira e Gafanha do Carmo) durante períodos de Outono, foram observados níveis de Hg (100 $\mu g\ L^{-1}$) superiores às Normas de Qualidade Ambiental (NQA) (70 $\mu g\ L^{-1}$). Assim, para certos locais da Ria de Aveiro, em determinadas épocas do ano, os níveis de Hg no compartimento abiótico podem ser um motivo de preocupação. O mesmo aconteceu na zona do Seixal (Estuário do Tejo) no período da Primavera. No que diz respeito à macrofauna, não houve uma clara tendência temporal de acumulação de Hg nos três ecossistemas. De um modo geral, verificou-se que os valores de Hg acumulados pelas espécies estudadas, não apresentaram uma preocupação para a saúde humana, uma vez que as concentrações totais de Hg se encontravam abaixo dos limites máximos permitidos pela legislação (< 0.5 ng g^{-1} peso húmido) sobretudo para espécies comestíveis, como os bivalves. Pelo contrário, verificou-se na Ria de Aveiro um moderado risco de contaminação por mercúrio (HQ > 1) associado à cadeia trófica em períodos de Outono, devido às elevadas concentrações nas águas superficiais. Paralelamente à avaliação das tendências temporais de Hg nos três ecossistemas mencionados anteriormente, foi também realizado um estudo piloto para perceber quais as concentrações das progestinas mais consumidas em Portugal. Para isso, foram recolhidas amostras de águas superficiais, nas três zonas estuarinas, mencionadas acima, para avaliar os níveis de drospirenona (DRO), desogestrel (DSG), gestodeno (GST) e levonorgestrel (LNG). Os resultados demonstraram que o DSG e a DRO foram as progestinas mais abundantes na costa. Assim, verificou-se que no Norte de Portugal (ou seja, Ria de Aveiro) o DSG foi a progestina mais abundante, enquanto a DRO foi a mais abundante na zona Sul, sobretudo em períodos de Verão. Estas diferenças espaciais podem estar relacionadas com os diferentes níveis populacionais (incluindo a população residente mais visitantes/turistas) associados às três áreas estudadas, bem como à hidrodinâmica de cada sistema estuarino (por exemplo, fluxo e volume de água de cada estuário). O estuário do Tejo e a Ria Formosa (no Verão) situam-se em zonas muito mais povoadas do que a Ria de Aveiro e caracterizadas também por um padrão sazonal de visitantes que pode justificar a ocorrência de níveis mais elevados de DRO no Verão. Para isso, assumimos que quase 50% dos turistas no Algarve correspondem a população Inglesa e que a maioria dos contracetivos administrados no Reino Unido têm DRO na sua composição. Paralelamente à monitorização ambiental dos níveis de mercúrio e progestinas ao longo da costa Portuguesa, foi realizado um estudo experimental, que procurou avaliar os efeitos combinados da temperatura e de misturas de DRO e Hg na ecologia e maturação das gónadas do gastrópode marinho *Nucella lapillus*, durante 21 dias. Os resultados mostraram que a sobrevivência de *N. lapillus* foi negativamente afetada pelos três fatores e foi exacerbada no caso das misturas de EDCs e sob o efeito do aumento da temperatura.

Relativamente ao impacto na dinâmica de maturação da gónada, observou-se que ambos os compostos, como fatores isolados não causaram qualquer efeito na maturação dos ovários e testículos de *N. lapillus*. Contudo, na presença de temperatura mais elevada observou-se um claro atraso na maturação dos ovários, mas não nos testículos, o que sugere um maior impacto negativo dos fatores de stress nas fêmeas do que nos machos.

Relativamente a este estudo, poderíamos concluir que a DRO, como fator isolado, parece ter um impacto menos negativo nas espécies aquáticas, como nos gastrópodes do que as progestinas mais antigas. No entanto, em combinação com outos EDCs e/ou temperatura pode ter graves implicações ambientais.

Alem disso, foi também feito um outro estudo para avaliar as respostas bioquímicas de *N. lapillus* aos mesmos fatores de stresse. Os resultados deste estudo, mostraram que os machos estudados, tiveram um aumento na defesa antioxidante contra o stresse oxidativo, induzindo a atividade da catalase (CAT) quando expostos a concentrações mais baixas de Hg, com um efeito oposto quando expostos a concentrações mais altas de DRO (inibição da CAT). Além disso, apesar de não terem sido observados efeitos significativos na peroxidação lipídica, os machos tenderam a apresentar níveis de MDA mais elevados do que as fêmeas, o que pode sugerir que o sistema de defesa antioxidante não era suficientemente forte para evitar a peroxidação lipídica e danos celulares. Este estudo preliminar sobre as respostas bioquímicas de *N. lapillus* aos efeitos combinados de temperatura e misturas de EDCs permitiu confirmar que as progestinas de última geração são menos ativas do que as mais antigas. Mas, em estudos futuros, é importante complementar esta informação com as respostas de outros biomarcadores (por exemplo, biomarcadores moleculares).

Concluindo, os nossos resultados demonstraram que existe uma forte necessidade de continuar a monitorizar os níveis de progestinas nos sistemas aquáticos e fazer uma caracterização mais profunda destes compostos na cadeia trófica, a fim de compreender a sua capacidade de bioacumulação e inferir sobre possíveis efeitos, nomeadamente para a saúde humana. Por outro lado, estes resultados permitiram também concluir que as progestinas de última

geração, como a DRO, são muito mais específicas e causam menos efeitos colaterais em espécies não-alvo, o que é um resultado positivo de muitos anos de investigação e desenvolvimento de fórmulas alternativas para contracetivos e tratamentos hormonais. No entanto, de forma holística, tendo em conta a interação de múltiplos fatores de stress, existe um risco para o meio aquático e a biota associada quando exposta a esta multiplicidade de fatores de stress. Assim, só através da implementação de mais estudos é possível deduzir sobre o impacto real destes compostos e, ao mesmo tempo, alertar as autoridades competentes para a importância da regulamentação das progestinas e, eventualmente, propor tratamentos mais eficazes na remoção destes compostos nas estações de tratamento de águas residuais.

Palavras-chave: Ambientes aquáticos, alterações climáticas, temperatura, disruptores endócrinos, metais pesados, mercúrio, progestinas, drospirenona,

Abstract

Coastal ecosystems are subject to multiple impacts of anthropogenic origin, being climate change and chemical contamination some of the most relevant ones. Regarding climate change, it is estimated that by 2100, the average temperature of the oceans will increase about 3 to 4 °C, which can cause serious problems in the aquatic ecosystems. One of the principal causes for this change is due to the increase in CO_2, which ends up altering the chemical balance and thus triggering a set of negative consequences for aquatic ecosystems.

It should be noted that in recent decades, with industrial development, aquatic pollution has increased dramatically, leading to many compounds of anthropogenic origin being released into the aquatic ecosystems. Some decades ago, the release of these compounds into the aquatic environments had less control or regulation capable of setting regulatory limits for ecosystems, which forced, over the years and with the emergence of serious ecological and public health problems, to rethink how they were treated. In this way, several classes of chemicals were identified, namely the endocrine disrupting compounds (EDCs), which for the world community were and continue to be, some of the most problematic ones. With the ability to interfere with the normal functioning of various metabolic pathways that are regulated by different receptors, EDCs can mainly affect the normal functioning of the endocrine system, affecting numerous biological processes (e.g., reproduction, survival and behaviour). Among the numerous EDCs that can be found in aquatic ecosystems, the steroid hormones, such as the synthetic progestins are some of the most potent ones. Progestins are micropollutants of special concern, not only because of its capacity as an EDC and its negative effects on non-target species, but also because of its growing use, worldwide and the environmental impact that they can have on the aquatic systems.

On the other hand, mercury (Hg), is a high priority pollutant that can be found in the same ecosystems, and whose impact on the endocrine system, particularly in aquatic species, is not well described in the literature.

So, attending to the fact that all these stressors (i.e., temperature, Hg and progestins) act at the same interconnecting axis (HPG axis), it is extremely relevant to study the combined effects of increased temperature and exposure to mixtures of Hg and progestins on the function of relevant coastal species.

So, this thesis includes, first, an environmental characterization of levels of mercury and progestins in different estuarine systems along the Portuguese coast (i.e., Ria de Aveiro, Tagus estuary and Ria Formosa) and then, an experimental approach to study the combined effects of temperature and mixtures of Hg and drospirenone (DRO) on the survival, fitness, gonads' maturation dynamics and oxidative stress responses of the marine gastropod *Nucella lapillus*.

To this end, the main objectives of this first phase of the study were to evaluate the temporal trends of Hg accumulation in the three different systems and respective trophic chains, analyzing mainly abiotic (water) and biotic components (i.e., primary producers and macrobenthic species). Subsequently, we tried to evaluate the risk of consumption of commercially important species (i.e., bivalves) for human health and, finally, to evaluate the ecological risk of exposure to Hg contamination.

Our results showed that the total Hg concentrations in surface waters were higher in Ria de Aveiro and in Tagus estuary than in Ria Formosa. In some sites of Ria de Aveiro (i.e., Torreira and Gafanha do Carmo), during autumn periods, were observed Hg levels (100 $\mu g\ L^{-1}$) higher than the Environmental Quality Standards (EQS) (70 $\mu g\ L^{-1}$). Thus, for certain systems, such as Ria de Aveiro, at certain times of the year, Hg levels in the abiotic compartment may be a cause for concern. The same occurred in the Seixal area (Tagus Estuary) in the spring period. Regarding macrofauna, there was no clear temporal trend of Hg accumulation in the three estuaries. In general, it was found that the Hg values accumulated by the studied species did not present a concern for human health, since the total Hg concentrations were below the maximum limits allowed by the legislation (< 0.5 ng g^{-1} ww) mainly for edible species, such as bivalves. On the contrary, it is verified that in Ria de Aveiro there is a moderate risk of contamination by Hg (HQ > 1), associated with the trophic chain in the autumn periods, due to the high concentration of Hg in surface waters.

In parallel to the evaluation of Hg temporal trends in the three estuarine areas mentioned above, a pilot study was also conducted to evaluate the concentrations of progestins most consumed in Portugal. For that, surface waters in the three estuarine areas were sampled to assess the levels of drospirenone (DRO), desogestrel (DSG), gestodene (GST) and levonorgestrel (LNG). Our results showed that DSG and DRO were the most abundant progestins on the coast. Thus, it was found that in the North of Portugal (i.e., Ria de Aveiro), the DSG was the most abundant, while in the South, the DRO was also relevant, particularly in summer periods. These spatial differences can be related to the different population levels (including the resident population plus visitors/tourists) associated to the three studied areas, as well as to the hydrodynamics of each estuarine system (e.g., water flow). Tagus estuary and Ria Formosa (in the summer) are located in areas much more populated than Ria de Aveiro and characterized also by a seasonal pattern of visitors that can justify the occurrence of higher levels of DRO in summer. For that, we assumed that almost 50% of the tourists in Algarve correspond to British people and that most of the contraceptive pills administered in UK have DRO in its composition.

Parallel to the environmental monitoring of the Hg and progestins levels along the Portuguese coast, an experimental study was carried out, which sought to evaluate the combined effects of temperature and EDCs (DRO and Hg) on the performance and gonads' maturation dynamics of the marine gastropod *Nucella lapillus,* for 21 days. Our data showed that survival of *N. lapillus* was negatively affected by the three factors and it was exacerbated in the case of mixtures of EDCs and under the effect of increased temperature.

Concerning the impact on the gonad's maturation dynamics, it was observed that both chemicals as single factors did not cause any effect on the maturation stage of ovaries and testis. However, in the presence of a higher temperature it was clear a delay in the maturation stage of the ovaries but not in the testis, suggesting a higher negative impact of the stressors in females than in males.

From this study we could conclude that DRO, as isolated factor seems to have a lower negative impact on aquatic species, such as in gastropods, than older progestins. However, in combination with other EDCs and/or temperature may have serious environmental implications.

In addition, it was also done another study to evaluate the oxidative stress responses of *N. lapillus* to the same stressors. The results of this study showed that males experienced an increase in antioxidant defense against oxidative stress by inducing catalase (CAT) activity when exposed to lower Hg concentrations, with an opposite effect when exposed to higher concentrations of DRO (CAT inhibition). Additionally, despite no significant effects on lipid peroxidation were observed, males tended to have higher MDA levels than females, which may suggest that the antioxidant defense system was not strong enough to avoid lipid peroxidation and cell damage. This preliminary study on the biochemical responses *of N. lapillus* to combined temperature and mixtures of EDCs allowed to confirm that last generation progestins are less active than the older ones. But it is important in future studies to complement this information with other biomarkers' responses (e.g., molecular biomarkers).

Concluding, our findings demonstrated that there is a strong necessity to continue to monitor the progestins' levels in the aquatic systems and do a deeper characterization of these compounds in the trophic web in order to understand their ability to bioaccumulate and infer about possible effects, namely to human health. On the other hand, these results also allowed to conclude that last generation progestins, such as DRO are much more specific and cause less side effects on non-target species, which is a positive result of many years of research and development of alternative formulas for contraceptives and hormonal treatments. However, in a holistic way, considering the interaction of multiple stress factors, there is a risk to the aquatic environment and associated biota when exposed to combined temperature and mixtures of EDCs. So, only through the implementation of more studies it is possible to infer about the real impact of these compounds and at the same time alert the competent authorities for the importance of regulation of progestins and possibly improve the treatments applied in wastewater treatment plants for these compounds and similar ones.

Keywords: aquatic environments, climate change, temperature, endocrine disruptors, heavy metals, mercury, progestins, drospirenone.

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List of abbreviations

AIC - Akaike Information Criterion

ANOVA - Analysis of variance

AvC - average consumption

CAT – Catalase

BMFs – Biomagnification Factors

CECs - Contaminants of Emerging Concern

CIIMAR – Interdisciplinary Centre of Marine and Environmental Research

CO_2 – Carbon dioxide

Ct - Control

CRMs - Certified Reference Materials

CV-AFS - Cold Vapour Atomic Fluorescence Spectroscopy

DGES – Direção Geral do Ensino Superior

DRO – Drospirenone

DRO1 – Low concentration of drospirenone

DRO2 – High concentration of drospirenone

DSG - Desogestrel

EDC - Endocrine Disrupting Compound

EDI - Estimated Daily Intake

EE_2 - 17α - Ethinylestradiol

EQS - Environmental Quality Standards

FCT – Fundação para a Ciência e Tecnologia

FCUP – Faculdade de Ciências da Universidade do Porto

FSH - Follicle-Stimulating Hormone

GHGs - Greenhouse gases

GLM - Generalized Linear Models

Glm.nb - Generalized linear model with negative binomial distribution

GST – Gestodene

GST - Glutathione S-transferase

HCl - Hydrochloric Acid

Hg – Mercury

Hg1 – Low concentration of Mercury

Hg2 – High concentration of Mercury

HNO3 - Nitric Acid

HQ - Hazard Quotient

HPG - Hypothalamic-Pituitary-Gonadal

IPCC - Intergovernmental Panel on Climate Change

LH - Luteinizing hormone

LNG -Levonorgestrel

LODs - Limits of detection

LMM - Generalized Linear Mixed Models

LPO - Lipid Peroxidation

LOQs - Limits of quantification

MeHg - Methylmercury

MDA – Malondialdehyde

NQA – Normas de Qualidade Ambiental

NIEHS - National Institute of Environmental Health

Mix 1 (Mixture: Hg1 + DRO1)

Mix 2 (Mixture: Hg1 + DRO2)

Mix 3 (Mixture: Hg2 + DRO1)

Mix 4 (Mixture: Hg2 + DRO2)

OA – Ocean acidification

PNEC - Predicted no-effect concentration

PT – Portuguese

P4 – Progesterone

RfD - Reference risk dose

RCRs - Relative Consumption Rates

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

SCt - Solvent control

SnCl2 - Stannous chloride

SPM - Suspended Particulate Matter

STPs - Sewage Treatment Plants

TBT - Tributyltin

THg - Total mercury

Temp – Temperature

T18 °C – Temperature 18 °C

T22 °C – Temperature 22 °C

T30 – 30 minutes

T60 – 60 minutes

UP – Universidade do Porto

ww - wet weight

WWTPe - Waste Water Treatment Effluents

WWTPs -Waste Water Treatment Plants

Chapter 1

General Introduction

General Introduction

1. Coastal ecosystems and their main impacts

According to the Millennium Ecosystem Assessment, the coastal zone can be defined as a narrow land strip dominated by the influence of the ocean, either under the direct action of seawater through the tides and an adjacent marine area, tend to be limited by the maximum depth of penetration of sunlight, with coastal systems limited, either by the depth of 50 m or by the distance of 100 km on the shoreline or at an altitude of 50 m, closest to the sea (UNEP, 2006).

Coastal ecosystems represent approximately 8% of the global ocean area (Bauer et al., 2013) and can be represented by various types of ecosystems, namely estuaries, coastal marshes, sandy beaches, coral reefs, rocky beaches and algae forests (Scales et al., 2014). The regional characteristics and the heterogeneity of the habitats of all coastal ecosystems support endemic fauna and flora, which makes them particularly vulnerable to the impacts of climate change with a high risk of biodiversity loss and changes in the structure and functioning of these same ecosystems and their interaction (Rilov, 2016, Chefaoui et al., 2018).

All these ecosystems are also important subsistence areas that ensure the survival of humans by supporting high fishing yields (Scales et al., 2014) consequently, the high density of human populations on coastal land causes most adjacent marine ecosystems to be affected by local anthropogenic disturbances such as eutrophication, coastal changes, chemical pollution and overfishing. Since the estuaries are some of the most productive areas on Earth, throughout the years, they have attracted more people which have explored intensely their resources. Consequently, various stressors have been identified in these ecosystems, such as nutrient enrichment (eutrophication), habitat loss and changes, chemical contamination, climate change, overexploitation of resources, and invasive species (Cardoso, 2021).

1.1. Climate changes

According to the Intergovernmental Panel on Climate Change (IPCC), the steady increase in global population and, consequently, the economic growth and later industrial development were the responsible for the increases in greenhouse gases (GHGs) in the last three decades (IPCC et al., 2014) As anthropogenic GHG emissions, the above all carbon dioxide (CO_2) has led to its increase in the atmosphere, which remains the most environmentally relevant GHG. Thus, current high levels of CO_2 have been the main cause of global warming and ocean acidification (OA) since the mid-20th century, presenting physical and indirect consequences for the marine environment and the organisms living there (Guinotte and Fabry, 2008, Doney et al., 2009, Bauer et al., 2013).

Direct consequences of cumulative post-industrial emissions include increasing global temperature, perturbed regional weather patterns, rising sea levels, acidifying oceans, changed nutrient loads, and altered ocean circulation (Brierley and Kingsford, 2009).

According to McKibben (2022) in the book “The End of Nature”, Man, having altered the composition of the atmosphere, irreversibly transformed the planet. There are several studies that show that changes in the atmosphere point to a multiplicity of possible impacts for the intensification of the greenhouse effect, making extreme phenomena more and more frequent, such as increased frequency and intensity of storms, loss of biodiversity and subsequent extinction of species, and reduction of water resources, which may later trigger other much more serious problems that compromise the balance of planet Earth.

It is true that there have always been climate changes on the planet, but this is the first time that these changes have been caused by a species. These changes are from a high concentration of GHG in the atmosphere, caused by the burning of fossil fuels - linked to transport, agriculture, industry, heating and human activities such as urbanization and deforestation, leading to changes in land use that affect the carbon cycle and also contribute to the greenhouse effect.

All this evolution over the centuries has brought benefits to human beings, but on the other hand, it has shown to have long-term consequences for the planet.

In parallel with all the phenomena described, there are other problems such as global warming, which translate into an increase in atmospheric temperature and, consequently, in water resources, which ultimately translate into the melting of glacial and icy areas across the planet, causing the sea level rises and consequently floods all over the coastal zones and low-lying areas closer (Ingels et al., 2012). This warming also ends up intensifying the acidification of marine environments, which consequently can cause modifications in the precipitation patterns, causing changes in marine ecosystems, deviations in migratory routes and subsequent imbalance in food chains and in many cases affecting reproduction, compromising survival for many species (Thomas et al., 2004, Hoegh-Guldberg and Bruno, 2010, Nagelkerken and Connell, 2015).

Several studies show that the high rate to which climate change occurs ends up jeopardizing the adaptability of fauna and flora and even the ecosystems in which they occur, although there is a constant adaptation to those changes.

This combination of stress factors, as it becomes worst, can trigger direct and indirect effects in marine ecosystems and their livelihoods, such as changes in phenology (altering life cycles at the level of fauna and flora) abundance and distribution of organisms, composition of communities, habitat structures and the normal processes of ecosystem functioning caused by humans at sea, leading to loss of biodiversity and the increase in dead zones in the oceans and also an uncontrolled spread of invasive species. In this way, changing the balance of species and habitats in ecosystems can have a huge impact on life on Earth (Thomas et al., 2004, Bauer et al., 2013).

1.2. Chemical contamination/Endocrine Disrupting Compounds (EDCs)

For the first time, in 1996 at the European workshop on the impact of Endocrine Disruptors on Human Health and Wildlife (Weybridge, UK) the concept of "Endocrine Disruptor" emerged being of interest for specific studies on their endocrine activity and the link between human health and environmental exposure. The term Endocrine Disrupting Compound (EDC) is used to define a structurally diverse class of synthetic and natural compounds that possess the ability to interact with the endocrine system (Carnevali et al., 2018).

These compounds are quite diverse being distributed by various groups, such as herbicides and pesticides, plastics, heavy metals, biocides, heat stabilizers, chemical catalysts, pharmaceutical and diathetic products, fire retardants, among others (Ribeiro et al., 2017, Illuminati et al., 2017). Due to their physicochemical characteristics and diverse biological effects, it is susceptive that these compounds interact with the endocrine system through a wide range of metabolic mechanisms and pathways of action (Henley and Korach, 2006). Besides that, action mechanisms, they can interfere with the neuroendocrine and endocrine functions involved in the reproduction and embryo development (Godfrey et al., 2017).

EDCs can reach the aquatic systems mainly through the excretion of (faeces/urine) via discharge of various wastewaters (Pirger et al., 2018a, Liang et al., 2019). The great concern about EDCs in the environment is that they exert their effects by mimicking, antagonizing, or altering endogenous steroid levels (androgens or estradiol) by changing rates of their synthesis or metabolism and/or expression or action at receptor targets. EDCs, in fact, share structural homologies with natural hormones, being able to bind their cognate receptors and triggering the same biological response (Gillio Meina et al., 2013, Ojoghoro et al., 2021).

Several studies have been conducted to understand the environmental distribution of these compounds, to understand whether despite the low concentrations existing in the environment can become a harmful factor for environmental health, since their persistence can be lasting and as such, due to bioaccumulation, chronic exposure and even a possible occurrence of synergistic/antagonistic interaction between them (Pirger et al., 2018a, Liang et al., 2019, Ojoghoro et al., 2021).

As such, it is essential to understand not only whether the action of these compounds can have harmful consequences for the environment, but also how they act, the concentrations in which they occur, what mechanisms of action are involved and whether the endocrine system will be affected or not, and whether it will have an ecologically relevant impact (Traversi et al., 2014, Ruhí et al., 2016, Carnevali et al., 2017).

1.2.1. Pharmaceuticals

A group of chemicals that has recently received a lot of attention from environmental scientists is pharmaceuticals (Ojoghoro et al., 2021). The problematic of the pharmaceuticals contamination is that their usage is increasing worldwide and their occurrence in the environment is a growing concern because the pharmaceuticals ingredients, including their metabolites and conjugates, are mainly excreted in urine and faeces hence, they enter municipal sewage treatment plants (STPs) where they can be degraded in part or adsorbed to sewage sludge, or eventually diluted into surface water (Carlsson et al., 2006).

In addition, the high frequencies of detection of these contaminants in aquatic environments and their incomplete removal from STPs may pose the greatest risk for the aquatic communities (Carlsson et al., 2006, Gillio Meina et al., 2013, Pirger et al., 2018a).

In the literature, there are many studies regarding the effects of different classes of pharmaceuticals in the aquatic life, being the steroid hormones, one of the groups that received greatest attention. Steroids are one of the most potent EDCs, since they can occur at quite low concentrations (ngL^{-1}), but they can trigger a wide range of negative effects on reproduction, metabolism, etc of both vertebrate and invertebrate species (Pirger et al., 2018a, Liang et al., 2019, Ojoghoro et al., 2021).

In the last decade, many studies have been performed with synthetic estrogens, like the 17α - ethinylestradiol (EE2), which have demonstrated its negative impact for example, may modify brain structure, function, and consequently, behaviours pattern during the female brain development (Pirger et al., 2018) on gonad development, sex differentiation, reproduction and transcription expression of *vtg* gene in fish at low concentrations (Brion et al., 2004, Fent, 2015a, Liang et al., 2019). These contaminants associated to climate drivers (temperature and pCO_2)

may produce a stronger and unpredictable effect on the aquatic habitat, since the latter can also interfere in the regulation of the Hypothalamus-Pituitary-Gonadal (HPG) axis of vertebrates and invertebrates (Miranda et al., 2013), impairing the reproductive system.

However, a great lack of information exists on the study of another class of steroid hormones which are the synthetic progestins.

1.2.1.1. Progestins

Steroids with progestogenic activity are called gestagens, progestogens, or progestins. These compounds are potent endocrine disruptors, with the ability to modify various physiological, hormonal and behavioural processes in wild species, and subsequently affect their ability to reproduce, develop and grow (Fent, 2015a, Pirger et al., 2018a).

Synthetic progestins are compounds that derive from Progesterone (P4) or the steroid androgenic hormone, testosterone and according to Fent (2015a), there are about 20 different progestins. These progestins are produced primarily as pharmaceutical compounds with contraceptive function and can be administered individually or in combination with other synthetic steroids (e.g.,, EE2). However, these compounds can be used for other purposes, such as hormone replacement therapy, endometrial cancer prevention, treatment of dysfunctional uterine haemorrhage, stimulation of palliative appetite and growth promoters in animal agriculture (Fent, 2015a, Kumar et al., 2015).

Several recent studies have shown that several metabolites are eliminated through human and animal urine/faeces and then treated at STP where the generally applied treatment process is not suitable for eliminating them perfectly (Heberer, 2002, Liu et al., 2011, Pirger et al., 2018a, Liang et al., 2019, Ojoghoro et al., 2021). Consequently, synthetic residues of progestins enter the aquatic environment (Pirger et al., 2018) and may cause adverse effects on different species. For example, Xu et al. (2023) observed negative effects on fertility of Pacific oyster *Crassostrea gigas* when exposed to norgestrel. Also, the reproduction of Japanese marine medaka and Fathead minnow was compromised when exposed to northindrone and a mixture of levonorgestrel, drospirenone, gestodene and desosgestrel, respectively (Zeilinger et al., 2009, Paulos et al., 2010, Runnalls et al., 2013). Moreover, females of the same species showed development of male secondary sexual characteristics (Zeilinger et al., 2009, Runnalls et al., 2013). Other studies showed modifications at different levels, such as, altered hormone levels in fish Fathead minnow (Runnalls et al., 2013), induced transcriptional effects in adults (Zucchi et al., 2013, Zucchi et al., 2014) and embryos of zebrafish (Zucchi et al., 2012), altered sex development in zebrafish and frog species *Xenopus tropicalis* (Kvarnyrd et al., 2011, Liang et al., 2015a, Liang et al., 2015b).

Since progestins can potentially interact at all levels of the hypothalamus-pituitary-gonad axis, and several additional mechanisms, it can cause deregulation and may even have an inhibiting function (Zucchi et al., 2013, Fent, 2015a, Carnevali et al., 2018). Several studies have shown that they act at the same axis level in both males and females, causing inhibition of gametogenesis, development of intersex gonads, alteration of the gonadosomatic index, and decreased fertility rate (Zucchi et al., 2013, Carnevali et al., 2018).

Based in the literature, the last generation progestins, such as drospirenone (DRO), were designed to bind the progesterone receptor with greater specificity and minimize side effects related to interactions with androgen, estrogen or glucocorticoid receptors, combining potent progestogenic and antiandrogenic activities (Marqueño et al., 2019). However, still there is a lack of knowledge about the effects of this progestin in non-target aquatic species.

1.2.2. Heavy metals / Mercury

Marine ecosystems are continuously loaded with xenobiotics produced by human activities, very often affecting aquatic organisms (Van der Oost et al., 2003). An example of these xenobiotics, are heavy metals, such as cadmium, lead, mercury (Hg) and tributyltin (TBT), which are also reported to have an endocrine disruptive potential (Zhu et al., 2000, Ingre-Khans et al., 2017).

Mercury is a neurotoxic heavy metal and considered a priority substance according to the Water Framework Directive (2013). It can be released into the environment by natural and anthropogenic sources, being almost 50% of its emissions supplied by fossil fuel combustion, transport and energy production and waste burning (Tan et al., 2009). After its release and deposition in the aquatic ecosystems, inorganic Hg is metabolized by bacteria becoming in organic methylmercury (MeHg) form (Bergman et al., 2013, Rice et al., 2014).

Due to the bioaccumulation processes, Hg can negatively affect marine fauna and human health given its high toxicity and persistence in the environment (Rice et al., 2014).

The MeHg is persistent and bioccumulative and consequently can accumulate to toxic levels in top predators and stay in the environment long after the chemical has ceased to be actively used (Bergman et al., 2013, Alves et al., 2016), that in most cases, in more than 90% of cases the total mercury (THg) present in the muscle of these animals is in the form of MeHg (Scheuhammer et al., 2007).

According to Minoia et al. (2009), low exposure levels of Hg may affect the endocrine system in animals and people, causing disruption of the pituitary, thyroid, adrenal and pancreas. Several authors also demonstrate that Hg can precipitate pathophysiological changes along the hypothalamus-pituitary-gonadal axis (Davis et al., 2001, Carnevali et al., 2018) that may affect reproductive function. Signs of reduced fecundity and fertility after Hg exposure have been observed in some aquatic species like organisms at lower trophic levels (such as plankton and invertebrates) and wild fish's populations although the fish populations are more vulnerable to effects of Hg due to bioaccumulation and biomagnification through food chains (Xie et al., 2020).

