DEPARTMENT OF CHEMISTRY

From Biomarkers to Transcriptomics: Assessing the Impact of PFOA, TBBPA, and EE2 on the Sentinel Species *Mytilus galloprovincialis*

SANDRA CRISTINA MARINHEIRO FERREIRA COPETO

Master in Biomedical Sciences

DOCTORATE IN FOOD SCIENCES

NOVA University Lisbon

September, 2025

NOVA SCHOOL OF SCIENCE & TECHNOLOGY

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In this work, generative artificial intelligence tools, namely chatgpt (https://chatgpt.com/), were used for grammatical review and slight text reformulation. These tools were employed under the author's supervision, and all generated content was verified for accuracy. The authorship and validation of the content remain entirely the responsibility of the author, and the use of artificial intelligence complies with academic integrity standards.

To my parents, my son, my daughter, and my husband, for their love and support

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"Research is to see what everybody else has seen, and to think what nobody else has thought." Albert Szent-Györgyi

ABSTRACT

Emerging contaminants, particularly endocrine-disrupting chemicals (EDCs), are increasingly detected in marine ecosystems, food, and consumer products, posing risks to both wildlife and human health. Among them, perfluorooctanoic acid (PFOA), tetrabromobisphenol A (TBBPA), and 17α-ethinylestradiol (EE2) stand out due to their environmental persistence, bioaccumulation potential, and ability to interfere with hormonal regulation, oxidative balance, and cellular homeostasis. However, their toxicological effects on marine bivalves, key sentinel species, remain poorly understood.

Mussels such as *Mytilus galloprovincialis* play a central ecological role and are widely used as bioindicators because of their capacity to accumulate contaminants through filter feeding. Assessing their biochemical and transcriptomic responses provides critical insight into the sublethal and realistic effects of EDCs at multiple levels of biological organization. This is particularly relevant given the high seafood consumption in Europe, which represents a major human exposure pathway to these contaminants.

Laboratory exposures of *M. galloprovincialis* were conducted using environmentally relevant concentrations of PFOA (1–100 $\mu g \cdot L^{-1}$), TBBPA (1–100 $\mu g \cdot L^{-1}$), and EE2 (10–300 $ng \cdot L^{-1}$). A multi-biomarker strategy was applied to evaluate oxidative stress (SOD, CAT, GST, TAC, MDA), protein degradation (UBI), apoptosis (caspase-3), neurotoxicity (AChE), and endocrine disruption (VTG). In parallel, transcriptomic analyses were performed to identify differentially expressed genes and disrupted pathways related to stress responses, detoxification, lipid metabolism, and endocrine regulation.

The results revealed concentration-dependent alterations in antioxidant defences and cellular homeostasis, with PFOA and TBBPA inducing oxidative stress and apoptosis, while EE2 primarily triggered estrogenic and gametogenic disruptions. Transcriptomic analysis confirmed the modulation of pathways associated with xenobiotic metabolism, energy balance,

apoptosis, and non-genomic estrogen signaling. Together, these results indicate that *M. galloprovincialis* exhibits coordinated biochemical and molecular responses upon exposure to EDCs, reinforcing its relevance as a sentinel species.

The study highlights potential risks of contaminant biomagnification through the food chain and provides mechanistic evidence to support ecotoxicological monitoring frameworks and regulatory measures aimed at protecting marine ecosystems and public health.

Keywords: Endocrine-disrupting chemicals (EDCs), *Mytilus galloprovincialis*, Biochemical biomarkers, Transcriptomics, Endocrine disruption, Human health risk

RESUMO

Os contaminantes emergentes, em particular os disruptores endócrinos (EDCs), têm sido cada vez mais detetados em ecossistemas marinhos, alimentos e produtos de consumo, representando riscos para a saúde ambiental e humana. Entre estes compostos, o ácido perfluorooctanóico (PFOA), o tetrabromobisfenol A (TBBPA) e o 17α-etinilestradiol (EE2) destacam-se pela sua persistência ambiental, potencial de bioacumulação e capacidade de interferir com a regulação hormonal, o equilíbrio oxidativo e a homeostase celular. Contudo, os seus efeitos toxicológicos em bivalves marinhos, espécies-chave sentinela, permanecem insuficientemente compreendidos.

Os mexilhões (*Mytilus galloprovincialis*) desempenham um papel ecológico fundamental e são amplamente utilizados como bioindicadores devido à sua capacidade de acumular contaminantes através da filtração. A avaliação das suas respostas bioquímicas e transcriptómicas fornece informações cruciais sobre os efeitos subletais e realistas dos EDCs em múltiplos níveis de organização biológica. Tal é particularmente relevante no contexto Europeu, dado o elevado consumo de marisco, que constitui uma via significativa de exposição humana a estes contaminantes.

Foram conduzidos ensaios laboratoriais com *M. galloprovincialis* expostos a concentrações ambientalmente relevantes de PFOA (1–100 $\mu g \cdot L^{-1}$), TBBPA (1–100 $\mu g \cdot L^{-1}$) e EE2 (10–300 $ng \cdot L^{-1}$). Foi implementada uma estratégia multibiomarcador para avaliar o stress oxidativo (SOD, CAT, GST, TAC, MDA), a degradação proteica (UBI), a apoptose (caspase-3), a neurotoxicidade (AChE) e a disrupção endócrina (VTG). Em paralelo, realizaram-se análises transcriptómicas para identificar genes diferencialmente expressos e vias moleculares afetadas,

relacionadas com respostas ao stress, metabolismo de xenobióticos, metabolismo lipídico e regulação endócrina.

Os resultados evidenciaram alterações dependentes da concentração nas defesas anti-oxidantes e na homeostase celular, com o PFOA e o TBBPA a induzirem stress oxidativo e apoptose, enquanto o EE2 desencadeou sobretudo disrupções estrogénicas e gametogénicas. A análise transcriptómica confirmou a modulação de vias associadas ao metabolismo de xe-nobióticos, ao balanço energético, à apoptose e à sinalização estrogénica não-genómica. Em conjunto, estes resultados demonstram que *M. galloprovincialis* desenvolve respostas bioquí-micas e moleculares integradas à exposição a EDCs, confirmando o seu valor como espécie sentinela.

Este estudo evidencia os potenciais riscos de biomagnificação destes contaminantes na cadeia alimentar e fornece evidência robusta que apoia programas de monitorização ecotoxi-cológica e medidas regulatórias destinadas à proteção dos ecossistemas marinhos e da saúde pública.

Palavras chave: Disruptores endócrinos (EDCs), *Mytilus galloprovincialis*, Biomarcadores bioquí-micos, Transcriptómica, Disrupção endócrina, Risco para a saúde humana

OUTPUTS OF THESIS

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Copeto, S., Ganço, S., Ferreira, I. J., Silva, M., Motta, C., & Diniz, M. (2024). The Effects of Tetrabromobisphenol A (TBBPA) on the Mussel *Mytilus galloprovincialis*: A Multi-Biomarker Approach. Oceans, 5(2), 181–195. https://doi.org/10.3390/oceans5020011

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Ganço, S., **Copeto, S.,** Ferreira, I. J., Silva, M., Motta, C., & Diniz, M. (2023, July 5–7). *Effects of exposure to tetrabromobisphenol A (TBBPA) on mussels (Mytilus galloprovincialis)*. Egas Moniz International Scientific Congress (6th Edition).

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Panel communications

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ACRONYMS

4-NP	4-Nonylphenol
ABTS	2,2-azino-bis 3- ethylbenzothiazoline-6-sulphonic acid
AChE	Acetylcholinesterase
ACN	Acetonitrile
ACTI	Actin, cytoskeletal protein / housekeeping gene
ADI	Acceptable Daily Intake
AFFFs	Aqueous film-forming foams
Akt	Protein Kinase B
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
AOPs	Adverse outcome pathways
APCI	Atmospheric pressure chemical ionization
APE1	Apurinic/Apyrimidinic Endonuclease 1
ARE	Antioxidant Response Element
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3-related

Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
BDE-209	Decabromodiphenyl Ether
BFRs	Brominated Flame Retardants
BI-1	Bax Inhibitor-1
BPA	Bisphenol A
BrPhs	Bromophenols
BSA	Bovine Serum Albumin
BSEF	Bromine Science and Environmental Forum
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CASP-3	Caspase
CAT	Catalase
Cd	Cadmium
CDNB	1-Chloro-2,4-dinitrobenzene
CECs	Contaminants of Emerging Concern
CH_3COONH_4	Ammonium Acetate
CI	Chemical Ionization
Cu	Copper
CYP19	Aromatase
CYP4Y1	Cytochrome P450 4Y1
DAD	Diode Array Detector
DBDPE	decabromodiphenyl ethane
DBP	Dibutyl phthalate
DDT	Dichlorodiphenyltrichloroethane

DDVP	Dichlorvos
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
Dff-A	CAD endonuclease
DGE	Differential Gene Expression
DGRM	Directorate-General for Natural Resources, Safety and Maritime Services
DMRT1	Doublesex and Mab-3 Related Transcription Factor 1
DNA	Deoxyribonucleic Acid
DNS-Cl	Dansyl Chloride
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
dw	Dry Weight
E1	Estrone
E2	17β-Estradiol
E3	Estriol
ECF	Electrochemical fluorination
ECHA	European Chemicals Agency
ECNI	Electron Capture Negative Ionization
EDCs	Endocrine-Disrupting Chemicals
EDI	Estimated Daily Intake
EDTA	Ethylenediaminetetraacetic Acid
EE2	17α-Ethinylestradiol
EFSA	European Food Safety Authority
EI	Electron Impact

ELISA	Enzyme-Linked Immunosorbent Assay
EOCs	Organic Emerging Contaminants
ER	Endoplasmic Reticulum
ERR	Estrogen-Related Receptor
ERs	estrogen receptors
ES	Endocrine System
ESI	Electrospray Ionization
ESR-like genes	Estrogen Receptor-like genes
FABP	Fatty Acid Binding Proteins
FD	Fluorescence Detection
FELASA	Federation of European Laboratory Animal Science Associations
FoxL2	Forkhead Box L2
FRs	Flame Retardants
FTOHs	Fluorotelomer alcohols
GABA Receptors	γ-Aminobutyric Acid Receptors
Gadd45	Growth Arrest and DNA-Damage Inducible 45
GC	gas chromatography
GC-IRMS	Gas Chromatography–Isotope Ratio Mass Spectrometry
GnRH-1	Gonadotropin-Releasing Hormone 1
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
GST	Glutathione S-transferases

H_2O_2	Hydrogen peroxide
HBB	Hexabromobiphenyl
HBCD	Hexabromocyclododecane
HCl	Hydrochloric Acid
Hg	Mercury
HPG axis	Hypothalamic–Pituitary–Gonadal axis
HPG	Hypothalamic-pituitary-gonadal
HPGL	Hypothalamus-pituitary-gonad-liver axis
HPLC-FD	High-Performance Liquid Chromatography with Fluorescence Detection
HRGC	high-resolution gas chromatography
HRMS	High-resolution mass spectrometry
HSD17β	17β-Hydroxysteroid Dehydrogenase
HSEs	Heat Shock Elements
HSF	Heat Shock Factor
HSP	Heat Shock Proteins
IARC	International Agency for Research on Cancer
IBR	Integrated Biomarker Response
ICNF	Institute for Nature Conservation and Forests
IRMS	isotope ratio mass spectrometry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JNK	c-Jun N-terminal Kinase
Kd	solid-liquid partition coefficient
LC	liquid chromatography
LC MS/MS	Liquid Chromatography–Tandem Mass Spectrometry

LC-HRMS	Liquid Chromatography–High Resolution Mass Spectrometry
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LC50	Lethal Concentration 50%
LOD	Limit of Detection
LOQ	Limit of Quantification
Log2FC	Log2 Fold Change
LPO	Lipid peroxidation
MAPK	Mitogen-Activated Protein Kinase
MDA	Malondialdehyde
MEKC	Micellar Electrokinetic Chromatography
MRE	Metal Response Element
mRNA	messenger RNA
MS	Mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	Methyl tert-Butyl Ether
mTOR	Mammalian Target of Rapamycin
MTs	Metallothioneins
MXR	Multixenobiotic Resistance
MyD88	Myeloid Differentiation Primary Response 88
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
NCBI GEO	Gene Expression Omnibus
NF- κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NH_4OH	Ammonium Hydroxide

Nrf2	Nuclear factor erythroid 2–related factor 2
O_2^-	Superoxide anion
OCs	Organochlorines
OH	Hydroxyl radical
OPEs	Organophosphate Esters
OSPAR	Convention for the Protection of the Marine Environment of the Northeast Atlantic
PAEs	Phthalate Acid Esters
PAHs	Polycyclic Aromatic Hydrocarbons
Pb	Lead
PBBs	polybrominated biphenyls
PBDEs	Polybrominated Diphenyl Ethers
PBS	Phosphate-Buffered Saline
PCBs	Polychlorinated Biphenyls
PDRP	p53-Downstream Regulated Protein
PEs	Phthalate Esters
PFASs	Per- and Polyfluoroalkyl Substances
PFBCl	Pentafluorobenzyl Chloride
PFCAs	Perfluoroalkyl carboxylic acids
PFCs	Perfluorinated organic compounds
PFDA	Perfluorodecanoic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic Acid
PFP	Pentafluorophenylpropyl

PFSAs	Perfluoro sulphonic acids
PI3K	Phosphoinositide 3-kinase
PIGE	Particle-Induced Gamma-ray Emission
pNPP	4-nitrophenyl phosphate disodium salt hexahydrate
POPs	Persistent organic pollutants
PPARs	Peroxisome proliferator-activated receptors
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PPCPs	Pharmaceuticals and Personal Care Products
PROT	Cytosolic protein concentration
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
qTOF	High-resolution quadrupole time-of-flight mass spectrometry
RAD51	DNA repair protein RAD51 homolog 1
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	Reactive oxygen species
RPLC	Reversed-Phase Liquid Chromatography
RXR	Retinoid X Receptor
SDGs	Sustainable Development Goals
SDS	Sodium dodecyl sulfate
SIM	Selected Ion Monitoring
SOD	Superoxide Dismutase
Sox	SRY-related HMG-box
SPE	Solid-Phase Extraction
SRA	Sequence Read Archive

TAC	Total antioxidant capacity
TBAHS	Tetrabutylammonium Hydrogen Sulfate
TBARS	Thiobarbituric Acid Reactive Substances
TBBPA	Tetrabromobisphenol A
bDiBPrE	Tetrabromobisphenol A bis(2,3-dibromopropyl ether
bMeE	Tetrabromobisphenol A bis(2-methoxyethyl ether
TBBPA-DHEE	TBBPA bis(2-hydroxyethyl) ether
TCA	Trichloroacetic acid
TDI	Tolerable Daily Intake
TLR	Toll-like Receptor
TLR4	Toll-like Receptor 4
TOP	Total Oxidizable Precursor Assay
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TWI	Tolerable weekly intakes
UBI	Ubiquitin
UniProt	Universal Protein Resource
VTG	Vitellogenin
WHO	World Health Organization
ww	Wet weight
WWTPs	Wastewater treatment plants
XOD	Xanthine Oxidase/Dehydrogenase
XRC	Xeroderma Pigmentosum Complementation Group Genes
Zn	Zinc
α-HBCD	α-Hexabromocyclododecane

β-HBCD	β-Hexabromocyclododecane
γ-HBCD	γ-Hexabromocyclododecane

1

CHAPTER 1- GENERAL INTRODUCTION

1.1 Endocrine -Disrupting Chemicals (EDCs)

EDCs are a class of environmental contaminants recognized for their ability to interfere with the endocrine system of both humans and wildlife. Since the late 20th century, there has been a growing awareness of the health risks associated with exposure to chemicals that can disrupt endocrine function. Over three decades ago, 21 experts convened at the Wingspread Conference Center in Racine, Wisconsin, USA (July 26–28, 1991) to assess the impact of industrial and agricultural chemicals on endocrine systems in wildlife and humans. This meeting, now commonly referred to as Wingspread, introduced the terms "endocrine disruption" and "endocrine disruptor" into scientific language, generated the first consensus statement on endocrine-disrupting chemicals, and laid the foundation for the field of endocrine disruption as it is understood today (Soto et al., 2021).

Numerous studies have identified substances that can alter hormonal activity in various species under laboratory conditions, with a growing body of evidence documenting similar effects in wildlife populations (Crisp et al., 1998; Tyler et al., 1998). The environmental impact of EDCs has sparked significant debate within scientific circles and among the public. Laboratory research suggests that estrogenic or estrogen-mimicking compounds can deeply affect the development of reproductive and nervous systems and influence behavior and immune responses in higher organisms (Hecker & Hollert, 2011; Kloas et al., 2009).

These chemicals can mimic, block, or alter hormonal functions, adversely affecting reproductive, developmental, and neurological health **(Figure 1-1)**.

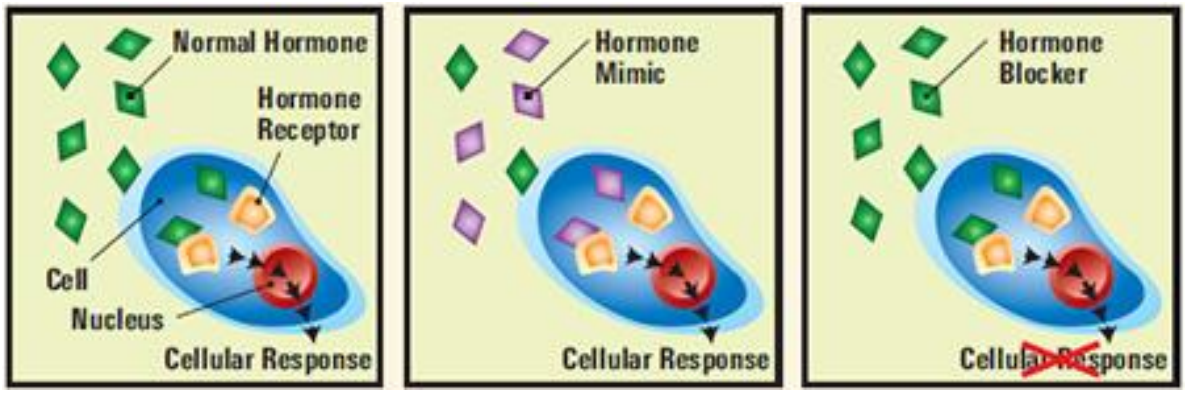

Figure 1-1 - Mechanisms of EDC Action. Source https://www.niehs.nih.gov/health/topics/agents/endocrine

EDCs comprise a diverse range of substances, including organochlorine compounds (e.g., DDT), halogenated aromatic hydrocarbons (e.g., PCBs), brominated flame retardants (BFRs), per- and polyfluoroalkyl substances (PFASs), alkylphenols, phthalates, bisphenol A (BPA) and its analogues, as well as pharmaceuticals, illicit drugs, and steroid hormones (Wang et al., 2024).

These compounds are found across various environmental media, including water, soil, air, and biota, and their persistence and widespread presence raise significant concerns for public health and ecosystems. EDCs are known to disrupt critical biological processes, such as growth, reproduction, and metabolism, by altering endocrine functions (Metcalfe et al., 2022). Reproductive success, defined as an organism's ability to produce viable offspring capable of reproducing, ensures the continuation of its genetic lineage. While reproductive success can be easily measured in humans and domesticated animals, it is more challenging to assess in wild species due to various abiotic factors (e.g., temperature, photoperiod) and biotic factors (e.g., parental investment, nutritional status) that influence reproductive outcomes. Despite these complexities, considerable evidence demonstrates that environmental contaminants, particularly EDCs, negatively impact reproductive success across a wide range of vertebrates and invertebrates (Marlatt et al., 2022).

EDCs disrupt key components of the endocrine system, such as those involved in reproductive development (Marlatt et al., 2022).Growing concerns regarding EDC-related risks have

led to international efforts to enhance risk assessment methodologies (Barton-Maclaren et al., 2022). EDCs, primarily originating from anthropogenic activities, are chemically diverse and ubiquitous in the environment (Metcalfe et al., 2022). Legacy pollutants, such as polychlorinated biphenyls (PCBs), are known for their persistence in all environmental compartments and their long-lasting adverse effects on growth and reproduction (Bergman et al., 2015; El-Shahawi et al., 2010; Kortenkamp et al., 2011). Other sources of EDCs include alkylphenol ethoxylates, commonly used in cleaning products and industrial processes, which are frequently detected in wastewater effluents (Acir & Guenther, 2018). Moreover, PCBs and brominated flame retardants are found in both indoor and outdoor environments (Qi et al., 2014; Rudel & Perovich, 2009).Assessing the risks posed by EDCs is further complicated by fluctuations in chemical concentrations over time and concurrent exposure to multiple chemicals (Metcalfe et al., 2022).

Recent studies highlight widespread exposure to indoor and outdoor contaminants in both humans and wildlife (Chen et al., 2011; Gao & Kannan, 2020). Biomonitoring efforts have confirmed the presence of EDCs such as parabens, bisphenols, and phthalates in human biological samples, including blood, urine, breast milk, and adipose tissue (Lambertino et al., 2021; Pan et al., 2020; van der Meer et al., 2021). Additionally, evidence of placental transfer of these chemicals underscores the risk of early-life exposure to EDCs (Mitro et al., 2015; Plante et al., 2022). The impact of chemical exposure on reproductive success is further complicated by the diversity of reproductive strategies across species and the varying levels of understanding of the endocrine systems involved in reproduction.

While human reproductive systems are relatively well-studied, isolating the effects of EDCs from other confounding factors, such as co-exposures to different chemicals and lifestyle factors, remains challenging (Marlatt et al., 2022). Vertebrates share key components of the hypothalamic-pituitary-gonadal (HPG) axis, which regulate reproduction through sex steroids and neurohormones. EDCs frequently disrupt this system and its associated targets.

Invertebrate species, including molluscs, insects, and crustaceans, are also vulnerable to EDC-induced neuroendocrine disruptions, although the endocrine systems in these organisms are less well-understood (deFur, 2004).

There is growing recognition that EDCs, found in the environment, food, and consumer products, pose significant health risks. These chemicals interfere with hormone biosynthesis, metabolism, and action, leading to deviations from standard homeostatic control and reproductive functions (Corsini et al., 2018). According to the World Health Organization (WHO), approximately 10% of global deaths are attributable to environmental factors, and 40% of the global population is exposed to dangerous pollutants (WHO, 2024).

Contaminants of emerging concern (CECs) persist in the environment and include synthetic and natural substances that, although not consistently monitored, pose known or unknown risks to ecosystems and human health. Among the most dangerous persistent organic pollutants, several EDCs, such as phthalate esters (PEs), organophosphorus esters (OPEs), flame retardants (FRs), and perfluoroalkylated substances (PFASs), have been identified as priority chemicals (Wang et al., 2024).

EDCs are a heterogeneous group of substances that can be either natural or synthetic in origin, and they vary widely in their structure and properties (Diamanti-Kandarakis et al., 2009; Muncke, 2011). Natural EDCs include plant compounds (phytoestrogens), animal steroid hormones, and metals. In contrast, synthetic EDCs include industrial chemicals such as lubricants, solvents, plastics, plasticizers, pesticides, and pharmaceuticals (Diamanti-Kandarakis et al., 2009; Mezcua et al., 2012; Vandenberg et al., 2012). These substances alter the endocrine and homeostatic systems by interfering with endocrine mechanisms, often as small molecules with masses of less than 1000 Daltons (Diamanti-Kandarakis et al., 2009; Vandenberg et al., 2012).

Synthetic EDCs encompass a broad range of chemicals, including organochlorinated pesticides, industrial chemicals, plastics, plasticizers, fuels, and numerous other environmental substances widely used in everyday life (Diamanti-Kandarakis et al., 2009).

Beyond EDCs, other classes of organic emerging contaminants are of increasing concern. These include pharmaceuticals and personal care products (PPCPs), food and feed additives, surfactants, plastic additives, solvents, pesticides, and cyanotoxins.

Each class comprises compounds with distinct applications, sources, and toxicological profiles. A detailed overview of the main categories and representative examples of organic emerging contaminants (EOCs) is provided in **Table 1.1-1**.

The endocrine system (ES) is essential for the health of humans and animals (Diamanti-Kandarakis et al., 2009). Through its various hormones, it is responsible for the normal development of the organism, including cellular differentiation during tissue formation and maturation, maintenance of the functions of different organs and tissues, and reproduction (Diamanti-Kandarakis et al., 2009). All cells in the body are regulated by hormones, which can be peptides/proteins, lipids, or amino acid derivatives. Once secreted, these hormones act on receptors that trigger intracellular reactions.

Over the past 50 years, there has been an observed increase in the incidence of endocrine-related diseases and disorders. The rapid pace of this rise, which cannot be fully explained by genetic mechanisms or improved diagnostic rates, has raised the possibility of environmental factors playing a role. Among these, anthropogenic substances, either newly created or previously present only in minimal amounts on Earth, are highlighted due to their ability to interfere with the ES. As evidence linking EDC exposure to adverse health effects continues to grow, the need for improved risk assessment, monitoring, and mitigation strategies becomes increasingly urgent (Ruiz et al., 2018).

Table 1.1-1 - Emerging contaminants: A One Health perspective. Adapted from Wang et al. (2024)

Category of Organic Emerging Contaminants (EOCs)	Examples
Endocrine-disrupting compounds (EDCs)	17-Alpha-ethinylestradiol (EE2), 17-Beta-estradiol (E2), Estrone (E1), Steroid hormones, Phthalate acid esters (PAEs), Bisphenols
Food and Feed Additives	2,6-Di-tert-butyl-4-methylphenol
Persistent Organic Pollutants (POPs)	Brominated flame retardants, PCBs, Polycyclic aromatic hydrocarbons (PAHs), DDT, Perfluorinated chemicals, Polybrominated diphenyl ethers (PBDEs), Per- and Polyfluoroalkyl Substances (PFAS)
Pharmaceuticals and Personal Care Products (PPCPs)	Disinfectants: Disinfection byproducts, Chlorate, Formaldehyde, Diclofenac; Cosmetics: 2-Ethylhexyl-4-methoxycinnamate; Analgesics and anti-inflammatories: Flumequine, Trimethoprim, Ketorolac; Antibiotics, Illicit drugs
Surfactants	Nonylphenols
Organic Solvents/Plastic Additives	Hexachlorobutadiene, Dechlorane (both cis and trans forms), Dichloromethane, Chloroform
Pesticides and Herbicides	Methiocarb, Neonicotinoids, Oxadiazon, Triallate, Perchlorate, Dicofol
Cyanotoxins	Microcystin(s), Cylindrospermopsin, Anatoxin(s), Saxitoxin(s)

Table 1.1-2 summarizes the main categories of organic emerging contaminants of environmental and public health concern, including steroid hormones (EDCs), brominated flame retardants (BFRs), per- and polyfluoroalkyl substances (PFAS), phthalates, and polychlorinated biphenyls (PCBs). For each group, representative compounds, common sources, chemical structures, environmental persistence, ecological impacts, and human health effects are described.