Also, according to Wang et al. (2016), Hg may induce severe lesions at the level of fish testis, mainly affecting the size and abundance of mature sperm and can alter the circulating levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, progesterone, and the androgens (Davis et al., 2001). These effects in the reproductive system can be explained by the estrogenic properties of Hg (Tan et al., 2009). So, despite in the literature there are many studies designed to understand what the real impact Hg can have on human life and even wildlife, it is even more relevant to study the combined effects of chemicals, such as Hg and progestins, acting both at the same interconnecting axis (HPG).

2. Benthic communities

Macrofauna plays a key role in the structure, functioning, and interactions between benthic marine ecosystems (Dreujou et al., 2020). Benthic organisms play an important ecological role by reworking the sediments, which affects the flux of nutrients across the sediment-water interface.

When multiple stressors act in opposite directions on a biological response, the interpretation of these interactions becomes even more complex. To this end, it is important to identify generalities about stress responses and types of interaction that can better inform decision-makers in their conservation and legislative actions (Carrier-Belleau et al., 2021).

Generally, macrobenthic organisms, like gastropods and bivalves have been used as non-target model organisms in studying environmental disturbances (e.g.,, climate changes, chemical contamination, eutrophication) for a long time (Pirger et al., 2018a). They proved to be effective model animals because they are ubiquitous, have highly conserved control and regulatory biochemical pathways that are often homologous to vertebrate systems, and they are extremely sensitive to anthropogenic inputs (Rittschof and McClellan-Green, 2005, Duft et al., 2007).

3. General Goals

The main goals of this thesis were: 1) to do an environmental characterization of levels of total mercury and different progestins in three main estuarine systems along the Portuguese coast and 2) evaluate the effects of combined multiple stressors (temperature and a mixture of drospirenone and mercury) on the gastropod *Nucella lapillus*.

3.1. Specific Objectives

Regarding the first goal, the main objectives of the work were: 1) to evaluate temporal trends of Hg accumulation in three different estuarine ecosystems (i.e., different Hg sources, water basins and estuarine characteristics) and respective trophic webs, along the coast. For that, different ecosystem compartments were analysed (abiotic - water and biotic - primary producers and macrobenthos) in three different coastal areas: Ria de Aveiro, Tagus estuary and Ria Formosa; 2) to evaluate the risk of consumption of certain seafood species (e.g., bivalves) to human health; and 3) to evaluate the ecological risk of exposure to Hg contamination. Moreover, another objective was to do a pilot study about the environmental characterization of levels of progestins in surface waters of the same estuarine systems along the Portuguese coast.

Concerning the second goal, it was evaluated the combined effects of temperature and EDCs (mercury and drospirenone) on several biological aspects of *N. lapillus*, such as survival, condition index, consumption rate, gonads maturation dynamics and oxidative stress response.

4. Thesis Outlines

This thesis is structured in two main chapters, each one composed by two sub-chapters and one general discussion. Each sub-chapter corresponds to one manuscript submitted, in preparation or under review on scientific journals.

I declare that I was totally involved in the works described in this thesis, always with the guidance of my supervisor, co-supervisors and co-authors that participated in all the works developed and described in the different chapters.

- **Chapter 2.1**
  Cardoso, PG, Morais, H, Crespo, D, Tavares, D, Pereira, E, Pardal, MA 2023. Seasonal characterization of mercury contamination along the Portuguese coast: human health and environmental risk assessment. *Environmental Science and Pollution Research 30: 101121-101132.* https://doi.org/10.1007/s11356-023-29495-5

- **Chapter 2.2**

  Morais, H, Cruzeiro, C, Pardal, MA, Cardoso, PG. 2023. Baseline progestins characterization in surface waters of three main Portuguese estuaries. *Marine Pollution Bulletin*, *194*, 115352. https://doi.org/10.1016/j.marpolbul.2023.115352

- **Chapter 3.1**

  Morais, H, Arenas, F, Cruzeiro, C, Galante-Oliveira, S, Cardoso, PG. 2023. Combined effects of climate change and environmentally relevant mixtures of endocrine disrupting compounds on the fitness and gonads' maturation dynamics of *Nucella lapillus* (Gastropoda). *Marine Pollution Bulletin*, *190*, 114841. https://doi.org/10.1016/j.marpolbul.2023.114841

- **Chapter 3.2**

  Morais H, Barreiro A, Amorim VE, Cardoso PG. Oxidative stress responses of the marine gastropod *Nucella lapillus* to increased temperature and mixtures of endocrine disruptor chemicals. (In preparation).

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Chapter 2

Environmental characterization of mercury and synthetic progestins levels in the Portuguese coast

Environmental characterization of mercury and synthetic progestins levels in the Portuguese coast

2.1. Temporal characterization of mercury contamination along the Portuguese Coast: human health and ecological risk assessment

Abstract

A seasonal characterization of mercury (Hg) accumulation in three different estuaries along the Portuguese coast (i.e., Ria de Aveiro, Tagus estuary and Ria Formosa) was done. For that, it was evaluated: 1) Hg concentrations in abiotic (water) and biotic matrices (flora and fauna); 2) the risk of consumption of local seafood species (e.g., bivalves) to human health; and 3) the environmental risk to Hg exposure. During 1 year, water and biological samples were collected during low tide, in each system for Hg quantification. Our findings revealed that total Hg concentrations in surface waters were higher in Ria de Aveiro and Tagus estuary than in Ria Formosa. In Ria de Aveiro, a particular attention should be given in autumn periods, where Hg levels ($\approx$ 100 µg L^{-1}) were considered quite high according to European quality parameters. The same was observed for the Tagus estuary during spring time. Regarding macrofauna Hg levels, no clear seasonal trend was observed. Also, total Hg concentrations in edible species (<0.5 µg. g^{-1} ww) represent no risk for consumption. However, considering the risk assessment, in Ria de Aveiro there is a moderate risk (RQ > 0.1) in autumn periods, which can be a matter of concern.

Keywords

Metal contamination, coastal areas, risk assessment, seafood consumption, edible bivalves

2.1.1 Introduction

Mercury (Hg) is a pollutant of worldwide concern due to its persistence in the environment and strong ability to bio-accumulate and magnify throughout the food web, reaching elevated concentrations in biota, such as top predators (e.g., seafood), which can threaten both ecological and human health (Zhang et al. 2012). Both inorganic and organic Hg (methylmercury) exist in the environment, being the latter the most toxic form to biota and lately to humans. Both forms can be stored in bottom sediments and water column and accumulated in the sediments. Considering that the benthic fauna is in close contact with sediments, it can be particularly vulnerable (Elliott and Quintino 2007), especially the bivalves (Cardoso et al. 2013a, Oliveira et al. 2016, Gao et al. 2016), among others.

Mercury is subject to complexation and reduction with dissolved organic matter and suspended particulate matter in the water column, affecting its speciation, bioavailability and mobility in aquatic systems (Chakraborty and Babu 2015, Chakraborty et al. 2019). All these processes may vary seasonally due to modifications in physical and biological factors, which can affect the methylmercury bioaccumulation (Diaz-Jaramillo et al. 2013). Therefore, defining temporal patterns in mercury biomagnification rates in food webs can contribute to minimize the monitoring efforts, leading to a decrease in human and ecological risk from Hg exposure (Zhang et al. 2012).

Along the Portuguese coast, Ria de Aveiro and confined areas of the Tagus estuary are historically contaminated with mercury from industrial (e.g., chlor-alkali plants) sources (Cardoso et al. 2019). Currently, despite the efforts to minimize Hg sources (by modification of the electrolysis process), historic emissions of Hg were deposited in the sediments of both systems (Canário et al. 2007, Cardoso et al. 2013b) having the potential to affect the respective trophic webs and lately the human health (Cesário et al. 2017).

In the south coast of Portugal, Ria Formosa is not a heavily industrialized area, and according to previous records, there is indication that Hg contamination in surface waters has become stable since the 1970´s (Bebianno et al. 2019). However, there are few recent records on the system (Coelho et al 2014b, Bebiano et al. 2019).

The characterization of Hg contamination and bioaccumulation in specific coastal areas/estuaries and/or biotic groups is a common topic in the literature. Nevertheless, there is a lack of information regarding a broader scenario, comparing distinct systems and food webs in a joint work. So, the main goal of the present work was to do a general temporal characterization of Hg levels in three different estuarine ecosystems (i.e., different Hg sources, water basins and estuarine characteristics) and respective trophic webs, along the coast. For that, different ecosystem compartments were analysed (abiotic – water and biotic – primary producers and macrobenthos) in three different coastal areas: Ria de Aveiro, Tagus estuary and Ria Formosa in order to: 1) evaluate bioaccumulation along the trophic web; 2) to evaluate the risk of consumption of certain seafood species (e.g., bivalves) to human health; and 3) to evaluate the ecological risk of exposure to Hg contamination.

2.1.2 Materials and Methods

2.1.2.1. Study sites

Sampling was performed in four different periods in three Portuguese estuaries along the coast (Ria de Aveiro: May (sp), July (su), November (au) 2019 and January (wi) 2020; Tagus estuary: March (sp), July (su), October (au) 2019 and February (wi) 2020; Ria Formosa: March (sp), July (su) and October (au) 2019) (Fig.1). The lack of data in some temporal points in Ria de Aveiro and Ria Formosa was due to some logistic issues related with COVID-19 constraints.

Ria de Aveiro

Ria de Aveiro is a shallow coastal lagoon located in the northwestern coast of Portugal (40º38'N, 8º45W) with a single connection to the Atlantic Ocean and covering an area of approximately 75 km^2 (Fig. 2.1.1A). It is considered one of the most Hg-contaminated systems in Europe due to continuous discharges of Hg from a chlor-alkali industry between 1950 and 1994 (Pereira et al. 1998). During this period high concentrations of Hg were deposited in sediments of the Ria and inclusive, a recent work from Coelho et al. (2014a) related the observed high suspended particulate Hg concentrations with the bottom erosion of most contaminated sediment layers.

Five sampling stations (i.e., Ovar, Torreira, Murtosa, Gafanha do Carmo, and Gafanha da Boa Hora) were selected along a transect over the coast (see Fig. 2.1.1).

Tagus estuary

Tagus estuary is the largest estuary in Portugal, and one of the biggest in Europe, covering an area of approximately 350 km^2 (Rusu and Guedes Soares 2008) (Fig. 2.1.1B). It experienced high levels of Hg contamination as consequence of past industrial activity mainly related with pyrite processing (South margin) and chlor-alkali production (North channel) (Figueres et al. 1985).

Five sampling stations were selected along the estuary, two in the North Margin (Alhandra and Trancão), two in the South margin (Seixal and Samouco) and one in the mouth (Trafaria) (see Fig. 2.1.1).

Ria Formosa

Ria Formosa is a mesotidal coastal lagoon with 180 km^2 of area, in permanent connection with the sea through six channels. Located in the South of Portugal, represents the largest lagoon of the Portuguese coast (Said et al. 2019) (Fig. 2.1.1C).

Five sampling stations were selected along the lagoon (i.e., Aeroporto de Faro, Faro, Olhão, Fuseta, and Tavira) (Fig. 2.1.1).

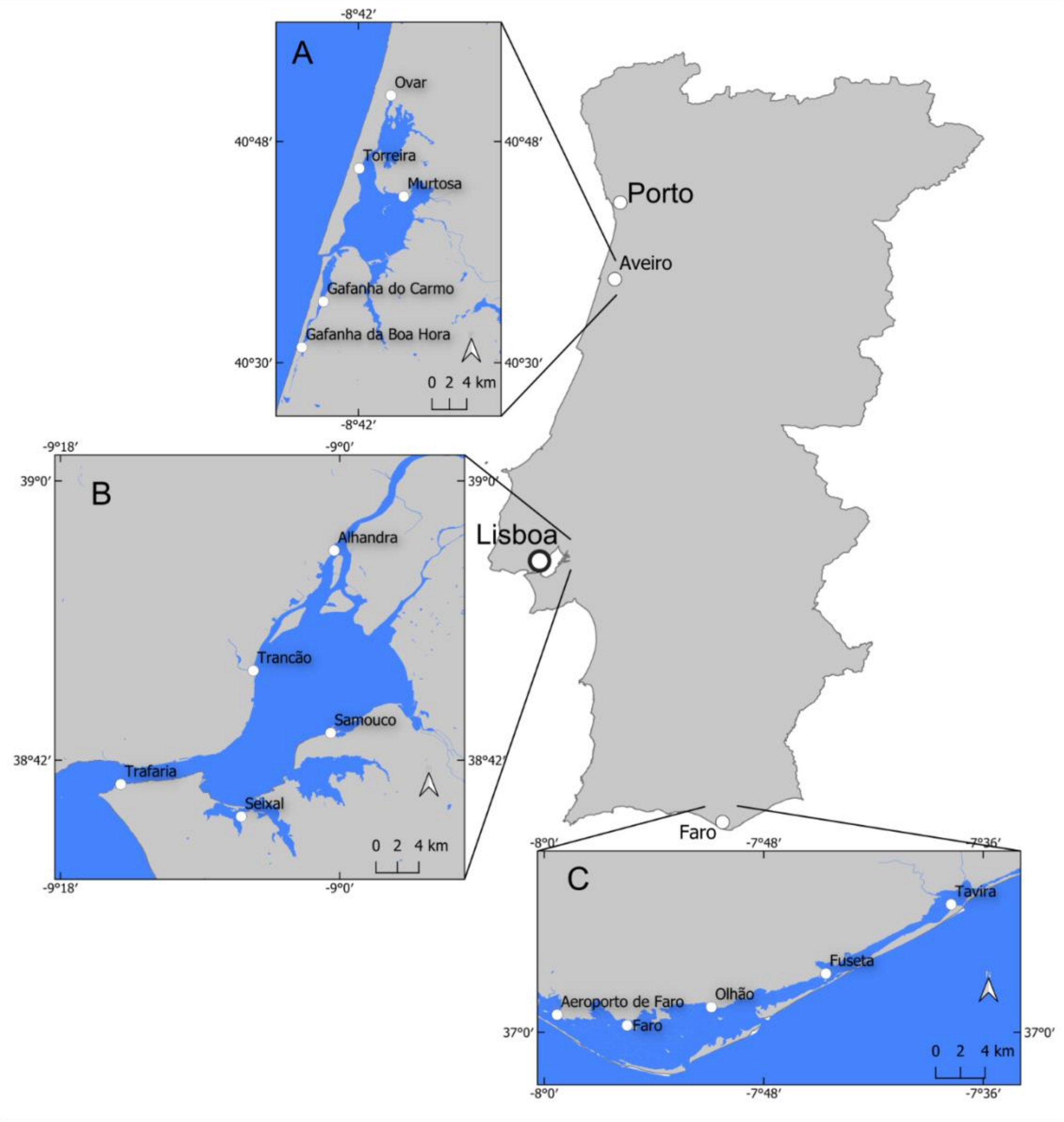


Figure 2.1.1 – Location of the study sites along the Portuguese coast: A) Ria de Aveiro; B) Tagus estuary and C) Ria Formosa.

2.1.2.2. Sampling procedure

At each coastal system 5 sampling stations were selected (mentioned above) for water collection during low tide and, of those, 3 were also sampled for biota. At each sampling station and occasion, *in situ* measurements of water temperature, salinity, dissolved oxygen, and pH were taken (multiparameter meter HI 98194).

Parallel to the macrobenthic sampling, water from the low intertidal water pools was also collected, in glass bottles (n = 3), for analysis of total dissolved Hg.
Macrobenthic samples were collected with a core (141 cm^2 surface area) to a depth of 20 cm and plants were collected by hand during low tide. Both macrobenthic samples and plants (i.e., different macroalgae, such as *Ulva* sp, *Gracilaria* sp and *Fucus* sp.) and the seagrass *Zostera noltii*) were washed *in situ* through a 500 µm mesh sieve bag and then placed into plastic bags and transported in a cool box. In the laboratory, organisms were separated in main species and kept in filtered-seawater (adjusted to field salinity) for 24 h in order to eliminate sediment particles. Afterwards, they were separated under a dissecting microscope, identified to the lowest possible taxon, frozen (-20ºC) and posteriorly freeze-dried for mercury analyses.
The primary producers, mentioned above, were washed to eliminate sediment particles and separated in different organs: roots, stem and leaves. Posteriorly, they were freeze-dried for later Hg analyses.

2.1.3. Mercury quantification

2.1.3.1. Water

Water samples were filtered through 0.45 µm pore size Millipore cellulose filters and acidified with concentrated nitric acid (HNO_3 65%) ‘‘mercury free’’ to pH < 2, as in Sturgeon et al. (1987). Samples were stored in borosilicate glass bottles (according to Sturgeon et al. 1987, there are lower Hg adsorptive losses than in other materials) and maintained in a refrigerated room at 4 ºC. Total dissolved Hg analysis in water samples was performed by cold vapour atomic fluorescence spectroscopy (CV-AFS), on a PSA Merlin atomic fluorescence spectroscope coupled with a cold vapour generator, model 10.023, associated with a Merlin PSA detector, model 10.003, and using tin chloride ($SnCl_2$ 2% m/v in HCl 10 % v/v) as reducing agent. The Hg (II) concentration in water samples was quantified through a calibration curve ($r^2 \geq 0.999$) of five standards (0.0 to 100 ng L^{-1}) prepared by dilution from the certified standard stock solution of mercury nitrate (998 ± 2 mg L^{-1}) in a HNO_3 solution (2% v/v). For quality control, triplicate samples, blanks and standards were analysed in the sample batch. The detection limit of the method is 1.6 ng L^{-1} and the precision and accuracy, expressed respectively, as relative standard deviation and relative error were <5%. This analytical methodology is highly sensitive, allowing the measurement of 1 ng L^{-1} of mercury (Mucci et al. 1995).

2.1.3.2. Biota

For total mercury quantification in plants and fauna, freeze-dried samples were analysed in triplicate by thermal decomposition atomic absorption spectrometry with gold amalgamation, using a LECO AMA-254 (Advanced Mercury Analyzer). The detection limit was 0.01 ng. Analytical quality control was performed using Certified Reference Materials (CRMs). For the plants, ERM CD200 was used, while TORT-3 was used on the fauna. The values obtained for the whole CRM analysis ranged from 99–150% for the plants and 80–112% for the fauna (at 0.05 significance level). Analyses of CRMs were always performed in triplicate and coefficient of variation was lower than 10% for all the analyses.
A summary table of all the species found in the three estuarine systems can be seen in supplementary material (table S4).

2.1.4. Risk assessment

2.1.4.1. Health risk

The human health risk associated to the consumption of edible bivalves was assessed through the estimation of the hazard quotient (HQ), which is used to estimate the non-carcinogenic effects of Hg through food ingestion. It is calculated by the ratio between the estimated daily intake (EDI) and the reference risk dose (RfD) of Hg (Eq. 1):

$$HQ = EDI/RfD \qquad (1)$$

EDI (ug kg^{-1} bw/day) was calculated with the following equation (Vinceti et al. 2014):

$$EDI = [C \times AvC]/bw \qquad (2)$$

Where C (μg g^{-1} ww of bivalve) is the mean Hg concentration in the bivalve tissue, AvC is the average consumption of bivalves per day, and bw is the average body weight. The EDI values were compared to the established values of reference doses (RfD), 0.1 μg g^{-1} wet weight of fish for Hg (US-EPA, 2017).

For the calculation of EDI we have converted the total Hg concentration in methylmercury (MeHg), assuming that the proportion of MeHg in the total Hg body burden is higher than 80% (Andersen and Depledge 1997). So, a fraction of 90% of MeHg was considered for all the samples, as in Costa et al. (2020).

HQ < 1 indicate no risk in terms of health effects, while HQ > 1 indicates high probability for long-term health effects (Copat et al., 2012).

Regarding AvC, it was considered an average of 6.8 g d^{-1} of bivalves consumed per capita per day, according to Anacleto et al. (2014).

The mean body weight of Portuguese population was fixed on 70 kg, considering that the average weight in the European population is about 70 kg (Stevens et al. 2012).

2.1.4.2. Ecological risk

For the characterization of the potential risk of a toxic pollutant it was used the index risk quotient (RQ), calculated by equation 3:

$$RQ = Ce/PNEC \qquad (3)$$

Where Ce (ugL^{-1}) is the environmental concentration in surface waters and PNEC is the predicted no- effect concentration.

In the present study, it was decided to use the PNEC value (0.39 μgL^{-1}) for Hg(II) in the aqueous phase, calculated by Du et al. (2015).

According to Tulcan et al. (2021) values higher than 1 represent a high risk, values below 0.1 represent a low risk, and values between 0.1–1 represent moderate risks.

2.1.5. Data analysis

One-way (factor: season) analysis of variance (ANOVA) on ranks were applied to assess statistical differences in total Hg concentrations in surface waters and biota for each ecosystem, separately. All data were previously checked for normality using the Kolmogorov–Smirnov test and for homogeneity of variances using the Levene's test (Zar, 1999). These analyses were done using Statistica 7 software.

Consumption risk assessment was evaluated for the general population, according to the method described in Vinceti et al. (2014).

Biomagnification factors (BMFs) were calculated for selected prey-predator scenarios as: BMF = Metal Predator / Metal Prey where Metal Predator and Metal Prey are the concentrations of mercury in micrograms per gram wet weight of the predator and prey, respectively (adapted from Hoekstra et al., 2003). For invertebrates and green macroalgae (Ulva sp.), trace element concentrations of total body homogenates were used for calculations. Prey-predator relationships were defined based on the information from Cardoso et al. (2014). In addition, this information was complemented with information from WoRMS database (www.marinespecies.org).

2.1.6. Results

2.1.6.1. Physicochemical characterization and total dissolved mercury

A physicochemical characterization of each ecosystem was done and the results can be seen in the supplementary material (tables S1-S3).

Total dissolved Hg concentrations were generally higher in Ria de Aveiro (22.2 ± 38 ngL^{-1}) than in Tagus estuary (14.9 ± 17.7 ngL^{-1}) and Ria Formosa (10.3 ± 6.7 ngL^{-1}). In Ria de Aveiro, the highest concentrations were recorded in Torreira (127.3 ± 7.9 ngL^{-1}), Gafanha do Carmo (132.6 ± 9.2 ngL^{-1}) and Murtosa (41.6 ± 5.2 ngL^{-1}) and there was a significant temporal variation with higher values during autumn campaigns than in other periods, in all the study areas (1-Way ANOVA on ranks, $F = 14.9$, $p < 0.05$) (Fig. 2.1.2A).

For the Tagus estuary, the highest concentrations were observed in Alhandra (41.6 ± 7.3 ngL^{-1}) and Seixal (72.2 ± 0.5 ngL^{-1}) during spring period (1-Way ANOVA on ranks, $F = 6.8$, $p < 0.05$) (Fig. 2.1.2B).

Ria Formosa was the aquatic system with the lowest Hg concentrations (< 20 ngL^{-1}) being significantly higher during spring period (1-Way ANOVA on ranks, $F = 3.7$, $p < 0.05$) (Fig. 2.1.2C).

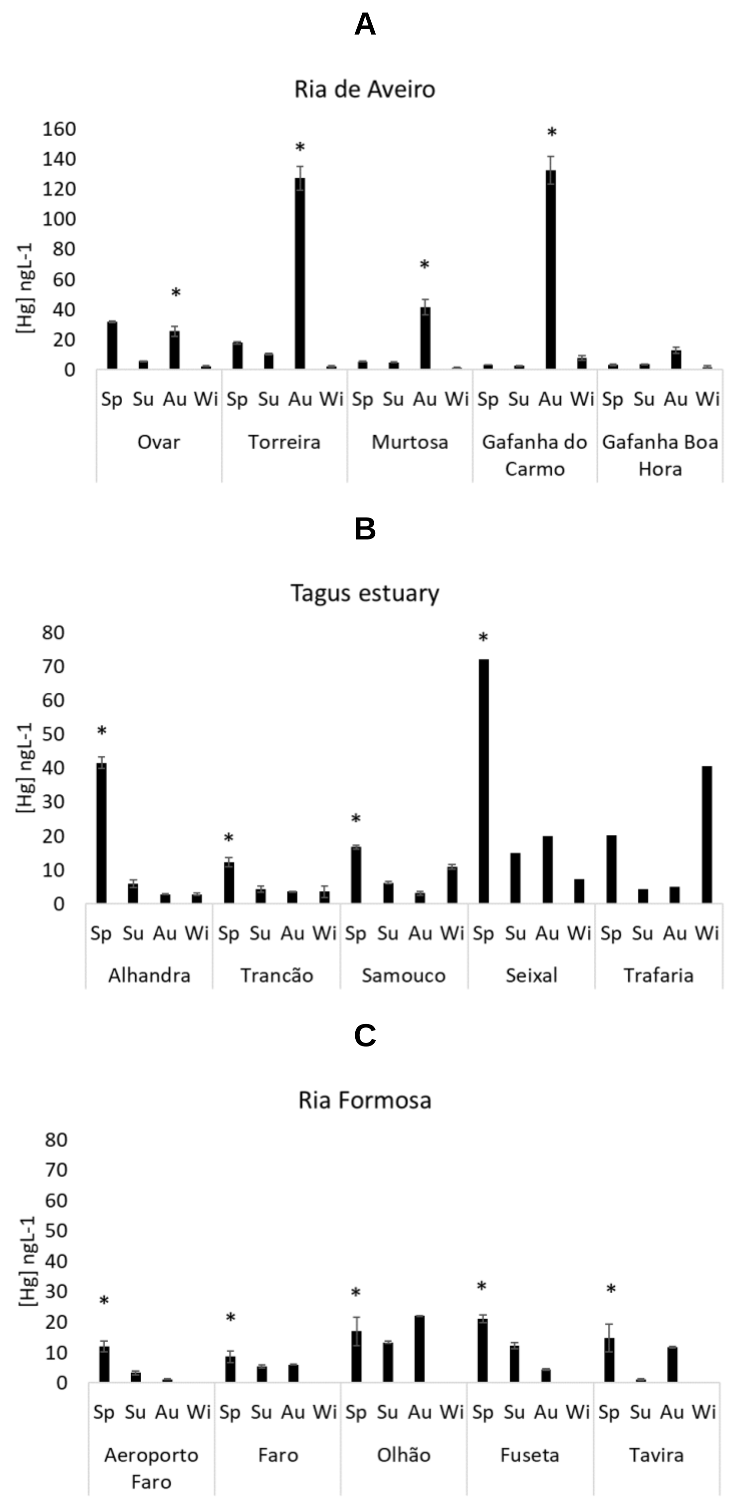


Figure 2.1.2– Total dissolved mercury concentrations (ngL^{-1}) in surface waters of the three study sites. * Indicates significant differences among sites. Sp - spring, Su-summer, Au – autumn, Wi – winter.

2.1.6.2. Flora and Fauna

The diversity of the macroalgal and seagrass species, associated to the macrobenthic community, was similar in the three coastal systems. The commonly found species were: *Ulva* sp, *Gracilaria* sp, *Fucus* sp and the seagrass *Zostera noltii*.

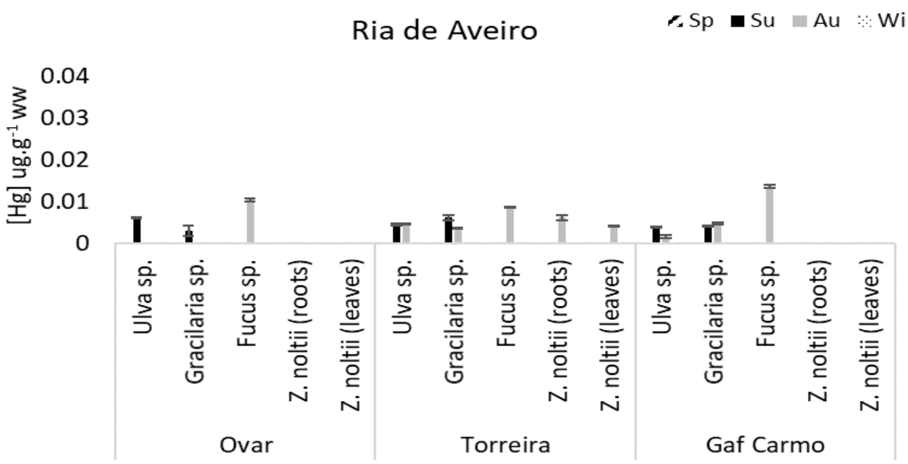
The average total Hg concentration in the Tagus estuary species ($\approx$ 0.014 µg g^{-1}) was clearly higher than in the other two systems ($\approx$ 0.006 µg g^{-1}). The same pattern was observed for the macrobenthic communities, having higher mean Hg concentrations in the Tagus estuary ($\approx$ 0.05 µg g^{-1}) than in Ria de Aveiro and Ria Formosa ($\approx$ 0.03 µg g^{-1}) (Figs. 2.1.3-2.1.5).

The macrobenthic species collected in the three systems were similar (e.g., *Carcinus maenas*, *Hediste diversicolor*, *Scrobicularia plana*, *Ruditapes decussatus*, *Littorina littorea*, *Peringea ulvae*, *Cerastoderma edule*) despite in Ria Formosa were identified some particular species (e.g., *Nassarius* sp., *Haloa* sp., *Pagurus bernhardus*, *Gibbula* sp.), typical of that system.

A resume table of the list of species found in each estuarine system can be seen in supplementary material (tables S4-S6).

In a temporal perspective, there were significant differences in the Hg concentrations in the plants of Ria de Aveiro between summer and autumn periods (1-Way ANOVA on ranks, $F = 4.76$, $p < 0.05$) (Fig. 2.1.3A). For fauna no significant differences between sampling periods were observed.