Table 1.1-2 - Comprehensive Overview of Organic Emerging Contaminants: Sources, Persistence and Impacts

Category	Key Compounds	Sources	Chemical Structure	Persistence in the Environment	Environmental Impact	Health Impact	Authors
Steroid Hormones (EDCs)	Estrone (E1), 17β-estradiol (E2), Estriol (E3), 17α-ethinylestradiol (EE2)	Wastewater, contraceptives, pharmaceutical industry	Steroid structure with a cyclopentanoperhydrophenanthrene ring. EE2 contains an ethinyl group at carbon 17.	Persistent in wastewater, soil, and water; bioaccumulates in aquatic systems.	Bioaccumulates in food chain; disrupts reproduction and development; induces oxidative stress in aquatic organisms.	Interferes with hormone regulation, reproduction, puberty, fertility, and development; detected in drinking water.	(Manickum & John, 2014)
Brominated Flame Retardants (BFRs)	Tetrabromobisphenol A (TBBPA), Polybrominated diphenyl ethers	Electronics, textiles, plastics, construction materials.	Aromatic rings with bromine substituents; structurally similar to BPA with four bromine atoms.	Highly persistent; accumulates in sediments and dust; bioaccumulates in marine environments.	Accumulates in marine organisms; disrupts thyroid and reproduction;	Disrupts thyroid function, increases cancer risk, and	(Zhang et al., 2025)

	(PBDEs), Hexabromocyclododecane (HBCD)				induces oxidative stress.	causes neurodevelopmental disorders and reproductive issues.	
Per- and Polyfluoroalkyl Substances (PFAS)	Perfluorooctanesulfonic acid (PFOS), Perfluorooctanoic acid (PFOA), Fluorotelomer alcohols (FTOHs)	Firefighting foams, non-stick cookware, textiles, food packaging	Stable C–F bonds; PFOA has carboxyl group, PFOS has sulfonate group.	Extremely persistent; bioaccumulates in protein-rich tissues; resists degradation in water and soil.	Causes oxidative stress; immune and reproductive toxicity in aquatic species; biomagnifies in food chains.	Linked to kidney/testicular cancer; thyroid disruption; immune dysfunction; reproductive and developmental toxicity.	(Wee & Aris, 2023b)

Phthalates	Diethyl phthalate (DEP), Dibutyl phthalate (DBP), Di(2-ethylhexyl) phthalate (DEHP)	Plastics, food packaging, cosmetics, personal care products	Dialkyl or alkyl aryl esters of phthalic acid.	Moderately persistent; biodegrades under aerobic conditions; continuously reintroduced into environment.	Disrupts reproductive development in fish/amphibians; reduces sperm production; alters sexual development.	Linked to reproductive issues; reduced sperm quality; hormone disruption; developmental effects; suspected carcinogen.	(Dutta et al., 2020)
Polychlorinated Biphenyls (PCBs)	Aroclor 1254, Aroclor 1260, Kanechlor, Phenochlor	Electrical equipment, transformers, lubricants	Chlorinated biphenyl rings.	Highly persistent; remains in soil, water, and sediments for decades; bioaccumulates in fatty tissues.	Bioaccumulates in fatty tissues; disrupts immune and reproductive systems in marine mammals and fish; causes	Causes cancer; neurodevelopmental delays; immune	(Montano et al., 2022)

		developmental issues.	suppres-sion; liver damage; probable human carcino-gen.

1.2 Tetrabromobisphenol A (TBBPA)

Flame retardants (FRs) are a group of synthetic chemicals commonly used in a wide range of industrial and household products, including plastics, polymers, textiles, and electronic devices, to enhance their fire resistance.

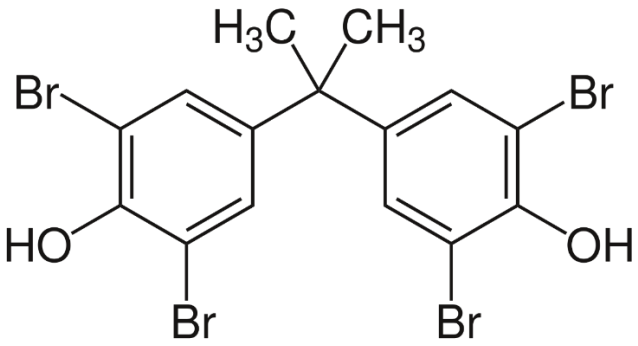

Figure 1-2 - Chemical structure of TBBPA

These compounds can be classified into organic (halogenated), inorganic, organophosphorus, and nitrogen-based categories (Poma et al., 2018). Among these, brominated flame retardants (BFRs) are the most commercialized due to their cost-effectiveness and high performance, with the most common BFRs being hexabromocyclododecane (HBCDs), polybrominated diphenyl ethers (PBDEs), brominated phenols (BrPhs), and tetrabromobisphenol A (TBBPA) (Poma et al., 2018; Yang et al., 2023), **Table 1.2-1** summarizes the key physicochemical properties, environmental behavior, and toxicological profile of TBBPA, while **Figure 1-2** illustrates the chemical structure of the molecule.

Table 1.2-1 - Chemical Profile and Properties of Tetrabromobisphenol A (TBBPA). Adapted from EFSA (2011), WHO (1995), and PubChem (CID 6618)

Property	Description
Chemical Name	Tetrabromobisphenol A
Chemical Formula	$C_{15}H_{12}Br_4O_2$
Molecular Weight	543.91 $g.mol^{-1}$
CAS Number	79-94-7
Chemical Structure	Two phenolic rings connected by a methylene group (-CH_2-) substituted with bromine atoms
Physical State	Solid (at room temperature)
Color	White or slightly yellowish
Melting Point	181–184 °C
Water Solubility	Low (< 0.1 $mg{\cdot}L^{-1}$ at 25 °C)
Solubility in Solvents	Soluble in acetone, methanol, and other organic solvents
Chemical Stability	Stable under normal conditions but may degrade at high temperatures or under UV radiation
Main Use	Flame retardant in plastics, epoxy resins, and electronic equipment
Log Kow	4.5–5.0 (indicates high lipophilicity)
Toxicity Class	Potentially toxic to aquatic organisms; suspected endocrine disruptor

TBBPA is the most widely produced brominated flame retardant globally, as reported by the Bromine Science and Environmental Forum (BSEF) in 2010. It is commonly used as a reactive or additive flame retardant across various industries, including polymers, resins, adhesives, textiles, and printed circuit boards (Lévy-Bimbot et al., 2012). Due to its high production volume and broad application, TBBPA plays a prominent role in the global market for flame retardants (Liu et al., 2019; Sun et al., 2008). TBBPA has been widely found in the aquatic environment, such as water, sediment, sewage sludge and biota (Ashizuka et al., 2008; Gerecke et al., 2006; Sellström & Jansson, 1995). Although no current restrictions exist on the production and use of TBBPA, its inclusion in the list of substances requiring priority action by the Convention for the Protection of the Marine Environment of the Northeast Atlantic (OSPAR) highlights the need for immediate attention and regulation (OSPAR, 2007).

1.2.1 Sources and transport pathways

The **Figure 1-3** Illustrates that TBBPA is released from industrial production, e-waste recycling, and wastewater treatment into soil, air, dust, sediments, and water, exposing humans via ingestion, inhalation, and skin contact, leading to endocrine, reproductive, developmental, neuro-, immuno-, and hepatotoxic effects (Sunday et al., 2022).

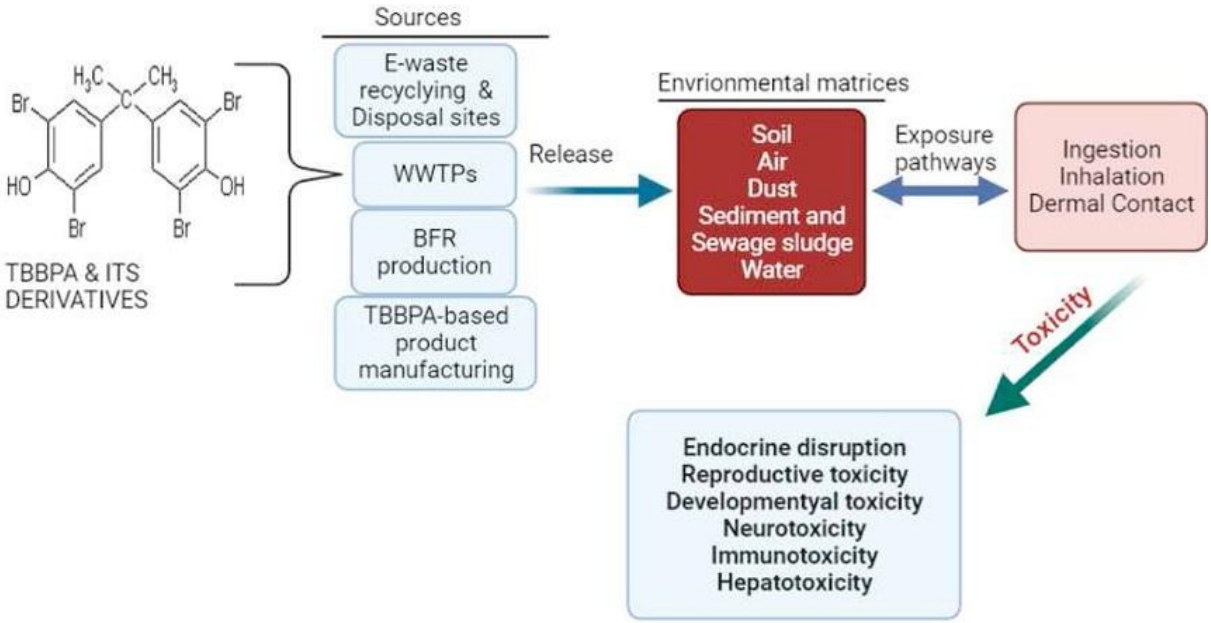

Figure 1-3 - Sources and exposure pathways of tetrabromobisphenol A (TBBPA). Adapted from Sunday et al. (2022)

TBBPA can be released into the environment from both additive- and reactive-treated consumer products. The release of these compounds into aquatic environments poses a significant risk to aquatic organisms due to their persistence, leading to bioaccumulation in sediments and marine life (Álvarez-Muñoz et al., 2015; Kotthoff et al., 2017).

Water samples from different regions have reported TBBPA concentrations ranging from non-detectable (n.d.) levels to as high as 4.87 $\mu g \cdot L^{-1}$ (Blanco et al., 2005; Yang et al., 2012). In contrast, sediment samples have shown levels ranging from n.d. to 500 $\mu g.kg^{-1}$ (Saint-Louis & Pelletier, 2004; Yang et al., 2012).

Since TBBPA concentrations reported in sediments have exceeded the maximum theoretical sorption amount, there is a significant potential risk of TBBPA being released from sediment to overlying water (Huang et al., 2013).

TBBPA's high lipophilicity and thermal stability contribute to its bioconcentration and biomagnification through food webs (Sun et al., 2024; Wang et al., 2023), potentially impacting human health through the consumption of contaminated seafood. However, there is still a lack of sufficient studies on its presence in food, which calls for regular monitoring (Lee et al., 2020).

1.2.2 Effects of TBBPA on Exposed Organisms

TBBPA has been identified as a potential environmental contaminant and endocrine disruptor, with studies highlighting its ability to induce various toxic effects. These include neurotoxicity (Eriksson et al., 2001; Mariussen & Fonnum, 2003; Reistad et al., 2006) immunotoxicity (Pullen et al., 2003), nephrotoxicity, and hepatotoxicity (Fukuda et al., 2004). The European Chemicals Agency (ECHA) classifies TBBPA as a carcinogen, emphasizing the need for further research into its toxicological impacts (Fukuda et al., 2004; Wang et al., 2021).

Although debate continues over the full extent of TBBPA's toxicity (He et al., 2015), its harmful effects on aquatic biota, including algae, bivalves, crustaceans, and fish, have been well documented (Pittinger & Pecquet, 2018). This highlights the need to address potential human exposure through fisheries, where consumption of TBBPA-contaminated food could disrupt endocrine systems and lead to various health issues (Gerecke et al., 2008; Wang et al., 2023). **Table 1.2-2** compiles data from various studies on the toxicological effects and environmental concentrations of brominated flame retardants (BFRs), including legacy and novel compounds, across different biological matrices and organisms.

For instance, in toxicity studies, Jiang et al. (Jiang et al., 2019) reported a 96-hour lethal concentration (LC50) value of 7.4 $mg \cdot L^{-1}$ for juvenile clams (*Ruditapes philippinarum*), while Yang et al. (Yang et al., 2012) found LC50 values ranging from 0.006 to 3.0 $mg \cdot L^{-1}$ for freshwater fish species such as *Pimephales promelas* and *Danio rerio*. However, due to variations in experimental conditions, caution is advised when interpreting these results. Elevated levels of PBDEs, a related class of BFRs, were first detected in the serum of workers at an electronics dismantling facility in 1999 (Sjödin et al., 1999).

Over the next two decades, PBDEs and other BFRs were commonly found in environmental and human samples worldwide. These compounds, including hexabromobiphenyl

(HBB) and hexabromocyclododecane (HBCD), were eventually classified as persistent organic pollutants (POPs) under the Stockholm Convention, leading to their phased elimination. However, concerns remain regarding the endocrine-disrupting effects of newer BFRs, such as TBBPA and decabromodiphenyl ethane (DBDPE) (Zuiderveen et al., 2020). A recent review identified 63 novel BFRs in indoor air, dust, consumer products, and food, highlighting the limited data available on many of these compounds (Zuiderveen et al., 2020).

Novel BFRs have been detected in biota and human tissues, raising concerns that they may not be inherently safer than legacy BFRs (Gyllenhammar et al., 2016; Ho et al., 2017).

Given the structural similarity between BFRs and thyroid hormones, these compounds exhibit significant endocrine-disrupting potential, particularly affecting thyroid function. Consequently, lipid-rich tissues, umbilical cord blood, breast milk, and body fluids such as urine and plasma are considered priority matrices for monitoring BFRs in the environment (Zuiderveen et al., 2020).

Table 1.2-2 - Toxicological Effects and Environmental Presence of BFRs Across Different Matrices and Species

Organism/Matrix	Concentration	Effects	Authors
Juvenile clams (*Ruditapes philippinarum*)	LC50: 7.4 $mg \cdot L^{-1}$ (96-hour)	Mortality at acute exposure levels	(Jiang et al., 2019)
Freshwater fish (e.g., *Pimephales promelas, Danio rerio*)	LC50: 0.006–3.0 $mg \cdot L^{-1}$	Acute toxicity, mortality	(Yang et al., 2012)
Humans (workers in electronics dismantling)	Elevated PBDE levels (serum)	Potential neurotoxicity, endocrine disruption	(Sjödin et al., 1999)
Algae, bivalves, crustaceans, fish	Various exposure levels	Endocrine disruption, neurotoxicity, hepatotoxicity, and immunotoxicity	(Fukuda et al., 2004; Pittinger & Pecquet, 2018)
Human matrices (e.g., breast milk, plasma, cord blood)	Trace levels of BFRs detected	Endocrine disruption, thyroid function impairment	(Ho et al., 2017; Zuiderveen et al., 2020)

Indoor air, dust, consumer products	63 novel BFRs detected	Potential for human and environmental exposure; unknown toxicological profiles	(Zuiderveen et al., 2020)
Marine and terrestrial biota	Detected in various tissues	Bioaccumulation of novel BFRs; endocrine-disrupting effects like legacy BFRs	(Gyllenhammar et al., 2016; Ho et al., 2017)

1.3 Per-and polyfluoroalkyl substances (PFASs)

Perfluorooctanoic acid (PFOA) belongs to the larger class of per- and polyfluoroalkyl substances (PFASs), which are characterized by their remarkable thermal stability, chemical inertness, and surfactant properties, mainly due to their structure (**Figure 1-4**) containing one or more fluoroalkyl chains (Krafft & Riess, 2015).

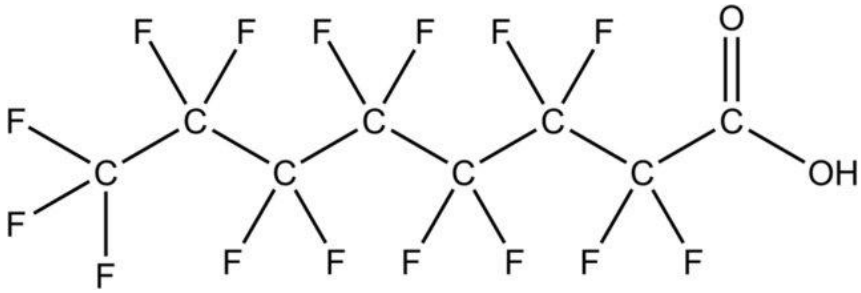

Figure 1-4 - Chemical structure of PFOA

According to **Table 1.3-1**, PFOA, is a highly stable perfluoroalkyl substance composed of a linear chain of eight fully fluorinated carbon atoms with a terminal carboxylic acid group.

Table 1.3-1 - Chemical Profile and Properties of PFOA. Adapted from EFSA (2018), WHO (2017), and PubChem (CID 9554)

Property	Description
Chemical Name	Perfluorooctanoic Acid (PFOA)
Chemical Formula	$C_8HF_{15}O_2$
Molecular Weight	414.07 $g.mol^{-1}$
CAS Number	335-67-1
Chemical Structure	Linear chain of 8 fully fluorinated carbon atoms with a terminal carboxylic acid group
Physical State	Solid (at room temperature)
Color	White
Melting Point	50–54 °C
Boiling Point	189 °C (decomposes)
Water Solubility	3.4 $g.L^{-1}$ at 25 °C
Log Kow	5.3 (indicates high bioaccumulation potential)
Vapor Pressure	0.525 mmHg at 25 °C
pKa	< 1 (indicates strong acidity)
Chemical Stability	Extremely stable due to the strength of C-F bonds
Main Use	Surfactant in industrial processes; manufacturing of Teflon and other fluoropolymers
Toxicity Class	Persistent organic pollutant (POP); toxic to humans and wildlife, potential carcinogen

These unique properties have made PFAS highly valuable for industrial and commercial applications since their large-scale synthesis began in the 1940s with electrochemical fluorination (ECF) developed by 3M (Baran, 2001). The development of the telomerization process by DuPont in the 1960s further expanded the production of various PFAS compounds (Buck et al., 2011; Garcia & Harbison, 2015). However, due to their environmental persistence and toxicity, PFAS have become pollutants of global concern (Lau et al., 2007).

PFAS are widely used in diverse applications, particularly in aqueous film-forming foams (AFFFs), employed for fire suppression at airports, military bases, and industrial sites (Moody & Field, 2000). PFAS compounds like perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have applications in industries such as oil drilling, electronics, and metal plating

(Buck et al., 2011; Löfstedt Gilljam et al., 2016). Polymeric derivatives, such as polytetrafluoroethylene (PTFE) and polyvinylidene fluoride (PVDF), have also been extensively used in manufacturing carpets, cookware, and food packaging (Buck et al., 2011; Robel et al., 2017). In 2009, PFOS and related compounds were included in Annex B of the Stockholm Convention to restrict their use.

Over time, more than 3,000 PFAS compounds have been identified in the global market, contributing to increasing environmental concerns due to their persistence and potential for bioaccumulation (Wang et al., 2017).

1.3.1 Sources and transport pathways

PFASs are produced and incorporated into a wide range of consumer goods, enter the environment through industrial emissions, waste mismanagement, and product degradation. Due to their high persistence, they contaminate water bodies, undergo bioaccumulation in aquatic and terrestrial organisms, and ultimately reach humans via consumption of contaminated food, drinking water, and contact with PFAS-containing products.

Chronic exposure has been associated with a spectrum of adverse health outcomes, including metabolic disorders (cholesterol impairment, overweight, obesity), hepatic toxicity, reproductive complications (preeclampsia, miscarriage, low birth weight), neurotoxicity, immunosuppression, organ damage, and increased cancer risk (Araújo et al., 2022) (**Figure 1-5**).

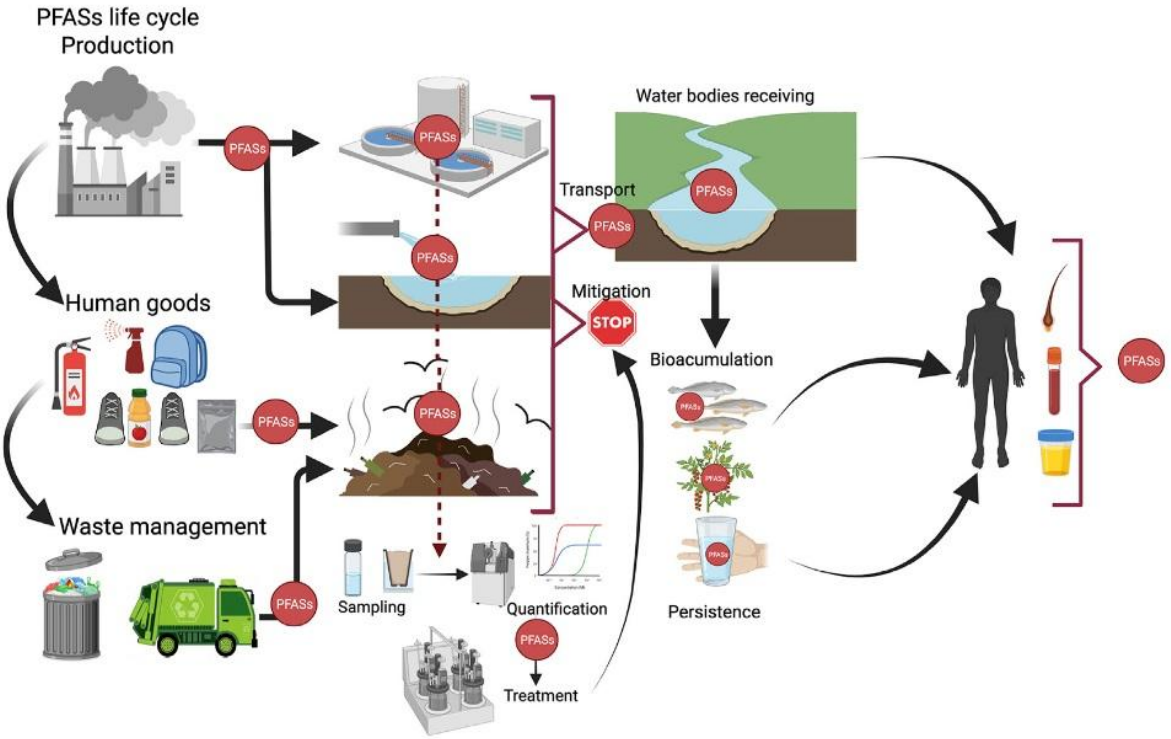

Figure 1-5 - PFAS: Sources, Fate, and Human Exposure. Adapted from Araújo et al. (2022)

PFASs contamination is prevalent in surface water and groundwater, with concentrations typically ranging from ng·L^{-1} to µg·L^{-1}. Numerous studies have shown that PFOA and PFOS levels in surface water can vary significantly. PFOA levels have been documented between 0.09 and 1270 ng·L^{-1}, while PFOS levels have been observed to range from 0.06 to 144 ng·L^{-1} (Ahrens et al., 2009; Hansen et al., 2002; Loos et al., 2008; Quinete et al., 2009; Teng et al., 2009; Wei et al., 2007).

Recognizing dietary intake as a major exposure route, the European Commission proposed directive 2017/0332, setting drinking water limits at 0.4 µg·L^{-1} for PFOS and 4 µg·L^{-1} for PFOA (Knutsen et al., 2018). Directive 2017/0332, later amended and implemented as Directive 2020/2184, addresses PFAS concentrations in drinking water, focusing only on these substances' total and sum concentrations. It sets a new group limit of 0.5 µg·L^{-1} for PFAS and a limit of 0.1 µg·L^{-1} for 16 specific individual PFAS (European Parliament and the Council of the European Union, 2020).

The European Food Safety Authority (EFSA) further highlighted fish, meat, eggs, milk, dairy products, and drinking water as significant sources of exposure (Knutsen et al., 2018).

Drinking water, particularly in areas contaminated with PFAS, remains a significant source of PFAS exposure (Rogers et al., 2021). Tolerable weekly intakes (TWI) of 13 ng.kg-^{1} bw for PFOS and 6 ng.kg-^{1} bw for PFOA have been established (Knutsen et al., 2018). Military firefighting activities, particularly the use of AFFF, have been identified as significant contributors to PFAS transport between the lithosphere (soil) and hydrosphere (surface water and groundwater). Studies have shown that PFOA (6.98–287 µg.kg^{-1} dry weight) and PFOS (118–8520 µg.kg^{-1} dry weight) can persist in soil for over 30 years and subsequently leach into groundwater (Filipovic et al., 2015). This contamination is especially notable in countries like Sweden, the United States, Germany, and Australia, where firefighting activities have been more prevalent. PFOA tends to be detected more frequently and at higher concentrations than PFOS, likely due to PFOS's higher adsorption onto particles, as indicated by its solid-liquid partition coefficient (Kd) (Høisæter & Breedveld, 2022; Milinovic et al., 2015).

Beyond firefighting foams, PFAS contamination arises from various sources, including industrial discharges, pipeline leaks, landfill leachates, atmospheric transport and deposition, precipitation, and urban runoff (Bao et al., 2019; Chen et al., 2016; Dong et al., 2020; Eschauzier et al., 2013; Harrad et al., 2020; Hepburn et al., 2019; Podder et al., 2021). The adsorption of PFAS onto atmospheric particulates explains its presence in remote or rural areas with little to no industrial development (Chen et al., 2016). Both point and non-point sources contribute to PFAS contamination, influenced by both anthropogenic and natural factors. Therefore, monitoring and managing PFAS sources and transport pathways is crucial to minimize environmental and human health impacts (Wee & Aris, 2023a).

PFAS contamination stems from multiple sources, including industrial emissions, wastewater discharges, and landfill leaching (Chen et al., 2018; Galloway et al., 2020; Wang et al., 2019).

AFFFs are a significant source of PFAS in groundwater and surface water near firefighter training and emergency response sites (Mejia-Avendaño et al., 2017; Nickerson et al., 2020). Wastewater treatment plants (WWTPs) are also a key source of PFAS emissions, as conventional treatment methods are ineffective at removing these substances (Lenka et al., 2021; J. Liu & Mejia Avendaño, 2013). Additionally, PFAS precursors can degrade into more persistent compounds, further contributing to environmental contamination (Nakayama et al., 2019).

1.3.2 Effects of PFOA on Exposed Organisms

PFOS and PFOA have been widely detected in aquatic environments across the globe (Ahrens, 2011; Ankley et al., 2021; Huang et al., 2021), as well as in aquatic organisms (Houde et al., 2011; Lee et al., 2020; Li et al., 2008; Ma et al., 2022). These compounds are known to act as endocrine-disrupting chemicals (EDCs) by interfering with hormone systems at the molecular level, despite their high degree of ionization in water (pKa < 1) (Xiao, 2017). PFOS and PFOA have a high bioaccumulation potential, particularly in long-chain compounds like perfluorocarboxylates (PFCAs), which accumulate in protein-rich tissues such as blood and liver (Ahrens, Siebert, et al., 2009; Ng & Hungerbühler, 2013). Studies have demonstrated that PFAS can biomagnify through food webs, with top predators, including marine mammals in remote Arctic regions, exhibiting elevated concentrations (Houde et al., 2011). Furthermore, PFAS are known to interact with peroxisome proliferator-activated receptors (PPARs), disrupting metabolic processes, particularly adipogenesis, thus earning the classification of "obesogens" (Nappi et al., 2016; Tian et al., 2019).

The adverse effects of PFOS and PFOA on aquatic organisms are well documented, with studies reporting reproductive toxicity (Ankley et al., 2005; Wang et al., 2011), oxidative stress (Arukwe & Mortensen, 2011), metabolic disturbances (Lee et al., 2017), immune toxicity (Han et al., 2012), and developmental toxicity (Ankley et al., 2005; Sant et al., 2018).

PFOS and PFOA can disrupt the endocrine system by binding directly to estrogen receptors (ERs) in the hypothalamus-pituitary-gonad-liver (HPGL) axis (Zhang et al., 2024), leading to altered gene expression related to reproduction (Ankley et al., 2005; Shi et al., 2008).

As shown in **Table 1.3-2**, PFAS exposure has been linked to a diverse range of adverse effects across various taxa. In *Cyprinus carpio*, for example, atretic oocytes and sperm paucity were observed after exposure to 2 $mg \cdot L^{-1}$ of PFOA for 56 days (Giari et al., 2016). At the population level, PFOS and PFOA exposure can lead to significant reductions in fecundity, decreases in hatching rates, and changes in sex ratios (Ankley et al., 2005; Lee et al., 2017; Wang et al., 2011). More evidence on these effects can be found in a review by Lee et al. (2020).

In bivalves, particularly the Mediterranean mussel (*Mytilus edulis*), subchronic exposure to PFOA altered the nutritional quality of tissues (amino acid and fatty acid composition) and induced oxidative stress and metabolic disruption (Zhou et al., 2024). Field studies have

confirmed bioaccumulation of PFAS in coastal mussel populations, with PFOA and PFOS being dominant (Giffard et al., 2022).

Human exposure to PFAS has also become a growing concern. Epidemiological studies suggest that PFAS exposure is linked to cancer, immune dysfunction, and metabolic and neurological issues (Lindh et al., 2012; Zhou et al., 2014). High PFAS concentrations have been found in populations frequently consuming seafood, such as those in Greenland, the Faroe Islands, and China (Christensen et al., 2017; Weihe et al., 2008).

Moreover, seafood has been identified as a major source of PFAS exposure, with cooking methods such as boiling shown to reduce PFAS concentrations (Del Gobbo et al., 2008). The European Food Safety Authority (EFSA) estimated that fish and seafood contribute up to 86% of PFAS exposure in the diet of adults (Knutsen et al., 2018). Similarly, high serum concentrations of PFAS compounds with a chain length of C≥9 have been used to identify seafood as a primary source of exposure (Hu et al., 2018).

Table 1.3-2 - Toxicological Effects and Environmental Presence of PFASs Across Different Species

Organism	Concentration	Effects	Authors
Cyprinus carpio	2 mg·L^{-1} of PFOA (56 days)	Atretic oocytes, sperm paucity, impaired reproduction	(Giari et al., 2016)
Marine Mammals (dolphins, seals, whales, polar bears)	Biomagnified PFAS (Arctic regions)	High PFAS accumulation in protein-rich tissues (liver, blood); biomagnification in food webs	(Ahrens et al., 2009; Houde et al., 2011)
Zebrafish (*Danio rerio*)	PFOS (50-200 µg·L^{-1})	Oxidative stress, disrupted embryonic development, immune dysfunction	(Sant et al., 2018; Shi et al., 2008)
Humans (general)	Serum concentrations >10 ng/mL PFOS/PFOA	Linked to cancer, immune dysfunction, metabolic disorders, reduced vaccine efficacy	(Lindh et al., 2012; Zhou et al., 2014)
Humans (coastal)	High serum PFAS (C≥9; seafood diet)	Seafood consumption was identified as a dominant exposure source; long-chain PFAS bioaccumulation	(Hu et al., 2018; Knutsen et al., 2018)
Rainbow Trout	PFOS (1-100 µg·L^{-1})	Altered liver metabolism, disrupted endocrine function, and oxidative stress	(Arukwe & Mortensen, 2011)

Cyprinus carpio	PFOA (10 μg·L^{-1} for 28 days)	Estrogen receptor disruption; altered gene expression in the hypothalamus-pituitary-gonadal axis	(Shi et al., 2008)
Mediterranean mussel *(Mytilus galloprovincialis)*	PFOA (10–100 μg·L^{-1}, 21 days)	Altered nutritional quality (amino acids and fatty acids), oxidative stress, disrupted metabolism	(Zhou et al., 2024)
Green mussel *(Perna viridis)*	PFOS/PFOA (1–50 μg·L^{-1})	Reduced phagocytosis, altered hemocyte function, immunotoxicity	(Liu & Gin, 2018)
Marine mussels (field studies)	0.5–12 ng·g^{-1} wet weight (coastal mussel tissues)	Bioaccumulation proportional to coastal contamination; PFOA and PFOS dominant	(Giffard et al., 2022)

1.4 17α-Ethinylestradiol (EE2)

Estrogens primarily contribute to estrogenic activity in aquatic environments among endocrine-disrupting compounds (EDCs). EDCs present a significant threat because they can interfere with the endocrine system, disrupting hormone synthesis and enzyme function (Grobin et al., 2024; Sukatis et al., 2024).

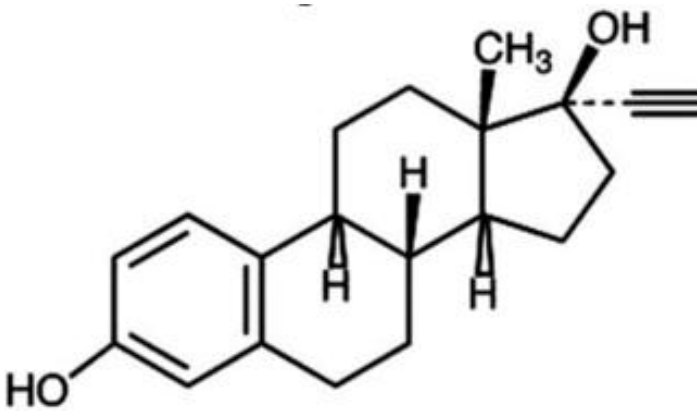

Figure 1-6 - Chemical structure of EE2

Among EDCs, steroid hormones, including natural estrogens such as estrone (E1), 17β-estradiol (E2), and estriol (E3), as well as the synthetic estrogen 17α-ethinylestradiol (EE2), are commonly found in wastewater, primarily due to their use in contraceptives. These compounds interfere with cellular apoptotic processes, influence epigenetic regulation in specific target

cells, and disrupt the cell cycle (Sifakis et al., 2017). Despite their harmful effects, EDCs persist in the environment, released through industrial, urban, and agricultural effluents. Once in the environment, these substances infiltrate soil, contaminate groundwater, and eventually affect drinking water, posing severe risks to human growth, development, and reproduction (Saravanakumar et al., 2022; Sukatis et al., 2024).

Steroid hormones, a subset of EDCs, are molecules synthesized from cholesterol and are characterized by the cyclopentanoperhydrophenanthrene ring structure (Ying et al., 2002). Natural estrogens (E1, E2, E3), along with synthetic estrogen (EE2), commonly used in contraceptives, are frequently detected in wastewater. These estrogens differ primarily in their configuration at carbon 17 (**Figure 1-6**), resulting in varied physicochemical properties (Dias et al., 2023).

Table 1.4-1 provides an overview of the primary physicochemical and toxicological characteristics of EE2, highlighting its relevance as a potent endocrine-disrupting compound and environmental contaminant. EE2 is a stable, poorly water-soluble compound with moderate lipophilicity, high estrogenic activity, and persistence in the environment, making it a significant risk to aquatic organisms even at low concentrations.

Table 1.4-1 - Chemical Profile and Properties of EE2. Adapted from EFSA (2010), WHO (2003), and PubChem (CID 5991)

Property	Description
Chemical Name	Ethinylestradiol
Chemical Formula	$C_{20}H_{24}O_2$
Molecular Weight	296.41 g/mol
CAS Number	57-63-6
Chemical Structure	Synthetic estrogen with a 17α-ethynyl group attached to the estradiol molecule
Physical State	Solid (at room temperature)
Color	White to off-white crystalline powder
Melting Point	182–186 °C
Water Solubility	Poorly soluble (4.8 mg/L at 20 °C)
Log Kow	3.67 - 4.15 (high lipophilicity)
pKa	10.4

Chemical Stability	Stable under normal conditions; sensitive to light and heat
Main Use	Active ingredient in oral contraceptives and hormone replacement therapy
Biological Activity	High estrogenic activity; synthetic analogue of the natural hormone estradiol
Toxicity Class	Potential endocrine disruptor; environmental pollutant affecting aquatic organisms

These compounds are highly lipophilic, enabling them to pass through the plasma membrane and bind to intracellular receptors (Silva et al., 2012). Due to their lipophilicity, they undergo conjugation with sulphate groups and glucuronic acid in the liver, which reduces their estrogenic potency. However, environmental microorganisms can deconjugate these compounds, restoring their estrogenic activity (Zayyat et al., 2024). Additionally, the lipophilic nature of these molecules also promotes bioaccumulation and biomagnification within trophic chains, potentially leading to higher concentrations that impact humans (Chiu et al., 2018).

EE2, a synthetic derivative of E2, is used in oral contraceptives and in treatments for osteoporosis, cancer, and alopecia. Due to its high estrogenic activity and resistance to degradation, EE2 is a persistent contaminant in aquatic environments. EE2 concentrations vary globally, with substantial levels detected in wastewater treatment plants and coastal regions (Islam & Tanaka, 2004; Sukatis et al., 2024). This compound is highly toxic, with harmful effects observed at concentrations as low as 0.1 $ng \cdot L^{-1}$, and the European Union has recognized it as an emerging aquatic pollutant (Zayyat et al., 2024). EE2 bioaccumulates in trophic chains, profoundly affecting marine organisms, particularly bivalves, which are especially vulnerable to prolonged exposure, thereby increasing its toxicity (Wojnarowski et al., 2021).

In bivalves, exposure to EE2 induces the overproduction of reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2). This oxidative stress activates antioxidant enzymes such as catalase and superoxide dismutase, which aim to mitigate cellular damage and prevent membrane disintegration (Lopes et al., 2022). Furthermore, EE2 disrupts lysosomal membrane stability and induces gonadal damage in *M. galloprovincialis* mussels, while affecting vitellogenin gene expression in *Saccostrea glomerata* oysters, and altering metabolic pathways related to anaerobic metabolism, osmotic

stress, and reproduction in *Ruditapes philippinarum* clams (Brew et al., 2020; Fabrello et al., 2021; Rodrigues et al., 2023).

In fish, EE2 interferes with sexual development, as observed in male western mosquitofish (*Gambusia affinis*), where it inhibits DNA repair gene transcription and increases vitellogenin levels in rainbow trout (*Oncorhynchus mykiss*) (Wojnarowski et al., 2021). The effects extend to other species, such as snails, where EE2 reduces heart rates in embryos, and amphibians, where it disrupts metamorphosis in leopard frogs (*Rana pipiens*) (Hogan et al., 2008; Schirling et al., 2006). These disruptions are linked to reduced fertility rates and the feminization of males, with organisms exhibiting the production of female-associated hormones like vitellogenin (VTG), a protein produced by ovoviviparous organisms (Wojnarowski et al., 2021).

Among EDCs, estrogens are the predominant contributors to estrogenic activity in aquatic ecosystems, with major sources being wastewater from municipal and hospital treatment plants, as well as livestock operations (Ying et al., 2002).

Wastewater effluent containing estrogenic compounds is commonly discharged into rivers, and residual sludge is often applied as fertilizer (Vulliet & Cren-Olivé, 2011). Research emphasizes the environmental significance of EE2, which binds to organic matter, accumulates in sediments, and bioaccumulates in aquatic organisms (Wit et al., 2010).

Even at low concentrations ($ng \cdot L^{-1}$), EE2 has been shown to disrupt the endocrine systems of exposed organisms, leading to alterations in sex determination, delays in sexual maturity, and reductions in secondary sexual characteristics (Chen et al., 2010; Duong et al., 2010; Dzieweczynski & Hebert, 2013; Liu et al., 2012; Saaristo et al., 2010; Silva et al., 2012; Vethaak et al., 2005). The toxicity of EE2 varies among species and is influenced by the type of assay (*in vitro vs. in vivo*) used for assessment (Van den Belt et al., 2004). EE2 (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol) is a synthetic derivative of natural estradiol (E2), widely used in modern contraceptives. It has low solubility in water (4.8 $mg \cdot L^{-1}$ at 20°C) and a higher affinity for estrogen receptors compared to natural estradiol, making it more potent in some fish species (Laganà et al., 2000; Lima et al., 2012).

EE2's resistance to degradation and high bioavailability make it an effective contraceptive but also allow it to persist in the environment. Its stability and resistance to oxidation due to the ethynyl group at the 17-position, enhance its environmental persistence (Li et al., 2013;

Partridge et al., 2010; Picazo et al., 2010; Zhang et al., 2011). EE2 has demonstrated the highest estrogenic potency in *in vitro* tests, ranking as EE2 > E2 > E1, with EE2 being 11–30 times more potent than E2 in fish (Colman et al., 2009; De Mes et al., 2005). In addition to its use in contraceptives, EE2 is employed for treating menopausal symptoms, hormone replacement therapy, and certain cancers, and is also used in livestock and aquaculture to manipulate growth and reproduction (Kuster et al., 2006; Lima et al., 2011; Moriyama et al., 2004).

1.4.1 Sources of EE2 in the environment

Human and animal activities are the primary sources of EE2 in the environment. Hormones, including EE2, are excreted by humans and livestock and enter water bodies through sewage treatment plants, septic systems, and agricultural runoff when manure is used as fertilizer.

Wastewater from industries that produce synthetic hormones also contributes to environmental contamination. Estrogenic compounds accumulate in sediments, where they can persist for extended periods (Matozzo et al., 2008).

As we can see in **Figure 1-7**, once released into aquatic systems, EE2 persists in surface waters and sediments, where it undergoes bioaccumulation and biomagnification in aquatic organisms (Tang et al., 2021). These processes are associated with endocrine disruption, reproductive impairment, population decline, and potential risks to human health through trophic transfer and water reuse.

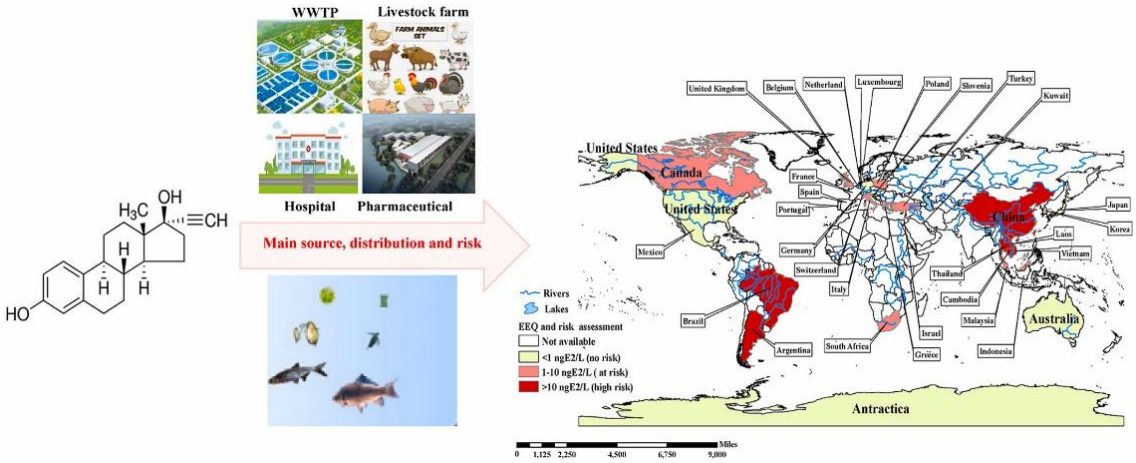

Figure 1-7 - Sources, environmental distribution, and risk assessment of 17α-ethinylestradiol (EE2). Adapted from Tang et al. (2021)

Human urine is the main source of EE2, as the compound is metabolized into inactive, water-soluble forms prior to excretion. In contrast, feces often contain the parent compound due to bacterial deconjugation in the gut (Pauwels et al., 2008; Vethaak et al., 2005). In wastewater treatment plants, EE2 can be reactivated into its free form. Due to its stability, it often remains intact throughout the treatment process, ultimately being released into the environment and contributing significantly to EE2 pollution (Al-Ansari et al., 2010; Forrez et al., 2009). Livestock farming also contributes to EE2 pollution, especially from wastewater runoff in areas of intensive animal farming (Briciu et al., 2009; J. Liu et al., 2012). Furthermore, aquaculture introduces estrogens into surrounding waters, as they are used to control fish sex differentiation (Körner et al., 2008; Kuster et al., 2006).

The increasing use of synthetic estrogens, particularly EE2 in contraceptives and livestock management, has resulted in growing environmental contamination.

High concentrations of EE2 have been detected near agricultural and livestock operations, and the compound is frequently found in sediments where it can accumulate at levels up to 1,000 times higher than in the overlying water (Lai et al., 2000).

Research by Labadie and Hill (2007) reported estrogen levels in bed sediments ranging from less than 0.12 to 22.80 $ng.g^{-1}$ in rivers and up to 3.6 $ng.g^{-1}$ in coastal environments, indicating that estrogen compounds, including EE2, are prone to bioaccumulation and biomagnification in aquatic organisms (Labadie & Hill, 2007).

EE2 has also been detected in various aquatic species. Dussault et al. (2009) studied the bioaccumulation of EE2 in benthic invertebrates, showing that species such as *Chironomus*

tentans and *Hyalella azteca* can accumulate significant amounts of EE2, particularly in sediment environments. This bioaccumulation poses a risk to predators higher in the food chain (Dussault et al., 2009).

1.4.2 Effects of EE2 on Exposed Organisms

Numerous studies have demonstrated the toxic effects of EE2 on aquatic species, particularly its capacity to disrupt endocrine systems at concentrations as low as a few nanograms per liter (Robinson & Hellou, 2009) as shown in **Table 1.4-2**. Exposure to EE2 has been shown to lead to the feminization of male fish, reduced gamete quality, and decreased reproductive success (Aydin & Talinli, 2013; Yan et al., 2012). In fish, exposure to EE2 consistently results in elevated plasma vitellogenin levels, a biomarker of estrogenic exposure, and adverse effects, including intersex development and behavioral changes that negatively impact reproduction (Andersson et al., 2007; Hallgren et al., 2012).

Early life stages of aquatic organisms are susceptible to EE2, making them useful indicators of water quality (Liu et al., 2012). Studies on species such as *Oryzias latipes* (medaka), *Danio rerio* (zebrafish), and *Gambusia affinis* (mosquitofish) have demonstrated sex-reversing effects following exposure to EE2 (Angus et al., 2005; Örn et al., 2003; Scholz & Gutzeit, 2000). In addition, EE2 has been linked to tumor formation and changes in gene expression in species like zebrafish (Hoffmann et al., 2006; Notch et al., 2007).

Behavioral changes due to EE2 exposure have also been observed in species such as sand gobies (*Pomatoschistus minutus*) and zebrafish (*Danio rerio),* where reproductive and social dominance behaviors are altered, potentially affecting population stability (Colman et al., 2009; Saaristo et al., 2010). In amphibians, EE2 has been shown to influence metamorphosis and sex ratios, as demonstrated in studies on northern leopard frogs (*Rana pipiens*) and African clawed frogs (*Xenopus laevis*) (Hogan et al., 2008; Tompsett et al., 2012). In mammals, exposure to EE2 during development has been shown to significantly impact reproductive systems, leading to altered growth and reproductive outcomes. Male rats exposed to EE2 have exhibited reduced fertility and diverse reproductive abnormalities (Delclos et al., 2009; Vosges et al., 2008). Furthermore, EE2 has also been shown to impact the development of gonadotropin-

releasing hormone (GnRH-1) neurons, which are crucial for reproductive function in mammals (Pillon et al., 2012).

Table 1.4-2 - Toxicological Effects and Environmental Presence of EE2 Across Different Species

Organism	Concentration	Effects	Authors
Fish *(general)*	Nanograms per liter ($ng \cdot L^{-1}$)	Feminization of males, reduced gamete quality, decreased reproductive success	(Aydin & Talinli, 2013; Robinson & Hellou, 2009; Yan et al., 2012)
zebrafish *(Danio rerio)*	Low $ng \cdot L^{-1}$	Elevated vitellogenin, intersex development, tumor formation, behavioral changes	(Andersson et al., 2007; Hoffmann et al., 2006; Notch et al., 2007)
medaka *(Oryzias latipes)*	Sub-$ng \cdot L^{-1}$ to $ng \cdot L^{-1}$	Sex-reversing effects, altered gene expression	(Örn et al., 2003; Scholz & Gutzeit, 2000)
mosquitofish *(Gambusia affinis*	Nanograms per liter ($ng \cdot L^{-1}$)	Sex reversal, reproductive behavior alterations	(Angus et al., 2005)
Sand Gobies	Nanograms per liter ($ng \cdot L^{-1}$)	Altered reproductive and social dominance behaviors	(Colman et al., 2009; Saaristo et al., 2010)
leopard frog *(Rana pipiens)*	Low $ng \cdot L^{-1}$	Altered metamorphosis, skewed sex ratios	(Hogan et al., 2008)
clawed frog *(Xenopus laevis)*	Low $ng \cdot L^{-1}$	Changes in sex ratios, disruption in gonadal development	(Tompsett et al., 2012)
Mammals *(general)*	Varying developmental exposure	Altered reproductive systems, reduced fertility, abnormalities in gonadotropin-releasing hormone neurons	(Pillon et al., 2012; Vosges et al., 2008)
Male Rats	Developmental exposure to EE2	Reduced fertility, reproductive abnormalities	(Delclos et al., 2009; Pillon et al., 2012)

Few studies have investigated EE2 levels in natural environments, though recent research has provided some insights. For example, Wang et al. (2015) analyzed EE2 concentrations in Taihu Lake, China, while Zhang et al. (2011) examined samples from Yundang Lagoon in Xiamen City (Wang et al., 2015; Zhang et al., 2011).

Zhang et al. reported EE2 concentrations ranging from 0.16 to 0.34 mg·kg^{-1} in various fish species. In contrast, Wang et al. found EE2 concentrations in biota between 21.3 and 417 mg·kg^{-1} dw. These findings suggest that EE2 levels in aquatic organisms may have increased over time, as earlier studies documented lower concentrations in comparable environments.

1.5 Detection and Quantification of EDCs

EDCs are typically analyzed using advanced chromatographic techniques such as gas chromatography (GC) or liquid chromatography (LC). These systems are commonly paired with ionization sources and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detectors, which identify positive or negative charged ions through various ion monitoring modes (Jeannot et al., 2002; Jiménez-Díaz et al., 2015; Wille et al., 2012). High-resolution mass spectrometry (HRMS) is increasingly being adopted due to its enhanced sensitivity and specificity (Hernández et al., 2011).

However, variability in extraction efficiency and potential interference from co-extracted matrix components with MS detectors (i.e., "matrix effects") demands the routine use of internal standards to ensure accurate quantification of target analytes. Commonly, these internal standards are the target compounds' stable isotope analogues (also known as "surrogate standards").

1.5.1 Detection and Quantification of TBBPA

Analytical techniques for certain classes of brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs), are often comparable to those used for other hydrophobic chemicals, like organochlorines (OCs) and polychlorinated biphenyls (PCBs) (Covaci et al., 2011). These BFRs are frequently incorporated into multi-class hydrophobic organic contaminant analysis methods for environmental and biological samples (Svarcova et al., 2019; Zhang et al., 2015a). Review articles have discussed analytical approaches for emerging BFRs (Covaci et al., 2011; Papachlimitzou et al., 2012).

Common extraction methods for BFRs from tissues or biota include solvent extraction (Zhong et al., 2018), ultrasound-assisted extraction (Zhang et al., 2015b), microwave-assisted extraction (Bayen et al., 2004), and pressurized liquid extraction (Neugebauer et al., 2018). Solid-phase extraction (SPE) has proven effective for isolating various BFRs from blood serum (Megson et al., 2016) and for extracting certain urinary biomarkers of BFR exposure (Lin et al., 2020). Given that hydroxy-metabolites of PBDEs can disrupt thyroid function, specific extraction and analysis methods have been developed for detecting PBDE metabolites in urine (Feng et al., 2016).

For the analysis of BFRs in biological extracts, GC-MS and GC-MS/MS are the preferred techniques for quantification. Special precautions are required during GC analysis to prevent thermal degradation of highly brominated PBDEs (e.g., BDE 209), such as using a temperature-programmable injector (e.g., 75–300°C) and a short GC column (e.g., 15 m). Both Electron Capture Negative Ionization (ECNI) and Electron Impact (EI) are commonly used ionization techniques for BFR analysis.

The US EPA Method 1614A outlines protocols for extracting PBDEs from soil, sediment, water, and biological tissues and analyzing them via high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) using ECNI or EI ionization. Recently, Megson et al. (2016) demonstrated that a method using HRGC with atmospheric pressure chemical ionization (APCI) and high-resolution quadrupole time-of-flight mass spectrometry (qTOF/MS) provided performance comparable to, and in some cases superior to, existing HRGC-EI-HRMS methods (Megson et al., 2016).

For specific BFRs, such as tetrabromobisphenol A (TBBPA), hexabromocyclododecane diastereomers (α-HBCD, β-HBCD and γ-HBCD), and brominated phenols, LC-MS/MS with electrospray ionization (ESI) is the method of choice (Lankova, Kockovska, et al., 2013). Additionally, LC-MS/MS methods have been developed to simultaneously analyse PBDEs, HBCD isomers, and other BFRs in wastewater (Zhou et al., 2010).

Recent advances in BFR analysis have focused on reducing sample size, preparation time, and costs, while including BFRs in multi-analyte methods. These approaches often target both parent compounds and their transformation products or metabolites. For instance, Tan et al. (2020) developed a rapid and straightforward Liquid Chromatography–High Resolution

Mass Spectrometry (LC-HRMS) method for the qualitative and quantitative analysis of TBBPA and its metabolites in fish samples (Tan et al., 2020). Furthermore, isotope ratio mass spectrometry (IRMS) has provided new insights into the fate of BFRs.