A



Season	1	2
Su	**a**	
Au		**b**

B

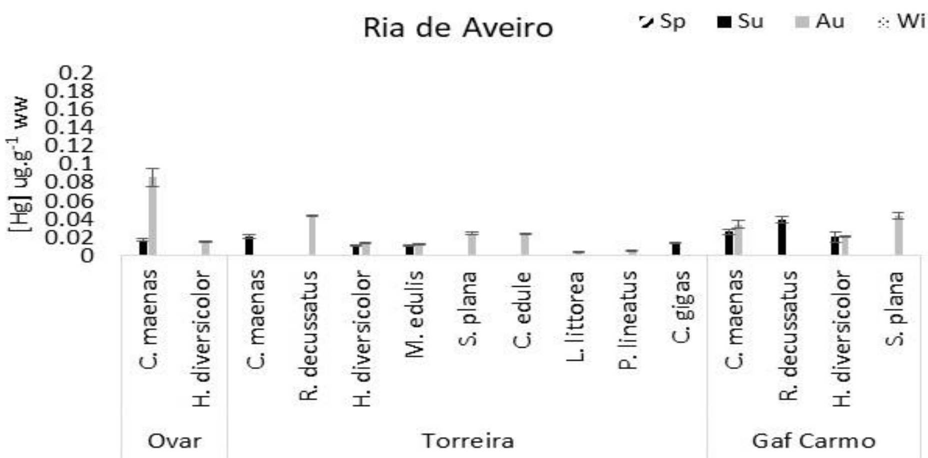
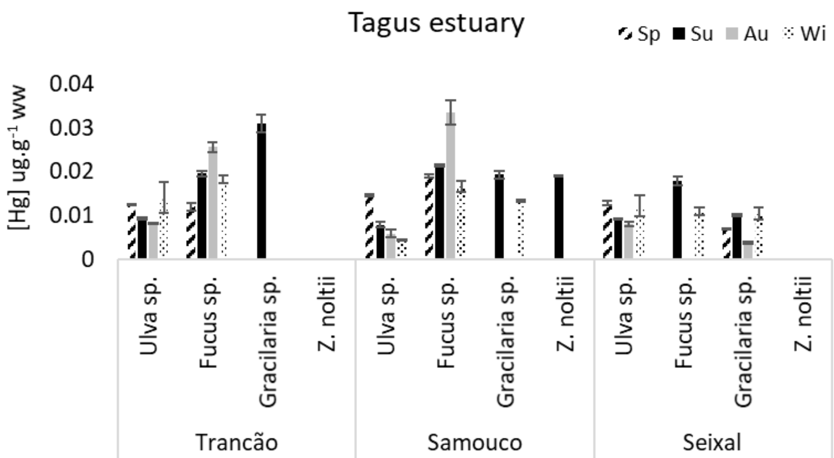


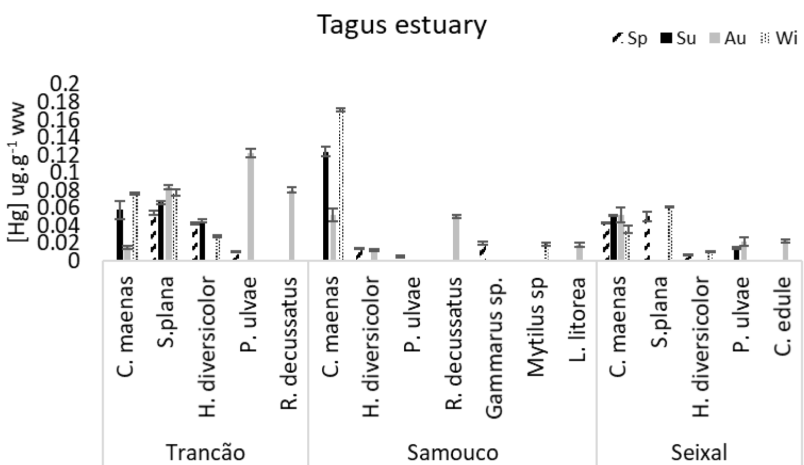
Figure 2.1.3 – Total mercury concentrations (µg g^{-1} ww) in the flora (A) and fauna (B) of Ria de Aveiro. The table close to the figure represents the statistical differences (indicated by different letters) between seasons for the plants' community.

For the Tagus estuary, there were significant differences in the Hg concentrations of the macrobenthic species between seasonal periods. In general, mean Hg values in spring periods were lower than in summer and winter periods (1-Way ANOVA on ranks, $F = 3.27$, $p < 0.05$) (Fig. 2.1.4B).

A



B

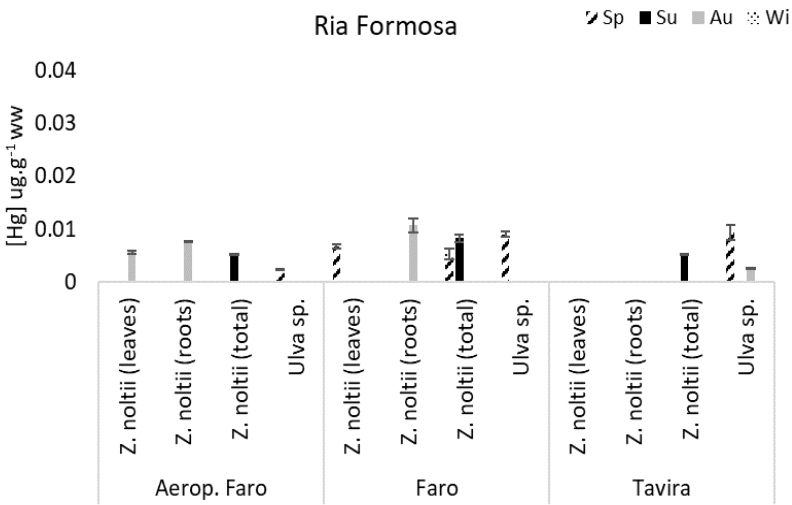


Season	1	2
Sp		b
Su	a	b
Au	a	
Wi	a	

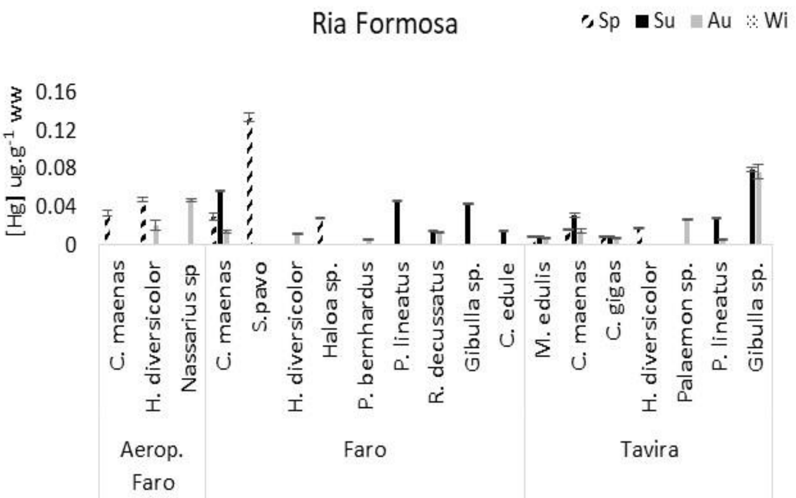
Figure 2.1.4 – Total mercury concentrations (µg g^{-1} ww) in the flora (A) and fauna (B) of Tagus estuary. The table close to the figure represents the statistical differences (indicated by different letters) between seasons for the macrofauna community.

In Ria Formosa, the Hg concentrations in the macrobenthic community were significantly higher in spring and summer periods than in autumn (1-Way ANOVA on ranks, $F = 6.87$, $p < 0.05$) (Fig. 2.1.5B).

A



B



Season	**1**	**2**
Sp	**a**	
Su	**a**	
Au		**b**

Figure 2.1.5 – Total mercury concentrations (µg g^{-1} ww) in the flora (A) and fauna (B) of Ria Formosa. The table close to the figure represents the statistical differences (indicated by different letters) between seasons for the macrofauna community.

2.1.6.3. Biomagnification factors (BMFs)

The bioaccumulation analysis was restricted to the data available for the trophic webs of the different estuarine systems, since it was just sampled the macrobenthic community. Based in our results, there is a Hg biomagnification from the lower to the higher trophic levels, which can be seen by the BMF >1.

In Ria de Aveiro, the BMFs ranged between 1 and 5.4. The highest value corresponded to Hg transfer from *H. diversicolor* to *C. maenas* in Ovar in the autumn period (Table 2.1.1).

Table 2.1.1 - Biomagnification factors (BMF) of mercury in the Ria de Aveiro estuarine food web. Sp – spring; Su – summer; Au-autumn; Wi – winter; *C. maenas – Carcinus maenas; H. diversicolor – Hediste diversicolor; L. littorea – Littorina littorea; P. lineatus – Phorcus lineatus; Ulva sp.*

(predators/grazers) (preys)	***C. maenas*** ***H. diversicolor*** **Sp/Su/Au/Wi**	***L. littorea*** ***Ulva* sp.** **Sp/Su/Au/Wi**	***P. lineatus*** ***Ulva* sp.** **Sp/Su/Au/Wi**
Ovar	-/-/5.4/-	-	-
Torreira	-/1.89/-/-	-/-/1.03/-	-/-/1.18/-
Gafanha do Carmo	-/1.29/1.59/-	-	-

In the Tagus estuary, BMFs ranged between 0.3 and 14.9. The highest values corresponded to the highest trophic levels, with exception of the Hg transfer from *Ulva* sp. to *P. ulvae* in Trancão in the autumn period that reached a BMF ≈15 (Table 2.1.2).

Table 2.1.2 - Biomagnification factors (BMF) of mercury in the Tagus estuary food web. Sp – spring; Su – summer; Au-autumn; Wi – winter; *C. maenas – Carcinus maenas; H. diversicolor – Hediste diversicolor; L. littorea – Littorina littorea; P. ulvae – Peringea ulvae; Gammarus sp.; Ulva sp.*

(predators/grazers) (preys)	***C. maenas* *H. diversicolor* Sp/Su/Au/Wi**	***P. ulvae* *Ulva* sp. Sp/Su/Au/Wi**	***Gammarus* sp. *Ulva* sp. Sp/Su/Au/Wi**	***L. littorea* *Ulva* sp. Sp/Su/Au/Wi**
Trancão	-/1.27/-/2.72	0.92/-/14.85/-	-	-
Samouco	-/-/4.25/-	0.36/-/-/-	1.37/-/-/-	-/-/3.12/-
Seixal	6.5/-/-/3.3	-/1.63/2.76/-	-	-

In Ria Formosa, BMFs varied between 0.7 and 30.3 and curiously the highest values were observed for the grazers *P. lineatus* and *Gibbula* sp in Tavira in summer and autumn months (Table 2.1.3).

Generally, the highest BMF corresponded to the highest contaminated sites.

Table 2.1.3 - Biomagnification factors (BMF) of mercury in the Ria Formosa food web. Sp – spring; Su – summer; Au-autumn; Wi – winter; *C. maenas – Carcinus maenas; H. diversicolor – Hediste diversicolor; P. lineatus – Phorcus lineatus; Haloa sp; Gibbula sp.; Ulva sp.*

(predators/grazers) (preys)	***C. maenas* *H. diversicolor* Sp/Su/Au/Wi**	***P. lineatus* *Ulva* sp. Sp/Su/Au/Wi**	***Gibbula* sp. *Ulva* sp. Sp/Su/Au/Wi**	***Haloa* sp. *Ulva* sp. Sp/Su/Au/Wi**
Aeroporto Faro	0.7/-/-/-	-	-	-
Faro	-/-/1.19/-	-	-	3.17/-/-/-
Tavira	0.9/-/-/-	-/4.84/2.66/-	-/13.37/30.3/-	-

2.1.6.4. Human health risk assessment and Ecological risk assessment

Since HQ was always lower than 1 for all the coastal systems, the health risks for the general population associated to the consumption of bivalves from the three studied systems are reduced. No significant seasonal variations were observed for the three studied areas.

Considering the environmental concentrations of total Hg in surface waters and the PNEC value for inorganic Hg, the RQ values obtained for the different aquatic systems represented low risk for the respective trophic chains, except during autumn in Ria de Aveiro where the highest concentrations in water were found, and which could be classified as moderate risk (RQ = 0.17).

2.1.7. Discussion

The present work allowed to see that Ria de Aveiro recorded the highest average values of total dissolved Hg in surface waters, even considering study areas located apart from the Laranjo basin, which is considered the most contaminated area in the lagoon (Pereira et al. 2009). For example, in Torreira and Gafanha do Carmo were registered values (≈ 100 ng L^{-1}) similar to the ones observed in the most inner area of Laranjo (Cardoso et al. 2014). The highest concentrations were observed during autumn period while in the previous study from Cardoso et al. (2014), the highest values were observed during the summer sampling.

In Tagus estuary, the highest concentrations of dissolved Hg were observed during spring sampling, in Seixal (≈70 ng L^{-1}) and Alhandra (≈ 40 ng L^{-1}), presenting higher values than in a previous study from Cesário et al. (2018). In the latter study, the highest values recorded in Seixal were in the range of 40-50 ng L^{-1} and in Alhandra ranged between 12-24 ng L^{-1}. These differences can be justified based on the sampling conditions, since in our study the water samples were collected during low tide while in Cesário et al. (2018), were collected in flooding or ebbing tide which means that the Hg is more diluted.

Lastly, the Ria Formosa, is the least contaminated system reaching the highest values (≈ 20 ng L^{-1}) in Olhão during spring sampling. There are very few studies addressing metals contamination in the Ria Formosa. A recent work from

Bebianno et al. (2019) only refers that Hg trends in the Ria Formosa water are stable since the 1970s, with no indication of values. A previous work from Coelho et al. (2014b), indicated values of 30-40 ng L^{-1} in the region of Faro (late autumn), which is in accordance with our results.

Comparing the results observed in the three estuarine systems with the environmental quality standards (EQS) (according to the Water Framework Directive), it is possible to highlight that in two sites of Ria de Aveiro (i.e., Torreira and Gafanha do Carmo) and in Tagus estuary (Seixal), during autumn and spring periods respectively, the values were above the EQS established for mercury (70 ng L^{-1}) in surface waters. Also, according to the ecotoxicological assessment criteria (EAC), total dissolved Hg levels in those sites were much higher than the EAC threshold (> 50 ng L^{-1}). This means that these areas, in those particular periods can be more problematic and constitute a matter of concern. In fact, despite the efforts to reduce the Hg sources in the last decades, the Hg accumulated through the years in the sediments of the different estuaries, can still be more or less available to the ecosystem depending on physical disturbance (e.g., dredging), or weather events (e.g., increase of temperature or flooding events) which can lead to sediment re-suspension and enhance metal mobilization from the sediments (Coelho et al. 2014a). However, in Ria the Aveiro the last Hg records (Oliveira et al. 2018, 2022) reveal an ongoing natural recovery of the most contaminated area of Aveiro lagoon associated with natural attenuation by plants, leaching of sediments and through deepening of most contaminated sediments due to the natural sedimentation rates. No signs of any kind of dredging activities in the system were recorded. In the Tagus estuary, even after the end of Hg inputs in the system, the contamination still persists and can spread along the estuary. External factors (e.g., low pH, high organic matter, low dissolved oxygen) can affect Hg methylation in estuarine environments facilitating its spread (Couto and Ribeiro 2022).

Regarding the Hg levels accumulated in flora and fauna of the three systems, the Tagus estuary was the one that, generally, revealed higher Hg concentrations than Ria de Aveiro and Ria Formosa. This can be related to the Hg concentrations in the sediments of respective areas. Since most of the collected species are benthonic, they are in closer association with the sediment than with the water column. In this study the sediments were not analysed, but by comparing with previous works, the total Hg in sediments of the regions of Trancão and Seixal presented higher concentrations (0.2-1.5 µg g^{-1}) (Canário et al. 2005) than those in Ria de Aveiro (e.g., Torreira - <1 µg g^{-1}) (Pereira et al. 2009) or Ria Formosa (0.05-0.1 µg g^{-1}) (Coelho et al. 2014b). However, the Hg concentrations found in the macrofauna, inclusive in the edible bivalves, of the three systems were considered low, with values far below the legislation values (0.5 µg g^{-1} ww) established by the European food safety legislation (Commission Regulation 2006).

In a temporal scale, we could observe some significant differences between sampling periods particularly for the macrobenthic community in Tagus and Ria Formosa, but there is not an evident pattern common to all the systems. For example, in Ria Formosa, total Hg concentrations were higher in spring/summer periods than in autumn sampling. Nonetheless, in Ria de Aveiro were not detected seasonal differences. Also, in a previous study from Cardoso et al. (2014), in Ria de Aveiro were not observed clear temporal differences in the Hg content of the studied macrobenthic species and this can be explained based in part on the life span of these species. Since they are long-lived species (i.e., they can live from months to years) and most of the individuals collected were adults (e.g., *S. plana* ≈ 2-3 years; *C. maenas* ≈ 1-2 years; *P. ulvae* ≈ 15-20 months) (Verdelhos et al. 2005, Baeta et al. 2005, Cardoso et al. 2002) they will incorporate Hg in a cumulative way during their life, so it is not dependent on a seasonal variation. This pattern is in part in agreement with the results obtained by Diaz-Jaramillo et al. (2013) for the macroinvertebrates regarding total Hg concentrations. For example, according to the latter study, just in one site were found significantly higher concentrations of total Hg in the summer relative to winter for some of the studied taxa.

However, Gao et al. (2022) found seasonal differences in Hg accumulation in the food web in the coastal waters of Jiangsu (China). According to their findings, Hg concentrations were higher in summer than in spring and autumn. And, the justification for this pattern could be related with the methylation process that tends to be higher in warmer months, favouring the accumulation of the metal in the trophic web. Yet, the difference found between systems can be related to the composition of the trophic web and the food availability that can change seasonally, as well as its Hg content. For example, if a trophic web is constituted mainly by deposit feeders that are in straight connection with the sediment, which is the case of Ria de Aveiro, its Hg content will not change too much along the time. But if the trophic web is more diverse, like in Ria Formosa, and is constituted by different trophic groups including suspension feeders, herbivores, etc, this means that the Hg sources are different (e.g., suspended particulate matter, microalgae, macroalgae), and its content can vary more seasonally, which will influence the entire trophic web. For example, Diaz-Jaramillo et al. (2013) and Reichmuth et al. (2010) observed that in different species of crabs the accumulation of metals was highly variable and often followed environmental concentrations. Also, in the present study, the crab *Carcinus maenas*, presented oscillations in its Hg concentrations according to the time of the year.

Concerning the Hg biomagnification through the macrobenthic community, in general the highest BMF values were related to higher trophic levels, like the predator *C. maenas* and its prey *H. diversicolor* (omnivore). While for lower trophic levels, such as for example, between the green macroalga *Ulva* sp. and the herbivore *P. ulvae* the Hg transfer was lower. This pattern was common for Ria de Aveiro and Tagus estuary and it is corroborated by previous studies (Cardoso et al. 2014). However, for Ria Formosa, the BMF values were quite opposite, since for higher trophic levels there was a Hg biodilution and not biomagnification. This can be related to the fact that the crabs *C. maenas* collected at Ria Formosa during field campaigns belonged to class 1+ (around 30 mm width) (Baeta et al. 2005), which means that they were young individuals, so the accumulation of Hg was lower. Attending that Hg tends to increase with size/age, this can explain some of the results obtained. Also, biodilution of Hg in the macrofauna can be a result of higher organic matter and detritus levels and growth dilution across different trophic levels (Kidd et al 2012). On the other hand, the higher values obtained in lower trophic levels can be associated to a higher primary productivity, namely production of microalgae, since the species studied are also grazers. Also, the Ria Formosa being a system with warmer waters can be susceptible of higher methylation rates and higher Hg availability to superior trophic levels.

Considering the analysis of risk assessment to the edible bivalve community found in the three ecosystems, the hazard quotients (HQ) were always lower than 1 which means that there is no risk in terms of health effects (Copat et al. 2012). In fact, the concentrations of total Hg found in the biota were quite low, so they do not represent a concern to the human health. On the other hand, the ecological risk assessment highlighted that the high Hg values in surface waters of Ria de Aveiro could have medium risks for aquatic species in specific sites and periods of the year (i.e., autumn periods).

2.1.8. Conclusions

Our findings demonstrated a clear seasonal trend in Hg concentrations in the water column that can be variable according to the system. Ria de Aveiro and Tagus estuary presented the highest Hg concentrations. In some particular sites of Ria de Aveiro, even far from the most contaminated area of Laranjo, still present quite high Hg values in certain periods of the year.

For the macrofauna species (in particular the bivalves), there is no concern relative to their Hg accumulation levels and possible health effects. But, in those particular sites with high dissolved Hg values, there is a medium risk for the aquatic species in certain periods of the year.

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2.2. Baseline progestins characterization in surface waters of three main Portuguese estuaries

Abstract

Synthetic progestins are micropollutants of special concern, due to their growing use in human and veterinary therapies and their risks to aquatic life. Currently, there is a lack of environmental information on these compounds, worldwide. The main objective of this work was to characterize the levels of the most consumed progestins in Portugal. For that, Ria de Aveiro, Tagus estuary and Ria Formosa were sampled in a temporal perspective to evaluate levels of drospirenone (DRO), desogestrel (DSG), gestodene (GST) and levonorgestrel (LNG). Drospirenone and desogestrel were the most abundant progestins. In the North of Portugal, DSG was the most abundant (Aveiro: 193.9 ng L^{-1} in summer), while DRO was more representative in the South (Tagus: 178.9 ng L^{-1}; Formosa: 125.7 ng L^{-1}) and also in summer. These spatial differences can be associated with the hydrodynamics of each estuarine system as well as the distinct population and tourist levels associated with each site.

Keywords: Water quality, progestogens, coastal areas, synthetic hormones, pollution

2.2.1. Introduction

Coastal ecosystems face an ongoing and significant threat from a variety of stressors, and among them, the contamination by endocrine-disrupting chemicals (EDCs) emerges as a major concern for aquatic environments. EDCs pose a substantial risk to the health and integrity of these ecosystems. Many of them, such as the synthetic progestins are active ingredients used in women's contraceptives but also in other hormonal preparations (Zeilinger et al. 2009, Fent 2015). In the last two decades, different generations of progestins have been produced, being the last ones (e.g., drospirenone) designed for better progestogenic action and less androgenic activity to prevent side-effects (Sitruk-Ware and Nath 2010). As a consequence of the increase of growing population, the consumption of these compounds tends to increase, by approximately 13% in the next five years, according to the “Progesterone Market” (Intelligence, 2023). Some records from European countries reveal large amounts of progestins being consumed. For example, the total annual consumption of progestins in France and the United Kingdom is estimated to be about 12.800 and 1.700 kg year^{-1}, respectively (Besse and Garric 2009, Runnals et al. 2010) and in the Czech Republic 2.400 kg year^{-1} (Golovko et al. 2018b). Most of the progestins’ residues reach the aquatic systems through wastewater treatment plants (WWTPs) whose treatments are not efficient to retain these compounds (Fent 2015, Sauer et al. 2018, Wang et al 2021). In the literature, many studies are advertising for the risks caused by progestins, particularly the oldest ones, in the aquatic species too (Zeilinger et al. 2009, Runnalls et al. 2013, Zhao et al. 2015a,b, Cardoso et al. 2018). Despite recent studies testing the effects of new-generation progestins admitting that they show lower activity than the previous ones (Schmid et al. 2020), it is extremely relevant to evaluate the levels of progestogens that are discharged into the aquatic systems that can affect non-target species. Synthetic progestins occur usually in the range of ng L^{-1} and can vary depending on the country. There are some studies worldwide evaluating the environmental concentrations of progestins. For example, in Germany, a study from Weizel et al. (2018), registered concentrations of dienogest of up to 2.3 ng L^{-1} in surface waters in rivers. While in the Czech Republic, Golovko et al. (2018a) detected concentrations in surface waters below 1 ng L^{-1} for the different studied progestins. The highest concentrations were observed in the influent of some WWTPs that reached up to 100 ng L^{-1} (e.g., in the case of progesterone).

In Portugal, due to demographic differences between regions (local and touristic and/or fertile female population) and different average river flow, it is expected:

- A constant and high progestin concentration, over the year, in the Tagus River and Ria Aveiro due to high population densities;
- A high seasonal touristic peak in the Ria Formosa;

So, the main goal of this work was to do a pilot study on a temporal characterization of the levels of most prescribed progestins in Portugal (i.e.,, drospirenone (DRO), desogestrel (DSG), gestodene (GST) and levonorgestrel (LNG), based on the list of names of contraceptives authorized for each EU member state, according to the Directive 2001/83/CE), on three main estuarine systems along the coast.

2.2.2 Material and methods

2.2.2.1. Studied areas and sampling procedure

Sampling was performed in four sampling periods in three Portuguese estuaries along the coast (Ria de Aveiro: May (sp-spring), July (su-summer), November (au-autumn) 2019 and January (wi-winter) 2020; Tagus estuary: March (sp), July (su), October (au) 2019 and February (wi) 2020; Ria Formosa: March (sp), July (su) and October (au) 2019) (Fig.1). These estuaries are subjected to different stressors and their catchment areas are occupied by distinct population clusters. The lack of data in some temporal samples in Ria de Aveiro and Ria Formosa was due to some logistic constraints related to the COVID-19 pandemic.

Ria de Aveiro: is a shallow coastal lagoon located on the northwestern coast of Portugal (40º38'N, 8º45W) with a single connection to the Atlantic Ocean and covering an area of approximately 75 km^2 (Fig. 2.1.1A). The catchment area drained by the lagoon is densely populated, summing-up to approximately 370.000 inhabitants (Fidelis et al. 2019). Over the last decades, population pressure, industrialization, pollution issues related to diffuse source pollution and wastewater treatment plants, industrial activities and sediment contamination, especially with heavy metals, such as mercury have increased (Cardoso et al. 2014; Stoichev et al. 2019). The Aveiro region has three main WWTPs (Cacia, Ilhavo and S. Jacinto). Cacia is the one with the highest capacity to receive effluents from 272K inhabitants (https://www.aguasdocentrolitoral.pt/aveiro/); Ilhavo is prepared to serve a population of 160K inhabitants (https://www.adp.pt/pt/?id=61&img=90&bl=6) and S. Jacinto is the smallest (9.4K inh.) (http://www.simria.pt/gca/popup_2.php?id=97).

Five sampling stations (i.e.,, Ovar, Torreira, Murtosa, Gafanha do Carmo, and Gafanha da Boa Hora) were selected along a transect over the coast (see Fig. 2.2.1).

Tagus estuary: is the largest estuary in Portugal, and one of the biggest in Europe, covering an area of approximately 350 km^2 (Dias et al. 2013) (Fig. 2.1.1B). It is surrounded by the large metropolitan area of Lisbon with a population of approximately 3 million and it is the largest urban centre at the Atlantic coast of Europe (Vaz et al. 2020). It experienced the deposition of several pollutants for decades due to local industries. The Lisbon region comprises many WWTPs. The most relevant ones that serve population close to our study sites are: Alverca (serves 153K inh. https://www.aguasdotejoatlantico.adp.pt/content/alverca); Beirolas (213K inh., https://www.aguasdotejoatlantico.adp.pt/content/beirolas); Alcântara (756K inh., https://www.aguasdotejoatlantico.adp.pt/content/alcantara); Afonsoeiro (48K inh.); Seixal (156K inh.) and Seixalinho (64.5K inh., https://www.simarsul.adp.pt/content/infraestruturas-0).

Five sampling stations were selected along the estuary, two in the North Margin (Alhandra and Trancão), two in the South margin (Seixal and Samouco) and one in the mouth (Trafaria) (see Fig. 2.2.1).

Ria Formosa: is a mesotidal coastal lagoon with 180 km^2 of area, in permanent connection with the sea through six channels. Located in the South of Portugal, represents the largest lagoon of the Portuguese coast (Said et al. 2019) (Fig. 2.2.1C). Ria Formosa is located in the region of Algarve which is an area whose population varies considerably between summer and winter months (i.e., three times more in the summer). Regarding the main WWTPs in the region, we can highlight, Albufeira-Poente (133K inh., https://www.aguasdoalgarve.pt/content/etar-de-albufeira-poente), Faro-Olhão (113K inh., https://www.aguasdoalgarve.pt/content/etar-de-faroolhao), Almargem (serves the population of Tavira and surroundings, 48K inh., https://www.aguasdoalgarve.pt/content/etar-de-almargem-0), Vila Real de Sto

António (58K inh., https://www.aguasdoalgarve.pt/content/etar-de-vila-real-de-santo-antonio).

Five sampling stations were selected along the lagoon (i.e., Aeroporto de Faro, Faro, Olhão, Fuseta, and Tavira) (Fig. 2.2.1).