Cheng et al. (2019) recently developed a Gas Chromatography–Isotope Ratio Mass Spectrometry(GC-IRMS) method to determine stable carbon isotope ratios of HBCD diastereomers for tracking abiotic or biological transformation processes (Cheng et al., 2019). Similarly, Zhu et al. (2020) used compound-specific carbon isotope analysis with an offline elemental analyzer to study the debromination of decabromodiphenyl ether (BDE-209) (Zhu et al., 2020). Ongoing research is crucial for developing methods to detect emerging BFRs in environmental matrices (Zuiderveen et al., 2020).

1.5.2 Detection and Quantification of PFAS

Analytical methods for PFAS have been extensively reviewed in recent literature (Liu et al., 2019; Mullin et al., 2019; Munoz et al., 2019; Nakayama et al., 2019; Valsecchi et al., 2013).

The transition from GC-MS to LC-MS in the early 2000s significantly enhanced detection limits (LODs) by more than three orders of magnitude, facilitating the analysis of PFAS at ultra-trace levels and reducing the required sample volumes. In particular, the introduction of dried blood spot (DBS) techniques has provided a practical and minimally invasive approach for biomonitoring. (Ma et al., 2013).

Background contamination from sampling equipment is generally of minimal concern in highly contaminated areas, as the PFAS concentrations in these samples far exceed potential contamination from field materials (Rodowa & Reiner, 2021). However, when low concentrations are expected, background contamination should be minimized, for example, by implementing washing protocols for sampling and laboratory materials.

To further reduce PFAS background levels from LC-MS/MS mobile phases or tubing, a PFAS trap column (or 'delay' column) is typically installed after the injector (Vestergren et al., 2012). Additionally, each batch should include field, laboratory, and analytical injection blanks to prevent false positives. Water sample preparation methods commonly involve off-line solid-phase

extraction (SPE) with weak-anion exchange cartridges (e.g., Oasis WAX, Strata X-AW) to achieve adequate concentration factors, particularly for low-level contaminants in drinking and surface water.

The US EPA has set a drinking water health advisory limit of 70 $ng \cdot L^{-1}$ for PFOS and PFOA combined, later revised to even lower levels in 2020 (Post, 2020). The European Commission proposed directive 2017/0332, setting drinking water limits at 0.4 $\mu g.L^{-1}$ for PFOS and 4.0 $\mu g.L^{-1}$ for PFOA (Knutsen et al., 2018). Directive 2017/0332, later amended and implemented as Directive 2020/2184, addresses PFAS concentrations in drinking water, focusing only on these substances' total and sum concentrations. It sets a new group limit of 0.5 $\mu g \cdot L^{-1}$ for PFAS, along with a limit of 0.1 $\mu g \cdot L^{-1}$ for 16 specific individual PFAS (European Parliament and the Council of the European Union, 2020).

Direct injection is possible for contaminated samples, but it requires adding an organic co-solvent (e.g., methanol) to prevent sorption artefacts (Martin et al., 2019). Large-volume aqueous injections, such as on-line SPE methods, is less recommended due to potential sorption issues. Some researchers, like Backe et al. (2013), have bypassed this limitation using large-volume non-aqueous injection with orthogonal columns (Backe et al., 2013).

Soil sample preparation presents unique challenges due to the specific properties of various PFAS. Anionic PFAS are generally well-extracted using methanol combined with a weak base, such as ammonium hydroxide (NH_4OH), or acetonitrile mixtures in water. However, methods that target zwitterionic and cationic PFAS often report low recoveries (<10%) due to strong sorption.

Alternative methods using methanol mixed with strong bases (e.g., NaOH), strong acids (e.g., HCl), or salts (e.g., CH_3COONH_4) have been shown to improve recovery rates (Liu et al., 2021; Nickerson et al., 2020). However, these methods may also promote unwanted analyte transformations. Post-extraction cleanup is typically performed with ENVI-Carb cartridges, widely used across environmental matrices to reduce interferences, including in biota studies (Barhoumi et al., 2022).

For biota tissues, one of the most widely used extraction methods involves ion-pairing extraction with tetrabutyl ammonium hydrogen sulfate (TBAHS) and methyl tert-butyl ether (MTBE), as proposed by Hansen et al. (2001). Extracts are typically dried and reconstituted in

an appropriate solvent for instrumental analysis. Other extraction methods include alkaline digestion with strong bases (e.g., potassium hydroxide or sodium hydroxide) in methanol or ultrasonic extraction with methanol or acetonitrile mixtures (Valsecchi et al., 2013). Cleanup steps often involve solid-phase dispersion with silica or graphite or combining both methods (Bertin et al., 2014).

Despite the use of internal standards, matrix effects are occasionally observed in biota extracts, and these can be addressed through matrix-matched calibration. Certified reference materials for biological tissues containing PFAS are available from institutions such as NIST (Rodowa & Reiner, 2021).In most cases, PFAS quantification relies on isotopic dilution, with internal standards introduced at the start of the preparation process.

Isotopologues are available for most major PFCA, PFSA homologues, and some anionic and neutral precursors. However, internal standards for zwitterionic and cationic PFAS remain limited (Antell et al., 2025).

Reversed-phase liquid chromatography (RPLC) is typically used for separation, with a C18 chromatographic column and mobile phases such as water and acetonitrile (ACN) or methanol/ACN mixtures modified with acids (formic or acetic acid, 0.1–0.5%) or ammonium acetate (2–20 mM). Pentafluorophenylpropyl (PFP) stationary phases can also improve the separation of linear and branched PFOS isomers (Y. Liu et al., 2019).While most labs focus on a select group of anionic PFAS, such as PFCAs, PFSAs, and precursors from the C8 electrochemical fluorination (ECF) process, a broader range of PFAS classes may need to be analyzed to avoid underestimating total PFAS levels.

For example, PFCA/PFSA may account for only 1–3% of total PFAS in soils impacted by AFFF foams (Mejia-Avendaño et al., 2017; Nickerson et al., 2020). Furthermore, the lack of standardized methods and reference chemicals for emerging PFAS remains a significant limitation. Semi-quantitative methods using calibrants matched with similar structures have been proposed as an interim solution (Nickerson et al., 2020).

In addition to targeted PFAS analysis, total PFAS can be estimated using surrogate parameters, such as total organofluorines (Koch et al., 2020) and total fluorine via particle-induced gamma ray emission (PIGE) spectroscopy (Ritter et al., 2017). The total oxidizable precursor

(TOP) assay, introduced by Houtz and Sedlak (2012), is another important tool, converting precursors into measurable perfluoroalkyl carboxylic acids (PFCAs) (Houtz & Sedlak, 2012). While it has been applied mainly to water samples, more complex matrices like soils may require additional cleanup to remove competing organic matter (Casson & Chiang, 2018).

The growing availability of high-resolution mass spectrometry (HRMS) has driven the adoption of suspect-target and non-target PFAS analysis. Post-acquisition strategies such as mass defect filtering are widely used, while other techniques, like in-source fragmentation flagging, have also been employed (Liu et al., 2015). Due to the high number of fluorine atoms in PFAS, their mass defects fall within specific ranges, making mass defect filtering an effective strategy for PFAS detection (Myers et al., 2014).

1.5.3 Detection and Quantification of EE2

Several methods have been described for identifying and quantifying estrogens in biological matrices. Immunoassays have long been considered the gold standard for measuring estrogens in biofluids such as serum or plasma (Borges et al., 2009; Zhang et al., 2009). However, these techniques are often time-consuming and susceptible to cross-reactivity with other analytes, resulting in reduced accuracy and limited sensitivity (Al-Ansari et al., 2011; Borges et al., 2009; Zhang et al., 2009). Moreover, antibody-based methods typically require separate assays for each biomarker, meaning each estrogen must be measured individually.

In response to these limitations, gas chromatography or liquid chromatography coupled with mass spectrometry (GC–MS or LC–MS, respectively) has emerged as the preferred method for determining estrogens in biological samples. Both techniques, particularly when combined with tandem mass spectrometry (MS/MS), offer high specificity, minimal cross-reactivity, and the ability to simultaneously analyze multiple compounds in addition to E2 and EE2 (Zhang et al., 2011).

Other emerging methods include capillary electrophoresis (Contin et al., 2014), micellar electrokinetic chromatography (MEKC) (Gañán et al., 2014), biosensors (Huang et al., 2014; Wang et al., 2014), high-performance liquid chromatography with fluorescence detection (HPLC-FD) (Kumar & Srivastava, 2014; Lu et al., 2014), modified electrochemical sensors (Wang

et al., 2014), electrochemiluminescence (Zhang et al., 2014), and chronoamperometry (Zhang et al., 2014).

Several recent biosensors have been developed for biological samples, such as a label-free aptamer sensor for E2 using vanadium disulfide nanoflowers and gold nanoparticles, with detection via differential pulse voltammetry in spiked urine samples (Huang et al., 2014). Another example is a biosensor utilizing thiol-capped E2 aptamers in a competitive detection scheme, relying on complementary DNA targeted to unbound E2 aptamers (Zhang et al., 2014). A biosensor based on a recombinant human estrogen receptor fragment was also developed, employing the competitive displacement of coumestrol, a fluorescent phytoestrogen (Wang et al., 2014).

Due to the low molecular weight and volatility of steroid estrogenic hormones, gas chromatographic analysis often requires derivatization to increase target compounds' volatility, improving sensitivity and selectivity. For example, Al-Ansari et al. (Al-Ansari et al., 2011, 2013) optimized a method for detecting estrogens in whole fish tissues using pentafluorobenzoyl chloride (PFBCl) derivatization following extraction and cleanup. In contrast, Park et al. (2009) proposed derivatization with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), followed by heating at 65°C for 20 minutes, for estrogen determination in aerial invertebrates. However, derivatization steps can be time-consuming and may lead to analyte loss (Fayad et al., 2013).

Consequently, liquid chromatography methods, which are not limited by volatility or thermolability, have gained significant traction, with LC–MS/MS becoming the most widely used analytical tool for detecting estrogens in biological samples.

The increasing adoption of LC–MS/MS is evident from the rising number of publications employing this technique to quantify E2 and EE2 in biofluids and tissues (Bichon et al., 2012; Borges et al., 2009; Chen et al., 2012; Farlow et al., 2009; Keski-Rahkonen et al., 2013; Liu et al., 2014; State et al., 2009; Wheaton et al., 2012; Xiong et al., 2010; Zhang et al., 2009, 2011). Among studies published between 2009 and 2014 that described the quantification of E2 and/or EE2 in biological samples, 86% employed LC-based methods, while only 14% utilized GC.

Although mass spectrometry is the primary detection method, diode array (DAD) (Socas-Rodríguez et al., 2013; Zhong et al., 2012) and fluorescence (FD) (Socas-Rodríguez et al., 2013) detectors have also been used. In GC–MS analyses, chemical ionization (CI) is generally performed in negative mode (Al-Ansari et al., 2011, 2013), while LC–MS methods typically use electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or atmospheric pressure photoionization (APPI) in both positive and negative polarities. Derivatization with dansyl chloride (DNS-Cl) has been shown to enhance ionization efficiency in the positive mode (Bichon et al., 2012; Farlow et al., 2009; Liu et al., 2014; Wheaton et al., 2012; Xiong et al., 2010; Zhang et al., 2009).

Recent studies have employed both ionization modes to analyze 12 endogenous estrogens and their intact conjugates in blood and urine, detecting dansylated estrogens in positive mode and their conjugates in negative mode (Zhao et al., 2014).

For quantifying estrogens in biological matrices using GC–MS (Al-Ansari et al., 2011, 2013), selected ion monitoring (SIM) mode is often employed, targeting ions such as m/z 490 for pentafluorobenzoyl-derivatized EE2 and m/z 492 for the internal standard 13C-EE2 derivative. In LC–MS/MS, selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) is typically used, with common precursor-to-product ion transitions for E2 including m/z 271.2 → 145.1(W. L. Chen et al., 2012; Keski-Rahkonen et al., 2013), 255.3 → 159.2 (Keski-Rahkonen et al., 2013), and 271.1 → 183.0 (W. L. Chen et al., 2012). For EE2, transitions such as m/z 295.1 → 269.1 and 295.0 → 145.0 (W. L. Chen et al., 2012; State et al., 2009) are frequently applied. Dansylated derivatives are monitored at ion transitions like m/z 506.4 → 171.0 for dansylated E2 (Farlow et al., 2009; F. Zhang et al., 2009, 2011) and m/z 530.0 → 171.0 for dansylated EE2 (Bichon et al., 2012; Xiong et al., 2010; Zhang et al., 2009).

Since estrogens are often present in biological matrices at nanogram levels, analytical methods must provide low limits of detection (LOD) and quantification (LOQ). For solid matrices, GC–MS and LC–MS/MS methods typically achieve LOQs at the ng.g-1 level, while for liquid matrices, LOQs can range from ng/mL to as low as $pg.mL^{-1}$. However, UV and fluorescence detection methods generally have higher LOQs, still in the ng/mL range.

Matrix effects must also be considered, especially in mass spectrometry, where impurities can affect ionization efficiency, leading to either signal suppression or enhancement. To mitigate these effects, proper sample cleanup and the use of isotopically labelled internal standards, such as deuterated compounds, are essential to mitigate these effects. Reported recoveries for E2 and EE2 in biological matrices range from 70% to 130% for plasma, 90% to 120% for fish tissues, and 75% to 100% for milk samples, demonstrating the feasibility of these methods for complex sample types (Barreiros et al., 2016).

Applying these techniques has enabled the detection of E2 and EE2 in various biological matrices, sometimes at concentrations that raise concerns for both human health and wildlife (Barreiros et al., 2016).

For instance, EE2 has been detected in human plasma at concentrations as low as 1 $pg.mL^{-1}$ and as high as 85 $ng.mL^{-1}$ (Borges et al., 2009; Wheaton et al., 2012). E2 concentrations in female serum and endometrial tissue have been reported at 81 and 158 $pg.mL^{-1}$, respectively (Keski-Rahkonen et al., 2013).

1.6 Presence of TBBPA, PFAS, and EE2 in food

Environmental contaminants such as TBBPA, PFAS, and EE2 represent significant challenges to food safety and public health. These compounds are widespread in the environment due to industrial and agricultural activities and inadequate waste management systems. Their persistence, bioaccumulation potential, and toxicological effects make them critical subjects for study in food contamination.

As reported by the European Food Safety Authority (EFSA) (Schrenk et al., 2024), the highest concentrations of TBBPA were detected in the "Fish and seafood" category. The highest level was quantified in "Fish liver" (13 $\mu g.kg^{-1}$ ww), followed by "Ocean perch" and "Pollack," which showed concentrations of 2.87 $\mu g.kg^{-1}$ ww and 1.73 $\mu g.kg^{-1}$ ww, respectively.

Outside the "Fish and seafood" category, the highest concentration was found in "Animal fresh fat tissue" (up to 0.1 $\mu g.kg^{-1}$ ww) within the "Meat and meat products" food category. TBBPA has been studied extensively in food matrices across various regions, revealing a low

overall prevalence and concentration in most instances. Rivière et al. (2019) reported TBBPA detection in 30% of 169 infant food samples in France, with concentrations of 21.7 $ng.kg^{-1}$ ww in growing-up milk and up to 60.1 $ng.kg^{-1}$ ww in follow-on formulas (Rivière et al., 2019). Similar studies in Spain by Martínez et al. (2019) identified TBBPA in five of 50 infant formula samples, with levels ranging from <1 to 1.9 $\mu g.L^{-1}$ (Martínez et al., 2019). Conversely, no TBBPA was detected in six infant formula samples analyzed by Lankova et al. (2013) (LOQ = 50–150 $ng.kg^{-1}$ ww) (Lankova, Lacina, et al., 2013).

Venisseau et al. (2018) measured TBBPA and its derivatives in a broad range of food products in France, detecting TBBPA most frequently in sheep liver, fish, and seafood (<LOD to 68 $ng.kg^{-1}$ ww) (Vénisseau et al., 2018). Detection rates for TBBPA derivatives were higher in seafood (TBBPA-bMeE: 54%) and fish (TBBPA-bDiBPrE: 19%).

Poma et al. (2018) detected TBBPA in 2% of Belgian composite food samples, with concentrations up to 96 $ng.kg^{-1}$ ww in meat products (Poma et al., 2018). Studies from the Netherlands (de Winter-Sorkina et al., 2003; Gebbink et al., 2019) reported sporadic TBBPA detections in cheese, fish, and meat, with concentrations below 3.4 $\mu g.kg^{-1}$ ww.

Other European studies focused on seafood and wild fish, reporting varying TBBPA levels depending on species and fat content (Aznar-Alemany et al., 2017; Chessa et al., 2019). In freshwater samples, Kotthoff et al. (2017) detected TBBPA in breams and soles (0.5–1.2 $\mu g.kg^{-1}$ ww) (Kotthoff et al., 2017), while Hlouskova et al. (2013) and Svihlikova et al. (2015) reported mean values of 1.29–1.39 $\mu g.kg^{-1}$ ww in Czech fish samples (Hloušková et al., 2013; Svihlikova et al., 2015).

Outside Europe, TBBPA levels were higher in Chinese foods (Zhao et al., 2023), with mean concentrations of 19.0–97.7 $ng.kg^{-1}$ ww across aquatic food, meat, eggs, and dairy products, and trace levels in vegetables and fruits (Zhao et al., 2023). Similarly, Lee et al. (2020) reported comparable TBBPA levels in Korea (Lee et al., 2020) with high concentrations in fish (anchovy, eel, flounder, Spanish mackerel), cephalopods (squid, octopus), and shellfish (manila clams, oysters) compared to vegetables and cereals. TBBPA concentrations globally remain low, as evidenced by most studies reporting low detection frequencies and concentrations, which is consistent with data submitted to EFSA.

As reported by the European Food Safety Authority (EFSA) (Knutsen et al., 2018) PFASs are often found in higher concentrations in fish and shellfish, making them significant markers of environmental quality and valuable environmental indicators (Berger et al., 2009; Bossi et al., 2008; Del Gobbo et al., 2008; Ericson et al., 2008; Furdui et al., 2007; Gulkowska et al., 2006; Hoff et al., 2003; Hölzer et al., 2011; Kannan et al., 2005; Nania et al., 2009; Ostertag et al., 2009; Tittlemier et al., 2007). PFOS is the most abundant PFAS in these food sources, with concentrations typically ranging from <0.5 to 23 $\mu g.kg^{-1}$ in non-contaminated areas. Lean predatory fish generally exhibit the highest PFOS levels, strongly influenced by the species' trophic level (Bossi et al., 2008; van Leeuwen et al., 2009). Conversely, PFOA is detected at lower levels, usually between <0.1 and 0.25 $\mu g.kg^{-1}$, with much of the data censored due to limits of detection (LOD/LOQ) (Fromme et al., 2007; Schecter et al., 2010).

Wild fish tend to have higher PFOS concentrations than farmed fish, while industrial and water contamination can significantly increase PFAS levels (Berger et al., 2009). For example, PFOS concentrations exceeding 100 $\mu g.kg^{-1}$ have been reported in areas with known local pollution sources (Hoff et al., 2003; Kannan et al., 2005). Processed foods, like microwave popcorn, have also shown elevated PFOA levels, reaching 3.6 $\mu g.kg^{-1}$ (Tittlemier et al., 2007).

Temporal studies have shown decreasing PFOS concentrations in fish and eggs over time, particularly between 1999 and 2010 (Johansson et al., 2014; Vestergren et al., 2012). Archived samples from these periods demonstrated a 10-fold decrease in fish PFOS concentrations and a 40-fold decrease in eggs. Similar findings were reported for the decline in PFOS in meat products. However, specific food categories, like dairy and vegetable-based products, increased PFOA concentrations (Vestergren et al., 2012).

EFSA data confirm that fish is the most studied dietary source of PFAS, with mean PFOS concentrations ranging from 2.2 to 2.8 $\mu g.kg^{-1}$, far exceeding those of PFOA (<0.9 $\mu g.kg^{-1}$) (Stahl et al., 2012). Furthermore, wild animals, such as boars, have been shown to accumulate PFASs in liver and muscle tissues, influenced by exposure to contaminated soils and waste (Brambilla et al., 2016; Stahl et al., 2012).

The potential risks associated with EDCs to human health have prompted regulatory authorities to implement strict limits on their consumption. The Joint FAO/WHO Expert

Committee on Food Additives (JECFA) has established acceptable daily intake (ADI) levels of 0–0.05 μg.kg^{-1} body weight (bw) for 17β-estradiol, 0–30 μg.kg^{-1} bw for progesterone, and 0–2 μg.kg^{-1} bw for testosterone. Furthermore, both 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are currently listed on the European Commission's watch list for potential inclusion in the priority substance list under the European Water Framework Directive (Council, 2016)(Directive 2013/39/EU).

Recent investigations into seafood contamination have largely been concentrated in China, where natural hormones and synthetic estrogens like EE2 have been identified in various fish and mussel species. Notably, concentrations as high as 315.6 ± 6.5 ng.g^{-1} dry weight (dw) of EE2 were reported in native mussels from Shenzhen, China (Chiu et al., 2018).

Additionally, diethylstilbestrol (DES), a synthetic estrogen classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC), has been detected in wild fish sold in southern China, raising significant concerns about human exposure to this hazardous compound (Zhou et al., 2019).

Mussels, a key dietary component in certain regions, are a noteworthy vector for EDC exposure. For example, elevated concentrations of 4-nonylphenol (4-NP) and bisphenol A (BPA) 158 ng.g^{-1} wet weight (ww) and 404 ng.g^{-1} ww, respectively, were reported in *Mytilus galloprovincialis* from the Aegean Sea, Greece, highlighting potential public health implications (Gatidou et al., 2010). Similarly, in urbanized coastal regions of China, mussels collected from five polluted sites exhibited average EDC levels that translated to estimated daily intake (EDI) values of 0.03 μg.kg^{-1} bw for 4-NP, 0.04 μg.kg^{-1} bw for BPA, 0.13 μg.kg^{-1} bw for estradiol (E2), and 0.11 μg.kg^{-1} bw for EE2, based on a per capita annual mussel consumption of 12 kg and an average body weight of 50 kg (Chiu et al., 2018).

Although the EDIs for 4-NP and BPA were below the tolerable daily intake (TDI) levels established by the Danish Institute of Safety and Toxicology and the European Food Safety Authority, the estimated daily intake of E2 (6.5 μg/person/day) and EE2 (5.5 μg/person/day) from mussels significantly exceeded ADI thresholds set by international bodies (Caldwell et al., 2010). **Table 1.6-1** provides a synthesis of the information previously outlined. The WHO and regulatory authorities in the USA and Australia have set the ADI for E2 at 3 μg/person/day. In

contrast, for EE2, the limits are far lower, 0.007 µg/person/day in the USA and 0.0026 µg/person/day in Australia (Caldwell et al., 2010). These findings underscore the urgent need for comprehensive monitoring programs and stringent regulatory measures to mitigate EDC contamination in seafood, safeguarding public health.

Table 1.6-1 - Occurrence of TBBPA, PFAS, EE2 in food products, with emphasis on seafood

Compound	Food matrix	Reported concentrations	Authors
TBBPA	Fish and seafood (fish liver, ocean perch, pollack)	Up to 13 µg.kg^{-1} ww (fish liver); 2.87 µg.kg^{-1} ww (perch); 1.73 µg.kg^{-1} ww (pollack)	(Schrenk et al., 2024)
TBBPA	Infant food and formulas	21.7–60.1 ng.kg^{-1} ww (France); <1–1.9 µg/L (Spain)	(Martínez et al., 2019; Rivière et al., 2019)
TBBPA	Meat, sheep liver, cheese	Up to 96 ng.kg^{-1} ww (Belgium); <3.4 µg.kg^{-1} ww (Netherlands)	(de Winter-Sorkina et al., 2003; Gebbink et al., 2019; Poma et al., 2018)
TBBPA	Freshwater fish (bream, sole, Czech species)	0.5–1.39 µg.kg^{-1} ww	(Hloušková et al., 2013; Kotthoff et al., 2017; Svihlikova et al., 2015)
TBBPA	Aquatic food, meat, eggs, dairy, shellfish (China, Korea)	19–97.7 ng.kg^{-1} ww (China); higher in anchovy, eel, clams, oysters (Korea)	(Lee et al., 2020; Zhao et al., 2023)
PFAS (PFOS, PFOA, others)	Fish and shellfish (predatory species)	PFOS: <0.5–23 µg.kg^{-1} (unpolluted); >100 µg.kg^{-1} (polluted sites)	(Hoff et al., 2003; Kannan et al., 2005; Knutsen et al., 2018)
PFAS (PFOS)	Average fish (Europe)	2.2–2.8 µg.kg^{-1} (PFOS); <0.9 µg.kg^{-1} (PFOA)	(Stahl et al., 2012)

PFAS (PFOA)	Processed foods (microwave popcorn)	Up to 3.6 µg.kg^{-1}	(Tittlemier et al., 2007)
EE2 and other estrogens (EDCs)	Mussels (Shenzhen, China)	Up to 315.6 ng.g^{-1} dw EE2	(Chiu et al., 2018)
DES (synthetic estrogen)	Wild fish (China)	Detected in commercial samples	(Zhou et al., 2019)
BPA and 4-NP	Mussels (Greece)	404 ng.g^{-1} ww (BPA); 158 ng.g^{-1} ww (4-NP)	(Gatidou et al., 2010)
E2 and EE2 (estimated intake)	Mussels (China, polluted coastal areas)	EDI: 0.13 µg.kg^{-1} bw (E2); 0.11 µg.kg^{-1} bw (EE2)	(Caldwell et al., 2010; Chiu et al., 2018)

1.7 *Mytilus galloprovincialis* Genome and Stress-Response Genes

The Mediterranean mussel *M. galloprovincialis* has a large and highly variable genome. A recent assembly (~1.28 Gb) revealed an open pan-genome with a core set of ~45,000 genes and ~20,000 additional genes that are either present or absent in different individuals (Gerdol et al., 2020). Remarkably, about 25–30% of the mussel's genes are dispensable, showing presence/absence variation – a phenomenon unprecedented at such a scale in animals (Gerdol et al., 2020; Rey-campos et al., 2021). Many of these variable genes belong to young, expanded families linked to survival functions (e.g., immunity and stress tolerance) and likely contribute to the mussel's resilience in polluted and changing environments (Gerdol et al., 2020).

The genome sequence and gene annotations are publicly available through NCBI (BioProject PRJNA262617) and other databases (Murgarella et al., 2016). Key gene and protein sequences are cataloged in repositories like NCBI GenBank and UniProt, with functional annotations for many mussel genes (e.g., antioxidant enzymes, metallothioneins, heat shock proteins). Transcriptomic datasets (e.g., on NCBI GEO) provide gene expression profiles under various conditions (Banni et al., 2011). These resources enable researchers to link gene sequences with their expression patterns and functions in *M. galloprovincialis*. Below, we focus on major genes and regulatory elements related to oxidative stress, hormone/endocrine processes, apoptosis, neurotoxicity, and cell damage, and how they respond to environmental contaminants.