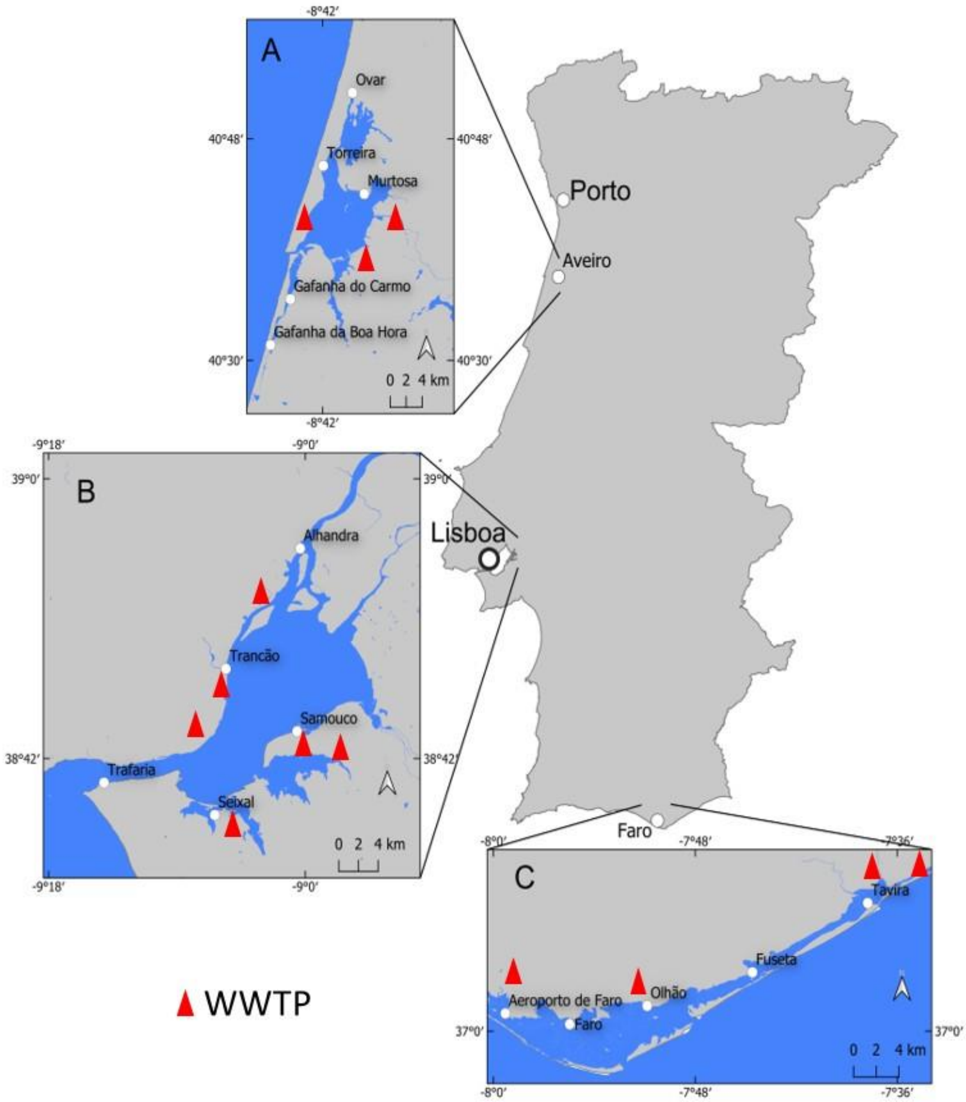

Figure 2.2.1 - Location of the study sites along the Portuguese coast: A) Ria de Aveiro; B) Tagus estuary and C) Ria Formosa.

At each coastal system were selected 5 sampling stations (mentioned above) to collect water (n = 3) in glass bottles from the low intertidal water pools for analysis of the four progestins mentioned above.

2.2.2.2. Sample Extraction Method

Environmental samples (1 L) were filtered with GF/C ™ Glass Microfiber Filters (1.2 µm; 47 mm; REF: 1822-047, Lot No:16836949, Whatman) and stored at -20 °C until analysis. Filtered samples were then submitted to solid phase extraction (SPE) using Oasis® HLB (6cc;200mg) cartridges (Waters, Milford, Massachusetts USA). HLB cartridges were first conditioned with 12 mL acetonitrile: ethyl acetate (50:50) and 12 mL deionized water, 1 L sample was loaded and washed with 6 mL deionized water. Cartridges were then dried for 20 min in a nitrogen stream and eluted with 6 mL acetonitrile: ethyl acetate (50:50). Finally, eluted products were vacuum-dried in a speed-vac, resuspended in 500 µL of methanol with 0.1% (v/v) formic acid and analyzed in an ESI-LC-MS/MS equipment. The samples were concentrated 2000 times through the SPE procedure.

2.2.3. Analytical methodology

Mass Spectrometer Tune Method parameters optimization was performed through direct injection of four progestin standard solutions with a concentration of 2 mgL^{-1} in methanol acidified with 0.1% (v/v) formic acid. The target molecules were Drospirenone (DRO) (CAS nº 67392-87-4, 98% purity), Desogestrel (DSG) (CAS nº 54024-22-5, 98% purity), Gestodene (GST) (CAS nº 60282-87-3, 98% purity) and Levonorgestrel (LNG) (CAS nº 797-63-7, 97% purity), all acquired from TCI (Tokyo Chemical Industry).

Standards and samples were injected in a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA) coupled to a LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA). Separation was achieved using a column Avantor ® ACE Excel (50 mm× 2.1 mm i.d., 1.7 µm; LotV19-3430; Serial No: A210214175), kept at 40 ºC. The mobile phases 0.1% formic acid in Mili-Q water (A) and 0.1% formic acid in acetonitrile (B) were used to apply a linear gradient of 0–2 min 5% buffer B, 2-8 min 5-100% B, 8-9 min 100% B, 9-9.1 min 100-5% B, 9.1-10 min 5% B. Injection volume was 10 µL, with a flow rate of 0.35 mL min^{-1}.

The mass spectrometer was operated at a capillary voltage of 30 V; a capillary temperature of 350 °C; a sheath gas pressure of 50 psi, auxiliary gas pressure of

10 psi, a capillary voltage of 30 V, a source voltage of 7 kV and tube lenses of 100 V. Nitrogen was used as sheath and auxiliary gas. Positive ionization in Total Ion Current mode (TIC, 30-500 m/z) was used together with the collision-induced dissociation mode (CID) at the corresponding progestin transition (See Table 2.2.1 for retention time, collision energy and mass transitions for each target progestin). The program used for data acquisition and processing is Xcalibur™ version 2.

Following the integration process, the samples underwent mathematical quantification utilizing a matched-matrix calibration curve based on a linear model. The calibration curve consisted of five nominal concentrations (previously pre-concentrated 2000 times) ranging from 5 to 250 µg L^{-1} for DRO, GST, and LNG, and from 60 to 500 µg L^{-1} for DSG. These concentration ranges correspond to 2.5 to 125 ng L^{-1} for DRO, GST, and LNG, and 30 to 250 ng L^{-1} for DSG. To mimic as close as possible the natural conditions, a spring water sample collected from Coimbra, Portugal (free of progestins) was used, as in Cruzeiro et al (2015).

Table 2.2.1- Quantification, diagnostic ions and limits of detection and quantification (LOD and LOQ, respectively) of each target compound analysed by LC-CID-MS/MS

Target compound	**Retention time**	**Base Peak $[M+H]^+$**		**Other confirming fragments**	**CID**	**LOD**	**LOQ**
	(min)	**(m/z)**		**(m/z)**	**(eV)**	**(ug/L)**	
Drospirenone	13.95	367	→	285,239,159	20	48.1	146.0
Desogestrel	17.24	293	→	265,197,161	35	38.8	117.0
Gestodene	13.80	293	→	275,271,149	20	13.7	45.5
Levonorgestrel	14.25	295	→	277,245,185	35	11.8	35.7

2.2.4. Quality assurance and quality control procedures

The performance of the equipment was checked using blanks at the beginning and end of the runs (solvent controls: methanol acidified with 0.1% (v/v) formic acid) and matrix-matched calibration curves for each set of 10 samples. The limits of detection (LODs) and quantification (LOQs) as LOD = 3.3(α/S) and LOQ= 10(α/S); with α as the standard deviation of the blank (n=10) and S as the average slope of the regression line (calibration curve) for each target compound. Recoveries (%) were determined by analyzing matrix blank samples that were spiked with the target compounds at two different concentration levels: 5xLOQ and 10xLOQ. Spiking was performed both before and after the extraction process to assess the recovery efficiency (EMEA 1995). Results are described in Table 1.

1-Way ANOVAs were applied for each estuarine system (whenever possible) to check for statistical differences among progestins. This was only possible to do in summer due to the lack of information in the other sampling campaigns. For all data, we first checked for normality using the Kolmogorov–Smirnov test and for homogeneity of variances using the Levene's test (Zar, 1999). All the analyses were performed in Statistica 7 software.

2.2.5. Results and Discussion

Our results demonstrated that in Ria de Aveiro the most abundant progestin in surface waters was DSG (wi: 126.5 ± 16.8; sp: 96.0; su: 193.9 ± 85.7; au: 142.3 ± 50.4 ng L^{-1}) followed by DRO (wi: 53.7; sp: 47.3; su: 37.5 ng L^{-1}) (Fig. 2.2.2A). There were no temporal statistical differences in the DSG concentrations but, slightly higher values seemed to occur during summer and autumn most probably associated with the small freshwater discharge from the catchment areas associated to a small increase in population due to some touristic activity.

In the Tagus estuary, the mean concentrations of DRO (su: 178.9 ± 70.5 ngL^{-1}) and DSG (wi: 125.8 ± 27.5; au: 246.8 ± 128.8 ngL^{-1}) were higher than in Ria de Aveiro. From a temporal perspective, DRO was only present in the summer while DSG was more abundant in the autumn (Fig. 2.2.2B). The other two progestins (i.e., GST and LNG) were relatively constant throughout the year. During summer, DRO was significantly more abundant than the others (1-Way ANOVA, $F_2 = 17.5$, $p < 0.0001$). The patterns observed are also most probably associated with the small freshwater discharge from the catchment areas in summer associated with a large increase in population due to high touristic pressure. Lisbon received between 1-2 million tourists in 2019/2020. Due to the Covid-19 pandemic, the tourism volume declined precisely in those years, even so, it is a considerably high number. In 2022, for example, the numbers increased significantly to approximately 5 million (https://www.statista.com/statistics/1155150/domestic-tourists-portugal-by-destination).

In Ria Formosa the pattern was similar, being DRO (max. - 125.7 ± 48.1 ng L^{-1}), and DSG (máx. – 410.8 ng L^{-1}) the most abundant progestins. Also, in summer, DRO was significantly more abundant than the others (1-Way ANOVA, $F_2 = 15.7$, $p < 0.05$ (Fig. 2.2.2C). DSG was more abundant in the autumn campaign. No data was available for winter and spring.

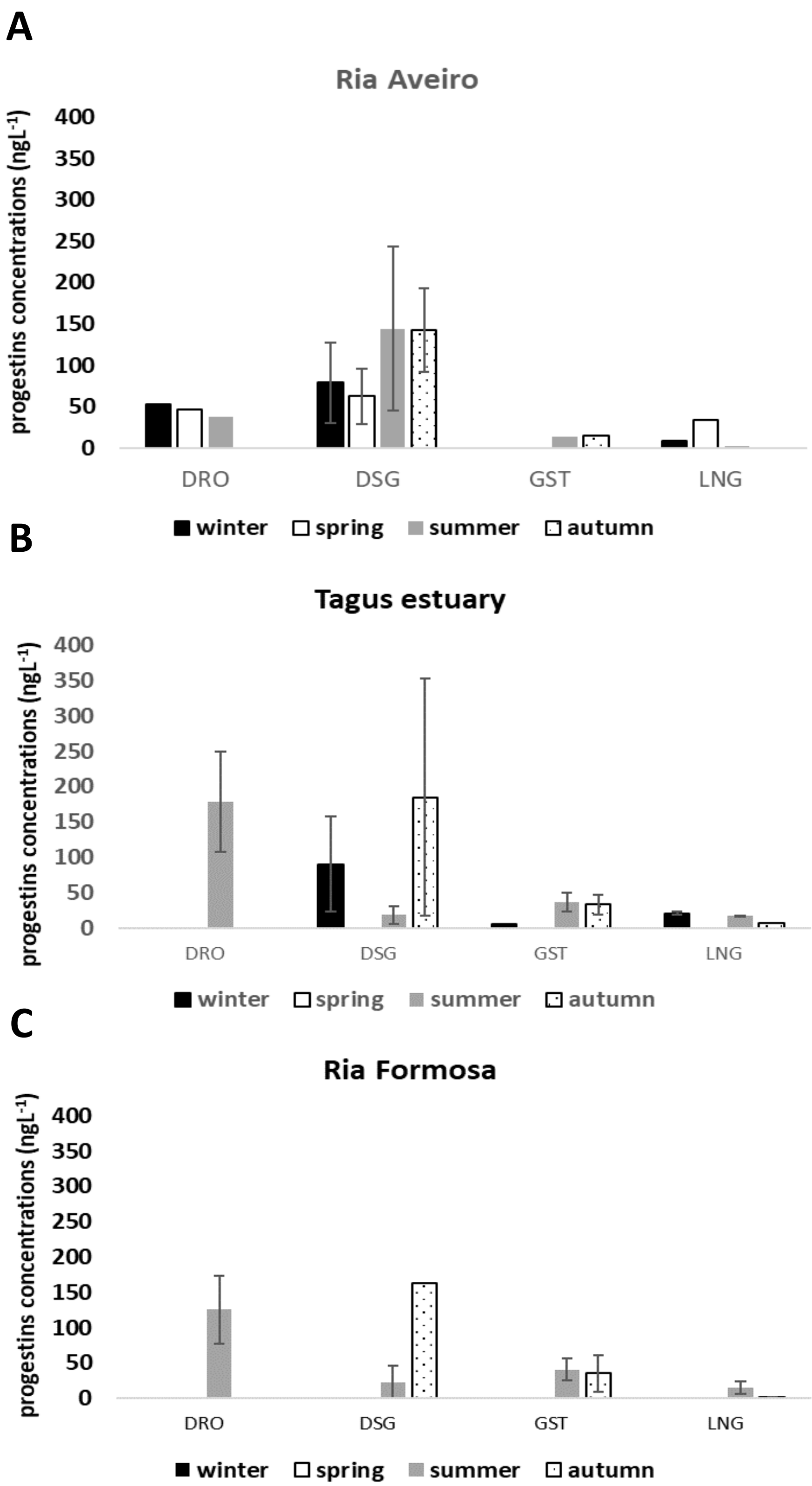

Figure 2.2.2 – Average progestins concentration (ng L^{-1} ± SE) in surface waters of three Portuguese estuaries, Ria de Aveiro (A), Tagus estuary (B) and Ria Formosa (C). DRO – drospirenone, DSG – desogestrel, GST – gestodene and LNG – levonorgestrel.

Progestins' concentrations found on the Portuguese coast are in the range of those found in surface waters in other systems worldwide (see Wang et al. 2021 and references therein), with average concentrations below 50 ng L^{-1}, with some extremely high peaks (100-200 ng L^{-1}) in the summer and autumn. These peaks can be comparable to the concentrations found in livestock farms or WWTPs effluents (Zhang et al., 2019) which must be a matter of concern attending to the negative effects that these compounds can cause in the aquatic biota, even at very low concentrations. For example, previous studies have demonstrated that zebrafish exposed to 10 ng L^{-1} of levonorgestrel showed a significant decline in the fecundity values compared to the control (Cardoso et al. 2017). In the present study concentrations of progestins much higher than 10 ng L^{-1} were found in all estuaries, attesting the level of concern of this topic.

Comparing the three systems, the Tagus estuary seems to have slightly higher progestins' concentrations than the other two systems. This could make sense attending to the higher population but also with a higher catchment area. The view that population and discharge flow drives the concentrations of progestins in the environment is also supported by Zhu et al. (2018) and Wang et al. (2021). Nevertheless, the progestins' concentrations found in the Tagus estuary are not proportional to the number of inhabitants in the surrounding areas compared to the Ria de Aveiro or Ria Formosa due to the river flow and volume of water of each estuary. For example, the average Tagus River flow is 331 m^3s^{-1}, with maximum monthly discharges averaging ~ 2200 m^3s^{-1} (Fernadez-Nóvoa et al. 2017). And, the Tagus estuary occupies a volume of 1900×10^6 m^3. So, it is a large estuary and even though the population in surrounding areas is tremendous, the contamination becomes diluted attending to the volume of water of the estuary.

The hydrographic system of Ria de Aveiro is dominated by the Vouga River whose average flow is approximately 40 m^3 s^{-1}, corresponding to a volume of 1.8 x 10^6 m^3 (Dias et al. 1999) which is much lower than the total tidal prism volume for neap and spring tide conditions, which is estimated to be 65.8×10^6 m^3 and 139.7×10^6 m^3 (Lopes et al. 2013).

In Ria Formosa, the maximal tidal volume is 140 x 10^6 m^3 (Newton and Mudge, 2003). So, in the case of Ria Formosa, in the summer, the progestins' concentrations are similar to the Tagus estuary. Despite the number of inhabitants being lower than in the Lisbon region, the volume of water of Ria Formosa is also much smaller, so there is less dilution than in the Tagus estuary.

Moreover, it seems that there is a geographical difference in the type of progestins that occurred between the North and South of Portugal. While in the North, DSG is the most abundant and consistent throughout the year, in the South, DRO had higher concentrations (3 times more) and it was present in the summer. This can eventually be related to a time of the year when the number of visitors in the south of Portugal triplicates (e.g., Algarve region). This is in accordance with Bebianno et al. (2019) that demonstrated that Ria Formosa is characterized by higher inputs of estrogens in the lagoon in the summer indicating that these compounds are linked to an increase in population from visitors and tourist activities. Regarding the DSG, since it has a consistent pattern in the North all over the year it seems that is more associated with the resident population while DRO seems to be more related to visiting population, mainly in the South region. According to the literature, different countries have different prevalences of progestins (Rocha and Rocha 2022). Therefore, it is plausible that visitors to the southern region of Portugal may be using different types of contraceptives that have compositions distinct from those most commonly used in Portugal. This is evident when considering the list of authorized contraceptive names for each member state of the European Union, as outlined in Directive 2001/83/CE. It is worth noting that a significant number of contraceptive pills administered in the United Kingdom contain drospirenone as an ingredient. Taking into account the fact that British tourists represent the largest foreign nationality in the Algarve region, accounting for 29% of the guests and 33% of the overnight stays (according to Turismo do Algarve 2016), it is highly plausible that this situation can explain the observed peak in drospirenone (DRO) levels in the waters of Ria Formosa.

2.2.6. Final Remarks

Concluding, it is important to continue monitoring the aquatic systems, particularly in terms of progestins levels, not only in surface waters but also in other matrices (e.g., sediment and biota), to understand the real impact of these compounds and contribute with more information as possible to create regulations to control the levels of these hormones in the environment. Also, due to the high touristic loads in some areas, it is relevant to alert for the worsening of the treatment of the WWTP during high touristic peaks. Also, it would be interesting in future works to consider other pharmaceuticals, including other progestins that are commercialised in other countries.

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Chapter 3

Interactive effects of climate change and mixtures of EDCs on a key coastal species

Interactive effects of climate change and mixtures of EDCs on a key coastal species

3.1. Combined effects of climate change and environmentally relevant mixtures of endocrine disrupting compounds on the fitness and gonads' maturation dynamics of *Nucella lapillus* (Gastropoda)

Abstract

Coastal areas are affected by multiple stressors like climate change and endocrine disruptors (EDCs). In the laboratory, we investigated the combined effects of increased temperature and EDCs (drospirenone and mercury) on the fitness and gonads' maturation dynamics of the marine gastropod *Nucella lapillus* during 21 days.

Survival was negatively affected by all the stressors alone, while in combination a synergistic effect was observed.

Both chemicals as single factors did not cause any effect on the maturation stage of ovaries and testis. However, in the presence of a higher temperature it was clear a delay in the maturation stage of the ovaries but not in the testis, suggesting a higher negative impact of the stressors in females than in males.

In summary, drospirenone caused a low negative impact in aquatic species, like gastropods, but in combination with other EDCs and/or increased temperature can be a matter of concern.

KEYWORDS: Temperature; synthetic progestins; drospirenone; mercury; aquatic species; gastropods.

3.1.1. Introduction

In the context of climate change, it is known that marine ecosystems have undergone constant changes. According to the Intergovernmental Panel on Climate Change (IPCC), it was estimated that by 2100 the average global surface temperature will increase between 3.3 – 5.7 ºC and the average surface temperature of the oceans will increase between 3 – 4 ºC (Stocker, 2014, Arias et al., 2021), which will bring strong implications on the structure and functioning of coastal ecosystems (Wernberg et al., 2011, Arias et al., 2021). Temperature is one of the physicochemical variables which plays an important role in the general functioning of aquatic communities (Brown et al. 2004). It is proved that global warming can alter feeding and growth rates (Miller, 2013) as well as the reproduction of many species (DeCourten and Brander, 2017), which will, ultimately, have cascading effects on all the marine communities (Miller, 2013).
Besides climate change, marine ecosystems are constantly being threatened by contaminants of anthropogenic origin, being the endocrine disruptor compounds (EDCs) one of the main priority topics for the European Union (Peterson et al. 2007, EU Commission, 2011).

EDCs mostly act as mimicking natural hormones, but some of them can antagonize the action or modify the synthesis, metabolism and transport of the endogenous hormones, producing a range of developmental, reproductive, neurological, immune, or metabolic diseases in humans and wildlife (Khetan, 2014).

One of the types of EDCs with greatest impact on the aquatic systems are the pharmaceutical compounds, being one of the most critical the steroid hormones. Steroids are ubiquitous in the environment and can be potent endocrine disruptors, even at low concentrations (ng L^{-1}) (Fent, 2015). Steroids with progestogenic activity are called gestagens or progestins. These are synthetic compounds that mimic progesterone activity and are commonly used as oral contraceptives or being part of hormonal replacement therapies. It is proved their adverse effects on fertility and reproduction of aquatic species (Ojoghoro et al., 2021), altered sex development, induced transcriptional effects, etc, (Ojoghoro et al., 2021, Zhao et al., 2015). Drospirenone (DRO) is a fourth-generation progestin, which was designed to bind the progesterone receptor with greater specificity and minimize side effects related to interactions with androgen, estrogen or glucocorticoid receptors, combining potent progestogenic and antiandrogenic activities (Marqueño et al., 2019). Drospirenone can reach the aquatic systems through the effluents of wastewater treatment plants in a range of ng L^{-1} (Fent 2015, Marqueño et al., 2019). And, there is a lack of knowledge about the effects of this progestin in non-target aquatic species. When it comes to metals, mercury is a high-priority pollutant whose endocrine effects have not been well studied to date (Tan et al., 2009).

According to several authors, human activity manages annually to almost triple the amount of atmospheric mercury (Rice et al., 2014). Once released into the environment, mercury can bioaccumulate and biomagnify in the food web and in the long-term can cause adverse effects on human health (Tan et al., 2009, Plunk and Richards, 2020) and aquatic fauna (Rice et al., 2014). Although the mechanism(s) of mercury entry into the food chain is (are) still unknown, several studies have been developed to understand how it happens (Tan et al., 2009, Rice et al., 2014). About its estrogenic properties, mercury can cause strong negative effects at the reproduction level in both vertebrate and invertebrate species (Tan et al., 2009). Mercury can act at the hypothalamic–pituitary–gonadal (HPG) axis, deregulating its functions at the reproductive level. In the aquatic environment, several studies have demonstrated negative effects of mercury exposure on fish survival, growth rate and external morphology, but also on behavioural characteristics such as hunting, predator avoidance and long-distance migration (Webber and Haines, 2003, Mora-Zamorano et al., 2016, Mora-Zamorano et al., 2017,).

Both EDCs (DRO and Hg) can act at interconnecting endocrine axes (as HPG) so they can produce combined effects, impairing the reproductive system and delaying the maturation of the gonads (Zucchi et al., 2014, Fent, 2015). These contaminants associated with climate drivers (e.g.,, temperature) may produce a stronger and unpredictable effect on the aquatic habitat, since the latter can also interfere in the regulation of the HPG axis of vertebrates and invertebrates (Miranda et al., 2013) – Hypothesis 1 (H1).

Regardless of the existent information about the studied stress factors (i.e., temperature, mercury and progestins), individually, less attention has been paid to the environmental health effects of mixtures of EDCs (progestins and metals) under the influence of climate drivers (Cardoso et al. 2017b, 2018a, Mannai et al. 2022). So, it is crucial to evaluate the response of aquatic species to the complex interaction of multiple stressors. Generally, organisms subjected to multiple stressors exhibit one of three types of responses: additive, antagonistic, or synergistic (Todgham & Stillman 2013), depending also on the timing of occurrence of the stressors. For example, when stressors occur close in time or simultaneously, then interactive effects are more likely to occur (Crain et al., 2008, Gunderson et al., 2016).
Molluscs, in particular gastropods, are ubiquitous in the aquatic system and have been used as non-target models for laboratory studies as they are required to constantly adapt to the many changes that occur in the surrounding environment in which they inhabit (Pirger et al., 2018b). They proved to be effective model animals because they have highly conserved control and regulatory biochemical pathways that are often homologous to vertebrate systems and they are extremely sensitive to anthropogenic inputs (Pirger et al., 2018b). Particularly, *Nucella lapillus* (L.) is a marine gastropod species with strong ecological relevance, occupying the intertidal zone usually associated with rocky shores and common amongst barnacles (*Balanus* spp.) and mussels (*Mytilus* spp.) on which they preferentially feed (Galante-Oliveira et al., 2010). It occurs within a salinity range from 18 to 40 and between 0 and 20 ºC isotherms throughout the North Atlantic littoral zone: from the Arctic to the south of Portugal in the east, including Iceland and the Faroe Islands, and from the south-west of Greenland to the north of Long Island in the west (Crothers, 1985, Tyler-Walters, 2007). *N. lapillus* is a gonochoristic species (separated sexes) with internal fertilization. Individuals can reproduce throughout the year but, in some places, reproduction is restricted to a few months (Galante-Oliveira et al., 2010).

The main goal of this study was to evaluate the effects of combined multiple stressors (i.e., temperature and mixtures of the EDCs, Hg and DRO) on the gastropod *Nucella lapillus*. Thus, several endpoints such as survival, fitness (i.e., condition index), consumption rates, and gonads' maturation dynamics were evaluated after 21-day exposure.

3.1.2. Materials and Methods

3.1.2.1. Chemicals

Pharmaceutical The standard drospirenone (DRO, CAS 67392-87-4; purity = 98.0%) was purchased from TCI (Tokyo Chemical Industry, Japan). Stock solutions were prepared with analytical ethanol (CAS 64-17-5; purity 99.9%) supplied by Merck Millipore (Germany) and stored at -20 ºC.

Metal: Mercury standard solution (1000 mg L^{-1} Hg) in 10 % nitric acid (for Atomic Absorption Spectrometry) standard was supplied by Fisher Chemical, stored at ambient temperature.

3.1.3. Organisms' collection and acclimation

The species used in the experiment (i.e., *Nucella lapillus* and its prey, *Mytilus edulis*) were collected in Praia Norte in Viana do Castelo, Portugal (41º 41'33''N 8º 51'06''N) in October 2020 and transported to the CIIMAR facilities in a cool box for acclimatation period. The dog-whelk *N. lapillus* individuals were separated by size and adults (total length >1.5 cm) were selected for the experiment. Also, individuals of *M. edulis* (the most abundant prey species in the field) were selected by size (2.64 ± 0.38 cm, total length).

During the acclimation period (15 days) (according to Castro et al., 2007), individuals of *N. lapillus* were fed with *M. edulis* on an *ad-libitum* basis and maintained in a semi-static system whereby 100% of the water was changed twice a week. Photoperiod was set to 18 h light: 6 h dark to simulate summer conditions, at constant intensity (1700 lm). The organisms were acclimated under the ambient temperature and normocapnia (18 ºC, pH 8.1) and salinity 33-35.

3.1.4. Experimental design

The experimental set-up followed a factorial design manipulating temperature [ambient temperature (18 ºC) (mean sea surface temperature in summer - sSST) (https://pt.seatemperature.net/current/portugal/viana-do-castelo-viana-do-castelo-portugal) and warming (22 ºC – the future sSST warming scenario in 2100 (+ 4 ºC) (Russell et al. 2013)], the progestin drospirenone (DRO: DRO1 – 100 ng L^{-1} and DRO2 – 1000 ng L^{-1}), mercury (Hg: Hg1 – 1.5 µg L^{-1}, drinking water's limit, and Hg2 – 50 µg L^{-1}, residual waters' limit), Mixture 1 (Mix 1: Hg1 + DRO1), Mixture 2 (Mix 2: Hg1 + DRO2), Mixture 3 (Mix 3: Hg2 + DRO1), Mixture 4 (Mix 4: Hg2 + DRO2), the Control (Ct: Seawater) and the Solvent control (SCt: Seawater + vehicle ethanol – 0.01%), in a total of 20 treatments (Table 1), for 21 days

Table 3.1.1 – Description of the different treatments to which *N. lapillus* were exposed. Ct – control, SCt – solvent control, Hg1 – 1.5 µg L^{-1}, Hg2 – 50 µg L^{-1}, DRO1 – 100 ng L^{-1}, DRO2 – 1000 ng L^{-1}; Mix1 – 1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2 – 1.5 µg Hg L^{-1}

Treatments	Condition
T1	18 ºC, Ct
T2	18 ºC, SCt
T3	18 ºC, Hg1
T4	18 ºC, Hg2
T5	18 ºC, DRO1
T6	18 ºC, DRO2
T7	18 ºC, Mix1
T8	18 ºC, Mix2
T9	18 ºC, Mix3
T10	18 ºC, Mix4
T11	22 ºC, Ct
T12	22 ºC, SCt
T13	22 ºC, Hg1
T14	22 ºC, Hg2
T15	22 ºC, DRO1
T16	22 ºC, DRO2
T17	22 ºC, Mix1
T18	22 ºC, Mix2
T19	22 ºC, Mix3
T20	22 ºC, Mix4

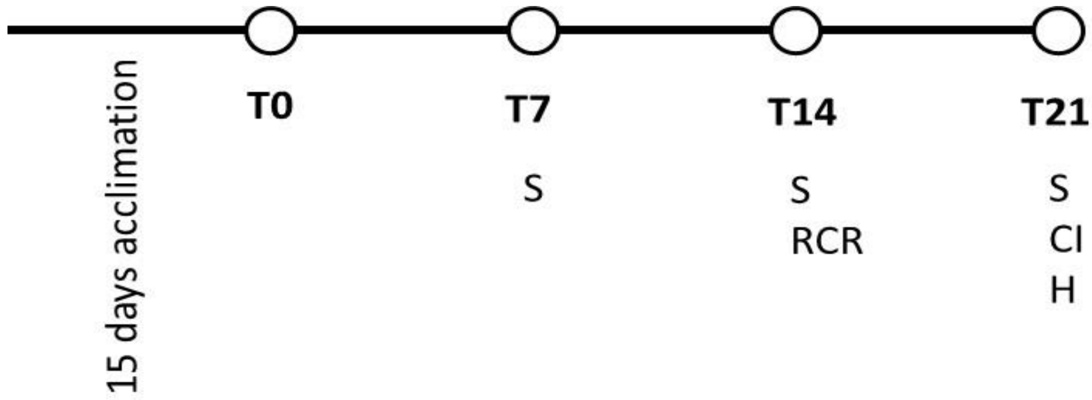


Figure 3.1.1 – Graphical representation of the timeline of the experiment with indication of the endpoints analysed at each sampling point. S – survival, RCR – relative consumption rate, CI – condition index and H – Histological analysis.

This experiment followed a similar model to the one already implemented in previous works (Cardoso et al., 2018a) in which a saltwater reservoir tank (500 L) was directly connected to the CIIMAR's internal saltwater network through a 10µm filter and the flow was maintained by a 1400 L h^{-1} flow pump (Eheim, Germany), that passed first through a UV filter Helix Max 2.0 9W (Aqua Medic, Germany). Seawater from this reservoir was directed to two tanks (50L each), whose water flow was later distributed to the 2nd level (i.e., experimental units).

The entire 2nd level was lit by artificial light suitable for marine setups (LED light v-tac, 18w, 240v, 50 Hz, 1700 lm) that was controlled by a timer to guarantee the photoperiod.

In all 2^{nd-}level flasks, aeration and the saltwater flow were maintained continuously (0.6 L h^{-1}flask^{-1}). Finally, every 4 flasks were linked by connecting vessels to another flask, external to the water baths, so that all the contaminated water passed through a set of particle filters with 3 different meshes (5, 10 and 25 µm), then a charcoal filter, before being eliminated.

Specimens were divided by treatments and each one was composed of four replicates in a total of eighty glass flasks (2 L-volume, 9.7 cm in diameter). Replicates were distributed randomly by ten water baths, to maintain the temperature constant (Figure 2). The temperature inside the water baths was maintained by 300 KW h^{-1} resistors that were regulated by a temperature sensor controlled by AT Control power box (Aqua Medic, Germany), which automatically heated the tanks whenever the temperature deviated from predetermined set points by 0.5 °C. Each flask had twenty-one *N. lapillus* (eighteen were maintained free in the flasks plus three in isolated perforated plastic flasks for control of consumption rate) that were maintained at salinity 33-35, and a photoperiod of 16h light : 8h dark (summer conditions), with a constant water renovation and aeration. During the experiment, the organisms in the flasks were directly exposed to Hg and DRO three times a day (200 µl of each stock solution for each contaminant concentration was injected directly into the flasks), to simulate episodic discharges from a contamination source.

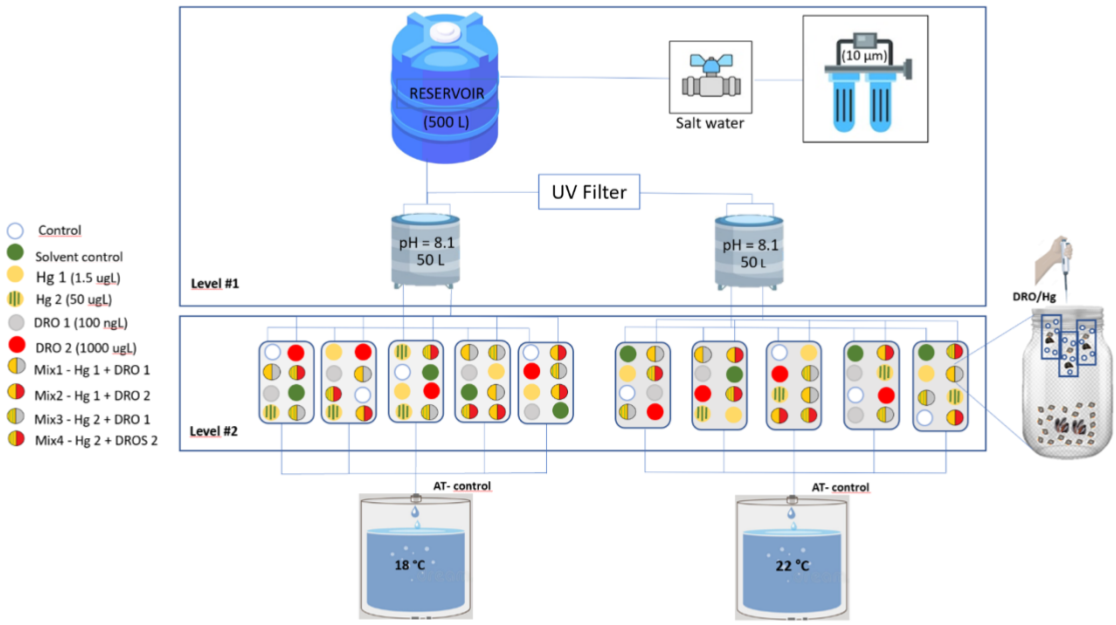


Figure 3.1.2 – Schematic representation of the experimental set-up of the mesocosm experiment with *N. lapillus*. Level #1 represents the saltwater reservoir tank (500 L) directly connected to the internal saltwater network through a 10µm filter and the flow is distributed to two main tanks that feed each temperature system (one for 18 ºC and the other for 22 ºC) of experimental units (2nd level). Level #2 represents the 10 water baths, for the 2 temperatures. Each water bath has 8 experimental units (i.e., flasks) corresponding to different treatments distributed randomly. In total, for each temperature there are 40 experimental units (10 treatments x 4 replicates). Drospirenone and mercury are injected directly in the flasks.

Following previous works (Cardoso et al., 2018a, Potts et al., 2021), the water temperature was increased gradually (1 ºC per day) until reaching the highest temperature of 22 ºC. After reaching the required temperature the experiment started. During the 21 days of exposure, individuals were fed with mussels (one mussel per three dog-whelks) replaced three times a week (corresponds approximately to 1 mussel/dog-whelk/week). Attending to the literature, the feeding rate can be very variable depending on the size of prey, temperature (Crothers, 1985). So, we considered an average value based on the literature (Crothers, 1985 and references therein: 0.28 – 0.77 mussels/week and 3.5 mussels/week; Hunt and Sheibling, 1998: 0.7-1.09 mussels/week).

Survival was checked on days 7, 14 and 21 (n = 21 individuals/replicate).

During the experiment, water physicochemical variables, such as temperature, pH, dissolved oxygen, ammonia and nitrites were measured in the experimental units three times a week, and the temperature was measured daily. During the experiment the seawater environmental variables were maintained as follows: temperature: 18.3 ± 0.11 ºC and 21.87 ± 0.23 ºC; pH: 7.92 ± 0.07 (18 ºC) / 9.81 ± 0.06 (22 ºC); dissolved oxygen: 98.9 ± 0.45% (18 ºC) / 97.6 ± 4 % (22 ºC); ammonia: 0.11 ± 0.03 mg L^{-1} (18 ºC) / 0.14 ± 0.04 mg L^{-1} (22 º C) and nitrites: 0.19 ± 0.04 mg L^{-1} / 0.21 ± 0.06 mg L^{-1} (22 º C) (please check tables S1 and S2 for more details). The experiment was carried out at CIIMAR aquatic animal facilities, according to the guidelines of the Directorate-General of Veterinary of Portugal (Decree-Law No. 113/2013), implementing the European Directive No. 2010/63/EU on animal welfare for scientific purposes.

3.1.5. *N. lapillus* condition index and consumption rate

For estimation of the *N. lapillus* condition (n = 4 individuals/replicate), the fresh weight (FW, i.e., body weight plus shell weight) and the wet tissue excluding the operculum (TW) were measured using an analytical precision scale (Acculab sartorius group, Germany) and shell weight (SW) was calculated as FW-TW. The condition index was estimated as (TW/SW) x 100, according to (Mamo et al., 2019) at the end of the experiment.

Relative consumption rates (RCRs) were evaluated during the second week of the experiment (n = 3 individuals/replicate). For that, were used three individualized *N. lapillus* (plus three *M. edulis*; one per dog-whelk) per replicate. The individuals selected for this assessment were starved for 24h before the consumption experiment that run for 3 days.

N. lapillus consumption rate (RCR) was determined according to (Guler and Lök, 2019) and calculated as mussel (g). snail (g)$^{-1}$. day $^{-1}$ as follows:

$$RCR = \left(\frac{initial\ average\ wet\ weight\ mussel\ (g) - final\ wet\ weight\ mussel\ (g)}{average\ wet\ weight\ snail\ (g)}\right) /3\ days$$

3.1.6. Histological procedures

Organisms selected for histological analysis (n = 3 individuals/replicate) were fixed individually in bouin solution (ITW Reagents, Spain) in small plastic flasks, for 48h. Afterwards, they were transferred to ethanol 70%. After 24-48h in ethanol, this was replaced with a clean one.

After fixation, tissues were processed in an automatic processor (Citadel 2000, Thermo Scientific, USA). Tissues were embedded in paraffin (Histoplast IM, Thermo Scientifc, USA) and left to cool. Embedded tissues were cut into 6 μm longitudinal semithin sections in a paraffin microtome (Jung RM 2035, Leica Biosystems, Germany). Finally, sections were stained in an automatic slide stainer (Shandon Varistain 24-4, Thermo Scientific, USA) with haematoxylin-eosin and mounted in Entellan new resin (Merck Millipore, Germany) for light microscopy observation to determine the individual gametogenic stage.

3.1.7. Gonads’ microscopic evaluation

For the microscopic examination of gonads’ maturation, it was used a classification based on the previous work of Galante-Oliveira et al. (2010), in which a 6-stage scale was established: I (immature), II (early recovering), III (late recovering), IV (ripe), V (partially spent) and VI (spent).

Attending to the variability in the maturity of the gonads it was decided to analyse 5 follicles from each gonad section in a total of 45 follicles (5 follicles x 3 slides x 3 sections) and the individual gametogenesis stage considered was the median value amongst those registered for the 45 observed follicles (following the same procedure as in (Cardoso et al., 2018b).

3.1.8. Hg quantification in the water

Water samples (150 mL, n = 2 replicates per treatment) were collected from all exposure groups just after the Hg addition (T30 – 30 minutes after and T60 – 60 minutes after) and preserved in glass flasks containing 0.7mL HCl 21%. Total dissolved mercury was quantified through ICP by Biogerm, S.A. (Portugal

3.1.9. Drospirenone (DRO) quantification in water samples by LC-MS

3.1.9.1. Water sampling

Water samples (1 L, n = 2 replicates per treatment) from all exposure groups were collected in amber flasks just after DRO addition (T30 – 30 minutes after and T60 – 60 minutes after). During sampling, all bottles were rinsed twice with the treated water before collection. Then, water samples were filtered with GF/C ™ glass microfiber filters from Whatman (1.2 um; 47 mm; Lot No.:16836949) and stored at -20 °C until analysis.

3.1.9.2. Extraction Procedure

The extraction method followed (Ribeiro et al., 2007), initially developed to extract phenolic compounds and steroids from water samples, with some modifications. The filtered samples were treated (cleaned and concentrated) by solid-phase extraction using SPE cartridges from Waters ™ Corporation (Milford, MA, USA; Oasis® HLB 6cc/200mg). Prior to use, cartridges were sequentially conditioned with 12 mL of acetonitrile:ethyl acetate (50:50, v/v) and 12 mL of ultrapure Milli-

Q water (VWR, EUA). Samples of 1L were, then, loaded onto SPE cartridges, at a constant flow rate of approximately 5 mL min^{-1} followed by a washing step with 6 mL of ultrapure Milli-Q water. Cartridges were dried under vacuum for 15 min and, then, eluted with 6 mL of acetonitrile:ethyl acetate (50:50, v/v), at 1 mL min^{-1}. Elution volume was completely evaporated under a nitrogen stream and reconstituted in 1 mL methanol (LC-MS grade) acidified with formic acid (0.1%), concentrating the original samples 1000 times.

3.1.9.3. Instrumental and methodological characteristics

Samples were injected (2 × 20 µL) into a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled to an ESI source-Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA). Separation was achieved with a column Avantor ® ACE Excel (50 mm × 2.1 mm i.d., 1.7 µm) (Avantor-LotV19-3430; Serial No.: A210214175) kept at 25 °C and at a flow rate of 0.15 mL min^{-1}.

The mass spectrometer was operated In positive mode at: spray voltage of 4.55 V; capillary voltage of 30 V; capillary temperature of 380 °C: tube lens of 55 V and normalised collision energy maintained at 35. Data were processed using XcaliburTM version 2 Software.

A summary of analysis detection parameters for Drospirenone can be seen in Table S1 of chapter 3.1 (in attachments).

After integration, samples were mathematically quantified by an external standard-matrix calibration curve (method of least squares) with eight nominal concentrations from 10 to 2000 µg L^{-1} (which is equivalent to 10 to 2000 ng L^{-1}).

Recovery tests were performed using sample-matrix doped with a three-level concentration, to have 500, 1000 and 2000 µg L^{-1} concentrations in LC-MS analysis. The recovery of the method was 69 ± 6 %. The limit of detection (LOD) is 10 µg L^{-1} and the limit of quantification (LOQ) is 20 µg L^{-1}. Nominal values below 20 µg L^{-1} and 10 µg L^{-1} were indicated as <LOQ and <LOD, respectively and no detected values as ND.

3.1.10. Data analysis

Experimental results were examined using linear models. All the statistical analyses were run in R environment (R Core Team, 2016). Initially, models for all the responses were constructed including fixed factors and random effects (i.e., generalized linear mixed models, LMM). However, when random effects did not improve the model fit, we applied the parsimonious principle, removed those terms and used generalized linear models provided by glm 2 package in R (Marschner, 2018). Hence, to examine the effects of the treatments on the survival of *N. lapillus* at the end of the experiment, we used GLM assuming a Binomial data distribution, as there were only two possible outcomes (alive and dead). To visualize the survival curves for all the treatments, which are represented as survival percentages, we used the function ggsurvplot providedby Survminer R package (Kassambara et al. 2021). The model included as predictors the three experimental factors: temperature level, Hg and DRO concentrations, but also their interactions. Analogous linear models were constructed to examine consumption rates, but this time data using a Gaussian data distribution. In the case of the analysis of gonads' maturation we used an ordered logist regression model, according to Galante-Oliveira et al. (2010), because the response was an ordered categorical outcome (6-stage condition scale). Model was constructed in the same way as described above.

In all the models, significant predictors were selected from the full models by removing sequentially those of higher-order, with the higher p values, and comparing the reduced model with the original one using analysis of variance (ANOVA). When significant interactions were found, treatments were compared using p-adjusted Tukey tests ($p < 0.05$). The Lsmeans package for R was used to perform these tests *a posteriori* (Lenth, 2016).

Generalized linear mixed models ran using nlme package (Pinheiro et al., 2012). Ordered logit linear models were constructed using the package MASS (Venables & Ripley, 2002). Assumptions for the linear models were checked by examining the residual plots. In the case of response variables with Gaussian probability distribution, and, when required, data were log-transformed to avoid heteroscedasticity.

3.1.11. Results

3.1.11.1. Mercury in water

Mercury concentrations measured at T30 (30 min) and T60 (60 min) after the first injection are indicated in Table 3.1.2. Measured concentrations in control (Ct) and solvent control (SCt) were close to zero. At T30, concentrations declined around 60% for the lowest concentration (Hg1) and approximately 80% for the highest concentration (Hg2). At T60 they decreased even more (» 40-50% in relation to T30) (Table 3.1.2). For the mixtures, the losses were more variable.

Table 3.1.2 – Nominal and measured concentrations of mercury (µg L^{-1}) in waters collected during the experiment. The water was sampled 30 min after the first injection (T30) and 60 min later (T60). The values are expressed as the mean (± SD). Ct – control, SCt: solvent control – 0.01 % ethanol; Hg1 – 1.5 µg L^{-1}, Hg2 – 50 µg L^{-1}; Mix1 – 1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2 – 1.5 µg Hg L^{-1} + 1000 ng DRO L^{-1}; Mix3 – 50 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix4 – 50 µg Hg L^{-1} + 1000 ng DRO L^{-1}.

Treatments		**Nominal** (µg L^{-1})	**T30** (µg L^{-1})	**T60** (µg L^{-1})
18 ºC	**Ct**	-	< 0.01	0.039 ± 0.025
	SCt	-	0.057 ± 0.028	< 0.01
	Hg1	1.5	0.487 ± 0.057	0.342 ± 0.095
	Hg2	50	8.475 ± 1.28	3.28 ± 0.82
	Mix1	1.5	-	-
	Mix2	1.5	0.231 ± 0.094	0.105 ± 0.05
	Mix3	50	0.485 ± 0.12	0.342 ± 0.205
	Mix4	50	4.29	0.86
22 ºC				
	Ct	-	0.017	0.020
	SCt	-	< 0.01	0.042 ± 0.034
	Hg1	1.5	0.75 ± 1.047	0.342 ± 0.095
	Hg2	50	7.76 ± 1.87	3.91 ± 1.527
	Mix1	1.5	0.16	0.114
	Mix2	1.5	0.527 ± 0.087	0.398 ± 0.035
	Mix3	50	14.2 ± 3.394	0.642 ± 0.06
	Mix4	50	16.3 ± 4.243	6.68 ± 1.047

3.1.11.2. Drospirenone in water

Drospirenone concentrations measured at T30 (30 min) and T60 (60 min) after the first injection are indicated in Table 3.1.3. In all control (Ct) and solvent control (SCt) samples, DRO was not detected. For the rest of the treatments, values at T30 were lower than the nominal values, except for DRO 2 (T18 ºC) and Mix 1 (T22ºC) which presented values slightly higher than nominal concentrations. At T60, the values declined considerably (Table 3.1.3).

Table 3.1.3 – Nominal and measured concentrations of drospirenone (ng L^{-1}) in waters collected during the experiment. The water was sampled 30 min after the first injection (T30) and 60 min later (T60). The values are expressed as the mean (± SD). Ct – control, SCt: solvent control – 0.01 % ethanol; DRO1 – 100 ng L^{-1}, DRO2 – 1000 ng L^{-1}; Mix1 – 1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2 – 1.5 µg Hg L^{-1} + 1000 ng DRO L^{-1}; Mix3 – 50 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix4 – 50 µg Hg L^{-1} + 1000 ng DRO L^{-1}.

Treatments		**Nominal (ng L^{-1})**	**T30 (ng L^{-1})**	**T60 (ng L^{-1})**
18 ºC	**Ct**	-	<LOD	<LOD
	SCt	-	<LOD	<LOD
	DRO1	100	86.31 ± 10.47	< LOD
	DRO2	1000	1249.16 ± 665.64	757.69 ± 35.21
	Mix1	100	129.15	< LOD
	Mix2	1000		
	Mix3	100	61.21 ±12.5	12.03
	Mix4	1000	965.06 ±56.88	583.08 ± 492.54
22 ºC	**Ct**	-	<LOD	<LOD
	SCt	-	<LOD	<LOD
	DRO1	100	54.41	<LOD
	DRO2	1000	583.01 ± 402.42	339.73
	Mix1	100	298.98	33.32±29.4
	Mix2	1000	892.52	244.04 ± 58.49
	Mix3	100	107.34 ± 1.10	19.34 ± 3.74
	Mix4	1000	749.06	59.75 ± 46.62

3.1.11.3. Survival

For all the endpoints analysed, was considered only the solvent control (SCt) against the other treatments, since no significant differences between control and solvent control were observed.

The survival rate was estimated along 21 days, with checking points on days 7, 14 and 21.

For the SCt group, no lethality was observed after 21 days of experiment. On the other hand, DRO and/or Hg treatments suffered a negative impact on survival rate. But, the warming condition associated with the presence of Hg and/or DRO had a greater negative impact on the survival of *N. lapillus*, since the treatments exposed to 22 ºC were particularly affected on day 21, reaching 65-80% survival rate compared with almost 90% of survival for those exposed to 18 ºC. Mix 2, 3 and 4 were the ones that presented the lowest survival rates (74-79%), except DRO1 (65%), (Figure 3.1.3).

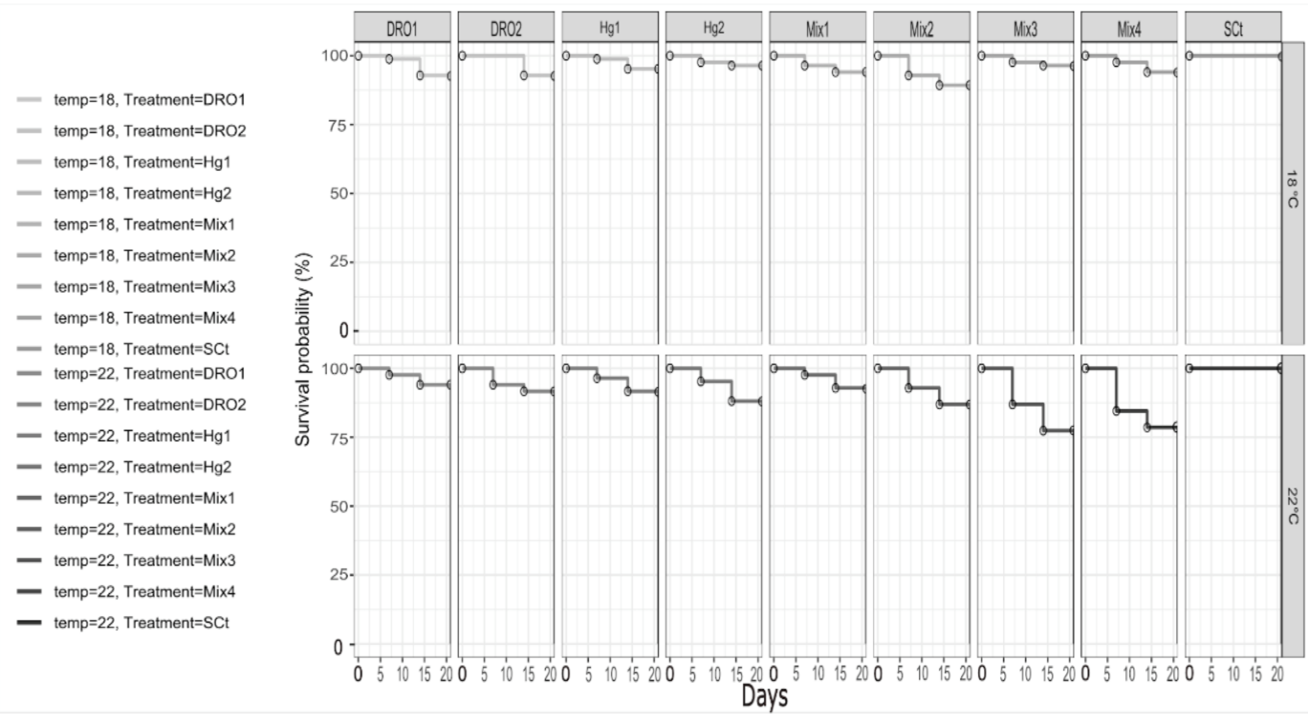


Figure 3.1.3 – Survival (%) of *N. lapillus* (n=21 individual/replicate) exposed to different combinations of temperature (18 and 22 ºC), Hg (Hg1 – 1.5 µg L^{-1}, Hg2 – 50 µg L^{-1}) and DRO concentrations (SCt – solvent control, DRO1 – 100 ng L^{-1}, DRO2 – 1000 ng L^{-1}); Mix1 – 1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2 – 1.5 µg Hg L^{-1} + 1000 ng DRO L^{-1}; Mix3 – 50 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix4 – 50 µg Hg L^{-1} + 1000 ng DRO L^{-1}. (n = 21 per treatment).

On day 7, the survival rates were quite similar for all the treatments (97-100%), except for Mix 2, 3 and 4 which were lower (84-92%). On day 7, significant effects of temperature, Hg and DRO, as well as a significant interaction between temp:Hg, were observed (GLM model, $p < 0.05$), (Table S2).

Table S2 - ANOVA results for the survival at day 7 (n=21 individuals/replicate).

	Df	**Deviance**	**Resid. Df**	**Resid. Dev**	**Pr(>Chi)**	
NULL			1511	523.77		
Temp	1	14.446	1510	509.33	0.0001442	***
Hg	2	18.181	1508	491.15	0.0001127	***
DRO	2	12.9	1506	478.25	0.0015806	**
Temp : Hg	2	7.113	1504	471.12	0.0282965	*

p<0.05;**p<0.01;p<0.001**

On day 14, significant effects of temp, Hg and DRO, as well as on the interaction between temp:Hg:DRO, were observed (GLM model, $p < 0.05$), (Table S3).

Table S3 - ANOVA results for the survival at day 14 (n=21 individuals/replicate).

	Df	**Deviance**	**Resid. Df**	**Resid. Dev**	**Pr(>Chi)**	
NULL			1511	857.77		
Temp	1	15.782	1510	841.98	7.11E-05	***
Hg	2	17.234	1508	824.75	0.000181	***
DRO	2	15.457	1506	809.29	0.00044	***
Temp : Hg	2	11.885	1504	707.41	0.002626	**
Hg : DRO	4	12.764	1500	784.64	0.012491	*

p<0.05;**p<0.01;p<0.001**

On day 21, a significant effect of temp, Hg and DRO as isolated factors, as well as interactions between Temp:DRO, and between Hg:DRO, were observed (Table S4).

Table S4 - ANOVA results for the survival at day 21 (n=21 individuals/replicate)

	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)	
NULL			1511	1313.1		
Temp	1	20.064	1510	1293.1	7.49E-06	***
Hg	2	14.646	1508	1278.4	0.0006601	***
DRO	2	22.208	1506	1256.2	1.51E-05	***
Temp : Hg	2	1.841	1504	1254.4	0.3982254	
Temp : DRO	2	9.195	1502	1245.2	1.01E-02	*
Hg : DRO	4	39.98	1498	1205.2	4.37E-08	***

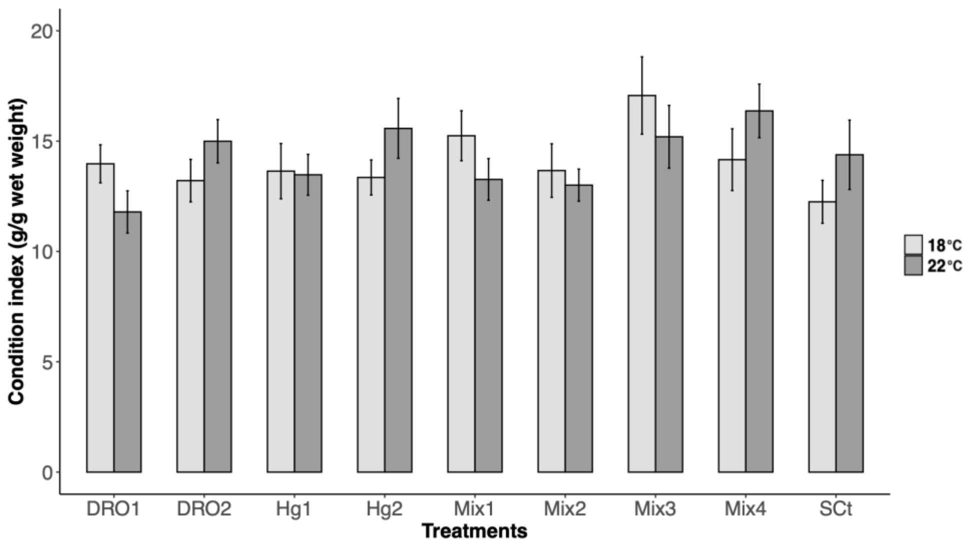
****p<0.05;**p<0.01;***p<0.001**

3.1.11.4. Condition index and consumption rate

The condition index, estimated at the end of the experimental period (i.e.,, after the 21 days) revealed no statistically significant differences among all the treatments (LMM model, $p > 0.05$), (Figure 3.1.4A).

According to the results obtained, consumption rates were very homogeneous throughout the treatments, with no significant differences among them (GLS model, $p > 0.05$), (Figure 3.1.4B).

A



B

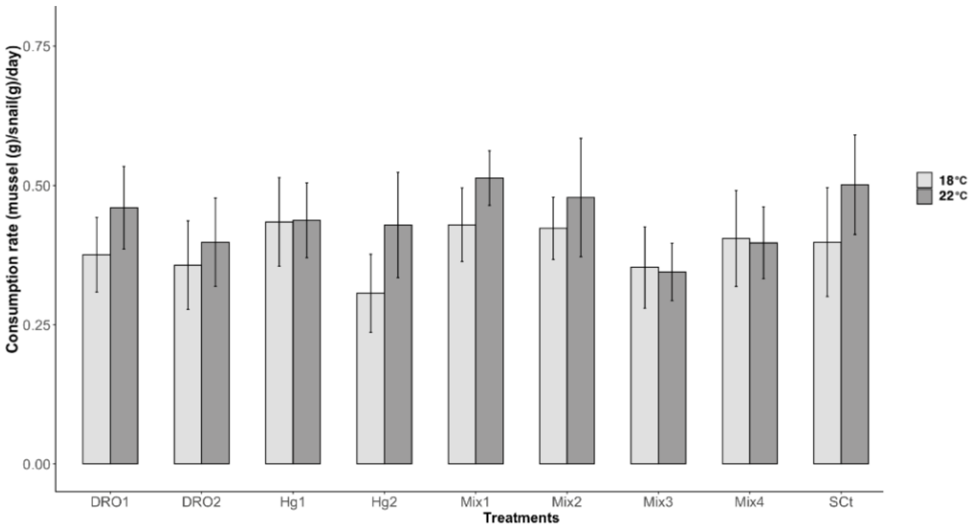


Figure 3.1.4 – Condition index (A) and consumption rates (B) of *N. lapillus* exposed to the distinct treatments (n = 12 per treatment) for the trial period (21 days). SCt: solvent control – 0.01 % ethanol; Hg1 – 1.5 µg L^{-1}, Hg2 – 50 µg L^{-1}; DRO1 – 100 ng L^{-1}, DRO2 – 1000 ng L^{-1}; Mix1 – 1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2 – 1.5 µg Hg L^{-1} + 1000 ng DRO L^{-1}; Mix3 – 50 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix4 – 50 µg Hg L^{-1} + 1000 ng DRO L^{-1}. Values represent mean (± SD).