M. galloprovincialis harbors a group of genes that protect against oxidative damage. Key enzymes include superoxide dismutase (SOD), which convert superoxide radicals to hydrogen peroxide, catalase (CAT), which breaks down hydrogen peroxide, and glutathione-related enzymes like glutathione S-transferases (GST) and glutathione peroxidases. These genes are conserved in sequence and function, encoding proteins that neutralize reactive oxygen species (ROS) to prevent cellular damage. For example, the mussel genome encodes multiple SOD isoforms (cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD) similar to those in other animals (Feidantsis et al., 2020).

CAT and GST genes have been identified and show inducible expression under stress (Banni et al., 2015). Their promoter regions likely contain regulatory motifs (e.g., antioxidant response elements, ARE) that enable upregulation when oxidative stress is sensed (analogous to the Nrf2-ARE pathway known in vertebrates, though the exact transcriptional regulators in mussels are still being characterized).

The mussel genome also encodes various heat shock protein genes that respond to proteotoxic stress, often associated with oxidative damage. *M. galloprovincialis* possesses multiple HSP families, for instance, Hsp70 and Hsp90, major folding chaperones and small Hsps like Hsp27/Hsp26, which protect cytoskeletal elements (Banni et al., 2015). These genes have conserved heat shock elements in their promoters that bind heat shock factor (HSF) to drive expression under stress. Indeed, environmental stress (e.g. temperature changes, pollutants) significantly alters HSP mRNA levels (Banni et al., 2015). Mussels exposed to toxic chemicals or thermal stress triggers an upregulation of Hsp70 and Hsp27 transcripts, reflecting the activation of cellular stress-response pathways designed to refold misfolded proteins and prevent their aggregation (Banni et al., 2015).

Antioxidant and HSP genes are typically expressed at basal levels in tissues such as gills and digestive gland, which are on the frontline of pollutant exposure. Under stress, their expression surges. For example, mussels exposed to an antibiotic (oxytetracycline) showed increased CAT and GST mRNA in gills at normal temperature, indicating an activated detoxification and ROS-scavenging response (Banni et al., 2015). However, combined stressors (e.g., pollutant plus heat) can modulate this, high temperature alone induced HSPs, like Hsp27, but in combination with a contaminant (oxytetracycline) the HSP response was altered (Banni et al., 2015).

Generally, oxidative stress genes are upregulated by pollutants that generate ROS including heavy metals, PAHs, and hydrocarbons (Dobal et al., 2022). For instance, extreme thermal stress or hypoxia leads to elevated SOD, CAT, and GST activities in mussel gills as the organism attempts to detoxify excess ROS (Feidantsis et al., 2020). This surge in antioxidants helps mitigate lipid peroxidation and DNA oxidation, thereby limiting cell damage.

Functional annotations classify these genes in oxidative stress defence. Many are homologous to vertebrate genes (e.g., mussel CAT shares high sequence similarity with other eukaryotic catalases) (Woo et al., 2013). Notably, *M. galloprovincialis* possesses metal-binding proteins like metallothioneins that also contribute to oxidative stress management by sequestering redox-active metals. Two main metallothionein genes (MT10 and MT20) are present, encoding cysteine-rich proteins ~60–70 amino acids long. They bind heavy metals (Cd, Cu, Zn), reducing metal-induced ROS generation. These MT genes are strongly and rapidly induced upon metal exposure (Banni et al., 2011). Field and laboratory studies show that mussel MT10/20 expression increases with tissue metal levels, making them excellent biomarkers for metal-induced oxidative stress (Banni et al., 2011).

Unlike vertebrates, mussels have a diffuse endocrine system, but they do possess genes responsive to hormonal cues and endocrine disruptors. Research indicates that steroid hormone signaling pathways in bivalves are present but modified. For example, while mussels take up estrogens from the environment, they lack a clear ortholog of the vertebrate aromatase (CYP19) enzyme needed to synthesize estrogen (Evensen et al., 2024). Instead, other enzymes may produce estrogen-like compounds in mussels (Evensen et al., 2024).

Mussel genomes do encode nuclear receptor proteins (e.g., retinoid X receptor, estrogen receptor-like proteins), and vitellogenin (yolk protein) genes for egg development. However, their regulatory control differs from vertebrates. A study in a related mussel *(M. edulis*) showed that exposure to 17β-estradiol did not induce vitellogenin or estrogen receptor gene expression (Evensen et al., 2024), suggesting that exogenous estrogen alone doesn't trigger a strong vitellogenic response in mussels as it does in fish. This highlights that mussel endocrine genes may require different stimuli or co-factors, and classical estrogen receptors may be either absent or unresponsive in these invertebrates.

Recent genomic studies have identified key genes controlling mussel sex differentiation and gonadal development, which can be targets of endocrine disruption. Notably, *M. galloprovincialis* has orthologs of DMRT1 (DMRT1L in mussels), SoxH (a Sox family transcription factor), FoxL2 (forkhead box L2), and β-catenin, all of which play roles in gonad development and sex determination (Evensen et al., 2024).

These genes' sequences show they encode transcription factors or signaling proteins similar to those in other animals that govern male/female differentiation. Expression patterns: in normal development, DMRT1L and FoxL2 are differentially expressed in male vs female gonads (as expected for sex-differentiation regulators). Under laboratory exposure to endocrine-active compounds, their expression can be dramatically altered. For instance, exposure to endocrine disrupting chemicals (EDCs) like the synthetic estrogen 17α-ethinylestradiol (EE2) or the fungicide ketoconazole caused mussels to undergo sex reversal and disrupted the expression of these genes. DMRT1L, SoxH, FoxL2, and β-catenin transcripts were misregulated in treated mussels, leading to impaired gonadal development. This demonstrates that steroid-like pollutants can hijack mussel molecular pathways, confirming that steroid hormones (endogenous or exogenous) influence bivalve endocrine systems (Evensen et al., 2024).

In the wild, contaminants such as estrogenic chemicals (EE2, nonylphenol, bisphenol A) and anti-androgens/biocides (e.g. tributyltin) may perturb these endocrine genes. Mussels chronically exposed to low-dose EDCs show skewed sex ratios and reproductive anomalies, likely from cumulative gene expression changes in pathways like Wnt/β-catenin and DMRT1/FOXL2 (Evensen et al., 2024). Although mussels do not have the same endocrine glands as vertebrates, they exhibit hormone-like signaling (e.g. neuroendocrine cells producing dopamine, serotonin that affect reproduction). Genomic scans have also found peptide hormone precursors and receptors in *M. galloprovincialis*, which could be analogs to insulin-like or GnRH-like pathways, though these are less studied.

Promoters of mussel sex genes may contain binding sites for conserved factors (e.g. DMRT1L likely has DM domain binding motifs). Disruptors might interfere via receptor binding (if mussels have an estrogen-related receptor, ERR, or RXR, those could be activated/inhibited by pollutants). For example, organotin (TBT) known for imposex in snails acts via RXR; *M. galloprovincialis* RXR gene could similarly be a target leading to reproductive toxicity (though mussels are gonochoric and don't exhibit imposex, TBT has been reported to reduce growth and gametogenesis) (Evensen et al., 2024).

The mussel genome encodes conserved sex differentiation genes and putative hormone receptors, and while their sequences mirror those of other eukaryotes, their regulation

is distinct making endocrine disruption effects observable in gene expression changes rather than classical vitellogenin induction. Environmental EDC exposure can feminize or masculinize mussel populations by altering the normal gene expression programs of these key developmental regulators (Evensen et al., 2024).

M. galloprovincialis has a well-developed set of cell death genes, many of which show high similarity to vertebrate apoptosis regulators (Estévez-Calvar et al., 2013). Notably, the intrinsic (mitochondrial) apoptosis pathway is conserved. Six key mitochondrial apoptotic genes characterized in mussel include p53, PDRP, Bcl-2, Bax, BI-1, and Dff-A.

The mussel p53 gene encodes a tumor suppressor protein containing all the characteristic domains, including the DNA-binding domain, proline-rich region, Mdm2-binding site, and nuclear localization/export signals. This indicates that mussel p53 can perform DNA damage sensing and transcriptional activation of target genes, much like human p53. It sits at the hub of stress-response pathways, deciding between cell repair or apoptosis. Mussel p53 is phylogenetically closer to vertebrate p53 than to other invertebrates, underlining its conserved function.

A p53-downstream regulated gene/protein (PDRP), identified in mussels for the first time. The mussel PDRP contains a Prefoldin domain seen in chordates, hinting at a role in protein folding or transcriptional complexes. While its precise function in apoptosis is unclear, it may interact with other proteins/DNA as part of the p53 response (Estévez-Calvar et al., 2013).

Bcl-2 and Bax, are central apoptosis regulators, Bcl-2 is anti-apoptotic and Bax is pro-apoptotic. Mussel Bcl-2 and Bax proteins contain the expected Bcl-2 homology (BH) domains and C-terminal transmembrane regions, indicating they localize to mitochondria and regulate cytochrome c release like their human counterparts. The balance of Bcl-2/Bax in mussel cells likely determines cell survival versus apoptosis under stress (Estévez-Calvar et al., 2013).

Mussel Bax Inhibitor-1(BI-1) has six transmembrane domains and an endoplasmic reticulum (ER) retention signal. This matches the known BI-1 structure in vertebrates, where it

resides in the ER membrane and suppresses apoptosis by regulating calcium and ER stress signaling. The conserved signature motif suggests mussel BI-1 functions to buffer ER stress-induced cell death (Estévez-Calvar et al., 2013).

Mussel DNA fragmentation factor, also known as CAD endonuclease (Dff-A), contains the caspase cleavage site and nuclease domain characteristic of this protein, which, once activated, fragmentates DNA during the final execution phase of apoptosis. Its presence shows that downstream executioners of apoptosis are encoded in the mussel genome to carry out controlled DNA degradation in dying cells (Estévez-Calvar et al., 2013).

These apoptosis genes are expressed at low levels in normal conditions but are upregulated in response to cell damage and stressors. Experimental exposure to DNA-damaging agents (e.g. UV radiation, genotoxic chemicals) triggers the transcription of p53 and its targets in mussel´s cells. In one study, UV exposure caused time-dependent increases in p53, Bcl-2, Bax, BI-1, and Dff-A mRNA, indicating activation of the intrinsic apoptosis cascade as a stress response (Estévez-Calvar et al., 2013).

The regulatory elements in these genes include p53 binding sites in promoters of PDRP, Bax, etc., allowing p53 to induce them when DNA damage is detected. Upstream of p53, there are likely mussel homologs of ATM/ATR kinases and checkpoint proteins that sense DNA breaks and stabilize p53. These specific upstream components are not yet fully annotated, but given the conservation of p53 pathway, they are expected to exist (Estévez-Calvar et al., 2013).

Environmental pollutants that cause cellular damage often activate the apoptotic pathway in mussels. For example, heavy metals like Cd or organic pollutants can induce DNA damage or mitochondrial dysfunction, leading to p53 stabilization and apoptosis. A recent toxicogenomic study exposed mussels to anticancer drugs (ifosfamide and cisplatin, which form DNA adducts) and observed significant upregulation of apoptosis and DNA-damage genes (p53, caspase-8, and Gadd45) in gill tissue. This upregulation was even more pronounced under elevated temperature, suggesting that pollution and climate stress together push cells toward apoptosis (Queirós et al., 2023).

Caspases (cysteine proteases executing apoptosis) are present in *M. galloprovincialis* (e.g. initiator Casp-8, Casp-9 and effector Casp-3, Casp-7). Their transcripts or activities increase

when mussels are exposed to toxins that trigger cell death pathways. For instance, bivalves exposed to polycyclic aromatic hydrocarbons (PAHs) or pesticides show caspase-3 activation in their hemocytes, indicating pollutant-induced apoptosis as a defence to remove damaged cells. At the functional level, measuring these gene expression changes is useful: Gadd45 (Growth arrest and DNA-damage-inducible protein) is one such gene induced by p53, and its high expression in contaminated mussels reflects DNA repair or apoptosis signaling (Queirós et al., 2023).

Overall, the mussel's apoptosis gene network behaves similarly to higher organisms – severe stress leads to a p53-mediated transcriptional response, activating BAX/cleavage enzymes and culminating in apoptosis if damage is irreparable.These genes serve as molecular biomarkers of cell damage, since their elevated expression often correlates with pollutant exposure and ensuing cellular injury (Estévez-Calvar et al., 2013; Queirós et al., 2023).

Marine mussels have a relatively simple nervous system, but they do encode key neural proteins that can be affected by neurotoxic contaminants. One of the best-known biomarkers is acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine at synapses. *M. galloprovincialis* has genes for multiple AChE isoforms – including a membrane-bound form in neural tissues and a soluble form in hemolymph – similar to other bivalves (Deidda et al., 2021). The gene sequences for mussel´s AChEs contain the typical cholinesterase active-site motif and cysteine loop, indicating comparable structure and function to insect or vertebrate AChE. AChE activity is crucial for neuromuscular function (e.g. controlling the mussel's shell closure and foot movement (Shi et al., 2012).

AChE is expressed in nerve-rich tissues like the ganglia and in peripheral tissues (gills, hemolymph cells) to some extent. Under normal conditions, AChE ensures proper neuronal signaling. However, many neurotoxic chemicals target this enzyme. Organophosphate and carbamate pesticides (e.g., dichlorvos, chlorpyrifos, carbaryl) inhibit AChE activity, leading to the accumulation of acetylcholine and neurotoxic effects.

In mussels, exposure to the organophosphate dichlorvos (DDVP) at increasing concentrations (1–10,000 $\mu g \cdot L^{-1}$) for 24 h caused a dose-dependent reduction in gill AChE activity (Deidda et al., 2021). Repeated low-dose exposures led to persistent AChE inhibition as well.

At high doses, this was accompanied by mussel mortality and abnormal behaviors (shell closure failure, loss of byssus attachment), underscoring the link between the gene/enzyme and organism-level neurotoxicity (Deidda et al., 2021).

Mussels sometimes upregulate AChE gene expression as a compensatory response when enzyme activity is inhibited. Some studies have noted an increase in AChE mRNA in mussel tissues after chronic low-level pesticide exposure, potentially an attempt by the organism to synthesize more enzymes to counteract inhibition (though this may not overcome the irreversible binding of organophosphates). The regulation of the AChE gene involves elements common to cholinesterase genes; no mussel-specific regulatory element is known, but stress can trigger general neuroprotective pathways. Besides AChE, the mussel genome encodes other neuro-signaling genes: voltage-gated ion channels (for neuron excitation), receptors for neurotransmitters (e.g. glutamate and GABA receptors), and neuropeptides (Murgarella et al., 2016). These genes could also be affected by pollutants, for example, heavy metals like lead and mercury can disrupt ion channel function and induce oxidative stress in neural cells. While detailed expression data on these is sparse, the presence of homologous genes suggests that mussels have the molecular components to be impacted by neurotoxicants like higher animals (Atchison, 2003).

In environmental monitoring, AChE inhibition in mussels is a widely used biomarker of neurotoxic pollution (D'Agata et al., 2014; Deidda et al., 2021). Because the enzymatic assay is easy, it's often measured, but the genomic context is that *M. galloprovincialis* possesses a multigene family for cholinesterase. Researchers have isolated two forms of cholinesterase from mussel tissues with different sensitivities to inhibitors (Deidda et al., 2021). This implies possibly two gene products: one that might be more sensitive to organophosphates and one less so, as seen in oysters (Deidda et al., 2021). Furthermore, neurotoxic algae and nanoplastics can also induce neurological stress; e.g., exposure to certain algal toxins (saxitoxin) or plastic nanoparticles led to decreased cholinesterase activity and oxidative stress in mussels (Acker & Systems, 2021).

The genes responding to such neurotoxins often overlap with general stress genes (HSPs and antioxidants) because neurotoxicity can cause secondary oxidative damage and inflammation (Deidda et al., 2021).

"Cell damage" encompasses a broad set of stress effects, including DNA damage, protein damage, and membrane damage. The *M. galloprovincialis* genome encodes numerous genes involved in DNA repair and maintaining cellular integrity. For instance, genes for DNA repair enzymes such as RAD51 (homologous recombination repair), XRC/XP (nucleotide excision repair), and APE1 (base excision repair) are present by homology, ensuring mussels can mend DNA lesions (Gerdol et al., 2020).

Although not all have been functionally studied in mussels, the existence of p53 and Dff-A (CAD endonuclease) described earlier implies that upstream DNA damage sensors and repair triggers are in place (Estévez-Calvar et al., 2013). One gene that has been noted is Gadd45 – a gene induced by genotoxic stress that can pause the cell cycle and aid DNA repair. Mussels exposed to DNA-damaging pollutants (like chemotherapy drugs, as mentioned) significantly upregulate Gadd45, reflecting activation of DNA damage response pathways (Queirós et al., 2023).

M. galloprovincialis cells also exhibit markers of general cell injury. For example, lysosomal membrane stability in digestive gland cells is a known cellular health indicator, when cells are damaged by pollutants, lysosomal membranes become destabilized, releasing enzymes. While this is a biochemical marker, it is linked to gene expression as well: stress can induce lysosomal enzymes and membrane proteins. Genes encoding lysosomal hydrolases (cathepsins, β-glucuronidase, etc.) are present and might be upregulated to handle damaged organelles via autophagy. Indeed, increased expression of some autophagy-related genes (LC3, beclin) has been observed in mussels under pollutant stress, suggesting activation of self-cleanup mechanisms for damaged cell components (Martínez-García & Mariño, 2020).

A comprehensive study of mussels transplanted to polluted sites found coordinated changes in a suite of cell-damage related genes: stress proteins (HSPs), antioxidant enzymes, metallothioneins, apoptosis regulators, and DNA repair genes all shifted in expression, corresponding with levels of contaminants (Feidantsis et al., 2020).

For example, heavy metal exposure not only elevates MTs but also can increase expression of HSP70 and heme oxygenase-1 (HO-1, an oxidative stress marker) to mitigate protein and heme damage. Polycyclic aromatic hydrocarbons and oil compounds often form DNA adducts; mussels respond by inducing DNA repair genes and phase I detoxification enzymes like cytochrome P450s (e.g. CYP4Y1).

In fact, M. *galloprovincialis* exposed to ifosfamide showed a strong induction of a CYP4 gene (cyp4Y1), indicating activation of xenobiotic metabolism to possibly hydroxylate and clear the pollutant (Queirós et al., 2023).

The promoters of many cell damage response genes contain stress-responsive elements. For instance, HSP gene promoters have HSF binding sites (HSEs) as mentioned, antioxidant genes have AREs, and DNA repair genes can have p53 response elements (for those that p53 regulates, like Gadd45, p21). Metallothionein gene promoters in mussels feature metal-responsive elements (MRE) – short DNA motifs to which a metal-regulatory transcription factor binds when metals are present, driving high MT expression. This has been confirmed in other bivalves and likely holds for *M. galloprovincialis*, given the rapid induction of MT10/20 upon metal exposure (Banni et al., 2011).

Additionally, mussel gene regulation might involve microRNAs: recent work on cadmium exposure in oysters and mussels shows that microRNAs (small regulatory RNAs) change in expression, which can downregulate certain mRNAs involved in cell proliferation or damage control. Such epigenetic regulation is an active research area, but it's clear that contaminants not only directly damage cells but also trigger complex regulatory responses at the gene expression level (Yu et al., 2020).

Metals such as cadmium (Cd), lead (Pb), mercury (Hg), and copper (Cu) induce oxidative protein damage and alterations in protein expression in *M. galloprovincialis*, reflecting a strong cellular stress response (Kournoutou et al., 2020). Upon metal exposure, metallothionein genes are among the first and most highly induced transcripts (sometimes increasing dozens-fold) to bind and detoxify metal ions (Banni et al., 2011).This response is often accompanied by induction of antioxidant enzymes (because metals generate ROS).

For example, co-exposure to cadmium and elevated temperature enhances stress protein expression in *M. galloprovincialis* (Banni et al., 2011), while metal exposure alone also induces Hsp70 and antioxidant genes (Boukadida et al., 2021).

Metals can also cause DNA strand breaks, so genes like p53 and repair enzymes become activated to maintain genome integrity. However, under chronic metal exposure, cellular defences may become overwhelmed, triggering apoptosis, for example, cadmium has been shown to activate caspases in the gill tissue of the freshwater mussel *Anodonta woodiana* (Li et al., 2022). Field studies along polluted coasts have validated that mussels from high-metal sites consistently show higher MT, HSP70, and antioxidant gene expression than reference sites, reflecting an acclimation at the molecular level to contamination (Banni et al., 2011). These gene expression patterns are leveraged as biomarkers for biomonitoring programs (Carella et al., 2018).

Synthetic estrogens can disrupt mussel reproductive gene networks. Evensen et al. (2024) showed that exposure to endocrine disruptors alters the expression of sex-related genes such as *DMRT1L* and *FoxL2* in *Mytilus edulis*, contributing to feminization effects. Evidence for similar effects from plastic additives or organotins is less direct but suggests potential interference with the same pathways (Evensen et al., 2024).

Over longer terms, EDC exposure might select for mussels with particular genotypes in hormone pathways – part of the mussel's open pan-genome could be an advantage here, providing a reservoir of gene variants that confer resilience to hormonal disturbances (Gerdol et al., 2020).

Oil spills, PAHs, and PCBs cause both oxidative stress and genotoxicity. Mussels respond by inducing Phase I/II detoxification genes: multiple cytochrome P450 genes in the genome (like CYP clan 4 and 3) help metabolize hydrophobic toxins, and conjugation enzymes (UDP-glucuronosyltransferases, sulfotransferases) further process them. These genes are part of large families in mussels, some of which may be in the dispensable gene set that expands their metabolic capacity (Gerdol et al., 2020). Additionally, multixenobiotic resistance (MXR) transporters such as P-glycoprotein (ABCB transporter genes) are present and can be upregulated to pump out toxins.

Genomically, mussels have multiple ABC transporter genes, whose expression (e.g. in gills) rises when encountering pollutants, reducing intracellular accumulation (Franzellitti et al., 2010).

Emerging contaminants like micro- and nanoplastics also activate stress responses. Ingested microplastics in mussels have been shown to cause inflammation-like reactions – genes for immune receptors (TLRs, cytokines) and oxidative stress are induced. Nanoplastics can penetrate tissues and lead to cholinesterase inhibition and lipid peroxidation (Acker & Systems, 2021). Thus, we see induction of both neurotoxicity markers (AChE down or compensatory up-regulation) and antioxidant defences (like other stressors).

High-throughput sequencing and omics have greatly advanced our understanding of these responses. RNA-seq studies provide a global view of how thousands of mussel genes change under pollutant stress, often highlighting networks of co-regulated genes (for instance, a cluster of antioxidant, chaperone, and apoptotic genes up together – a signature of oxidative damage). The 2020 genome study also noted that many immune and stress-related genes fall in the dispensable category, meaning different individuals may have unique complements of these genes (Acker & Systems, 2021). This suggests that some mussels may genetically lack certain detox genes while others have extra copies, potentially affecting their response to contaminants. Continuous pangenome analysis and resequencing of populations are uncovering how gene presence/absence might correlate with pollution tolerance (Gerdol et al., 2020).

To explore *M. galloprovincialis* genes in detail, several databases are available. The NCBI Gene and Protein databases list thousands of mussel gene sequences (e.g. sequences for catalase, metallothionein, AChE, p53, etc.), often with annotations transferred from model organisms (Agarwala et al., 2018). The UniProt database provides reviewed/unreviewed entries for mussel proteins with functional descriptions, for example, mussel metallothionein entries note their role in metal sequestration and have cross-references to gene ontology terms (Bateman et al., 2023). Specialized databases like MolluscanDB may host genome browsers for *M. galloprovincialis*, allowing one to find promoter regions and regulatory elements of interest (Liu et al., 2021).

Moreover, the raw and assembled genomic data from the 2020 genome project can be accessed via the European Nucleotide Archive (ENA) or NCBI under the project accession (as mentioned earlier), and the Genome Biology paper by Gerdol et al. (2020) provides supplemental datasets (Gerdol et al., 2020).

For gene expression, the NCBI Gene Expression Omnibus (GEO) contains mussel transcriptomic studies. For instance, a seasonal gene expression profiling dataset (GEO Series GSE23052) captures how mussel gene expression varies in nature (Banni et al., 2011). Other studies deposit RNA-seq reads in the Sequence Read Archive (SRA), which can be re-analyzed to find expression of genes under various contaminant exposures (Gerdol et al., 2014). In addition, KEGG and InterPro can be consulted for pathway mapping and protein domains of mussel genes (many *M. galloprovincialis* genes have been assigned KO entries via homology, placing them in pathways like "Glutathione metabolism" or "Apoptosis") (Blum et al., 2021; Kanehisa et al., 2023).

Ongoing research is identifying novel gene families in mussels that relate to stress adaptation. For example, new antimicrobial and stress-responsive peptides (e.g. mytimycins, mytticusins) have been discovered using the genome, often showing high variability among individuals (Rey-campos et al., 2021). These findings underscore the genomic plasticity of *M. galloprovincialis* – a likely reason why this species thrives in polluted waters. Scientists are particularly interested in regulatory elements controlling these genes; for example, the promoter analysis of immune genes revealed specific motifs that might be binding sites for stress-activated transcription factors (Rey-campos et al., 2021). As whole-genome methylation and chromatin studies begin on mussels, we will learn how gene regulation is epigenetically tuned in response to long-term pollutant exposure (e.g. metal-induced DNA methylation changes in MT promoters).

1.8 Thesis research questions and objectives

Human activities have introduced a wide range of chemical contaminants into the environment, many of which persist and accumulate in ecosystems, posing risks to ecological integrity and human health. Among these, PFAS, TBBPA, and EE2 have drawn significant attention due to their widespread use, environmental persistence, bioaccumulative potential, and demonstrated toxicity. These compounds, used respectively in industrial applications, flame retardants, and hormonal contraceptives, often find their way into aquatic environments, where they can interact with and disrupt biological systems.

Marine bivalves, such as the mussel *M. galloprovincialis*, are commonly employed as sentinel species in ecotoxicological studies. As filter feeders, they are exposed to high contaminants in their environments, making them ideal models for assessing the effects of chemical exposure. Furthermore, their ecological importance and wide distribution enhance their relevance for monitoring environmental health.

The primary goal of this thesis is to investigate the ecotoxicological effects of PFOA, TBBPA, and EE2 on *M. galloprovincialis*, employing both biomarker-based approaches and transcriptomic analyses. By integrating these methodologies, this study seeks to unravel the molecular and biochemical mechanisms underlying the toxicity of these contaminants, offering insights into their potential impacts on marine ecosystems.

This thesis seeks to address two critical research questions:

1. Can reliable biochemical biomarkers be used to identify the potential toxicity of PFOA, TBBPA, and EE2 in bivalves?
2. What are the toxicological effects of these compounds at the transcriptomic level, and how do they influence key metabolic and physiological pathways in *M. galloprovincialis*?

To answer these questions, the study is guided by the following objectives:

1. To evaluate the toxic effects of TBBPA, PFOA, and EE2 on the biochemical processes of *M. galloprovincialis*, focusing on biomarkers such as antioxidant responses, lipid peroxidation, enzymatic activity, and stress proteins.
2. To investigate the transcriptomic alterations induced by these contaminants, identifying disrupted gene expression pathways and elucidating their potential ecological and physiological consequences.
3. To integrate findings from biochemical and transcriptomic analyses, providing a comprehensive understanding of the mechanistic effects of TBBPA, PFOA, and EE2.

These objectives were accomplished through experimental work, resulting in the publication of two manuscripts in peer-reviewed international journals, the submission of one additional manuscript, and the preparation of another currently under elaboration.

The thesis is organized into four chapters, each aiming to assess the research objectives comprehensively. Chapter 1 provides the study's context, scope, and rationale, outlining the research questions and objectives. It introduces the contaminants of interest, the target organism, and the methodological approaches adopted.

Using a multi-biomarker approach, Chapter 2 investigates the biochemical and ecotoxicological impacts of three contaminants TBBPA, PFOA, and EE2 on the mussel *Mytilus galloprovincialis*. The study evaluates antioxidant defences, enzyme activity, oxidative stress markers, lipid peroxidation, and metabolic alterations induced by TBBPA and PFOA exposure. Additionally, it examines the endocrine-disrupting potential of EE2 by assessing estrogenic activity and stress-related biochemical responses. Collectively, these findings provide insight into the molecular and physiological disturbances caused by these contaminants, highlighting their potential risks to marine organisms.

Chapter 3 presents the transcriptomic analyses, highlighting gene expression changes induced by PFOA, TBBPA, and EE2 in *M. galloprovincialis*. The study identifies key pathways affected, such as stress response, detoxification mechanisms, and energy metabolism.

Finally, Chapter 4 integrates the findings from biomarker and transcriptomic analyses, providing a holistic interpretation of the results. The discussion emphasizes the broader implications for environmental monitoring, risk assessment, and future research directions.

This research contributes to the growing knowledge of persistent organic pollutants' environmental and ecological risks. Combining biomarker and transcriptomic approaches offers a detailed mechanistic understanding of how these contaminants affect marine bivalves. The findings are expected to inform environmental policies, guide pollution mitigation strategies, and enhance our ability to predict the ecological impacts of chemical pollutants in marine ecosystems.

The diagram (**Figure 1-8**) illustrates the structure of this thesis, linking the research questions to the objectives, chapters, and related publications. The work is organized into an introduction, two experimental chapters on biomarker and transcriptomic effects, a general discussion, and the final conclusions and perspectives.

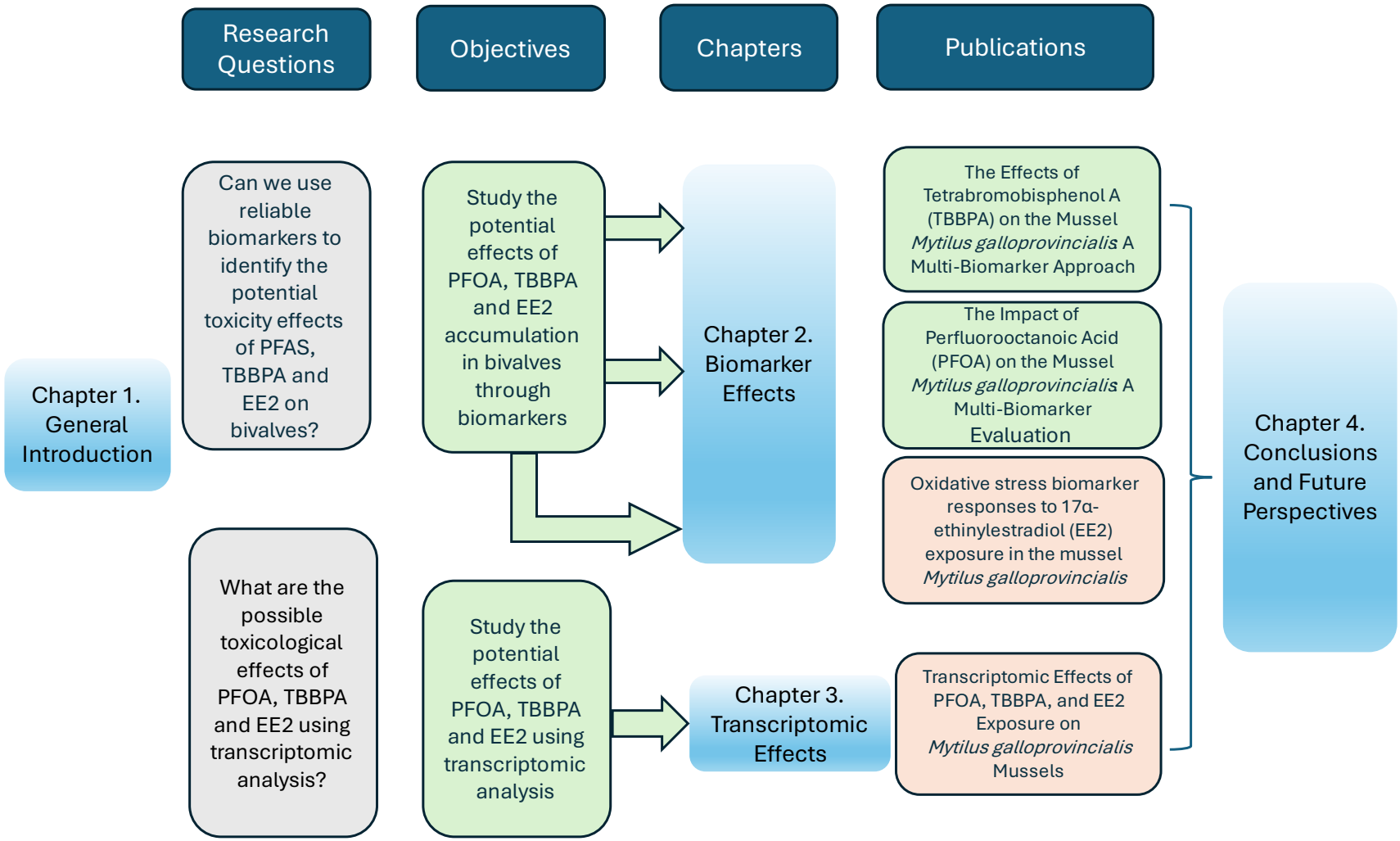

Figure 1-8 - Diagram of thesis structure

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2

Chapter 2 - Biomarker Analysis

2.1 The effects of Tetrabromobisphenol A (TBBPA) on the mussel *Mytilus galloprovincialis*: a multi-biomarker approach

Manuscript 1.

Copeto, S., Ganço, S., Ferreira, I. J., Silva, M., Motta, C., & Diniz, M. (2024). The Effects of Tetrabromobisphenol A (TBBPA) on the Mussel *Mytilus galloprovincialis*: A Multi-Biomarker Approach. Oceans, 5(2), 181–195. https://doi.org/10.3390/oceans5020011

Abstract

Tetrabromobisphenol A (TBBPA) is a fire-retardant containing bromine, produced in large quantities worldwide and extensively used in several industrial products. This compound was identified as a potential contaminant of the environment, causing toxicity to organisms. However, its toxicity remains poorly understood in marine bivalves. The first objective of this work was to evaluate the impact of TBBPA on mussels (*Mytilus galloprovincialis*) exposed for 28 days to various concentrations of TBBPA (0, 1, 10, and 100 $\mu g \cdot L^{-1}$), by assessing stress biomarkers' responses (Glutathione S-transferase, superoxide dismutase, catalase, lipid peroxidation, total antioxidant capacity, total ubiquitin, caspase-3 and acetylcholinesterase). The results showed that lower concentrations (1 and 10 $\mu g \cdot L^{-1}$) were efficiently detoxified, as suggested by GST activities, which were supported by the responses of the other biomarkers. The most pronounced effects were observed in animals exposed to the highest concentration of TBBPA (100 $\mu g \cdot L^{-1}$), suggesting oxidative stress. Additionally, significant strong correlations were found between total antioxidant capacity and some biomarkers (superoxide dismutase and lipid peroxidation), showing that processes involved in oxidative stress fighting are working to avoid cell injury. In brief, mussels' defence mechanisms were capable of dealing with exposure to the lower concentrations tested. Despite this, the risk of consuming shellfish or other fishery products contaminated with TBBPA should be a cause for concern.

Keywords: tetrabromobisphenol A; *Mytilus galloprovincialis*; biomarkers; oxidative stress

2.1.1 Introduction

Discharging various chemical substances into the environment has increased the number of emerging contaminants (e.g., endocrine disruptors, pharmaceuticals, brominated and perfluorinated compounds, and personal care products), which has raised significant concern worldwide (Fabbri et al., 2014; Gerecke et al., 2008). Flame retardants (FRs) constitute a group of synthetic compounds commonly incorporated into a wide range of industrial and household items, including plastics, polymers, textiles, and electronic equipment, to reinforce their resistance to combustion. They can be categorized into organic (halogenated), inorganic, organophosphorus, and nitrogen-based compounds (Poma et al., 2018). Brominated FRs (BFRs) are the most commercialized due to their low cost and high efficiency, comprising mainly hexabromocyclododecane (HBCDs), polybrominated diphenyl ethers (PBDEs), brominated phenols (BrPhs) and tetrabromobisphenol A (TBBPA) (Poma et al., 2018; Wang et al., 2023). TBBPA emerges as one of the most widely used FRs globally, exhibiting substantial production volumes and widespread use (Liu et al., 2019; Sun et al., 2008). Thus, water samples have shown the levels of TBBPA, from various regions of the world, ranging from non-detected (n.d.) to 4.87 $\mu g \cdot L^{-1}$ of magnitude (Blanco et al., 2005; Yang et al., 2012) and in sediments between n.d. and 500 of $\mu g \cdot kg^{-1}$ (Saint-Louis & Pelletier, 2004; Yang et al., 2012).

Despite the absence of current limitations on the manufacture and use of TBBPA, its inclusion by the Convention for the Protection of the Marine Environment of the Northeast Atlantic (OSPAR) on the substances requiring priority action (OSPAR, 2007) implies urgent assessment and intervention. Thus, releasing these compounds into aquatic ecosystems represents an increased risk to aquatic biota due to their persistent nature, promoting an accumulation in sediments and marine organisms (Álvarez-muñoz et al., 2015; Kotthoff et al., 2017). Furthermore, TBBPA's high lipophilicity and heat stability lead to bioconcentration and biomagnification across trophic levels (Sun et al., 2024; Wang et al., 2023). Moreover, the consumption of contaminated seafood could endanger human health. However, studies to detect their presence in food are still insufficient (Lee et al., 2020), and frequent monitoring should be carried out.

Additionally, identifying TBBPA as a potential endocrine disruptor (Wang et al., 2021), and its associated neurotoxic, immunotoxic, nephrotoxic, and hepatotoxic effects, highlights the urgency of understanding its toxicity (Fukuda et al., 2004; Mariussen & Fonnum, 2003; Pullen et al., 2003). Furthermore, it is recognized by the EU as carcinogenic (https://echa.europa.eu/, accessed on 6 February 2024). Although the debate surrounding the toxicity of TBBPA persists (He et al., 2015), evidence indicates its harmful impact on several aquatic organisms, including algae, bivalves, crustaceans, and fish (Pittinger & Pecquet, 2018), implicating fisheries as potential sources of human exposure. Consuming food contaminated with TBBPA can lead to diverse health problems, including its capacity to disrupt the endocrine system (Gerecke et al., 2008; Wang et al., 2023).

Regarding LC50 values found for TBBPA, Jiang et al. (Jiang et al., 2019) found 7.4 $mg \cdot L^{-1}$ (96h-LC50) in juvenile clams (*Ruditapes philippinarum*). However, we found no LC50 information for mussels. In a review by Yang et al. (S. W. Yang et al., 2012), it was reported that the 96 h LC50 for freshwater fishes (*Pimephales promelas and Danio rerio*) ranged from 0.006 to 3.0 $mg \cdot L^{-1}$. But we should be cautious about some of these results since, in some cases, the experiment conditions were not the same.

Mussels, such as *M. galloprovincialis*, are widely distributed in Europe from the Mediterranean to the U.K. and Norway coasts (Boukadida et al., 2021). Thus, mussels are often utilized in toxicity studies as biological models due to their filtration capabilities, wide distribution, and capability to bioaccumulate contaminants (Beyer et al., 2017), enabling the understanding of ecological disturbances induced by exposure to contaminants and functioning as indicators of aquatic pollution in environmental monitoring programs (Farrington et al., 2016).

Biomarkers of response, including indicators of oxidative stress and markers of cellular damage, are essential in strategies for monitoring and evaluating the effects of contaminants on living organisms. Biomarkers can, therefore, be utilized as early warning indicators to assess the condition of the environment (Cajaraville et al., 2000; Matozzo et al., 2008). Multi-biomarker methodologies have been recommended to assess the impacts of toxic substances and understand the impact on ecosystems (van der Oost et al., 2003). Exposure to various types of pollutants may result in an excess of reactive oxygen species (ROS) that can induce oxidative stress

and cell damage in cells, as the organism is unable to remove the excess of ROS produced (Afsa et al., 2022; Almroth et al., 2005; Borković et al., 2005; Rodrigues et al., 2024). To evaluate the consequences of exposure to contaminants, antioxidant enzymes can be helpful (Rodrigues et al., 2024) in addition to other biomarkers that suggest damage at a cellular level (e.g., lipid peroxidation, caspase, and ubiquitin) (Chora et al., 2008; Yoshida et al., 2013). However, there are few studies on antioxidant enzymes and other biomarkers of response (e.g., caspase, ubiquitin) in mussels exposed to TBBPA. Thus, multi-biomarker studies are essential for comprehending the effects of exposure to these compounds in marine biota.

The purpose of this work was to study the effects of TBBPA on the mussel (*Mytilus galloprovincialis*), a common marine model, by exposing the animal for 28 days to three different concentrations of TBBPA (1, 10, and 100 $\mu g \cdot L^{-1}$) and then analyzing oxidative stress biomarkers, such as GST (glutathione S-transferase), SOD (superoxide dismutase), CAT (catalase), LPO (lipid peroxidation), and TAC (total antioxidant capacity). Protein degradation signaling was examined by assessing total ubiquitin, cellular apoptosis was evaluated by measuring caspase (CASP-3) levels, and neurotoxicity was assessed by analyzing acetylcholinesterase (AChE) activity. In addition, an integrated biomarker analysis was also performed to provide further information on the effects on mussels, allowing a better understanding of the risk of exposure to the marine ecosystem.

2.1.2 Materials and Methods

2.1.2.1 TBBPA Stock Solution

To prepare the TBBPA (CAS No. 79-94-7; ≈99.3%; ref. 330396, Sigma-Aldrich, Shanghai, China), a stock solution was prepared, where 0.1 g of the compound was dissolved in 100 mL of methanol (≈99.8% (v/v); Honeywell, Seelze, Germany). An appropriate volume was then added to each aquarium to attain the concentrations to be tested (1, 10 and 100 $\mu g \cdot L^{-1}$). An additional aquarium with filtered seawater was used as a control. Considering the dilution factor, the methanol concentration in each aquarium was estimated to be ≤0.01%.

2.1.2.2 Experimental Trials

M. galloprovincialis (Lamarck, 1819) were manually gathered at Guincho coast (Cascais, Lisbon, Portugal) in January/February 2023. Only mussels of comparable lengths were collected to prevent variations in bioaccumulation and biomarker responses due to size or age. The animals (n = 40; 1.270 g ± 0.365) were transported in a thermal box to the laboratory facilities at NOVA School of Science and Technology. They were acclimatized in an aquarium with 50 L of seawater (from the same collection site "Guincho"), with filtration, water recirculation, and aeration (>6 $mg{\cdot}L^{-1}$ dissolved O_2). They were maintained at a pH of 8.1 ± 0.2; a temperature of 20.0 ± 1.0 ◦C; a salinity of 33 ± 1 $g{\cdot}L^{-1}$; a photoperiod of 12 h of light and 12 h of darkness; and continuously aerated. Water quality parameters were checked daily in each aquarium (temperature, pH, and salinity). The animals were randomly distributed by four aquariums containing 8 animals each. Then, for 28 days, mussels were tested at different concentrations of TBBPA (0, 1, 10, and 100 $\mu g{\cdot}L^{-1}$) diluted in seawater from the collection site. TBBPA concentrations selected for the exposure tests were based on values determined in seawater elsewhere (Blanco et al., 2005; Yang et al., 2012). However, higher concentrations were also assayed to better comprehend the effects on animals. In addition, a control aquarium was also used with ≤0.01% methanol in seawater. The animals were fed three times per week with *Chlorella sp*. (Shine superfood, Setúbal, Portugal).

Ethics

This work has been approved by the competent national authorities and has complied with all applicable laws regarding animal welfare. The researchers hold a level C certification from the European Federation for Laboratory Animal Science (FELASA) for carrying out experiments on animals. The published results are consistent with the ARRIVE recommendations, covering the 3 Rs of animal welfare.

2.1.2.3 Sample Treatment

Following the exposure period, the organisms were gathered, weighted, and the whole organism was removed.

Then, a tissue homogenizer (Tissue Master 125, Kennesaw, GA, USA) was used to homogenize the samples in a solution of 3.0 mL phosphate-buffered saline solution (PBS; comprising NaCl (140 mM; Panreac, Barcelona, Spain), Na_2HPO_4 (10 mM; Sigma-Aldrich, St. Louis, MO, USA), KCl (3 mM; Merck, Darmstatd, Germany), and KH_2PO_4 (2 mM; Sigma-Aldrich, Steinheim, Germany) at pH 7.3 ± 0.2. Subsequently, the samples were centrifuged for 10 min at 4 ◦C at 15,000× g using a centrifuge (VWR, model CT 15RE, Tokyo, Japan). The supernatant was transferred to microtubes (1.5 mL) and kept at −45 ◦C until biomarkers were analyzed.

Total Protein

Bradford method was used to determine the total cytosolic protein content (Bradford, 1976). Standards were prepared with Bovine Serum Albumin (BSA; Nzytech, Lisboa, Portugal) to construct a calibration curve, ranging from 0 to 4 $mg \cdot mL^{-1}$. Then, in a 96-well microplate (Greiner Bio-One, GmbH, Kremsmünster, Austria), 20 µL of each standard or sample and 180 µL of Bradford Reagent were pipetted into each well. A microplate reader (Synergy HTX, Multi-Mode Reader, BioTek, Winooski, VT, USA) was used to read the absorbance in each well at 595 nm. Total protein concentration in samples were obtained from the calibration curve, and the results were expressed as $mg \cdot mL^{-1}$. The cytosolic protein concentration (PROT) was determined for normalization purposes.

2.1.2.4 Biomarkers Analyses

2.1.2.4.1 Glutathione S-Transferase (GST) Activity

A protocol that was first published by Habig et al. (1974) but adapted and optimized for 96-well microplates was used. To determine the specific activity of GST, a molar extinction coefficient of 5.3 $mM^{-1} \cdot cm^{-1}$ for CDNB was utilized. Thus, 20 µL of the sample and 180 µL of the substrate solution (19.6 mL of PBS buffer, 200 µL of reduced L-Glutathione (200 mM; GSH; Sigma-Aldrich, St. Louis, MO, USA), and 200 µL at 100 mM of 1-chloro- 2,4-dinitrobenzene (CDNB; Sigma-Aldrich, St. Louis, MO, USA)) were pipetted into each well of the microplate. A microplate reader (Synergy HTX, BioTek) was used to read the absorbance at 340 nm, every minute, for six minutes. GST activity results were expressed relative to cytosolic protein content.

2.1.2.4.2 Superoxide Dismutase (SOD) Activity

The NBT method described by Sun et al. (1988) was performed to measure SOD activity, with adaptations for a 96-well microplate. Quickly, 200 µL of potassium phosphate buffer (50 mM; pH 8.0) was pipetted into each well, followed by 10 µL of EDTA (3 mM; Riedel- Haën, Seelze, Germany), 10 µL of xanthine (3 mM; Sigma-Aldrich, China), 10 µL of sodium chloride nitroblue tetrazolium (0.75 mM; NBT; Sigma-Aldrich, Germany), and 10 µL of sample. Next, 10 µL of xanthine oxidase (XOD, Sigma-Aldrich, Germany) was added to begin the reaction. The absorbance was monitored at 560 nm, every two minutes, for 20 min in a microplate reader (Synergy HTX, BioTek). Results were expressed in relation to cytosolic protein content.

2.1.2.4.3 Catalase (CAT) Activity

The method described by Johansson & Borg (1988) was used to determine CAT activity, after being adapted for use in 96-well microplates. Thus, 20 µL of sample, 30 µL of methanol (Honeywell), and 100 µL of potassium phosphate buffer (100 mM; pH 7.0; Sigma) were pipetted to each microplate well. Next, 20 µL of hydrogen peroxide (0.035 M; Sigma- Aldrich, Germany) was pipetted to each well to initiate the reaction. The microplate was then continuously shaken in an orbital shaker (Optic Ivymen System, JP Selecta, Barcelona Spain) for 20 min while incubating in the dark at room temperature. After adding 30 µL of KOH (10 M; ChemLab, Zedelgem, Belgium) and 30 µL of 4-amino-3-hydrazino-5-mercapto- 1,2,4-triazole (32.4 mM in HCl 0.5 M; purpald; Aldrich, Germany), the microplate was incubated again under the same conditions as mentioned previously but this time for ten minutes. Finally, 10 µL of potassium periodate (65.2 mM in KOH 0.5 M; Sigma-Aldrich) was pipetted into each well and incubated for 5 min in the dark at room temperature. Then, the microplate was measured with a Synergy HTX microplate reader (BioTek) at 540 nm. A calibration curve was built using formaldehyde (AppliChem, Darmstadt, Germany) as standards, ranging from 0 to 150 µM. The CAT results were then presented in relation to the cytosolic protein content.

2.1.2.4.4 Acetylcholinesterase (AChE) Activity

AChE activity was quantified by following the Ellman et al, (1961) method, adapted to 96-well microplates. Hence, 50 µL of sample and 250 µL of a mixture containing sodium phosphate buffer (50 mM; pH 8.0; Sigma-Aldrich), 5,5′-dithio-bis-2-nitrobenzoic acid (10 mM; DTNB; Sigma-Aldrich, St. Louis, MO, USA), and acetylcholine (75 mM; ACTI; Sigma, UK) were pipetted into the microplate wells. Next, the microplate wells were measured at 415 nm, each minute for ten minutes, with a microplate reader (Synergy HTX, BioTek) and the results were plotted as a function of the amount of cytosolic protein in each sample.

2.1.2.4.5 Lipid Peroxidation (LPO)

LPO assessment followed the thiobarbituric acid assay, as described by Madeira et al. (2018). A calibration curve was plotted for concentrations between 0 and 0.1 µM of Malondialdehyde (MDA, Merck, Germany) in Mili-Q ultrapure water. A volume of 5 µL of sample or standard, 45 µL of PBS buffer, 12.5 µL Sodium Dodecyl Sulfate (8.1% (w/v); SDS; Sigma-Aldrich, Germany), 93.5 µL Trichloroacetic Acid (20% (w/v); TCA; Panreac, Barcelona, Spain), 93.5 µL thiobarbituric acid (1% (w/v); TBA; Sigma-Aldrich, Germany), and 50.5 µL of ultrapure water were pipetted into 1.5 mL microtubes. This mixture was subjected to a brief centrifugation (3000× g rpm) for 30 s, followed by placing the microtubes, with punctured caps, in a dry bath (Thermobloc Digital, Labnet, Dusseldorf, Germany) for ten minutes at 100 ◦C. Next, samples were chilled on ice, 62.5 µL of ultrapure water was added to each microtube and subsequently centrifugated (3000× g rpm) for 30 s. Then, 150 µL of each sample was added to the wells of the microplate, and the absorbance was measured at 530 nm in a Synergy HTX microplate reader (BioTek). The results were then presented as a function of total cytosolic protein content.

2.1.2.4.6 Total Antioxidant Capacity (TAC)

Total antioxidant capacity assay was performed as described by Kambayashi et al. (2009). Standards of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Aldrich, Russian Federation) were diluted in potassium phosphate buffer (potassium phosphate monobasic (5mM; pH 7.4; Sigma), glucose (5.55 mM) and sodium chloride (154 mM; NaCl; Panreac)), with concentrations between 0 and 0.330 mM to construct a calibration curve. Afterwards, 10 µL of sample or standard, 10 µL of myoglobin (90 µM; Sigma, USA), and 150 µL of 2,2-azinobis 3- ethylbenzothiazoline-6-sulphonic acid (600 µM; ABTS; Alfa Aesar, Karlsruhe, Germany) were pipetted into the microplate wells (Greiner Bio-one, GmbH, Kremsmünster, Austria). To start the reaction, 40 µL of hydrogen peroxide (500 µM; Sigma- Aldrich) was pipetted into each well. The microplate was incubated for 5 min at room temperature and the absorbance was read on a microplate reader Synergy HTX (BioTek). Results were normalized to the total cytosolic protein content.

2.1.2.4.7 Caspase-3 (CASP-3)

Caspase-3 was determined by Enzyme-Linked Immunosorbent Assay (ELISA) (Lopes et al., 2018). Caspase-3 standards were prepared in PBS buffer (human caspase 3, recombinant human active cc119; Merck, Rahway, NJ, USA) with concentrations between 0 and 5 $\mu g \cdot mL^{-1}$ to obtain a calibration curve. A total of 50 µL of sample or standard was pipetted into each microplate well (Greiner Bio-one, Microlon 600 High Binding, Frickenhausen, Germany Germany), and the microplate was incubated at 4 ◦C for 24 h. Afterwards, a solution containing PBS-Tween solution (0.05% v/v; Panreac; Spain) was used to wash the microplate wells. Next, the microplate wells were blocked by pipetting 100 µL of BSA blocking solution (1% (w/v) in PBS; Nzytech, Lisboa, Portugal) and incubated for 90 min at ambient temperature. The microplate was rewashed three times with the same washing solution. Right away, a primary antibody (anti-caspase 3 antibody ab13847, Abcam, Amsterdam, the Netherlands) was prepared in 1% BSA (w/v) to obtain a final concentration of 1.5 $\mu g \cdot mL^{-1}$ and 50 µL was pipetted into the microplate wells. Then, the microplate was incubated at 4 ◦C for 24 h. Next, the microplate was rewashed three times and 50 µL of secondary antibody (anti-mouse IgG Fc specific-alkaline phosphatase), diluted in PBS (1% BSA w/v) to a concentration of 1.0 $\mu g \cdot mL^{-1}$, was pipetted into

the microplate wells. Then, the microplate was incubated for 90 min at 37 ◦C. The microplate was washed again and 50 µL of substrate solution (157 mg of trizma hydrochloride (Tris-HCl; Sigma, USA), 58 mg NaCl (Panreac), 50 µL $MgCl_2$ (5 mM; Fluka, BioChemika, Buchs, Switzerland), and 10 mg of 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP; Sigma-Aldrich, Gillingham, UK) prepared in 10 mL of distillate water, pH 9) was added to microplate wells. After the new incubation period (15 min) at ambient temperature, 50 µL of STOP solution (3MNaOH (Panreac, Spain)) was pipetted into the microplate wells and the absorbance was read in a microplate reader Synergy HTX (BioTek) at 405 nm. The results were expressed as a function of the total cytosolic protein content.