3.1.11.5. Histological analysis of gonads' maturation

According to Figure 3.1.5, at the ambient temperature (T1), the general maturation stage in females was III-IV while in males was IV-V. In the meanwhile, in a scenario of warming (T2), we could observe a general delay in the maturation stage of the ovaries, being observed a tendency for the occurrence of follicles in stage III to the detriment of IV. In the case of males, this pattern was not visible.

Analysing the percentage of occurrence of the distinct maturation stages in the ovary of *N. lapillus*, it seems that in the solvent control (SCt) condition at 18 °C, 80% of the females were classified as being in stage IV and 20% in stage V.

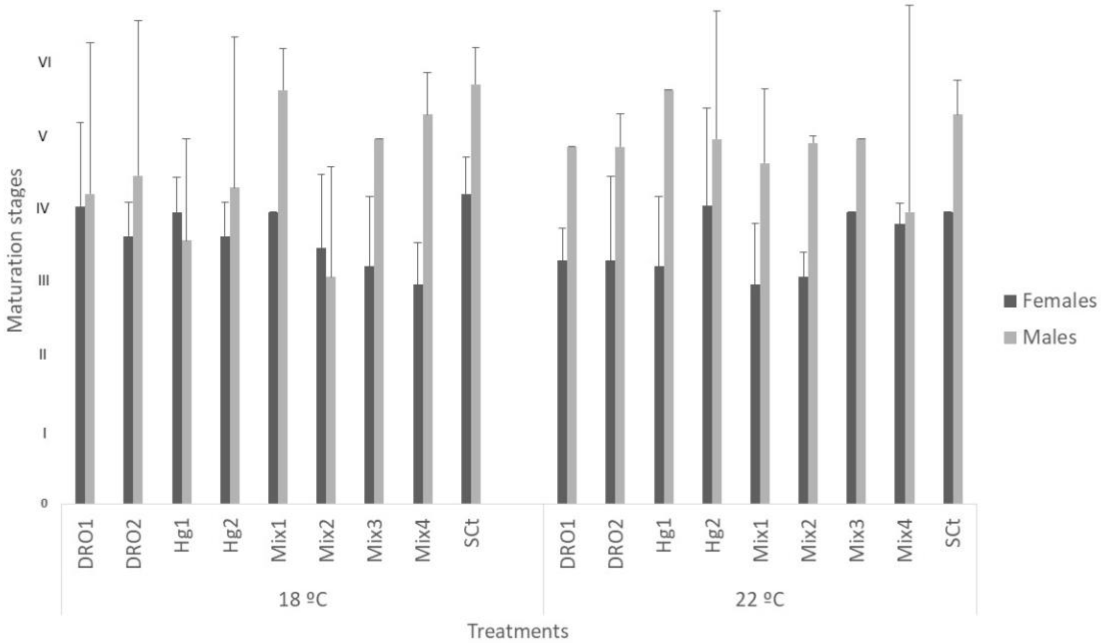


Figure 3.1.5 – Maturation stages – I (immature), II (early recovering), III (late recovering), IV (ripe), V (partially spent) and VI (spent) – of the gastropod *N. lapillus* (n=3 individuals/replicate) ovary and testis exposed to the different treatments. SCt: solvent control – 0.01 % ethanol; Hg1 – 1.5 µg L^{-1}, Hg2 – 50 µg L^{-1}; DRO1 – 100 ng L^{-1}, DRO2 – 1000 ng L^{-1}; Mix1 – 1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2 – 1.5 µg Hg L^{-1} + 1000 ng DRO L^{-1}; Mix3 – 50 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix4 – 50 µg Hg L^{-1} + 1000 ng DRO L^{-1}. Data are represented as median (± SD).

However, when they were exposed to the different chemicals (Hg and/or DRO) in combination with the increase of temperature (T2), the percentage of non-mature follicles increased (Figure 3.1.6), except for Mix1 (all females in stage IV). In males, a similar pattern was observed: in SCt most of the individuals were in stages V and VI while, in the presence of contaminants and/or elevated temperature, less mature stages occurred.

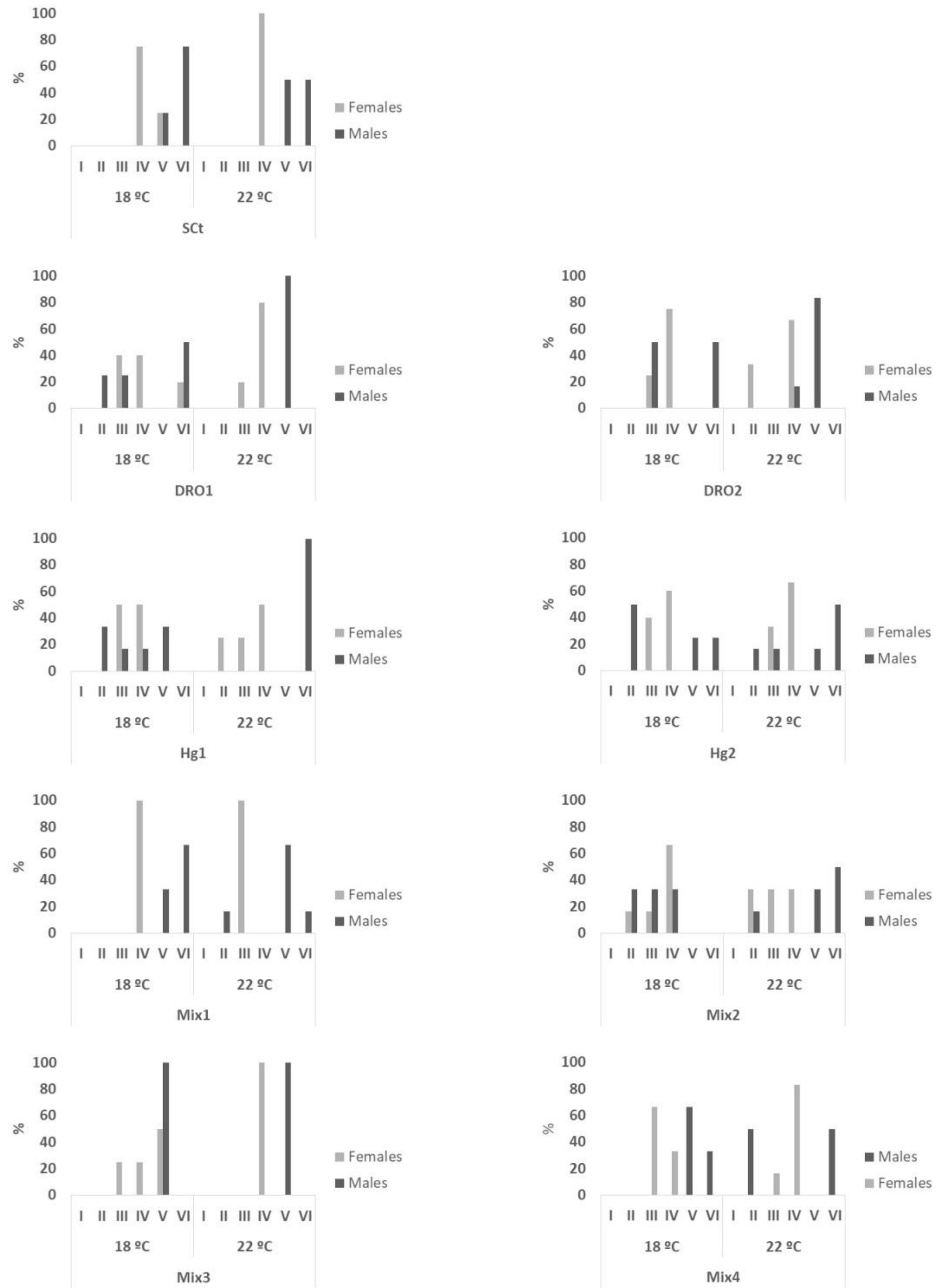


Figure 3.1.6 – Percentage of occurrence of the maturation stages of females and males of *N. lapillus* exposed to the different treatments (n=3 individuals/replicate). SCt: solvent control – 0.01 % ethanol; Hg1 – 1.5 µg L^{-1}, Hg2 – 50 µg L^{-1}; DRO1 – 100 ng L^{-1}, DRO2 – 1000 ng L^{-1}; Mix1 – 1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2 – 1.5 µg Hg L^{-1} + 1000 ng DRO L^{-1}; Mix3 – 50 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix4 – 50 µg Hg L^{-1} + 1000 ng DRO L^{-1}.

In Figure 3.1.7 are represented different stages of gametogenesis in which is visible a decline in the maturation stages, of both males and females, from SCt to other treatments (EDCs and/or warming), which confirms the pattern observed in Figure 3.1.5. For example, females of SCt were mainly in stage IV, but when exposed to mixtures of EDCs at higher temperature, other less mature stages I, and the frequency of occurrence of stage IV decreased. In males, a similar pattern was observed: in SCt males were in stages V and VI while, when exposed to EDCs, other less mature stages appeared (e.g., II, III and IV).

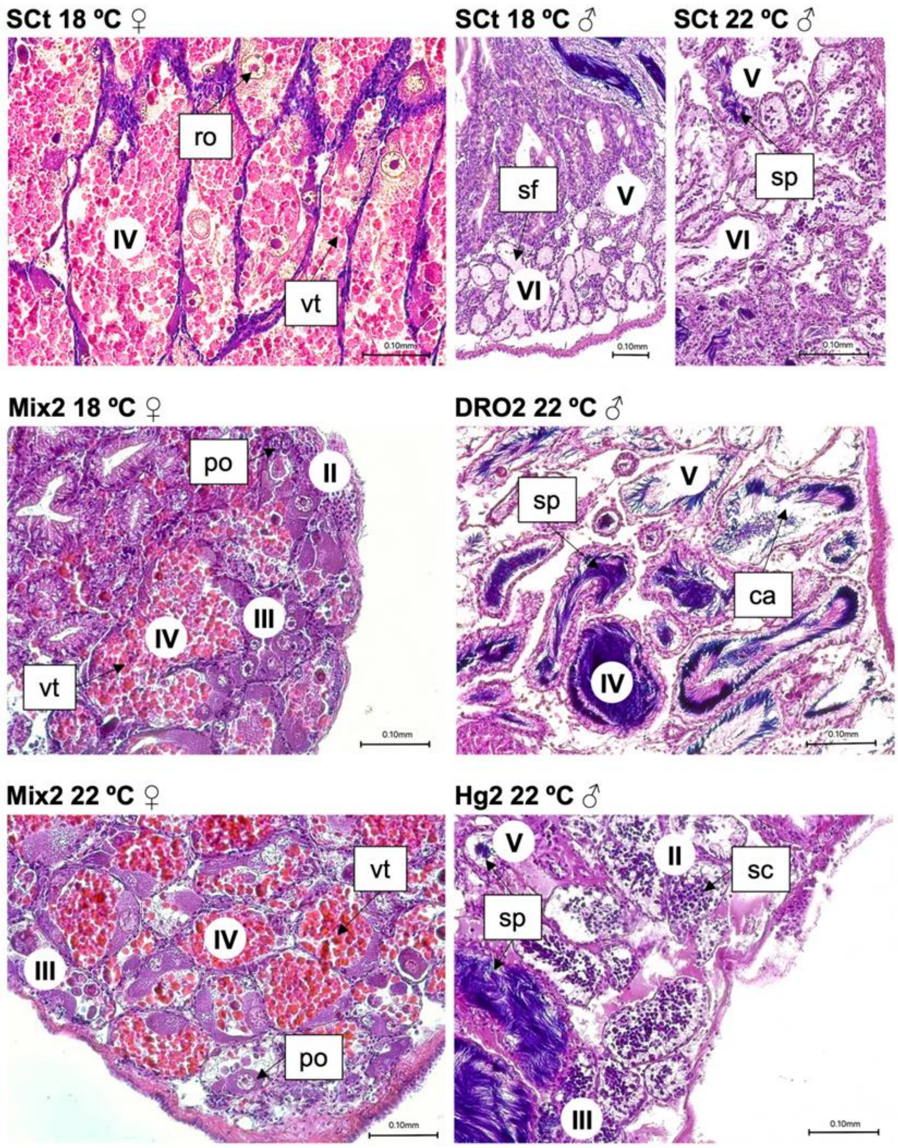


Figure 3.1.7 – Gametogenic stages (II to VI) identified in *N. lapillus* (n=3 individuals/replicate) ovaries (left column) and testis (right column) after exposure to the experimental treatment indicated on each picture top-left corner. Ca: cavity after sperm shed; po: previtellogenic oocyte; ro: ripe oocyte; sc: spermatocyte; sf: spent follicle; sp: spermatozoa; vt: vitelum.

The results of the ordered logit regression model are shown in Table S2 of chapter 3.1 (in attachments) and reinforce the previous results indicating that the males are in a more advanced gametogenic stage than females (Table S2, line 18). However, the highest temperature in combination with both concentrations of mercury (Hg1 and Hg2), (Table S2, rows 5-6), and mixtures of both contaminants (Table S2, row 9-10), had an inhibitory effect on female gonads' maturation.

In males, we found that the effect was also inhibitory at the lowest temperature (18 ºC) and in the presence of low Hg concentrations (Hg1), (Table S2, line 19). A similar effect was observed again at 22º C for Mix 1 (Hg1+DRO1), (Table 3.1.3 row 30), Mix 3 (Hg2+DRO1) and Mix 4 (Hg2+DRO2), (Table 3.1.3 rows 32-33).

The lower part of Table S2 (lines 34-37) specifies the thresholds (also referred to as cut-off points) that indicate the correspondence between a continuous variable and the different gametogenic maturation stages of the model species and the stages that were observed at the microscopic level. It should be noted that there was a negative effect of the compounds used concerning the development of the gonads, especially in the earlier stages of maturation (Table S2, rows 34-35) ($p < 0.001$).

3.1.12. Discussion

In the last decades, many studies have focused on the effects of progestins, first isolated and, more recently, as mixtures of steroid hormones, demonstrating the negative effects of these compounds on aquatic species (Fent, 2015, Liang et al., 2019, Schmid et al., 2020). However, to our knowledge, there is a lack of information about the combined effects of climate variables (e.g., temperature) and mixtures of EDCs, such as a progestin and a metal, namely mercury. So, this work breakthrough the state of the art, contributing with novel information regarding these hot topics.

According to our findings, the exposure of *Nucella lapillus* to DRO or Hg caused negative effects on the survival of the gastropod, but those effects were much more exacerbated in the presence of mixtures of both contaminants and at a higher temperature which confirms H1. This negative effect of warming on survival was similar to the observed by Falkenberg et al. (2021) for the gastropod species *Chlorostoma argyrostoma* and *Lunella granulate* in which was observed a decline of 10-20% in the survival rate when exposed to increased temperature (+ 2.5 ºC) after 40 days of exposure. A similar result was observed for the crustacean *Gammarus locusta* (Cardoso et al. 2018a). However, in the present work, there was a clear effect of DRO and/or Hg, even at low concentrations and ambient temperature, in the survival of *N. lapillus*. An opposite result was observed for *Daphnia magna* when exposed to a mixture of progestogens (Svigruha et al., 2021): no lethality was observed at any concentrations during 21 days of exposure.

Regarding possible effects of the studied stressors on the condition of *N. lapillus* and consumption rates, there were no significant impacts. Similar results were observed by Falkenberg et al. (2021) regarding effects of increased temperature on feeding and oxygen consumption rates of gastropods *C. argyrostoma* and *L. granulate*. However, in a previous study by Cardoso et al. (2018a), it was observed a significant positive effect of levonorgestrel (lowest concentration) on the consumption rates of the crustacean *Gammarus locusta*. Also, it was observed a significant negative effect of increased temperature on the condition of *G. locusta*.

Concerning the effects of studied EDCs on the gonads' maturation dynamics, it was clear that DRO and Hg as isolated factors did not affect *N. lapillus*, even at the highest concentrations tested. A similar effect of drospirenone was observed by Cappello et al. (2017) in the bivalve *Mytillus galloprovincialis*, when exposed to concentrations ranging from 20-10000 ng L^{-1}, for 7 days. However, in our study, we could observe that DRO and Hg in association with increased temperature had a synergistic effect, delaying the maturation stage of the gonads, particularly in females. So, once again, the temperature increase has a crucial impact on the vital functions of the organisms, exacerbating the effects of isolated factors, and affecting the reproductive system as expected (H1). Additionally, it was observed in both sexes an increase in the frequency of earlier maturation stages with the exposure to DRO and/or Hg under warming. In another study by Zeilinger et al.

(2009), they observed a decline in the percentage of mature vitellogenic stage oocytes of fathead minnow, from 26 to 2% after 21 days of exposure to 70 µg L^{-1} of drospirenone, which is a quite high concentration. So, compared to our study, we could see that the percentage of females in stage IV declined from 80% (SCt) to less (40%-DRO1; 50%-Hg1; Mix2-70%; Mix3-20%; Mix 4-30%), when exposed to much lower concentrations of DRO (100 and 1000 ng L^{-1}). However, the average maturation stage of the ovaries did not change when exposed to DRO or Hg. Just at a higher temperature, the effect was more visible. So, once again, these results demonstrate that the combination of stressors had a synergistic effect on the survival and gonads' maturation dynamics in the gastropod *N. lapillus*, since the combined effects of the three studied stressors were greater than the expected additive effect of the isolated stressors (according to Gunderson et al. 2016). In fact, from previous works, it was clear that the increase in temperature can exacerbate the negative effects caused by endocrine disruptors (e.g., Cardoso et al. 2017b, etc). These synergisms seem to be common in nature (Crain et al. 2008). However, some antagonistic responses can also be visible in other works. For example, Mannai et al. (2022) tested the effects of increased temperature and levonorgestrel (LNG) on the biochemical responses in the bivalve *Ruditapes decussatus* and observed that temperature diminished most of the responses to LNG. So, the responses can be variable.

Comparing the present work with Cardoso et al. (2018b), we could observe that drospirenone even at higher concentrations had a lower negative effect on *N. lapillus* gonads' maturation than a low concentration of levonorgestrel (10 ng L^{-1}) on zebrafish. So, drospirenone associated with increased temperature had a synergistic effect, being higher in females than in males. This pattern follows the literature, since high water temperature during pre-spawning phase may not be an impairment for normal gametogenesis in males (Miranda et al., 2013).

This study allowed us to infer that the last generation progestin drospirenone has, potentially, a lower negative impact on the aquatic species than other progestins, like levonorgestrel, that even at environmental concentrations (10 ng L^{-1}) caused stronger negative effects in the reproduction of certain species, like zebrafish (Cardoso et al., 2017b).

Also, from this work, it is important to highlight the relevance of studying the effects of mixtures of chemicals in comparison with isolated factors, since the results can be different and, in nature, all living organisms are exposed to highly complex mixtures of anthropogenic chemicals. In this work, it was demonstrated that compounds, on their own, may not produce significant effects but can add up to elicit substantial mixtures' responses. The same was observed in previous works by Thrupp et al. (2018). Additionally, the mixture effect was even more pronounced at higher temperature having a synergistic response.

3.1.13. Conclusions

Our findings suggest that drospirenone and mercury, as isolated factors, can negatively affect the survival of the gastropod *N. lapillus*. This effect can be synergistic when these chemicals are mixed and under warming. Nevertheless, the tested treatments (including the different concentrations of both chemicals) did not cause any effect on the condition index and consumption rates of the model gastropod.

Additionally, both chemicals did not cause an evident effect on the maturation stage of ovaries and testis of the *N. lapillus* as single factors. However, in mixture and under higher temperature, it was clear a delay in the maturation stage of the ovary but not of the testis. However, both sexes evidenced a decline in the frequency of higher maturation stages to the detriment of less mature ones with the exposure to contaminants and higher temperature. So, our results suggest that the tested stressors had a higher negative impact on females than on males.

Thus, under a medium to long-term projected scenarios of warming, the constant exposure to a higher temperature and mixtures of the studied chemicals can trigger negative consequences on the survival of the species as well as on the dynamics of maturation of the female's gonads. This means that in the long-term, the species can become more fragile and the delay in the maturation of the gonads can affect, for example, the reproduction process. Since all the processes of the organism occur in a specific timing and are connected with what occurs in nature, if the timing of one process fails others can be compromised or vice-versa. According to Gunderson et al. (2016), the way organisms respond to multiple stressors depends greatly on the intensity and relative timing of each stressor. So, if the population of *N. lapillus* will be affected, their prey can be affected too and consequently, modifications of whole trophic chain can occur. Therefore, the structure and functioning of intertidal rocky shore communities can be modified. So, the global ecosystem in which these gastropods are living can be compromised.

3.1.14. References

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3.2. Oxidative stress responses of the marine gastropod *Nucella lapillus* to increased temperature and mixtures of endocrine disruptor chemicals

Abstract

Climate change associated to the contamination by endocrine disruptor compounds (EDCs) deserves great attention due to the risks they cause to the aquatic ecosystems. This novel work evaluates the combined effects of temperature and mixtures of EDCs (i.e., mercury and drospirenone) on the biochemical responses of the dogwhelks, *Nucella lapillus*, for 21 days. Males of *N. lapillus* showed an enhancement of the antioxidant defense against oxidative stress (i.e., increase of CAT activity) when exposed to lower Hg concentrations while the exposure to increasing DRO concentrations caused CAT inhibition. Moreover, despite no significant effects on lipid peroxidation were found, males had higher MDA levels than females, meaning that the antioxidant defence system was not strong enough to prevent lipid peroxidation and cellular damage. This study reinforces that drospirenone is effectively less active than the oldest progestins, however, in future, it would be important to complement this information with other biomarker responses.

Keywords:

Warming; Progestins; Metal; mercury; *Nucella lapillus;* biochemical responses

3.2.1. Introduction

Marine ecosystems and associated biodiversity are continuously being subjected to constant changes caused by human activities (Harley et al., 2006, Wernberg et al., 2011). The warming of the climate system is unequivocal. The atmosphere and ocean have been warming in the last decades, the amounts of snow and ice decreased and sea level has been rising. As a consequence of these global changes, oceans and coastal systems have been suffering tremendous modifications, since water temperature, which is one of the most important environmental parameters, can affect the life cycle, physiology and behaviors of aquatic species (Harley et al., 2006).

Besides climate change, a large number of contaminants of emerging concern (CECs) have been reaching the environment on a global scale (Pironti et al., 2021). For example, CECs include different types of organic and inorganic chemical compounds such as endocrine disruptor compounds (EDCs) (Kasonga et al., 2021) like pharmaceutical compounds (e.g., progestins) and the metals (e.g., mercury).

Over the past decade, a great deal of research has provided new information on the mechanisms by which EDC's or mixtures EDC's (Scognamiglio et al., 2016, Ribeiro et al., 2017, Chen et al., 2022) under the influence of environmental climate conductors (DeCourten and Brander, 2017, Mannai et al. 2022) can interfere with the actions of different pathways of the endocrine system, the degree to which our environment is contaminated with these chemicals, and the relationship between chemical exposures and health outcomes in humans and wildlife(Damstra et al., 2002, Bergman et al., 2013, Lamb IV et al., 2014, Morais et al. 2023). From the large spectrum of EDCs, the synthetic progestins are considered some of the most potent endocrine disruptors even at environmental concentrations (ng L^{-1}) (Fent 2015). These steroid hormones are usually used as oral contraceptives or in human medicine and it is proved their negative effects on the reproduction of aquatic species (zebrafish, Cardoso et al. 2017, Ojoghoro et al. 2021), physiological and biochemical responses (e.g., clam *Ruditapes decussatus*, Mannai et al. 2022), sex development, metabolic responses (e.g., mussel *Mytilus galloprovincialis*, Capello et al. 2017), etc. Drospirenone is a last generation progestin, designed to minimize the side effects related to interactions with androgen, estrogen or glucocorticoid receptors (Marqueño et al. 2019). Till the moment there is scarce information regarding the effects of this hormone on aquatic species (Zucchi et al., 2014, Capello et al., 2017, Morais et al., 2023).

Concerning mercury (Hg), this is a high-priority pollutant with estrogenic properties which can cause strong negative effects on human health and aquatic fauna (Rice et al. 2014). Both EDCs (i.e., drospirenone and Hg) can act at the same hypothalamic-pituitary-gonadal (HPG) axis (Tan et al. 2009), impairing the reproductive system.

These contaminants associated with climate drivers (e.g.,, temperature) may produce a stronger and unpredictable effect on the aquatic habitat, and usually have an earlier effect at lower levels of biological organization, such as at the organism's level or even at the gene and cellular level, allowing the development of biomarkers to monitor Climate and toxic-related environmental changes (Miranda et al., 2013, Alves et al., 2016). For this, biomarkers act as an early warning of a specific harmful biological endpoint (Faheem and Lone, 2018).

The disruption of the metabolic homeostasis of the organisms by multiple stressors can occur due to the overproduction of oxidative radicals, such as reactive oxygen species (ROS) or reactive nitrogen species (RNS). These can damage DNA, proteins and lipids and consequently endanger cellular and organism fitness and functions (Pöhlmann et al., 2011). For example, lipid peroxidation, the most common cellular damage induced by oxidative stress, is the reaction of ROS with lipids, leading to the production of malondialdehyde (MDA). In order to cope with the adverse conditions, the aerobic organisms have developed cytoprotective mechanisms including antioxidant enzymes, such as catalase (CAT) which is considered an enzyme of the first line of defense that directly eliminate ROS and glutathione S-transferase (GST) enzyme that plays a role, in the process, where it facilitates the excretion of xenobiotics (Van der Oost et al., 2003, Buet et al., 2006). These enzymes are involved in the conversion of hydrogen peroxide into less reactive gaseous oxygen and water (Barber et al.,

2006) minimizing detrimental effects caused by ROS and strengthen the defence mechanism of species.

Some mollusc species, especially bivalves and gastropods are powerful model systems for a prudent and viable research approach because they are common, with high ecological and commercial importance on a global scale as food and as nonfood resources (Rittschof and McClellan-Green, 2005). Gastropods are known to be efficient accumulators of metals, organic pollutants and respond to pollution in a sensitive and measurable manner (Sarkar et al., 2014). Particularly, the dogwhelk *Nucella lapillus* (L.), is a marine gastropod species, an important predator of mussels (*Mytillus spp.*) and barnacles (*Balanus spp.*) of the Western European rocky intertidal areas that is regarded as harmless but with strong ecological relevance (Tyler-Walters, 2007).

The main goal of this study was to evaluate the effects of combined multiple stressors (i.e., temperature and mixtures of the EDCs, such as mercury (Hg) and the progestin drospirenone (DRO)) on the metabolism of the gastropod *N. lapillus*. For that, some biochemical markers indicative of oxidative stress (i.e., catalase – CAT, lipid peroxidation – LPO and glutathione S-transferase – GST) were used as a proxy to decipher the impact of the studied stressors on the functioning of *N. lapillus*, after a 21-day exposure.

3.2.2. Materials and Methods

3.2.2.1. Chemicals

Pharmaceutical: Drospirenone (DRO, CAS 67392-87-4; purity 98.0%) was purchased from TCI (Tokyo Chemical Industry, Japan). Stock solutions were prepared with analytical ethanol (CAS 64-17-5; purity 99.9%) supplied by Merck Millipore (Germany) and stored at -20 ºC.

Metal: The mercury (Hg) standard solution (1000 mg L^{-1}) in 10 % nitric acid (for Atomic Absorption Spectrometry) was supplied by Fisher Chemical (USA) and stored at ambient temperature.

3.2.3. Organisms' collection and acclimation

Nucella lapillus individuals were collected in the rocky shore of Praia Norte in Viana do Castelo, Portugal (41º 41'33''N 8º 51'06''N) in October 2020 along with *Mytilus edulis* (most relevant prey in the site) and transported to CIIMAR in a cool box. The organisms had an acclimatation period (15 days) after which they were separated by size. The same process was followed for *M. edulis*, where adults (± 3 cm, total length) were selected since they were the most abundant prey species in the field. Adults of *N. lapillus* (total length > 1.5 cm) were selected for the exposure experiment.

During the acclimation period, were simulated summer conditions, with a constant intensity photoperiod (18 h light: 6 h dark), water temperature (18 ºC), normocapnia (pH 8.1) and salinity 33-35 ppm. The organisms were fed with *M. edulis* on an *ad-libitum* basis and maintained in a semi-static system where 100% of the water was changed twice a week.

3.2.4. Experimental design

The experimental design was carried out as follows (according to a similar model used by Cardoso et al. 2018): a factorial design manipulating temperature [ambient temperature (18 ºC) and warming (+ 4 ºC)], the progestin drospirenone (DRO: DRO1 – 100 ng L^{-1} and DRO2 – 1000 ng L^{-1}), mercury (Hg: Hg1 – 1.5 µg L^{-1}, drinking water's limit and Hg2 – 50 µg L^{-1}, residual waters' limit), Mixture 1 (Mix 1: Hg1 + DRO1), Mixture 2 (Mix 2: Hg1 + DRO2), Mixture 3 (Mix 3: Hg2 + DRO1), Mixture 4 (Mix 4: Hg2 + DRO2), the Control (Ct: seawater) and Solvent control (SCt: seawater + ethanol – 0.01%), in a total of 20 treatments Table 3.2.1), for 21 days (same exposure time as in previous works (Cardoso et al., 2018, Morais et al., 2023)) Figure 3.2.1). Seawater was directly taken from the environment and filtered through a 10µm filter. Then, water flow was directed to two tanks (50 L each) and later distributed to the experimental units (2nd level). The flow was maintained at 1400 L h^{-1} with the aid of a flow pump (Eheim, Germany). The experimental units (2nd level) had artificial light, suitable for marine setups (LED light v-tac, 18w, 240v, 50 Hz, 1700 lm), controlled with a timer to guarantee the photoperiod (18 h light: 6 h dark).

In all 2nd level flasks (2 L glass flasks, diameter 9.7 cm), aeration and saltwater flow were continuous (0.6 L $h^{-1}$$flask^{-1}$). All eighty flasks were randomly distributed in ten water baths, with 8 flasks for 1 water bath (Figure 3.2.1). Finally, every line of 4 flasks (replicates) was linked via an individual communicating vessel to an external flask, so that all the contaminated water passed through a set of particle filters with 3 different meshes (5, 10 and 25 µm) then a charcoal filter, before being eliminated.