2.1.2.4.8 Total Ubiquitin (UBI)

To determine total ubiquitin, an ELISA assay was performed, following the same procedure as described before (Lopes et al., 2018). The ubiquitin standards (UBPBio; Dallas, TX, USA) were prepared in a range of concentrations between 0 and 0.8 $\mu g \cdot mL^{-1}$ to build a calibration curve. A primary ubiquitin antibody (P4D1; Sc-8017; Santa Cruz Biotechnology, Dallas, TX, USA) was diluted to 1.5 $\mu g \cdot mL^{-1}$ in 1% BSA (w/v) and pipetted into each microplate well. Next, the same steps were followed as described above for the caspase-3 assay. Results were expressed as a function of the total cytosolic protein content.

2.1.2.5 Statistical Analysis

Statistics were carried out with the software Prism 9 (GraphPad software; version 9.5.1). Depending on whether parametric assumptions were satisfied, statistical comparisons were carried out using either the Kruskal–Wallis or the one-way ANOVA test, which were both found using Dunnett's multiple comparison test. Whenever the parametric assumptions were fulfilled, then the one-way ANOVA test was carried out, whereas if these assumptions were not met, then the Kruskal–Wallis test was performed.

The non-parametric Spearman correlation was employed to measure the strength and direction of linear relationships between pairs of variables.

For IBR, to normalize the different biomarkers in the various concentrations, the difference between the mean of each group and the sample was calculated and divided for the standard deviation of each treatment as described in Madeira et al. (Madeira et al., 2018). Then, an integrated biomarker response (IBR) index was computed, and corresponding radar plots were created to compare the different concentrations. IBR was computed using Excel and the procedure was outlined by Beliaeff & Burgeot (2002).

2.1.3 Results

2.1.3.1 Mortality Rate

No deaths were recorded during the exposure assays.

2.1.3.2 Biomarkers of Oxidative Stress

2.1.3.2.1 Glutathione-S-Transferase (GST)

GST activity results (mean ± standard deviation) are depicted in Figure **2-1A**. Statistical analysis showed a significant increase in enzyme activity when comparing the control group with animals exposed to 100 $\mu g \cdot L^{-1}$ of TBBPA ($p < 0.05$) and between animals exposed to 1 $\mu g \cdot L^{-1}$ and animals exposed to 100 $\mu g \cdot L^{-1}$ of TBBPA ($p < 0.01$).

2.1.3.2.2 Superoxide Dismutase (SOD)

SOD activity, mean ± standard deviation, for each group is shown in Figure **2-1B**. Statistics revealed a significant increase between mussels tested for 1 $\mu g \cdot L^{-1}$ and animals tested for 100 $\mu g \cdot L^{-1}$ of TBBPA ($p < 0.05$).

2.1.3.2.3 Catalase (CAT)

Figure 2-1C shows CAT activity results (mean ± standard deviation) in animals tested to different tested TBBPA concentrations. A significant increase ($p < 0.05$) in this enzyme activity can be seen between the animals tested to the highest concentration of TBBPA (100 $\mu g \cdot L^{-1}$) and the other concentrations tested, as well as the control group.

2.1.3.2.4 Acetylcholinesterase (AChE)

AChE activity results (mean ± standard deviation) are shown in **Figure 2-1D**. Statistical analysis revealed a noteworthy increase in enzyme activity, when comparing each concentration (0, 1, and 10 $\mu g \cdot L^{-1}$) with the highest concentration ($p < 0.05$, $p < 0.0001$ and $p < 0.0001$, respectively). On the contrary, a significant decrease was observed comparing the animals tested to 1 $\mu g \cdot L^{-1}$ and control animals ($p < 0.05$) and between animals tested to 10 $\mu g \cdot L^{-1}$ ($p < 0.01$) and control animals.

2.1.3.2.5 Lipid Peroxidation (LPO)

LPO results (mean ± standard deviation), expressed in terms of MDA concentrations, are presented in **Figure 2-1E**. A notable increase in MDA concentration can be seen between control animals ($p < 0.05$) and mussels exposed to 100 $\mu g \cdot L^{-1}$ and between animals tested to 1 $\mu g \cdot L^{-1}$ and those tested to 100 $\mu g \cdot L^{-1}$ ($p < 0.001$) of TBBPA.

2.1.3.2.6 Total Antioxidant Capacity (TAC)

TAC values (mean ± standard deviation) determined in animals tested to the different TBBPA concentrations are presented in **Figure 2-1F**. Statistical analysis detected a significant increase in total antioxidant capacity between control animals and those tested to 100 $\mu g \cdot L^{-1}$ of TBBPA ($p < 0.05$) and between the animals tested to 1 $\mu g \cdot L^{-1}$ and those tested to 100 $\mu g \cdot L^{-1}$ ($p < 0.001$) of TBBPA.

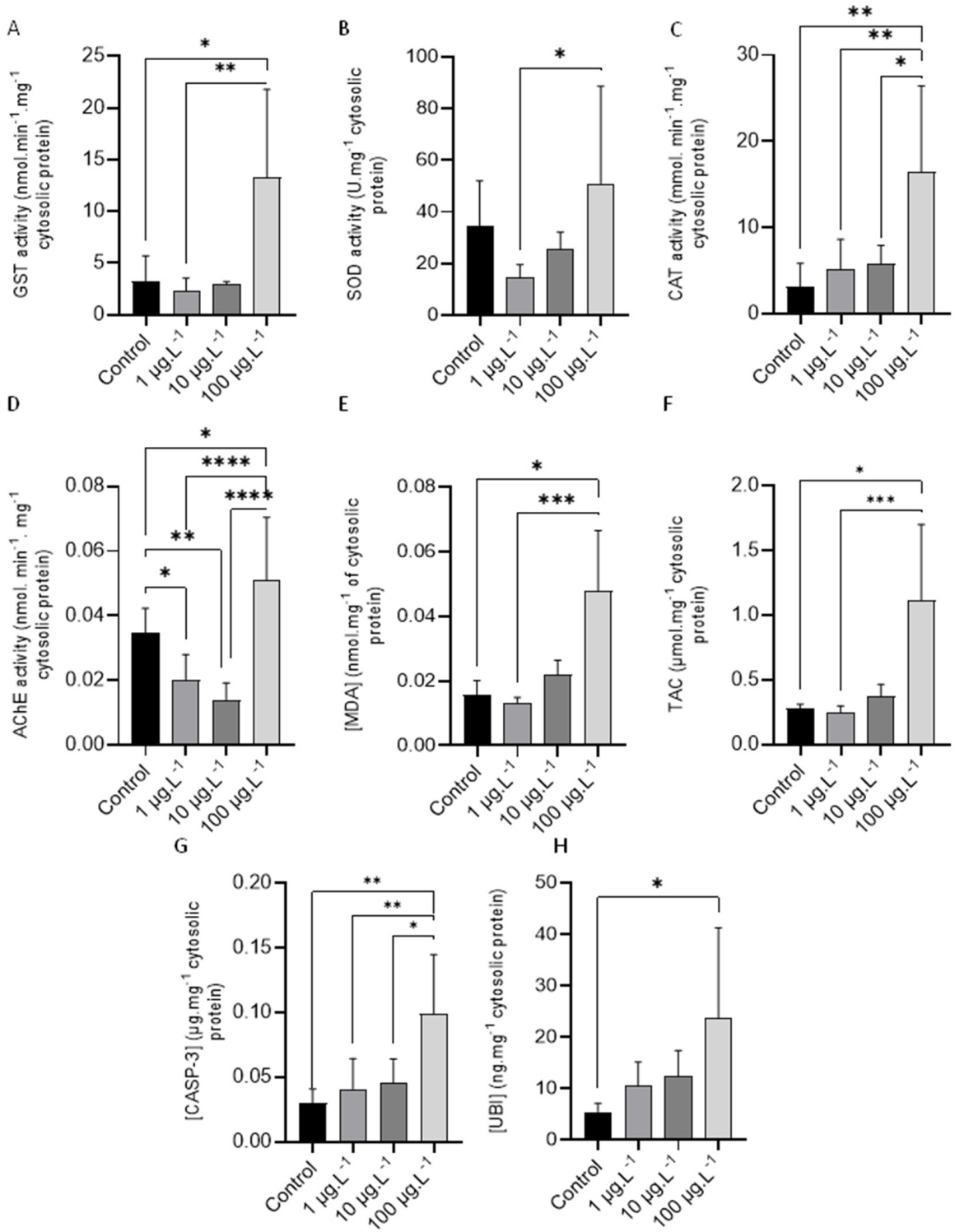

Figure 2-1 - (A) GST activity, (B) SOD activity, (C) CAT activity, (D) AChE activity, (E) MDA concentration, (F) TAC concentration, (G) CASP-3, and (H) UBI concentrations in mussels exposed to various concentrations (0, 1, 10, and 100 µg. L^{-1}) of TBBPA. One-way ANOVA was performed in CAT, SOD, AChE, and CASP-3. Kruskal–Wallis test was

performed in GST, TAC, and UBI. All data are presented as mean ± s.d. *—$p < 0.05$, **—$p < 0.01$, ***—$p < 0.001$ and ****—$p < 0.0001$.

2.1.3.2.7 Caspase-3 (CASP-3)

The levels of CASP-3 (mean ± standard deviation) determined in the animals exposed to TBBPA are shown in **Figure 2-1G**. A significant increase ($p < 0.05$) is evident when comparing the highest concentration (100 μg·L^{-1}) with the other tested concentrations (0 μg·L^{-1}, 1 μg·L^{-1}, and 10μg·L^{-1} of TBBPA).

2.1.3.2.8 Total Ubiquitin (UBI)

The results of the total UBI concentration (mean ± standard deviation) are presented in **Figure 2-1H**, where a significant increase ($p < 0.05$) can be observed between animals tested to 100 μg·L^{-1} of TBBPA and control animals.

2.1.3.3 IBR Index

The radar plot of IBR index is presented in **Figure 2-2**. Differences are only observed when comparing animals tested to the highest concentration (100 μg·L^{-1}) with all other tested animals. Thus, a decrease in MDA concentrations and in AChE activities can be observed, along with a slight decrease in UBI concentrations. GST showed no differences among all tested animals. For SOD, CAT, CASP-3, and TAC, there was a slight increase, as shown by the IBR radar plot.

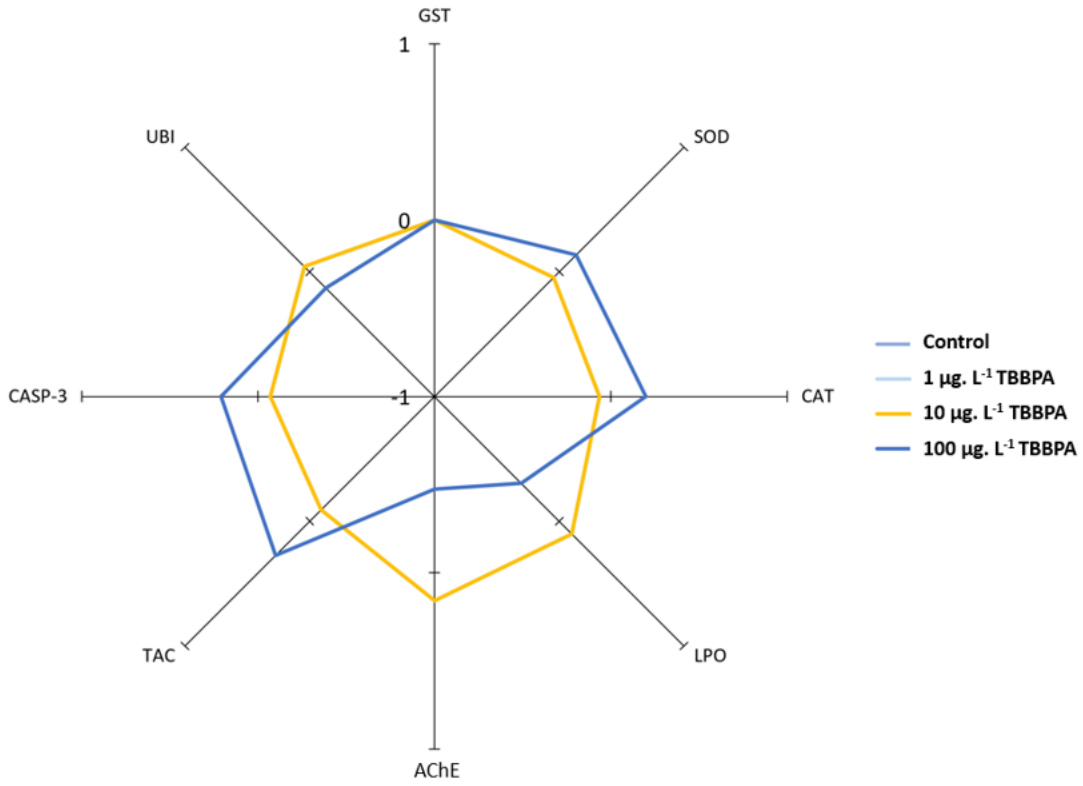

Figure 2-2 - Radar plot of IBR index in mussels exposed to different TBBPA concentrations. Control, 1µg. L^{-1}, and 10 µg. L^{-1} concentrations are overlapped (yellow line) and are, therefore, not distinguishable.

2.1.3.4 Correlation Analyses

The results of correlation analysis (Spearman) are presented in **Table 2.1-1**. GST shows a statistically significant moderate positive correlation with LPO ($p < 0.001$), while SOD exhibits a significant moderate positive correlation with TAC ($p < 0.0001$). Moreover, results indicate a strong significant correlation between LPO and TAC ($p < 0.0001$) and a significant moderate positive correlation with LPO and UBI ($p < 0.001$).

Table 2.1-1 - Spearman correlation matrix, with relevant correlations highlighted in bold. Significant differences are marked with asterisks. *—$p < 0.05$, **—$p < 0.01$, ***—$p < 0.001$ and ****—$p < 0.0001$.

	SOD	CAT	LPO	AChE	TAC	CASP-3	UBI
GST	0.26	0.37	0.71 ***	0.27	0.59 ***	0.52 *	0.10
SOD		0.14	0.60 **	0.33	0.78 ****	0.41	0.44 *
CAT			0.65 **	-0.05	0.55 *	0.40	0.31
LPO				0.48 *	0.87 ****	0.64 **	0.75 ***
AChE					0.50 *	0.20	0.18
TAC						0.69 ****	0.59 **
CASP-3							0.64 **

2.1.4 Discussion

Numerous research studies have focused on the detection of TBBPA and its effects on humans and other animals, including aquatic biota (Canesi et al., 2005; Jarosiewicz et al., 2019; Pittinger & Pecquet, 2018; Wu et al., 2016; Yu et al., 2022). However, to our knowledge, no studies are available on TBBPA effects in marine organisms combining the response of antioxidant enzymes, acetylcholinesterase, caspase-3, and ubiquitin, which are key markers of neurotoxicity (Feng et al., 2023), apoptosis (Crowley & Waterhouse, 2018), and the degradation of damaged proteins via the proteasome (Abbas & Larisch, 2021), respectively.

The evaluation of biomarker responses, such as enzymes involved in the biotransformation process (e.g., CYP1A, GST), antioxidant enzymes, and other specific biomarkers, is essential for assessing xenobiotic toxicity in biota and evaluating ecosystem health (Gil & Pla, 2001; Hook et al., 2014). Therefore, utilizing biomarkers to study the impact of xenobiotics in living organisms is a useful tool, as they provide an early indication on the presence of stressors (Lomartire et al., 2021).

The TBBPA concentrations selected for this study include previously reported environmentally relevant concentrations found in water (up to 4.87 $\mu g \cdot L^{-1}$) (Yang et al., 2012). Still, higher concentrations were also tested to help us assess toxicity in mussels.

GST, an enzyme mainly linked to the detoxification of xenobiotics, increased only in mussels tested to the most elevated concentration (100 $\mu g \cdot L^{-1}$ of TBBPA). This suggests that animals can detoxify TBBA at the lower concentrations tested. In a study by Hu et al. (2015), who exposed scallops (*Chlamys farreri*) for ten days followed by another ten days of depuration, it was demonstrated that TBBPA bioaccumulated in mussels' gills and digestive glands. Furthermore, GST increased in both organs analyzed, suggesting a detoxification response. However, the scallops were exposed to much higher levels than in the current work. Another study by the same author who exposed microalgae to 400 $\mu g \cdot L^{-1}$ of TBBPA, which was then used to feed scallops (Hu et al., 2015), also showed increased GST and SOD and suggested oxidative stress.

Yang et al. (2013) conducted exposure tests on freshwater fish (*Carassius auratus*), finding a significant increase in GST activity following an eight-day exposure assay to 0.5 $mg \cdot L^{-1}$ TBBPA. An alteration in the regulation of GST gene expression was reported by Gong et al. (2012), who observed a rise in GST expression in the gills of *C. farreri* exposed to TBBPA. Furthermore, Hu et al. (2015) exposed *C. farreri* to TBBPA (varying from 0.2 to 0.8 $mg \cdot L^{-1}$) and found that GST activity and gene expression levels, but also UDP-glucuronosyltransferase (UGT), increased accordingly with the time and concentration tested, demonstrating its role on TBBPA detoxification. Thus, various studies performed in different aquatic species seem to indicate a consistent increase in GST activity in response to exposure to TBBPA, suggesting activation of xenobiotic detoxification mechanisms. This is also corroborated by the rapid metabolization of TBBPA into hydrophilic conjugates of sulfate and glucuronide, which are easily eliminated by the organisms (Fini et al., 2012).

However, it should be emphasized that most studies in aquatic animals tested concentrations of TBBPA beyond the most elevated concentration tested in the current work. The activities of SOD and CAT also support the GST results, as only the highest concentration tested (100 $\mu g \cdot L^{-1}$) seems to increase the responses of both antioxidant enzymes determined in exposed mussels, suggesting a response to oxidative stress by eliminating the overproduction of reactive oxygen species (ROS) and neutralizing the excess of H_2O_2, as these enzymes act together to defend cells against oxidative stress.

Indeed, oxidative stress occurs when ROS production exceeds the ability of cells to remove them (Almeida et al., 2005; López-Barea & Pueyo, 1998). Noteworthily, TAC results followed the same pattern of antioxidant enzymes, suggesting that GST and antioxidant mechanisms are sufficient to deal with the exposure to lower TBBPA concentrations.

Antioxidant enzymes (e.g., SOD and CAT) act together to protect cells by converting reactive radicals into non-reactive molecules (Ellman et al., 1961) and, thus, avoid the establishment of stress oxidative damage. Studies by Hu, et al. (2015) also reported a substantial elevation in SOD activity in *C. farreri* tested to higher levels (0.2 to 0.8 $mg{\cdot}L^{-1}$) of TBBPA, suggesting that the response of SOD activity to TBBPA exposure may be concentration-dependent. The differences observed in SOD activity in these studies, and the present study, may be attributed to the different concentrations of TBBPA tested.

Studies by Wu et al. (2016) on zebrafish (*Danio rerio*) embryos and larvae tested to 0.1 to 1.0 $mg{\cdot}L^{-1}$ of TBBPA revealed no significant alterations in oxidative stress enzymes (SOD, CAT, and Glutathione Peroxidase—GPx), while animals tested at concentrations ranging from 0.4 to 1.0 $mg{\cdot}L^{-1}$ showed decreased enzyme activities. Feng et al. (Feng et al., 2023) reported developmental neurotoxicity in juvenile zebrafish exposed to different concentrations, varying from 0.86 to 193.5 $\mu g{\cdot}L^{-1}$ of TBBPA bis(2-hydroxyethyl) ether (TBBPA-DHEE). Interestingly, they found that females were more susceptible than males. Likewise, a proteomic and metabolomic study carried out in the gills of *M. galloprovincialis* exposed to TBBPA (10 $\mu g{\cdot}L^{-1}$), for 30 days, showed different responses between males and females, with females being more susceptible. They found changes in nine metabolites, mostly related to energy metabolism and osmotic regulation in females, while in males, alterations were related only to osmotic regulation.

Proteomic analyses revealed higher levels of proteins linked to energy metabolism and defence mechanisms in males (Ji et al., 2016). Lipid peroxidation (LPO) results from the action of free radicals on cell biological membranes (Oruç & Usta, 2007). Therefore, it can be used as a biomarker of cell damage upon exposure to xenobiotics (Ferreira et al., 2022). Oxidative damage following exposure to contaminants is often reported.

For instance, Emmanouil et al. (2008) showed oxidative damage in *Mytilus edulis* collected in a U.K. contaminated site.

However, the mussels were able to recover after depuration. In this work, LPO exhibited a trend to rise according to tested concentrations. However, this was only statistically significant for mussels tested to 100 $\mu g \cdot L^{-1}$ of TBBPA, which is in accordance with antioxidant enzyme levels and supports the hypothesis that cells' antioxidant mechanisms are enough to fight oxidative stress at the lower TBBPA concentrations tested but can lead to oxidative stress in mussels tested to the most elevated concentration.

Our findings are consistent with previous research conducted on zebrafish embryos, where an increase in LPO was observed, suggesting oxidative stress (J. Hu et al., 2009). AChE has a central role in the proper functioning of neurotransmission and muscle contraction, and is used as a marker of neurotoxicity (Matozzo et al., 2005). In the current work, mussels tested at lower levels of TBBPA (1 and 10 $\mu g \cdot L^{-1}$) displayed a reduction in AChE activity. However, after the animals were tested to 100 $\mu g \cdot L^{-1}$ of TBBPA, there was a significant increase in AChE activity. This behavior is not clear but may suggest that exposure to different levels of TBBPA can have different effects on enzyme activity. The elevation in AChE noticed in animals tested to the highest TBBPA concentration is consistent with findings by Zhu et al. (2018) in zebrafish and with Liu et al. (2016), who reported increased AChE in rat pheochromocytoma cells exposed to TBBPA. The rise in AChE activities was associated with ROS and apoptosis (Melo et al., 2003), which is consistent with our results in mussels exposed to the highest TBBPA concentration.

Interestingly, some studies reported increased AChE activity and lipid peroxidation in fish containing microplastics, which authors attributed to the disruption of vesicles containing acetylcholine (Barboza et al., 2020; De Marco et al., 2023). Caspase-3 holds a critical role in the regulation of apoptosis or programmed cell death, being activated during the apoptotic process, which is essential for removing unwanted or damaged cells from the body (Wang et al., 2005), while ubiquitin plays an important role in marking proteins for degradation (Hochstrasser, 1992).

In addition, the imbalance of the ubiquitin-proteasome system has been associated with various diseases (e.g., tumors, neurodegenerative disorders, and inflammation) (Shang et al., 1997). Research conducted in zebrafish larvae and embryos exposed to TBBPA showed developmental toxicity due to oxidative stress and apoptosis (Wu et al., 2016).

Moreover, Han et al. (2023) reported that TBBPA also caused apoptosis, mitochondrial dysfunction, and increased ROS in carp hepatocytes after exposure. Interestingly, it was also shown that degraded TBBPA (e.g., monoBr-BPA, diBr-BPA, and triBr-BPA) presented stronger toxicity to HeLa cells than parent TBBPA, leading to apoptosis (Suyama et al., 2023).

Our results suggest that exposing mussels to 100 $\mu g \cdot L^{-1}$ of TBBPA raised the production of both caspase-3 and ubiquitin, suggesting that this concentration can lead to apoptosis and increased damaged proteins. No-significant changes were observed for caspase-3 and ubiquitin in mussels exposed to 1 and 10 $\mu g \cdot L^{-1}$, which may suggest that these concentrations are not enough to cause significant apoptosis and protein damaging. Overall, the results suggest that when considering environmentally relevant concentrations, significant oxidative stress it is not expected to occur in mussels. Nonetheless, we must consider TBBPA bioaccumulation in the marine biota. While some authors reported moderate TBBPA bioaccumulation (H. Liu et al., 2018), others stated that this compound is rapidly absorbed and accumulates in several aquatic species (Miao et al., 2023). The potential for trophic transfer and biomagnification should also be emphasized as a risk to the aquatic ecosystem (Sun et al., 2024). Finally, there should be greater concern about the consumption of fishery products, as several studies have unequivocally shown levels of TBBPA that could impact human health health (Cunha et al., 2022; Poma et al., 2018).

2.1.5 Conclusions

The strong positive correlation between TAC and some biomarkers (SOD and LPO) indicates that the organism's global antioxidant capacity is linked to the response of these biomarkers, suggesting that when faced with oxidative stress, antioxidant mechanisms are activated to neutralize oxidative stress and its adverse effects. The biomarker responses of animals tested at 1 and 10 $\mu g \cdot L^{-1}$ are low or moderate and no noteworthy effects were detected at these concentrations. However, animals tested to the most elevated concentration of TBBPA (100 $\mu g \cdot L^{-1}$) showed more pronounced responses that can lead to oxidative stress and concomitant cellular damage. Although the higher concentrations tested are not considered environmentally relevant, they help to understand the effect on biota exposed to TBBPA and the risk if higher quantities of this compound are discharged into the environment.

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2.2 The impact of Perfluorooctanoic acid (PFOA) on the mussel *Mytilus galloprovincialis*: a multi-biomarker evaluation

Manuscript 2.

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Abstract

Perfluorooctanoic acid (PFOA) has been widely studied due to its environmental persistence and bioaccumulation potential, raising concerns about its effects on aquatic life. This research evaluates the impact of PFOA on the antioxidant defences and stress response systems of the mussel *Mytilus galloprovincialis*. Mussels were exposed to three concentrations of PFOA (1, 10, and 100 µg.L^{-1}) over 28 days. Several biomarkers, including glutathione S transferase (GST), superoxide dismutase (SOD), catalase (CAT), lipid peroxidation (LPO), total antioxidant capacity (TAC), vitellogenin (VTG), ubiquitin (UBI), and caspase-3 (CASP) were analyzed. The results suggest stress responses, particularly in animals exposed to higher concentrations, as shown by GST and SOD activities which increased according to PFOA concentrations. Additionally, oxidative stress markers such as MDA and CAT showed variable responses depending on the exposure concentration tested. This study underscores the need for further investigation into the effects of PFOA on mollusks but also the need to unveil gender-specific responses in aquatic organisms exposed to this contaminant. The concentrations of PFOA used in our research are lower than those examined in previous studies, providing crucial insights into the impacts of even minimal exposure levels. It highlights the potential of *M. galloprovincialis* as a bioindicator in environmental monitoring programs, providing crucial insights for environmental management and policy-making regarding regulating and monitoring PFOA in marine settings. Consequently, in a country where seafood consumption is the second largest in Europe, implementing environmental policies and regulatory measures to manage and monitor PFOA levels in marine environments is crucial.