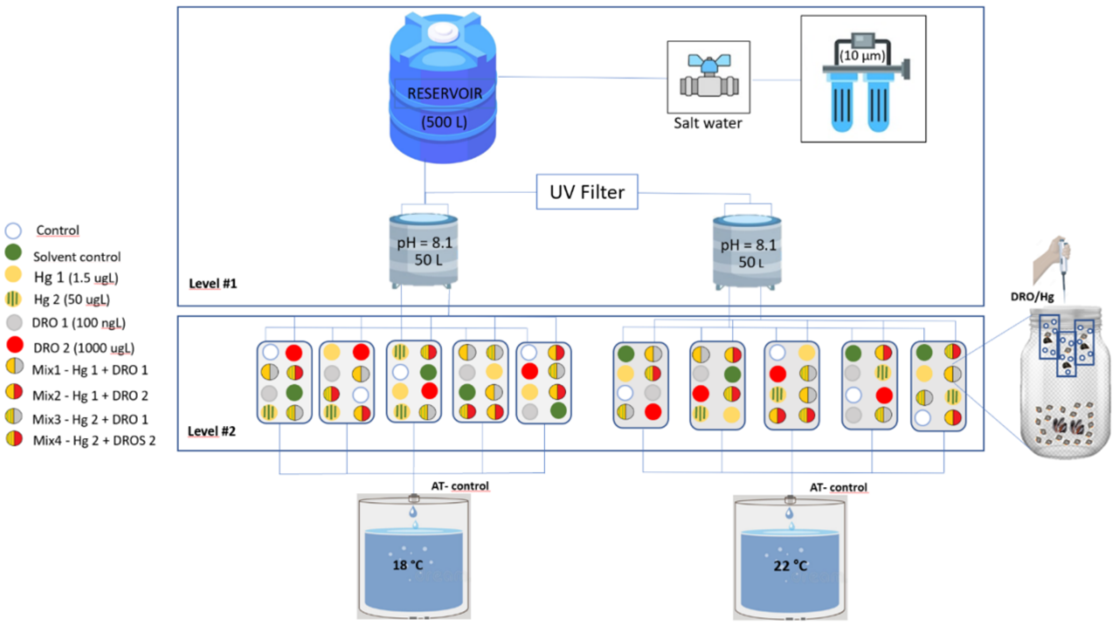

Figure 3.2.1 – Schematic representation of the experimental set-up of the mesocosm experiment with *Nucella lapillus* (L.). Level #1 represents the saltwater reservoir tank (500 L) directly connected to the internal saltwater network through a 10µm filter and the flow is distributed to two main tanks that feed each system (one for 18 ºC and the other for 22 ºC) of experimental units (2nd level). Level #2 represents the 10 water baths, for the 2 temperatures. Each water bath has 8 experimental units (i.e., flasks) corresponding to different treatments distributed randomly. In total, for each temperature there are 40 experimental units (10 treatments x 4 replicates).

According to the guidelines of the Directorate-General of Food and Veterinary of Portugal (Decreto-Lei n. º 113/2013), implementing the European Directive No. 2010/63/EU on animal welfare for scientific purposes, the experiment was carried out at CIIMAR's aquatic animal facilities.

First, the specimens were divided by treatments where each treatment was distributed randomly in the water baths, to maintain a constant temperature (Figure 1). Each group of 5 water baths was connected, in close circuit, to a reservoir with two 300 KW h^{-1} resistors, and each group was regulated by a temperature sensor controlled by AT Control power box (Aqua Medic, Germany), which automatically heated the tanks whenever temperature deviated from predetermined set points by 0.5 °C. Each replicate (flask) had twenty-one *N. lapillus* (eighteen were maintained free in the flasks plus three in isolated perforated plastic flasks for control of consumption rate) where all physicochemical parameters of the water in each replicate were controlled and measured in the experimental units three times a week and the temperature was controlled daily, as follows: salinity - 33-35 ppm, temperature - 18.3 ± 0.11ºC and 21.87 ± 0.23ºC; pH - 7.91 ± 0.05; dissolved oxygen - 99.18 ± 0.2%; ammonia - 0.13 ± 0.023 mg L^{-1} and nitrites - 0.20 ± 0.05 mg L^{-1}. The photoperiod was set at 16h light:8h dark (summer conditions), with constant water renovation. During the experiment, the organisms in the flasks were directly exposed to Hg and DRO three times a day, to simulate episodic discharges from a contamination source. During the exposure period, in order to evaluate the stability of the EDCs concentrations (DRO and Hg), water samples were collected regularly. Results were already described in Morais et al. (2023).

The water temperature was increased gradually (1 ºC per day), following the model of Cardoso et al., (2018) and Potts et al., (2021), until the highest test temperature of 22 ºC, to avoid a temperature shock to the organisms.

During the 21 days of exposure, the individuals were fed mussels (one mussel per three dogwhelk that were replaced three times a week (corresponds to approximately 6 mussels/dog-whelk/week). According to literature, the feeding rate can be very variable depending on prey size, temperature and even stress levels (Crothers, 1985, Matassa and Trussel, 2011). Thus, we considered an average value based on the literature, like 0.28 – 0.77 mussels/week and 0.7 – 1.09 mussels/week (Crothers, 1985, Hunt and Scheibling, 1998, Matassa and Trussell, 2011).

3.2.5. Oxidative stress biomarkers

Dog-whelks used for the oxidative stress biomarkers (n= 4 ind/replicate, total = 4 x 80 replicates = 320 individuals) were sampled at the end of the exposure experiment (day 21). Each individual was examined for sex identification and after that, the gonad and the digestive gland were separated from the rest, on ice, frozen in liquid nitrogen, and subsequently stored at -80 °C. Sex determination was possible only after death and based in visual characteristics. The sperm ingesting gland is used to establish sex of specimens since penis can occur on females affected by imposex (Crothers 1985, Smith 2014).

For the oxidative stress analysis, the tissues were initially weighted and the same amount of k-phosphate (HB buffer – 100 mM; pH 7.4) was added to proceed with its homogenization. After that, the protein quantification in the supernatant of the tissue homogenate was evaluated by the Lowry method (Lowry et al., 1951) and normalized to 1 mg/L. Then, all the samples were divided in aliquots (150 ml each), for the different oxidative stress tests according to (Pinheiro et al., 2021).

CAT activity was evaluated by measuring the consumption of H_2O_2 in 96-well UV microplates (Thermo Fisher Scientific) during 2 minutes at 15 seconds intervals at 240 nm and expressed as μmol/min/mg protein by adaptation from (Aebi, 1974, Ferreira et al., 2007).

GST activity was measured during 5 minutes in 20 seconds intervals at 340 nm in a 96 – well microplate (adapted from Habig et al., 1974) and using a reaction mixture of glutathione (GSH) 10 mM in HB buffer (0,1 M, pH 6.5) and 1-choloro-2,4-dinitrobenzene (CNDB) 60 mM in ethanol. The GST activity was expressed as nmol/min/mg protein.

Finally, LPO activity was determined according to (Ferreira et al, 2010) where the results were obtained by the quantification of malondialdehyde (MDA) by thio - barbituric acid (TBA) method at 530 nm and expressed as nmol MDA/mg protein. All the biochemical analyses were performed in triplicate for GST and CAT and duplicate for LPO analyses at 25 °C in a spectrophotometer (Synergy HT, Biotek, USA)

3.2.6. Data analysis

A statistical model was fit for each of the response variables (CAT, GTS and LPO activities). After a graphical exploration it was clear that all these variables showed more dispersion than assumable by a General linear model with Normal error distribution. Then, we fit Generalized Linear models using Poisson error distribution for each variable, implemented with *glm* function from *stats R* package. Even these models, for CAT and GTS activity, showed excess of dispersion (significant p-value of over-dispersion test with *dispersiontest* function from *AER R* package). In these cases then, we fit a Generalized linear model with negative binomial distribution, implemented with the *glm.nb* function from *MASS R* package. Negative binomial distribution belongs to the exponential distribution family (same as Poisson) and can fit larger dispersion than Poisson. In all these models, the independent variables employed were sex, treatment, gonad weight and their possible interactions. All the non-significant interactions were removed. With these ultimate model formulations, the significance of random factors was tested. We included as random factors the individual, nested in replicate, nested in tank. This was implemented with *glmmTMB* function from the homonym *R* package. The best model was selected based on the Akaike Information criterion (AIC), employing the *AIC* function from the *stats R* package. The formulations of the final selected models are shown in Table 3.2.1 For those independent variables that were categorical factors and showed significant effects, Tukey post-hoc tests were performed in order to test for significant differences between factor levels or combinations of factor levels. This was implemented with function *glht*, from *multcomp R* package.

3.2.7. Results

In the model for CAT activity, the random factor individual nested in replicate and nested in tank was found to significantly improve the model (AIC = 2010.2 for 11 *df* versus 2015.5 for 10 *df* for the model with immediately higher AIC). In this selected model, significant effects of sex, Hg and DRO concentrations were detected (Table 3.2.1). It was not detected a significant effect of temperature (Table 3.2.1).

Table 3.2.1 – Results of the analysis of variance/deviance for the models fitted with the CAT, GST and LPO activity (n= 4 individuals/replicate).

Independent variable	CAT		GST		LPO	
	***Chi^2*$_{df}$ and *p*-value**					
Sex	13.3_1	< 0.001	0.2_1	0.66	2.9_1	0.09
Hg	9.5_2	< 0.01	0.9_2	0.63	4.9_2	0.08
Drospirenone	15.4_2	< 0.001	4.7_2	0.10	1.3_2	0.53
Temperature	0.6_1	0.42	0.3_1	0.59	0.7_1	0.41
Tukey contrasts	***Z* value, *p*-value**					
Hg1 - C	2.8	< 0.05				
Hg2 - C	0.1	0.99				
Hg2 - Hg1	-2.6	< 0.05				
Drospirenone1 - C	-0.2	0.34				
Drospirenone2 - C	-0.5	< 0.001				
Drospirenone2 - Drospirenone1	-0.3	< 0.05				

In general, males presented a significantly higher CAT activity (16.42 ± 21.8 µmol min^{-1} mg protein^{-1}) than females (11.39 ± 13.47 µmol min^{-1} mg protein^{-1}) (Table 3.2.1, Figure 3.2.2). Moreover, individuals exposed to lower Hg concentrations (Hg1) also showed significantly higher CAT activity (16.9 ± 23.47 µmol min^{-1} mg protein^{-1}) than those individuals exposed to higher Hg levels (Hg2) (11.95 ± 10.36 µmol min^{-1} mg protein^{-1}) (Table 3.2.1, Figure 3.2.2). On the other hand, regarding the exposure to DRO, it was observed a decline in the response of CAT activity with the increase of DRO concentration (Table 3.2.1, Figure 3.2.2).

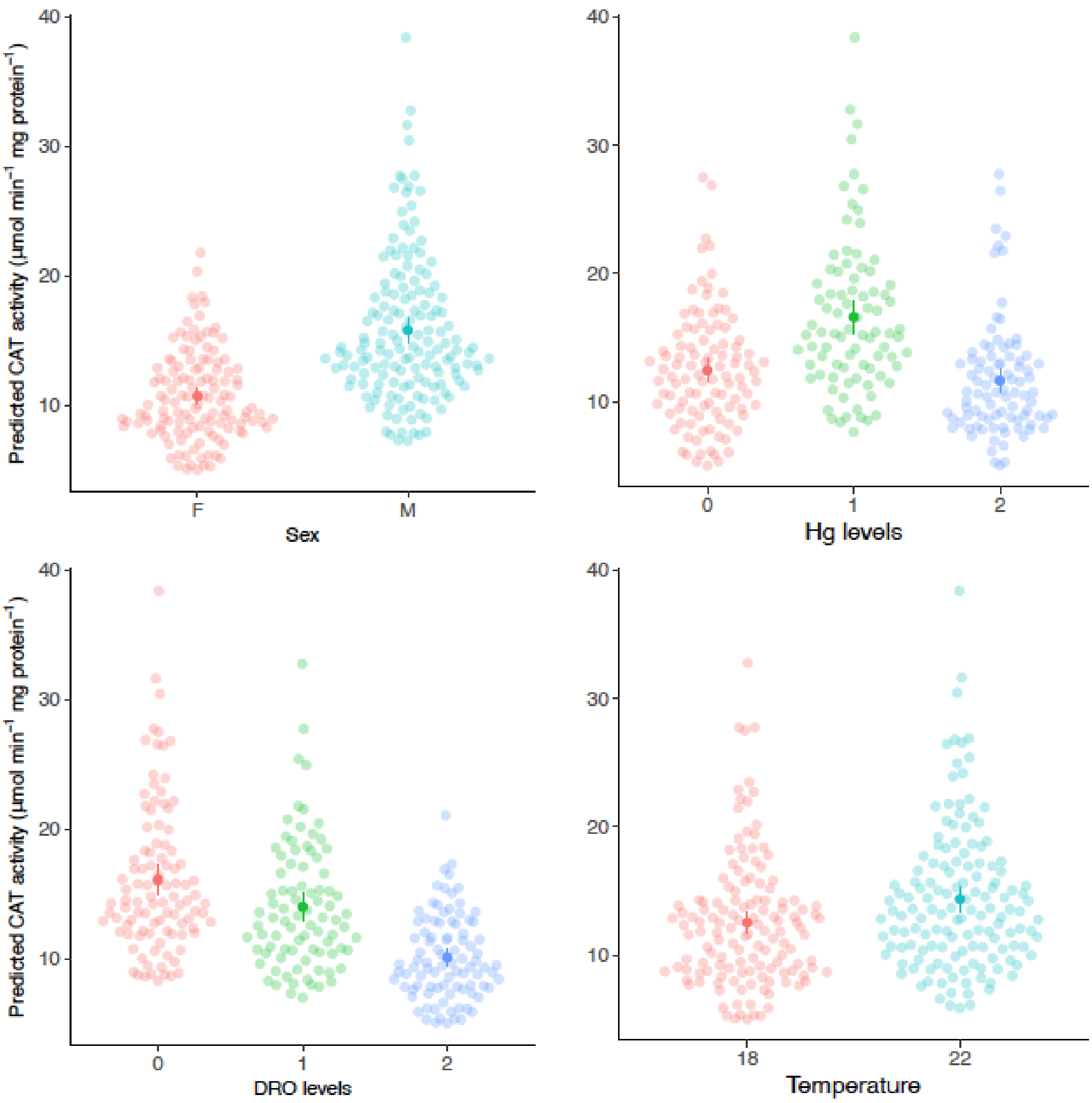

Figure 3.2.2 – CAT activity in *Nucella lapillus* (n= 4 individuals/replicate) exposed to combined factors of temperature, mercury and drospirenone for 21 days. F – females, M – males, Hg levels: 1 -1.5 µgL^{-1}, 2 – 50 µgL^{-1}; DRO levels: 1 – 100 ng L^{-1}; 2 – 1000 ng L^{-1}.

For GST and LPO, no significant improvement was detected by including random factors, and no significant effects were found for any independent variable, although DRO, for GST, was not far, as well as sex and Hg for LPO (Table 3.2.1) (Figures 3.2.3 and 3.2.4). In case of GST, despite no significant differences were observed for any of the variables, in case of DRO exposure, the individuals exposed to the highest concentration (DRO2) showed higher activity. In the later, despite no significant differences were obtained among factors, there was a tendency for the males to present a higher cellular damage (i.e., higher LPO levels) (0.15 ± 0.26 nmol MDA mg protein^{-1}) than females (0.13 ± 0.16 nmol MDA mg protein^{-1}), in agreement with the catalase results.

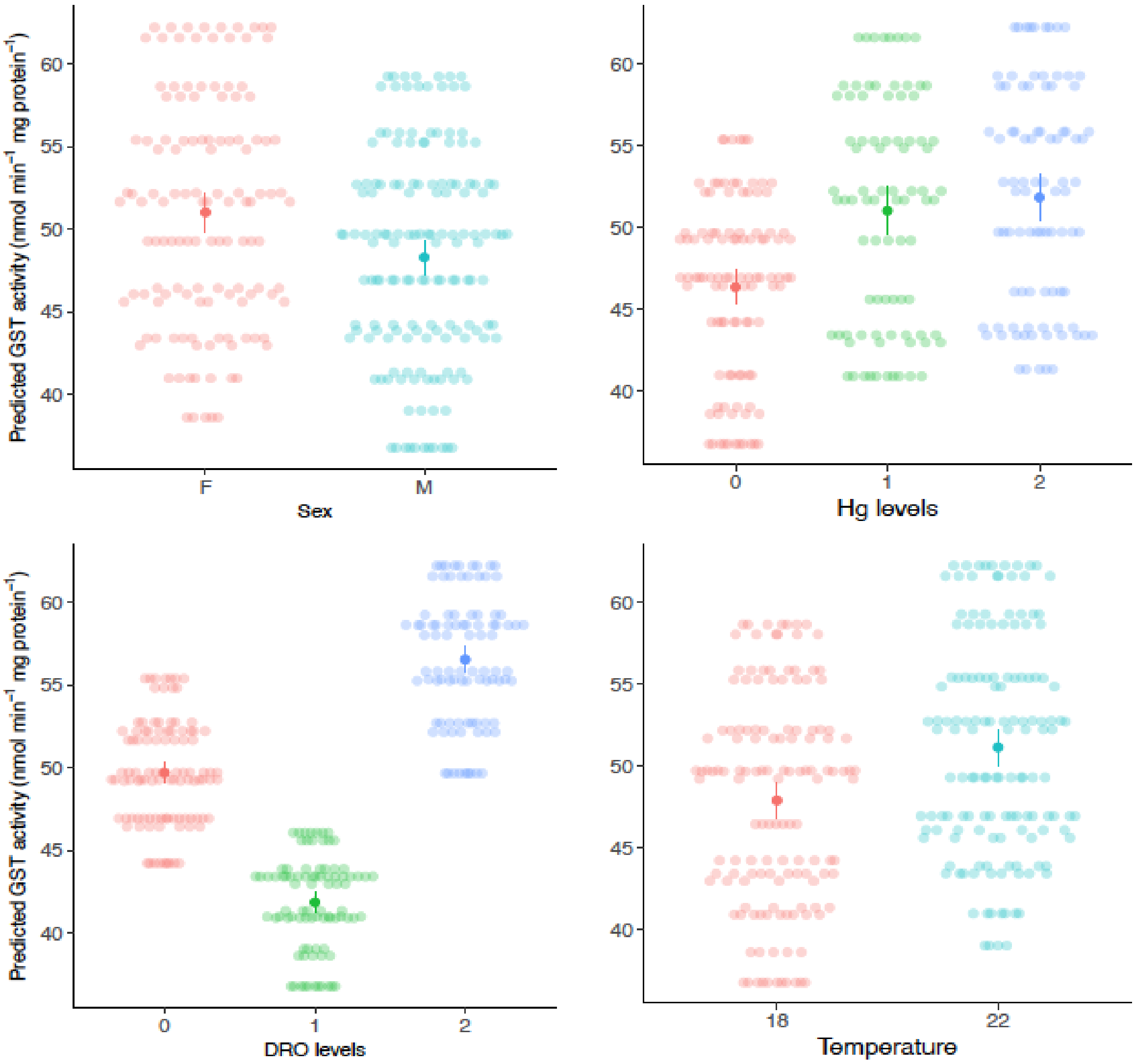

Figure 3.2.3 - GST activity in *Nucella lapillus* (n= 4 individuals/replicate) exposed to combined factors of temperature, mercury and drospirenone for 21 days. F – females, M – males, Hg levels: 1 -1.5 µgL^{-1}, 2 – 50 µgL^{-1}; DRO levels: 1 – 100 ng L^{-1}; 2 – 1000 ng L^{-1}.

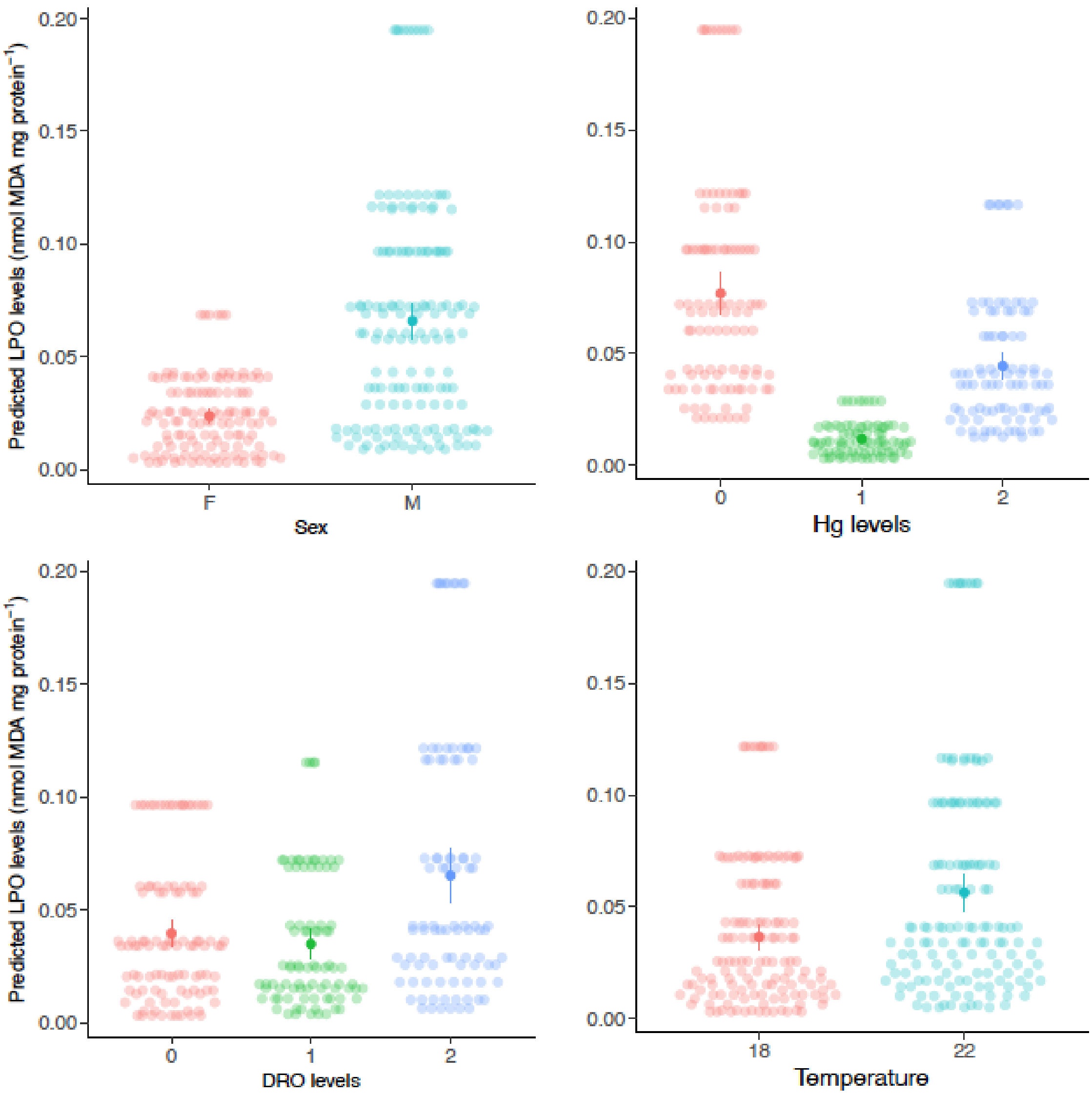

Figure 3.2.4 – LPO levels in *Nucella lapillus* (n= 4 individuals/replicate) exposed to combined factors of temperature, mercury and drospirenone for 21 days. F – females, M – males, Hg levels: 1 -1.5 µgL^{-1}, 2 – 50 µgL^{-1}; DRO levels: 1 – 100 ng L^{-1}; 2 – 1000 ng L^{-1}.

3.2.8. Discussion

Endocrine disruptor compounds like metals and synthetic progestins (Fent, 2015) have become a topic of global concern, as they are one of the main causes of pollution of the coastal environment and aquatic life (Minoia et al., 2009, Mora-Zamorano et al., 2017, Fuerst, 2019). Over the past few years some studies have been conducted on the effects of progestins on aquatic species, trying to understand the risks they cause at different functional levels (e.g., reproduction, growth, sexual maturity, etc) (Fent, 2015a, Cardoso et al., 2017a, Cardoso et al., 2018a).

However, there is a lack of information on the impact of mixtures of EDCs (e.g., DRO and Hg) associated to environmental variables (e.g., temperature) on the aquatic life, particularly in invertebrate species.

The present study breakthrough the state of the art, allowing to understand a little bit more about the combined effects of EDCs and temperature on the metabolism of the gastropod *Nucella lapillus*. Our results demonstrated that the dogwhelk *N. lapillus* showed to be quite resilient to the studied stress factors by enhancing their antioxidant protective mechanisms (i.e., CAT activity) while MDA levels remained almost unaltered. These modifications were more significant in males than in females. The exposure to Hg and DRO caused distinct responses. While the exposure to lower Hg concentrations caused an increase in the CAT activity, followed by a decline when exposed to the highest Hg concentrations. In the case of DRO exposure, the CAT activity was dose-dependent; it means, the higher the dose the lower the activity.

Mercury is known to be a potent natural oxidative agent that can produce ROS per redox cycle, generating oxidative stress in aquatic organisms as well as plucking antioxidant defenses (Sevcikova et al., 2011, Mahboob, 2013, Telahigue et al., 2019).

According to (Telahigue et al., 2019) they saw that when they exposed the *Holothurias forskali* to Hg, the response of CAT activity varied depending on the Hg dose. In fact, they found that CAT activity was significantly induced at the lowest Hg concentration and decreased at the highest concentration, justifying that the deterioration of enzyme activity at the highest dose was probably due to increased accumulation of Hg^{2+}.

In relation to the exposure to synthetic progestins, few studies have demonstrated that molluscs can be negatively affected particularly at molecular and biochemical levels.

For example, a previous study from (Contardo-Jara et al., 2011) has demonstrated that individuals of *Dreissena polymorpha* when exposed to levonorgestrel (LNG) showed modifications on the mRNA in terms of several genes belonging to metabolism and antioxidant defense.

Also, another study from (Mannai et al., 2022), demonstrated that organisms of *Ruditapes decussatus* when exposed to LNG (1000 ngL^{-1}), noted a significant increase of CAT activity, as well as GST activity, at ambient temperature. This result is contrary to ours, probably because drospirenone is a last-generation progestin with lower activity and less side-effects (Schmid et al., 2020a) than levonorgestrel. Contrarily, LNG was proved to cause negative effects on the functioning of aquatic species, even at quite low concentrations (Contardo-Jara et al., 2011, Cardoso et al., 2017a).

Regarding the GST activity, in the present study, no significant effects of the studied stressors were observed on the activity of this antioxidant enzyme, which means that both mercury and drospirenone, at the studied concentrations were not toxic for the gastropods. So, it seems that there was no need to activate the GST enzyme in order to promote the biotransformation of contaminants and facilitate their excretion, contrarily to what occurred with *R. decussatus* in the study of (Mannai et al., 2022) after exposure to LNG. However, in the latter when they combined LNG with increased temperature, the antioxidant activity (both CAT and GST) decreased.

In our study the increase of temperature did not cause significant effects on the oxidative stress response of *N. lapillus*. There are many studies that report increases in the oxidative stress responses after exposure to increased temperature (Solé et al., 1995, Ambekar et al., 2023), however in the present study that was not observed probably because *N. lapillus* is quite resilient to the increase of 4 ºC, since it is an intertidal species.

Regarding the LPO levels, and despite no significant differences were found for any of the stressors, the activity was incremented in the males, which is suggestive that they were subjected to higher cellular damage than females and as a consequence the antioxidant machinery, particularly the CAT enzyme was more activated in males than in females. However, CAT activity was probably not enough to reduce the LPO levels, due to an oversaturation of the enzyme, after some period subjected to an aggressor responsible for the oxidative stress. A similar response was observed in (Pinheiro et al., 2021). Also, in another study from (Jiang et al., 2019), it was observed that the bivalve *Ruditapes philippinarum* when exposed to mercury and benzo[*a*]pyrene, for 21 days presented an activation of the CAT activity as well as the MDA contents. This could mean that the antioxidant defense system was overloaded or the increased levels of antioxidant enzymes were equal to ROS overproduction.

So, the present results suggest that different sexes have different physiological strategies to cope with the oxidative stress. Since catalase is directly associated to the removal of ROS ions, its higher activity in males means that probably they are better prepared to cope with oxidative stress than females, having the capacity to minimize membrane lipid peroxidation.

3.2.9. Conclusions

Our results demonstrated that the studied EDCs had a significant impact on the physiological response of the dogwhelks *N. lapillus*, particularly on CAT activity. CAT response was sex-regulated, with males presenting significantly higher levels than females. On the other hand, exposure to Hg and DRO caused opposite antioxidant responses. While lower Hg concentrations caused higher CAT activity than higher concentrations, in case of DRO, the higher the dose the lower the CAT activity.

Moreover, despite no significant effects on lipid peroxidation were found, males tended to have higher MDA levels than females, which can be inferred that the antioxidant defence system was not strong enough to prevent lipid peroxidation and cellular damage.

This was a preliminary study on the physiological responses of *N. lapillus* to combined temperature and EDCs that allowed to confirm that last generation progestins are effectively less active than the oldest ones, however, in future studies, it would be important to complement this information with other biomarker responses.

3.2.10. References

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Chapter 4

General Discussion

General Discussion

Coastal ecosystems have been threatened by numerous stress factors, being the climate change and contamination by endocrine disruptor compounds (EDCs) some of the most relevant.

Over the last decades, these two topics have received a lot of attention especially as isolated factors (Runnalls et al., 2013, Rice et al., 2014, Pirger et al., 2018a, Carnevali et al., 2018), however, the main objective of this thesis was to break through the state of the art and bring some advances on the study of the interactive effects of climate drivers and EDCs on the structure and functioning of relevant coastal species.

Temperature is one of the most important environmental factors that control biochemical and physiological parameters, growth, reproduction, and survival (DeCourten and Brander, 2017) of aquatic organisms. However, with the constant climate changes, it is important to realize what are the main implications of the continuous increase in the average temperature of the oceans, since it is estimated that there will be an increase of about 4 °C by the end of the century (Wernberg et al., 2011, Arias et al., 2021), particularly in association with other stress factors, such as the EDCs. From the vast list of EDCs, for this study were selected two different groups of chemicals, the metal mercury and the synthetic hormones progestins.

Mercury (Hg) is still one of the most hazardous metals, whose endocrine effects are not well emphasized to date (Tan et al., 2009). Actually, despite reduction of Hg levels by developed countries, rapid industrialization in many parts of the world is responsible for the increment of Hg atmospheric burden in 1.5% per year (Rice et al., 2014). That is why it is important to continue to pay attention to the effects of this metal, particularly in combination with other EDCs, such as the synthetic progestins. The latter are considered an emerging problem due to the increasing worldwide use and their potential to disrupt non-target aquatic organisms. Some studies have already demonstrated the risks that they pose to the aquatic ecosystem (Carnevali et al., 2018, DeCourten et al., 2019, Chen et al., 2022), however, there is a complete lack of information about the combined effects of climate drivers and mixtures of EDCs. Therefore, the great novelty of this work lies, precisely, in the study of the combined effects of these multiple stressors.

4.1. Temporal characterization of mercury contamination along the Portuguese Coast: human health and ecological risk assessment

Chapter 2.1 had as main objectives: 1) to do a spatial characterization of mercury contamination in three distinct coastal areas along the Portuguese coast (i.e., Ria de Aveiro, Tagus estuary and Ria Formosa), and 2) to evaluate the ecological risk of exposure to Hg contamination in a temporal scale. For that, different ecosystem compartments (abiotic: water and biotics: primary producers and macrobenthos) were analysed in order to 3) evaluate, also, the possible risk of consumption of some marine species (i.e., bivalves) for human health.

According to the results obtained, the levels of total dissolved Hg in surface waters of Ria de Aveiro (i.e., Gafanha do Carmo and Torreira) were higher than in the other two systems, particularly in autumn periods. While in the Tagus estuary (particularly, Seixal and Alhandra), the highest concentrations were observed in the spring. In both of them, the Hg values were above the environmental quality standards established for mercury (70 ng L^{-1}) in surface waters and also the ecotoxicological assessment criteria (> 50 ng L^{-1}), which means that those areas are of particular concern in terms of ecological risk.

Both, Ria de Aveiro and Tagus estuary are coastal areas with an historical contamination by Hg due to the industrial activities associated (Fidélis et al., 2019, Couto and Ribeiro, 2022) Also, the continuous dredging and other port activities may be other factors that justify the availability of Hg in those systems (Eggleton and Thomas, 2004, Cabrita et al., 2020).

Regarding the Hg levels in the fauna and flora of these three different systems, the results showed that organisms from the Tagus estuary presented higher Hg concentrations than the ones from Ria Formosa and Ria de Aveiro. This was probably related with the Hg concentrations in the respective sediments, since most of the collected fauna is benthonic and deposit-feeder (i.e., lives close to

the bottom and feeds mainly on detritus). Despite in the present study, there were no data on Hg levels in the sediment, according to the literature, the studied sites in Tagus estuary tended to have higher Hg levels (0.2-1.5 $\mu g.g^{-1}$) (Canário et al., 2005) than those in Ria de Aveiro (e.g., Torreira - <1 $\mu g.g^{-1}$) (Pereira et al., 2009) or Ria Formosa (0.05-0.1 $\mu g.g^{-1}$) (Coelho et al., 2014). However, we found that although concentrations vary along the trophic chain, they do not represent a risk to public health, since values are far below the legislation values (0.5 $\mu g.g^{-1}$ ww) established by the European food safety legislation (Commission, 2006).
In a temporal point of view, Hg concentrations in macrobenthic communities of Ria de Aveiro, were constant throughout the year. On the other hand, in the Tagus estuary and in the Ria Formosa, significant differences between sampling periods were observed (higher concentrations in spring/summer than in autumn). These differences can be related with the type of trophic web characteristic of each site. In case of Ria de Aveiro, most of the collected species were deposit-feeders (i.e., feed on the detritus close to the sediment), so it is expectable that those Hg values do not fluctuate too much during the year. However, in case of Tagus estuary and Ria Formosa, the trophic webs are more heterogeneous, so it means that they are constituted by different trophic groups including suspension feeders, herbivores, etc. So, the Hg sources are different (e.g., suspended particulate matter, microalgae, macroalgae), and its content can vary more seasonally, which will influence the entire trophic web. Previous studies, corroborate our results, and admit that in different species of crabs, the accumulation of metals was quite variable and depended mainly on the environmental concentrations (Reichmuth et al., 2010, Díaz-Jaramillo et al., 2013).

Considering the risk assessment analysis to the edible bivalve communities, the present study demonstrated that there is no risk in terms of health effects, since hazard quotients were always lower than 1 (Copat et al., 2012). So, despite there is no special concern in terms of health effects, the ecological risk assessment revealed that high Hg levels in some sites in Ria de Aveiro can constitute a medium risk for the aquatic species.

4.2. Environmental characterization of progestins levels in surface waters of Portuguese coast

Chapter 2.2 had as main goal to do a pilot characterization of the levels of most consumed synthetic progestins in surface waters of three main estuaries along the Portuguese coast. This was a preliminary study but quite important to do a first characterization of these compounds in Portuguese surface waters. And at the same time allowed to develop an analytical method for the extraction and quantification of these compounds, which brings novelty to the literature.

Our findings demonstrated that desogestrel (DSG) and drospirenone (DRO) were the most abundant progestins in the Portuguese coast. On the other hand, it seemed to occur a spatial difference in the occurrence of these compounds. In the North (i.e., Ria de Aveiro), the dominant progestin is DSG while in the South (i.e., Tagus estuary and Ria Formosa) DRO was also quite representative, particularly in the summer.

Tagus estuary was the one that presented slightly higher values of progestins (i.e., DRO and DSG) in summer and autumn campaigns than the other two systems. In a temporal scale, DRO was only present in the summer months while DSG was more abundant in the autumn ones.

In Ria Formosa, the pattern of occurrence of progestins was similar to Tagus estuary and DRO was significantly more abundant in summer. So, it seems that Tagus estuary and Ria Formosa which are located in more populated areas (in Tagus estuary due to both residents and visitors while in Ria Formosa during summer the population more than triple due to visitors) presented a different pattern compared to Ria de Aveiro. Probably, the increase of DRO in summer is justified by the presence of foreign people that consume contraceptives whose composition has more DRO compared to those used by the resident Portuguese population. This is in accordance with the literature, since for different countries there are different inputs of progestins (see Rocha and Rocha (2022)). And it is well known that higher levels of progestins are associated to higher populational areas, as demonstrated in previous studies (Tan et al., 2015, Xu et al., 2016, Liu et al., 2017).

Progestins concentration found in the Portuguese coast are in the range of those found in surface waters in other systems worldwide (Wang et al. (2021) and references therein) with average concentrations below 100 ng L^{-1}, with some peaks in summer and autumn. These concentrations can be comparable to the values recorded in WWTP effluents, which constitutes a matter of concern, since the efficiency of the treatment plants to remove these compounds is poor (Runnalls et al., 2013, Kumar et al., 2015, Fent, 2015a, Rocha and Rocha, 2015, Ojoghoro et al., 2021).

So, it is particularly important to continue to monitor different aquatic systems, including different matrices (i.e., water, sediment, and biota) in order to understand if these compounds can be accumulated in the sediments and at the same time bioaccumulated in the trophic web and understand what is the risk of consumption of certain species (e.g., seafood) to human health. At the same time, only through a more exhaustive study of the synthetic progestins it is possible to alert the competent authorities for the necessity of regulation of those compounds.

4.3. Ecotoxicological effects of mercury, progestins and mixtures of both EDCs in aquatic ecosystems

Contaminants of anthropogenic origin, such as Hg, are a constant threat to marine ecosystems. Being one of the oldest neurotoxic heavy metals in the world, which over the years proved to be a very powerful EDC, became one of the major environmental problems (Zhu et al., 2000). Through the bioaccumulation process, Hg can cause negative effects on wildlife and, consequently, on human health (Rice et al., 2014). Its persistence in the environment can be long, due to its capacity for accumulation and distribution in the tissues of aquatic organisms (e.g., fish and bivalves) and biomagnify along the trophic web (Rivera-Hernández et al., 2019, Zheng et al., 2019).

To this end, several studies have contributed to understand what is the real impact of this compound on the aquatic environments. Some of these studies have shown that Hg can act at the hypothalamus-pituitary-gonad (HPG) axis

(Zhang et al., 2016), interfering on the expression of the genes that regulate the reproduction, causing negative effects at that level for example, in fishes (Crump and Trudeau, 2009, Zhang et al., 2019). Also, it can affect other organisms, such as bivalves, especially on physiological, metabolic and reproductive functions (Pytharopoulou et al., 2013). Other studies, have shown that Hg can negatively affect liver functions, for example in fishes (Chen et al., 2017).

Like Hg, the synthetic progestins (such as DRO), are also potent EDCs, derived from progesterone (P4) and other androgenic hormones like testosterone. They are emergent compounds that have raised much concern in the global scientific community due to the adverse effects they can cause on marine ecosystems, even at low concentrations (ng L^{-1}) (Matozzo et al., 2008).

Progestins can act at the same level of the HGP axis, managing to affect several physiological functions. Several studies have shown its ability to inhibit and deregulate the HPG axis, affecting males and females, and causing various adverse effects, such as fertility (Hua et al., 2015, Frankel et al., 2016), impair reproduction (Svensson et al., 2013), altered hormone levels (Runnalls et al., 2013, Kumar et al., 2015) and affect gonads' maturation dynamics (Cardoso et al., 2017a, Carnevali et al., 2018).

At the same time, in addition to wild organisms, human beings are also unintentionally exposed to these two types of compounds, either through contaminated waters or through the ingestion of contaminated animals (Rice et al., 2014, Zhao and Fent, 2016).

Attending to the lack of information in the literature regarding the interactive effects of multiple stressors and considering that both studied EDCs (i.e., Hg and progestins) act at the same level of the HPG axis, it is important to understand what are the effects that they can produce, isolated and in mixtures, and how they can act on the different pathways and thus understand what is the real impact of these compounds in the aquatic ecosystems.

So, the novelty of this thesis lies on the understanding of the impact of multiple factors (i.e., temperature, Hg and progestins) on the functioning of important coastal species.

Thus, chapter 3.1 had as main goal to evaluate the combined effects of mercury and drospirenone, under increased temperature (simulating warming scenario) on the performance and gonads' maturation dynamics of the marine gastropod *Nucella lapillus*.

To this end, our results showed that the exposure of *Nucella lapillus* to the chemicals caused negative effects on the survival of the gastropod, but those effects were much more exacerbated in the presence of mixtures of both contaminants and at a higher temperature. For example, treatments exposed to 22 ºC suffered a decline in the survival rate of 10-25 %, depending on the treatment. In general, the mixtures exposed to increased temperature were the ones that suffered a greater reduction (≈ 25%) in the survival rate.

The negative effect of increased temperature on the survival of aquatic organisms is well known (Edwards and Richardson, 2004). For example, according to the work of Ma et al. (2022), they verified that when they exposed adults of Asian green mussels (*Perna viridis*) at higher temperatures (20, 25 and 30 °C) and different salinities (23, 28 and 33 ‰) for 7 days, they had a decrease (between 20 e 45%) in survival rate at higher temperature (30 °C).

A similar result was observed in the study of (Rahman et al., 2019), with different species of molluscs, to different temperatures (15, 20 and 25 °C) during 14 days. The authors verified all species have a decrease in the survival rate, however the mud cockle *Katelysia rhytiphora wa*s the most affected species by the increase of temperature. So, our data also corroborates what is described in the literature.

Regarding the effects of Hg and DRO, even at low concentrations they caused some mortality on the studied species. According to the literature, there are some records that corroborate our results. For example, in the work of Penglase et al. (2014), they explored the effects of exposure to organic Hg and selenium on the growth, survival and reproduction of female zebrafish. They observed that when increased the amount of Hg in the diet of zebrafish, they suffered a reduction in the growth and survival rate.

On the other hand, in a work carried out by (Gilroy et al., 2014), they evaluated the toxicity and bioconcentration of three pharmaceuticals: moxifloxacin, rosuvastatin, and drospirenone (0–3 mg L^{-1}) on the mussel *Lampsilis siliquoidea. They verified that, in the case of DRO, it did not have a significant impact on mussel survival.*

In another study from (Teigeler et al., 2022), where they exposed individuals of zebrafish (from embryonic to adult stages) to levonorgestrel (0.06, 0.16, 0.47, 1.64 and 5.45 ng L^{-1}), they observed that survival rates were affected in the first stages of life at a concentration ≥ 0.47 ng/ L^{-1}.

Concerning the effects of studied EDCs in the gonads' maturation dynamics, it was evident that both Hg and DRO, even at highest concentrations did not affect the maturation of the gonads of *N. lappilus*. This result was also observed by Cappello et al. (2017a), for the mussel *Mytilus galloprovinciallis* when exposed to high concentrations of DRO (20 ng L^{-1} to 10 µg L^{-1}). However, in combination with increased temperature, they presented a synergistic effect leading to a delay in the gonads' maturation stage, particularly in females. So, again the increase of temperature revealed to have a relevant impact on the functioning of aquatic organisms, affecting the reproductive system in accordance with Miranda et al. (2013).

Regarding a possible effect of Hg on the gonads' dynamics, Wang et al. (2016) observed that females of zebrafish suffered a negative impact on the development of the gonads (i.e., delay on oocytes maturation) when exposed to Hg, (5µg L^{-1} or 10 µg L^{-1} (measured concentration 367 or 557 ng L^{-1}). Conversely, in males, when they exposed fish to the same concentrations of Hg, there was a delay on spermatogenesis. Other studies from Hatef et al. (2011) and Nowosad et al. (2018) found that when they exposed fish (*Perca fluviatilis* and *Anguilla anguilla,* respectively) to different concentrations of Hg, they observed changes on the reproductive system, namely on sexual indices (i.e.,, gonad maturation and gonadosomatic index) in the normal development of gonads, disturbances on the hormonal balance causing an interruption in the transcription of genes of the HPG axis and even a sex change.

Additionally, in our study it was observed in both sexes an increase in the frequency of earlier maturation stages with the exposure to DRO and/or Hg under warming In another study by Zeilinger et al. (2009a), they observed a decline in the percentage of mature vitellogenic stage oocytes of fathead minnow, *Pimephales promelas*, from 26 to 2% after 21 days of exposure to 70 µg L^{-1} of DRO, which is a quite high concentration. However, LNG significantly affected the number of eggs at a concentration of 0.8 ng L^{-1}.

Moreover, in our study, we could see that the percentage of females in stage IV declined from 80% (SCt) to less (40%-DRO1; 50%-Hg1; Mix2-70%; Mix3-20%; Mix 4-30%), when exposed to much lower concentrations of DRO (100 and 1000 ng L^{-1}). However, the average maturation stage of the ovaries did not change when exposed to DRO or Hg. Just at a higher temperature, the effect was more visible. In comparison with Cardoso et al. (2018a), we could observe that a low concentration of LNG (10 ng L^{-1}) had a much stronger effect on gonads' maturation of zebrafish than DRO at higher concentrations. Also, other works from Kumar et al. (2015) and Steinbach et al. (2019), comparing the effects of DRO with another progestin, such as LNG in fishes, they observed that the impact of DRO was lower, leading to the understanding that DRO may not have such harmful consequences for the aquatic environment like older progestins (e.g.,, LNG).

According to Brown et al. (2015), they suggested that continued exposure to EDCs and high temperatures can have a negative impact on survival, development and reproduction of zebrafish, causing population decline over time. They found that when they exposed zebrafish to increased water temperature and to the antifungal Clotrimazole independently, they caused male-skewed sex ratio, and the combined effects were greater.

Another study from Zhao et al. (2015), observed that when males of zebrafish were exposed to DRO (between 7 and 13650 ng L^{-1}), a greater amount of mature sperm was observed. In the case of females, it was found that at the same concentrations a degeneration of oocytes, was verified. Also, a decline on fertility rate was observed.

Concluding, our findings reinforce the results from previous studies, allowing to infer that consumption of last generation progestins has much less impact on the environment than oldest ones, which is a positive sign of evolution on the research and development of new chemical formulas.

On Chapter 3.2 were evaluated the oxidative stress responses of *Nucella lapillus* to increased temperature and exposure to mixtures of EDCs (Hg and DRO). The present study showed that *N. lapillus* showed to be quite resilient to the studied stress factors by enhancing their antioxidant protective mechanisms (i.e., catalase (CAT) activity) while MDA levels remained almost unaltered. These modifications were more significant in males than in females. On the other hand, these two chemicals caused different biochemical responses on the gastropod *N. lapillus*. While the exposure to lower Hg concentrations caused an increase in the CAT activity, followed by a decline when exposed to the highest Hg concentrations. In the case of DRO exposure, the CAT activity was dose-dependent; it means, the higher the dose the lower the activity.

As CAT is one of the enzymes of the first line of defence of the organisms to oxidative stress (Pandey et al., 2003), it is supposed to increase its activity in response to stress factors. According to Zhang et al. (2016) when they exposed zebrafish to different concentrations of Hg (15 and 30 $\mu g\ L^{-1}$) for a period of 4 months, the CAT and SOD activities increased. The authors verified that Hg can regulate the expression of sod1 and cat1 in the testis. Thus, they concluded that these enzymes are regulated by Hg at the predominant transcription level.

Similar results were observed by Verlecar et al. (2007), in which individuals of *Perna viridis* exposed to Hg (45 $\mu g\ L^{-1}$) and different temperatures (20, 26 and 32

°C) for 3, 7 and 14 days, presented an increase in the activity of CAT and GST mainly in the tissues of the gills and digestive gland.

Regarding the effect of DRO on the oxidative stress responses, it was observed an inhibitory response of the CAT activity with the increase of DRO concentration.

In the literature there are few studies that report the effects of progestins at biochemical levels. For example, (Mannai et al., 2022), demonstrated that organisms of *Ruditapes decussatus* when exposed to LNG (1000 ngL^{-1}) presented significant increases on CAT and GST activities, at ambient temperature. Although our results have not demonstrated a significant activity of GST, we can justify our results possibly because DRO is a progestin of 4th generation with lower activity and less side-effects (Schmid et al., 2020a) than older ones, such as LNG.

Finally, the oscillations in ambient temperature can trigger the activation of different mechanisms responsible for the induction and response to oxidative stress to which organisms are subjected (Lushchak, 2011). The stimulation of metabolic processes caused by the increase in temperature can trigger a response, such as increased oxygen consumption and consequent increase in ROS production, as a response to oxidative stress to which they are subjected.

However, in the present work, temperature did not affect significantly the homeostasis of the organisms. Probably, because due to the intertidal position of the *Nucella lapillus*, it is a quite resilient species to temperature.

Finally, with regard to the MDA levels, and even the results were not significant, it was observed an increasing trend, in relation to control, particularly in males. This suggests that males were probably more subjected to oxidative stress and some cellular damage than females and even CAT activity has increased in males, probably was not enough to avoid those damages., This pattern can be explained in the basis that in an oxidative stress scenario, CAT could be oversaturated and thus trigger LPO response to try to reduce ROS (Pinheiro et al., 2019). This integrates well with our results. Furthermore, in the work of Jiang et al. (2019) it was observed that *Ruditapes philippinarum* when exposed to mercury and benzo[a]pyrene during 21 days, presented significant increases in CAT activity as well as on MDA levels ($p<0.01$) which could mean that the antioxidant defense system was overloaded or that the increase in antioxidant enzymes was equal to that of ROS production. However, the question on whether antioxidant defenses would be able to give a stronger response in longer periods of exposure remains.

It should be noted that an increase in the period of exposure may increase the level of response in the defense of the organisms to oxidative stress.

This study on the physiological responses of *N. lapillus* to combined temperature and EDCs also confirmed the results of chapter 3.1, in which the progestin drospirenone, being a last generation progestin is more specific and cause less side effects than other progestins. However, in future studies, should be done other kind of analyses (e.g., molecular analysis) to validate these data.

4.4. Future recommendations

In the future, due to the lack of information on the impacts of some of the studied emergent compounds and their interactions with other stressors on the aquatic ecosystems, it would be important:

- To do an extended environmental characterization of the levels of synthetic progestins, reaching as much coastal systems as possible. This environmental characterization should be done not only at the level of the surface waters, but it would be important to characterize other matrices, such as the sediment and also different levels of the trophic web (planktonic, subrabenthic and macrobenthic community), in order to infer about the bioaccumulation and biomagnification ability of these compounds through different species life stages and in different trophic levels. Also, increasing the number of sampling campaigns could allow for a better seasonal characterization of the pattern of distribution of these compounds.

- To understand which are the effects of these multiple stressors not only at the organism level but also, at the level of the cell, for example. It would be relevant to study their effects at the molecular and physiological levels, both in vertebrates and invertebrates. These studies would improve the knowledge and understanding of toxicity mechanisms, allowing us to understand their modes of action and what impacts they can cause over time.
- From a more generational perspective, it would be important to understand the impact of the studied chemicals over generations, on different species (e.g., vertebrates vs invertebrates). Through this kind of studies, it will be possible to understand better if there is maternal transfer to the offspring and consequent generations, besides analysing the effects through generations.

- To combine more and more, environmental data with experimental data in order to have a holist view about the real impact of these emergent compounds associated to other stressors and this way contribute with new knowledge to promote a better management of the coastal ecosystems. Only through chronic partial or full life-cycle studies it is possible to confirm the negative effects of these compounds being able to discuss this topic with the regulatory entities, in order to create environmental quality standards, particularly for the case of progestins.

4.5. References

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Attachments

Chapter 2.1

Temporal characterization of mercury contamination along the Portuguese Coast: human health and ecological risk assessment

Table S1 – Temporal variation of the physicochemical parameters in Ria de Aveiro study sites.

		spring				summer				autumn				winter			
	Study areas	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**
Ria de Aveiro	Ovar	19.1	7.9	4.1	8.55	20.3	7.6	1.2	7.2	16.7	8.8	2.5	7.7	12.2	11.5	3.7	7.52
	Torreira	19.4	8.7	25.5	8.01	22.5	9	28.9	8.11	16.4	12.1	18.3	8.32	13.6	11.6	22	8.06
	Murtosa	19.7	9.6	14.1	7.87	22.7	6.9	30.7	8.01	15.1	8.7	9.6	7.58	11.8	11.9	14.8	7.54
	Gaf. Carmo	19.7	11	11.6	8.24	20.8	5.1	24.6	7.73	16.4	9.9	15	7.83	12.5	12.1	24.5	7.84
	Gaf. Boa Hora	18.9	7.3	0.8	9.17	21.9	5.6	11.5	7.89	16	8.7	1	7.95	11.2	12.3	1.5	7.93

Table S2 – Temporal variation of the physicochemical parameters in Tagus estuary study sites.

		spring				summer				autumn				winter			
	Study areas	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**
Tagus estuary	Alhandra	17.45	10.7	2.15	8.5	25.9	7.5	3	7.8	20.6	8	1.8	8.3	16.6	10.4	0.1	7.9
	Trancão	18.35	6.9	6.55	8.17	24.2	6.3	25.4	7.2	21.9	3.2	4	7.9	16.7	9.5	1.5	8.3
	Samouco	16.7	11.35	29.8	8.63	25.7	6.8	30.3	7.7	19.8	8.6	30.4	8.1	14.8	11	22.2	8.15
	Seixal	16.35	9.1	30.7	8.19	26.2	5.3	29.4	7.9	19.1	7.6	30.6	7.9	15.1	10.7	26.1	7.9
	Trafaria	13.8	6.3	18.4	6.68	24	9.9	31.9	8.0	18.7	9.4	34	8.1	15	11.2	31.2	8.0

Table S3 – Temporal variation of the physicochemical parameters in Ria Formosa study sites.

		spring				summer				autumn				winter			
	Study areas	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**	**Temp. (ºC)**	**Oxygen (mgL^{-1})**	**Salinity**	**pH**
Ria Formosa	Aeroporto	19.05	11.8	35.95	8.38	26.2	7.6	36.3	7.83	19.7	9.4	36.5	8.06	-	-	-	-
	Faro	17.8	10.55	35.15	8.36	28.1	6.8	35.3	7.66	19.9	8.5	34.6	8.2	-	-	-	-
	Olhão	18.05	9.35	34.6	8.38	27.3	9	35.1	8.08	20.6	8.3	34.6	8.21	-	-	-	-
	Fuseta	18.85	11.2	31.3	8.49	27.2	8.4	34.7	7.85	20.8	8.7	34.2	8.2	-	-	-	-
	Tavira	18.25	10.5	34	8.43	27.3	8	34.7	8.34	19.1	9.3	34.5	8.26	-	-	-	-

Chapter 3.1

Combined effects of climate change and environmentally relevant mixtures of endocrine disrupting compounds on the fitness and gonads' maturation dynamics of *Nucella lapillus* (Gastropoda)

Table S1- Detection conditions and limits of drospirenone analysis by LC-MS using the Drospirenone standard and sample signal/noise solutions; *collision induced dissociation (CID), limit of detection (LOD), limit of quantification (LOQ)*.

Compound (*m/z+1*)	Molecule Fragments (*m/z+1*)	CID	LOD ($\mu g\ L^{-1}$) s/n=3	LOQ ($\mu g\ L^{-1}$) s/n=10
Drospirenone (367.2)	349.1 331.2 307.2 231.3 197.1 149.3	20	10	20

Limit of Detection (LOD) and Limit of Quantification (LOQ) were found by using the signal-to-noise method as described in the literature (Sanagi et al., 2009), i.e., the peak-to-peak noise (N) around the analyte retention time was measured and subsequently, the concentration of the analyte that would yield a signal (S) equal to a certain value of S/N is estimated. The noise magnitude was measured by the auto-integrator of the instrument.

Table S2 - Results of the ordered logit model for the ordinal variable "maturation stage". SCt: solvent control – 0.01 % ethanol; Hg1–1.5 µg L^{-1}, Hg2–50 µg L^{-1}; DRO1–100 ng L^{-1}, DRO2–1000 ng L^{-1}; Mix1–1.5 µg Hg L^{-1} + 100 ng DRO L^{-1}; Mix2–1.5µgHgL^{-1}+1000ngDROL^{-1};Mix3–50µgHgL^{-1} +100ngDROL^{-1}; Mix4–50µgHgL^{-1} +1000ngDROL^{-1}.

	line	Coefficients	Value	*t*-Value	*p*- Value	
♀	1	**Sex : Mix1**	1.1675	1.1628	2.45E-01	
	2	**Sex : Mix2**	0.2465	0.2977	7.66E-01	
	3	**Sex : Mix3**	0.3254	0.3261	0.7444	
	4	**Sex : Mix4**	0.3814	0.4509	6.52E-01	
	5	**Sex : temp22 : Hg1**	-3.6189	-2.5879	9.66E-03	**
	6	**Sex : temp22 : Hg2**	-3.3652	-2.5835	9.78E-03	**
	7	**Sex : temp22 : DROS1**	0.1225	0.1470	0.8831	
	8	**Sex : temp22 : DROS2**	-0.0586	-0.0623	0.9503	
	9	**Sex : temp22 : Mix1**	-0.8569	-0.8155	0.4148	
	10	**Sex : temp22 : Mix2**	-0.2165	-0.2532	0.8001	
	11	**Sex : temp22 : Mix3**	2.0826	2.0779	0.0377	*
	12	**Sex : temp22 : Mix4**	1.4277	1.6910	0.0908	
♀ **vs** ♂	13	**Temp22**	2.9260	2.6634	7.74E-03	**
	14	**Hg1**	-1.0183	-1.2873	1.98E-01	
	15	**Hg2**	-1.0182	-1.7496	8.02E-02	
	16	**DROS1**	-0.4709	-0.7414	4.58E-01	
	17	**DROS2**	-1.0183	-1.6380	1.01E-01	
♂	18	**Sex**	2.0530	3.7915	1.50E-04	*
	19	**Sex : Hg1**	-2.1038	-2.0251	4.29E-02	*
	20	**Sex : Hg2**	-1.6872	-1.4519	1.47E-01	
	21	**Sex : DROS1**	-0.9304	-0.7246	4.69E-01	
	22	**Sex : DROS2**	-0.1252	-0.0827	9.34E-01	
	23	**Sex : temp22 : Hg1**	1.9881	1.1610	2.46E-01	
	24	**Sex : Mix1**	6.3153	4.6382	3.52E-06	**
	25	**Sex : Mix2**	-0.1361	-0.0889	9.29E-01	
	26	**Sex : Mix3**	4.2097	2.7155	6.62E-03	**
	27	**Sex : Mix4**	4.5639	2.6403	8.28E-03	**
	28	**Sex : temp22 : DROS1**	0.5406	0.3549	0.7226	
	29	**Sex : temp22 : DROS2**	0.0542	0.0360	0.9713	
	30	**Sex : temp22 : Mix1**	-6.9056	-4.7225	< 0.001	***
	31	**Sex : temp22 : Mix2**	1.9438	1.5716	1.16E-01	
	32	**Sex : temp22 : Mix3**	-4.9141	-3.4157	< 0.001	***
	33	**Sex : temp22 : Mix4**	-5.5307	-3.1325	< 0.001	***
	34	**2|3**	-2.7482	-6.7799	< 0.001	***
	35	**3|4**	-1.5633	-4.0091	< 0.001	***
	36	**4|5**	0.9199	2.4053	0.0162	**
	37	**5|6**	3.0743	7.4146	< 0.001	***

***p<0.05; **p<0.01; ***p<0.001**