Keywords: Perfluorooctanoic acid (PFOA); *Mytilus galloprovincialis*; Antioxidant defence; Oxidative stress; Endocrine disruptors; Environmental monitoring

2.2.1 Introduction

Perfluorinated organic compounds (PFCs) have emerged as significant environmental contaminants due to their stable carbon-fluorine bonds, which contribute to their persistence and potential for bioaccumulation in the environment, humans, and various organisms, including aquatic animals like fish and bivalves (Kodavanti & Loganathan, 2016; Koponen et al., 2015; Rodil et al., 2019). These compounds are distinguished by replacing all hydrogen atoms on the carbon chain with fluorine, resulting in extreme stability and unique properties such as low surface energy, hydrophobicity, and lipophobicity (Butenhoff et al., 2006; Chen et al., 2015; Prevedouros et al., 2006). Perfluorooctanesulfonic acid (PFOS) and Perfluorooctanoic acid (PFOA) were historically the most used PFCs until 2001, when PFOS production ceased, shifting the prevalence to PFOA (Zhao et al., 2017). Exposure to these compounds, particularly PFOS and PFOA, has been linked to a range of adverse health effects, including hepatic, immunological, reproductive, neurobehavioral, and hormonal disturbances, as well as genotoxic and carcinogenic potential (Squadrone et al., 2015). PFCs are used in a wide range of products and industries, such as textile treatment, metal planting, firefighting and semiconductors (Liu et al., 2021).

There are two ways that PFCs might enter the environment: directly or indirectly. The incorrect disposal or the discharge of waste into the atmosphere from the industrial facilities. PFCs are furthermore released directly into surface water through the effluent of wastewater treatment plants. Indirectly by precursors such as fluorotelomers alcohols that was transformed in perfluoroalkyl carboxylic acids (PFCAs) and perfluoro sulphonic acids (PFSAs) by abiotic or biological mechanisms (Khurana et al., 2022). PFCs are released into the environment, becoming widespread in water (Jin et al., 2009), sediment (Ferrey et al., 2012), dust (Björklund et al., 2009), and wildlife (Loos et al., 2017; Sedlak et al., 2017). Consequently, human exposure occurs through various routes, predominantly diet and inhalation (Ahrens, 2011).

Various studies have reported that the concentrations of PFOA and PFOS in surface water can vary considerably. PFOA levels have been documented between 0.09 and 1270 $ng.L^{-1}$, while PFOS levels have been observed to range from 0.06 to 144 $ng.L^{-1}$ (Ahrens et al., 2009; Hansen et al., 2002; Loos et al., 2008; Quinete et al., 2009; Teng et al., 2009; Wei et al., 2007).

Recognizing dietary intake as a major exposure route, the European Commission proposed directive 2017/0332, setting drinking water limits at 0.4 $\mu g.L^{-1}$ for PFOS and 4 $\mu g.L^{-1}$ for PFOA (Knutsen et al., 2018). Directive 2017/0332, later amended and implemented as Directive 2020/2184, addresses PFAS concentrations in drinking water, focusing only on these substances' total and sum concentrations. It sets a new group limit of 0.5 $\mu g \cdot L^{-1}$ for PFAS, along with a limit of 0.1 $\mu g \cdot L^{-1}$ for 16 specific individual PFAS (European Parliament and the Council of the European Union, 2020). The European Food Safety Authority (EFSA) further highlighted fish, meat, eggs, milk, dairy products, and drinking water as significant sources of exposure (Knutsen et al., 2018). Drinking water, particularly in contaminated areas, remains a significant source of PFAS exposure (Rogers et al., 2021). Tolerable weekly intakes (TWI) of 13 $ng.kg^{-1}$ bw for PFOS and 6 $ng.kg^{-1}$ bw for PFOA have been established (Knutsen et al., 2018).

Despite multiple exposure sources, diet remains the predominant route for most populations (Schrenk et al., 2020). In 2020, EFSA set a new TWI for PFOA, PFOS, perfluorononanoic acid (PFNA), and PFHxS at 4.4 $ng.Kg^{-1}$ bw per week, significantly lower than previous limits (Schrenk et al., 2020). The European Union and the United States Environmental Protection Agency have also explored alternatives to PFOA and its compounds (Schrenk et al., 2020). The presence of high PFOA concentrations near fluorochemical plants in Fuxin, China, as reported by Zhao et al (Zhao et al., 2017), is a significant cause for concern. For PFOA, milk, dairy products, drinking water, and fish are the main sources of chronic exposure. Associations between PFOA levels and kidney and testicular cancers have been observed (Barry et al., 2013; Vieira et al., 2013). In 2018, the International Agency for Research on Cancer classified PFOA as possibly carcinogenic (Group 2B). PFOA and PFOS are not metabolized but accumulate in the liver, kidneys, and blood (Cui et al., 2009; Fletcher et al., 2013). Exposure to PFAS during pregnancy has been linked to adverse health outcomes, including increased risks of preeclampsia, preterm birth, and low birth weight (Knutsen et al., 2018).

PFAS exposure induces oxidative stress in aquatic organisms, affecting antioxidant enzymes activities and detoxification pathways (Amraoui et al., 2018; Arukwe & Mortensen, 2011; Jeong et al., 2016; Liu et al., 2007; Liu et al., 2008; Sant et al., 2018; Yang et al., 2014). Disruption of nuclear receptors, fatty acid oxidation, and mitochondrial permeability contribute to oxidative stress (Lee et al., 2020a).

According to some studies (Tang et al., 2018; Tilton et al., 2008), this exposure to PFOA can have a negative effect on biological pathways related to detoxification, lipid metabolism, and xenobiotic metabolism. In a study performed on water flea (*Daphnia carinata*), the 48-hour lethal con-centration 50 (LC50) values for acute toxicity were 78.2 $mg.L^{-1}$ (with a confidence interval of 54.9-105) for PFOA and 8.8 $mg.L^{-1}$ (with a confidence interval of 6.4-11.6) for PFOS (Logeshwaran et al., 2021). Sentinel organisms, like bivalves, play a crucial role in monitoring environmental pollution and assessing ecosystem health, especially in coastal marine environments (Fabbri et al., 2014). They accumulate pollutants in their tissues by filtering large volumes of water for feeding. These organisms are highly sensitive to pollutants and provide early warning signs of environmental stress (Beyer et al., 2017).

Mussels, in particular, are extensively used as sentinel organisms to assess contamination levels in marine environments, as their efficient filtration abilities allow them to accumulate both organic and inorganic pollutants, making them effective bioindicators in aquatic ecosystems, and also, they have a broad distribution (Bruno et al., 2024). By examining their responses to contaminants, we can infer the potential impacts on the broader ecosystem.

The cellular antioxidant defence system protects cells from oxidative stress by activating specific enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). These enzymes are vital in mitigating oxidative damage and are frequently used as biomarkers to detect oxidative stress caused by exposure to environmental contaminants. Exposure to PFOA notably impacts the activity levels of these key antioxidant enzymes (Liang et al., 2020; Livingstone, 2001; Pytharopoulou et al., 2011; Rt et al., 1989). Specifically, the activities of SOD and CAT are reduced, whereas GPx activity significantly increases in response to PFOA exposure, indicating a shift in the oxidative balance within cells (Fabbri et al., 2014).

In certain studies, the activity levels of SOD, CAT, and GPx showed notable changes, which are crucial markers of oxidative stress. Specifically, CAT and SOD activities decreased, while GPx activity increased significantly in response to PFOA exposure (Fabbri et al., 2014; F. Li et al., 2021). Malondialdehyde (MDA), a byproduct of lipid peroxidation, is often used to measure the level of oxidative damage in cells.

According to some studies, an increase in MDA levels suggest increased oxidative stress in *M. edulis* following exposure to PFOA (Handke et al., 2011; Hao et al., 2019; Lee et al., 2020a; Mnkandla et al., 2019).

Exposure to PFOA notably impacts the activity levels of these key antioxidant enzymes (Liang et al., 2020; Livingstone, 2001; Pytharopoulou et al., 2011; Rt et al., 1989). Specifically, the activities of SOD and CAT are reduced, whereas GPx activity significantly increases in response to PFOA exposure, indicating a shift in the oxidative balance within cells (Fabbri et al., 2014). Previous studies have also shown that exposure to per- and polyfluoroalkyl substances (PFASs) triggers oxidative stress in various aquatic species (Amraoui et al., 2018; Jantzen et al., 2017; Lee et al., 2020a). In the hepatocytes of *Oreochromis niloticus*, the activities of SOD, CAT, and glutathione reductase (GR) increased following exposure to both PFOS and PFOA (C. Liu et al., 2007). Additionally, increased MDA concentration was observed in *Pimephales promelas* after PFOS exposure, demonstrating these substances' oxidative impact (Liu et al., 2008; Yang et al., 2014).

Although PFOA has been globally phased out due to its persistence and toxicity, rising concentrations have been observed in drinking water (with a mean of 3.55 $\mu g.L^{-1}$), seawater (ranging from 8.8 to 16.2 $ng{\cdot}L^{-1}$), and human blood (between 0.3 and 5.4 $\mu g.L^{-1}$). This increase is attributed to the degradation of precursor compounds, terrestrial runoff, and atmospheric transport (Shi et al., 2024; Wee & Aris, 2023; Yang et al., 2023; Zarębska et al., 2024). There have been a number of studies indicating the toxicity of exposure to PFAS in aquatic organisms, with various effects at cellular and molecular level, particularly in fish (Lee et al., 2020a; Ma et al., 2022). However, there are few or no studies on the toxicity of PFOA in other aquatic organisms, particularly in bivalves such as mussels. There is therefore an urgent need to assess the toxicity of PFOA in these organisms, which are also consumed by humans.

The current study aims to elucidate the biochemical mechanisms by which PFOA affects *Mytilus galloprovincialis*. The individuals were subjected to different PFOA concentrations (0, 1, 10, and 100 $\mu g.L^{-1}$) over 28 days. GST, SOD, CAT, LPO, and TAC were used to assess antioxidant responses. Ubiquitin (UBI) and caspase (CASP) were also evaluated, which are involved in damaged protein degradation and apoptosis.

Vitellogenin-like protein (VTG), a biomarker of endocrine disruption, was also measured. It is anticipated that the results of this study can help to understand the environmental impact of PFOA, particularly in marine species where these compounds can accumulate and pose risks to state exposure to various ecosystems.

2.2.2 Materials and Methods

2.2.2.1 PFOA Stock Solution

For the preparation of the stock solution of PFOA (CAS No 335-67-1, Sig-ma-Aldrich, Shanghai, China), 0.1g of the compound was dissolved in 100 mL of methanol (99.8% V/V; Honeywell, Seelze, Germany).

2.2.2.2 Experimental trial

The experimental setup used to expose mussels to PFOA was as described previously (Copeto et al., 2024; Wee & Aris, 2023; Yang et al., 2023; Zarębska et al., 2024). *M. galloprovincialis* (Lamarck, 1819) were gathered manually at "Guincho" coast (Cascais, Lisbon, Portugal) in February 2023. Mussels of similar sizes were collected to avoid variations in bioaccumulation and biomarker responses caused by differences in size or weight. The specimens were transported to the laboratory facilities at NOVA School of Science and Technology in a thermal container, to maintain the temperature constant and avoid additional stress. The mussels were then acclimatized for five days in a 50 L aquarium using seawater from "Guincho", equipped with filtration, recirculation, and aeration systems. They were maintained under controlled conditions with a pH of 8.1 ± 0.2, temperature at 20.0 ± 1.0 °C, and salinity of 33 ± 1 $g.L^{-1}$. The photoperiod was set to 12 hours of light and 12 hours of darkness, ensuring dissolved oxygen levels remained above 6 $mg.L^{-1}$ throughout the acclimatization process.

Mussels (0.735 ± 0.209 g) were randomly distributed among four aquariums, each with 8 individuals. Each tank was properly separated to avoid mixing or any risk of cross-contamination and the exposure assay was carried out under static conditions. Moreover, water quality parameters (temperature, pH, and salinity) were checked daily in each aquarium.

The appropriate volume of PFOA was taken from the stock solution (section 2.1) and added to each 8-litre aquarium to achieve the concentrations required for the exposure assay (1,10 and 100 $\mu g.L^{-1}$), and these conditions were renewed every 48 hours. For controls, an additional aquarium with filtered seawater was used. The methanol concentration in each aquarium was estimated to be < 0.01% according to the dilution factor.

The realistic criterion of 1000 $ng.L^{-1}$ for the studied concentrations is based on documented levels of PFOA ranging from 0.09 to 1270 $ng.L^{-1}$ and PFOS from 0.06 to 144 $ng.L^{-1}$, according to diverse studies(Antonopoulou et al., 2024), however, these concentrations can vary considerably. Recognizing dietary intake as a major exposure route, the European Commission proposed Directive 2017/0332 setting drinking water limits at 0.4 $\mu g.L^{-1}$ for PFOS and 4 $\mu g.L^{-1}$ for PFOA.

Higher concentrations to realistic concentrations were also tested to better understand their effects on the animals. In addition, a control aquarium was also used with ≤ 0.01% methanol in seawater. The animals were fed daily with 1.5 $mg{\cdot}L^{-1}$ of *Chlorella sp*. (Shine superfood, Portugal) previously dissolved in seawater.

Ethics

This work complied with the relevant policies and standards regarding animal experimentation and welfare (e.g. Directive 2010/63/EU) and ARRIVE guidelines. The collection of organisms and experimentation was authorized by the national authorities (DGRM and ICNF) and the faculty's ethics committee. The researchers who carried out the experiments on the mussels are certified at level C by the European Federation for Laboratory Animal Science (FELASA) and have extensive experience in animal experimentation.

2.2.2.3 Sample Treatment

At the end of the experiment, the animals were weighed using an analytical balance (Sartorius, Germany) and measured with a micrometer. Subsequently, the whole body of each mussel (9 males and 23 females) was separated from the shell using a scalpel and tweezers, collected, and stored in microtubes at -45°C.

Gender identification was based on the visual observation of gonads, with males showing a white-cream coloration and females exhibiting an orange-pink coloration. The specimens were then processed using a Tissue Master 125 tissue homogenizer (Kennesaw, GA, USA) in a 3.0 mL phosphate-buffered saline (PBS) solution (Guidelines et al., 2001). This PBS solution consisted of NaCl (140 mM; Panreac, Barcelona, Spain), Na_2HPO_4 (10 mM; Sigma-Aldrich, St. Louis, MO, USA), KCl (3 mM; Merck, Darmstadt, Germany), and KH_2PO_4 (2 mM; Sigma-Aldrich, Steinheim, Germany), adjusted to a pH of 7.3 ± 0.2. Following homogenization, the samples were centrifuged at 15,000× g for 10 minutes at 4°C using a VWR CT 15RE centrifuge (Tokyo, Japan). The resulting supernatant was then transferred to 1.5 mL microtubes (in duplicate) and stored at -45°C until biomarker analysis could be conducted. Samples were thawed as needed for each assay, and all procedures were carried out on ice to maintain sample integrity.

Total Protein

The Bradford method was employed to ascertain the total protein content (Bradford, 1976). Calibration standards were prepared using Bovine Serum Albumin (BSA; Nzytech, Lisboa, Portugal) to establish a calibration curve ranging from 0 to 4 $mg \cdot mL^{-1}$. Subsequently, 20 µL of each standard or sample was combined with 180 µL of Bradford Reagent in a 96-well microplate (Greiner Bio-One, GmbH, Kremsmünster, Austria). The absorbance of each well was measured at 595 nm using a Synergy HTX Multi-Mode Reader microplate reader (BioTek, Winooski, VT, USA). The total protein concentration in the samples was determined from the calibration curve, and the results were expressed in $mg \cdot mL^{-1}$. The cytosolic protein concentration (PROT) was calculated for normalization purposes.

2.2.2.4 Biomarkers Analyses

2.2.2.4.1 Glutathione S-Transferase (GST) Activity

The protocol initially described by Habig et al. (1974) and later optimized by (Kato & Naito, 1999) was adapted and optimized for 96-well microplates (Li et al., 2021). To determine the specific activity of GST, a molar extinction coefficient of 5.3 $mM^{-1} \cdot cm^{-1}$ for 1-chloro-2,4-dinitrobenzene (CDNB) was employed.

In each microplate well, 20 µL of the sample was combined with 180 µL of the substrate solution, which consisted of 19.6 mL of PBS buffer, 200 µL of reduced L-Glutathione (200 mM; GSH; Sigma-Aldrich, St. Louis, MO, USA), and 200 µL of 100 mM CDNB (Sigma-Aldrich, St. Louis, MO, USA). The absorbance at 340 nm was measured every minute for six minutes using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). GST activity results were normalized to cytosolic protein content and expressed as $nmol.min^{-1}.mg^{-1}$ cytosolic protein.

2.2.2.4.2 Superoxide Dismutase (SOD) Activity

To assess SOD activity, the Nitroblue Tetrazolium (NBT) method, as described previously (Sun et al., 1988), was adapted for use with a 96-well microplate following the protocol described by Copeto et al. (Copeto et al., 2024). In each well, 200 µL of potassium phosphate buffer (50 mM; pH 8.0) was combined with 10 µL of EDTA (3 mM; Riedel-Haën, Seelze, Germany), xanthine (3 mM; Sigma-Aldrich, China), NBT (0.75 mM; Sigma-Aldrich, Germany), and the same volume of the respective sample. The reaction was initiated by adding 10 µL of xanthine oxidase (XOD; Sigma-Aldrich, Germany). Absorbance was monitored at 560 nm every two minutes for 20 minutes using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). SOD activity results were normalized to the total protein content and expressed as $U.mg^{-1}$ cytosolic protein.

2.2.2.4.3 Catalase (CAT) Activity

CAT activity was determined following a method previously described (Johansson & Borg, 1988), with modifications for a 96-well microplate (Copeto et al., 2024). In each well, 20 µL of the sample, 30 µL of methanol (Honeywell), and 100 µL of potassium phosphate buffer (100 mM; pH 7.0; Sigma) were combined. The reaction was initiated by adding 20 µL of hydrogen peroxide (0.035 M; Sigma-Aldrich, Germany) to each well. The microplate was then placed on an orbital shaker (Optic Ivymen System, JP Selecta, Barcelona, Spain) and incubated in the dark at room temperature for 20 minutes. Subsequently, 30 µL of KOH (10 M; ChemLab, Zedelgem, Belgium) and 30 µL of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (32.4 mM in 0.5 M HCl; purpald; Aldrich, Germany) was added, followed by another incubation step under the same conditions for 10 minutes.

Finally, 10 µL of potassium periodate (65.2 mM in 0.5 M KOH; Sigma-Aldrich) was introduced to each well and incubated for 5 minutes in the dark at room temperature. Absorbance readings were taken at 540 nm using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). A calibration curve was established using formaldehyde (AppliChem, Darmstadt, Germany) standards ranging from 0 to 150 µM. CAT activity results were normalized to total protein content and expressed as $nmol.min^{-1}.mg^{-1}$ cytosolic protein.

2.2.2.4.4 Lipid Peroxidation (LPO)

LPO was evaluated using the thiobarbituric acid assay, as previously described (Copeto et al., 2024; Madeira et al., 2018). A calibration curve was constructed using malondialdehyde (MDA; Merck, Germany) concentrations ranging from 0 to 0.1 µM in Mili-Q ultrapure water. In 1.5 mL micro-tubes, 5 µL of the sample or standard was mixed with 45 µL of PBS buffer, 12.5 µL of sodium dodecyl sulfate (8.1% w/v; SDS; Sigma-Aldrich, Germany), 93.5 µL of trichloroacetic acid (20% w/v; TCA; Panreac, Barcelona, Spain), 93.5 µL of thiobarbituric acid (1% w/v; TBA; Sigma-Aldrich, Germany), and 50.5 µL of ultrapure water. Following a brief centrifugation at 3000× g for 30 seconds, the microtubes caps were punctured and then heated in a dry bath (Thermobloc Digital, Labnet, Dusseldorf, Germany) at 100 °C for 10 minutes. After cooling on ice, 62.5 µL of ultrapure water was added to each microtube, followed by another centrifugation at 3000× g for 30 seconds. Subsequently, 150 µL of each sample was transferred to the microplate wells, and the absorbance was read at 530 nm using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). The LPO results were expressed relative to the total protein content, being the [MDA] expressed as $nmol.mg^{-1}$ cytosolic protein.

2.2.2.4.5 Total Antioxidant Capacity (TAC)

The total antioxidant capacity was determined following the protocol described by Kambayashi et al. (2009).

Calibration curves were prepared using standards of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Aldrich, Russian Federation) diluted in a potassium phosphate buffer, which included potassium phosphate monobasic (5 mM; pH 7.4; Sigma), glucose (5.55 mM), and sodium chloride (154 mM; NaCl; Panreac), with concentrations ranging from 0 to 0.330 mM. Subsequently, 10 µL of the sample or standard, 10 µL of myoglobin (90 µM; Sigma, USA), and 150 µL of 2,2-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (600 µM; ABTS; Alfa Aesar, Karlsruhe, Germany) were dispensed into the microplate wells (Greiner Bio-one, GmbH, Krems-münster, Austria). The reaction was initiated by adding 40 µL of hydrogen peroxide (500 µM; Sigma-Aldrich) to each well. After incubating for 5 minutes at room temperature, absorbance readings were taken using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). The TAC values were then standardized based on the total cytosolic protein content and expressed as µmol.mg^{-1} cytosolic protein.

2.2.2.4.6 Caspase-3 (CASP-3)

Caspase-3 levels were measured using an indirect ELISA (Lopes et al., 2018). To create a calibration curve, standards of caspase-3 (recombinant human active cc119; Merck, Rahway, NJ, USA) were prepared in PBS buffer at varying concentrations from 0 to 5 µg.mL^{-1}. Each microplate well (Greiner Bio-one, Microlon 600 High Binding, Frickenhausen, Germany) received 50 µL of sample or standard and was incubated at 4°C for 24 hours. The wells were washed with a 0.05% PBS-Tween solution (Panreac, Spain). A blocking step followed, involving 100 µL of 1% BSA in PBS (Nzytech, Lisboa, Portugal) added to each well and incubated at room temperature for 90 minutes. After three more washes with PBS-Tween, 50 µL of the primary antibody (anti-caspase 3 ab13847, Abcam, Amsterdam, the Netherlands) diluted to 1.5 µg·mL^{-1} in 1% BSA was added, and the wells were incubated again at 4°C for another 24 hours. Following three washes, 50 µL of the secondary antibody (anti-mouse IgG Fc specific-alkaline phosphatase, Sigma, State of Israel) diluted to 1.0 µg·mL^{-1} in PBS with 1% BSA was added and incubated at 37°C for 90 minutes.

After a final wash, 50 µL of substrate solution consisting of 157 mg Trizma hydrochloride (Tris-HCl; Sigma, USA), 58 mg NaCl (Panreac), 50 µL $MgCl_2$ (5 mM; Fluka, BioChemika, Buchs, Switzerland), and 10 mg 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP; Sigma-Aldrich, Gillingham, UK) in 10 mL distilled water, pH 9, was added to each well. The reaction was stopped by adding 50 µL of 3M NaOH (Panreac, Spain) after 15 minutes at room temperature. Absorbance at 405 nm was recorded using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA). Results were normalized to the total protein content and results are expressed as $\mu g.mg^{-1}$ cytosolic protein.

2.2.2.4.7 Total Ubiquitin (UBI)

Total ubiquitin levels were measured via an indirect ELISA, following the protocol previously described (Lopes et al., 2018). Ubiquitin standards from UBPBio (Dallas, TX, USA) created a calibration curve with concentrations ranging from 0 to 0.8 $\mu g \cdot mL^{-1}$. The volume of sample or standard in each well was 50 µL. The primary ubiquitin antibody (P4D1; Sc-8017; Santa Cruz Biotechnology, Dallas, TX, USA) was diluted to 1.5 $\mu g \cdot mL^{-1}$ in 1% BSA (w/v) and dispensed into each microplate well. The results were adjusted for total cytosolic protein content and expressed as $\mu g.mg^{-1}$.

2.2.2.4.8 Vitellogenin (VTG)

The vitellogenin levels were extracted and measured by following the Ueda (1970) method (Ueda & Wada, 1970), adapted for a 96-well microplate. Briefly, 20 µL of either sample or standard was added to each well along with 140 µL of distilled water and 35 µL of vanadate molybdate reagent (Sigma-Aldrich, St. Louis, USA). A calibration curve was set using potassium dihydrogen phosphate (inorganic phosphate) concentrations ranging from 0 to 30 $\mu g.mL^{-1}$. Absorbance readings were taken with a Synergy HTX microplate reader at 470 nm. The results were expressed in µg of inorganic phosphate per mg of protein.

2.2.2.5 Statistical Analysis

Statistical analyses were conducted using Prism 9 (GraphPad Software, version 9.5.1). The choice of test was determined based on whether the data met parametric assumptions. For data satisfying these assumptions, a one-way ANOVA followed by Dunnett's multiple comparison test was performed. In cases where parametric assumptions were not met, the Kruskal–Wallis test, also followed by Dunnett's multiple comparison test, was used. Additionally, the non-parametric Spearman's rank correlation was employed to assess the strength and direction of linear relationships between pairs of variables.

2.2.3 Results

Mortality Rate

From the original 32 mussels that initiated the experience all survived at the end of the exposure period.

2.2.3.1 Biomarkers of Oxidative Stress

2.2.3.1.1 Glutathione S-Transferase (GST)

Figure 2-3A presents GST activity (mean ± standard deviation) results and shows an increasing activity trend in response to the various concentrations of PFOA. At a concentration of 1 $\mu g.L^{-1}$, there is a slight rise in GST activity compared to the control, although this difference is not statistically significant. At 10 and 100 $\mu g.L^{-1}$, GST activity increases significantly compared to control.

2.2.3.1.2 Superoxide Dismutase (SOD)

Figure 2-3B presents SOD activity, expressed as mean ± standard deviation, and reveals an upward trend in activity across varying concentrations tested over time. The tested concentrations significantly differ from the control. At 1 $\mu g.L^{-1}$, SOD activity significantly increases compared to the control. Although SOD activity is further elevated at 10 $\mu g.L^{-1}$, it is not significantly different from the 1 $\mu g.L^{-1}$ level but are significant different from the control. The 100 $\mu g.L^{-1}$ concentration shows similar SOD activity to the 10 $\mu g.L^{-1}$ level, with a significant increase compared to controls.

2.2.3.1.3 Catalase (CAT)

Figure 2-3C displays CAT activity results (mean ± standard deviation) for mussels exposed to varying concentrations of PFOA. The control group and lower concentrations (1 µg.L^{-1} and 10 µg.L^{-1}) exhibit relatively consistent and low levels of CAT activity, with only minor variations that are statistically non-significant when compared with the control group. At the 100 µg.L^{-1} exposure concentration, a marked increase but not statistically significant in CAT activity was observed, suggesting an antioxidant response at this higher concentration.

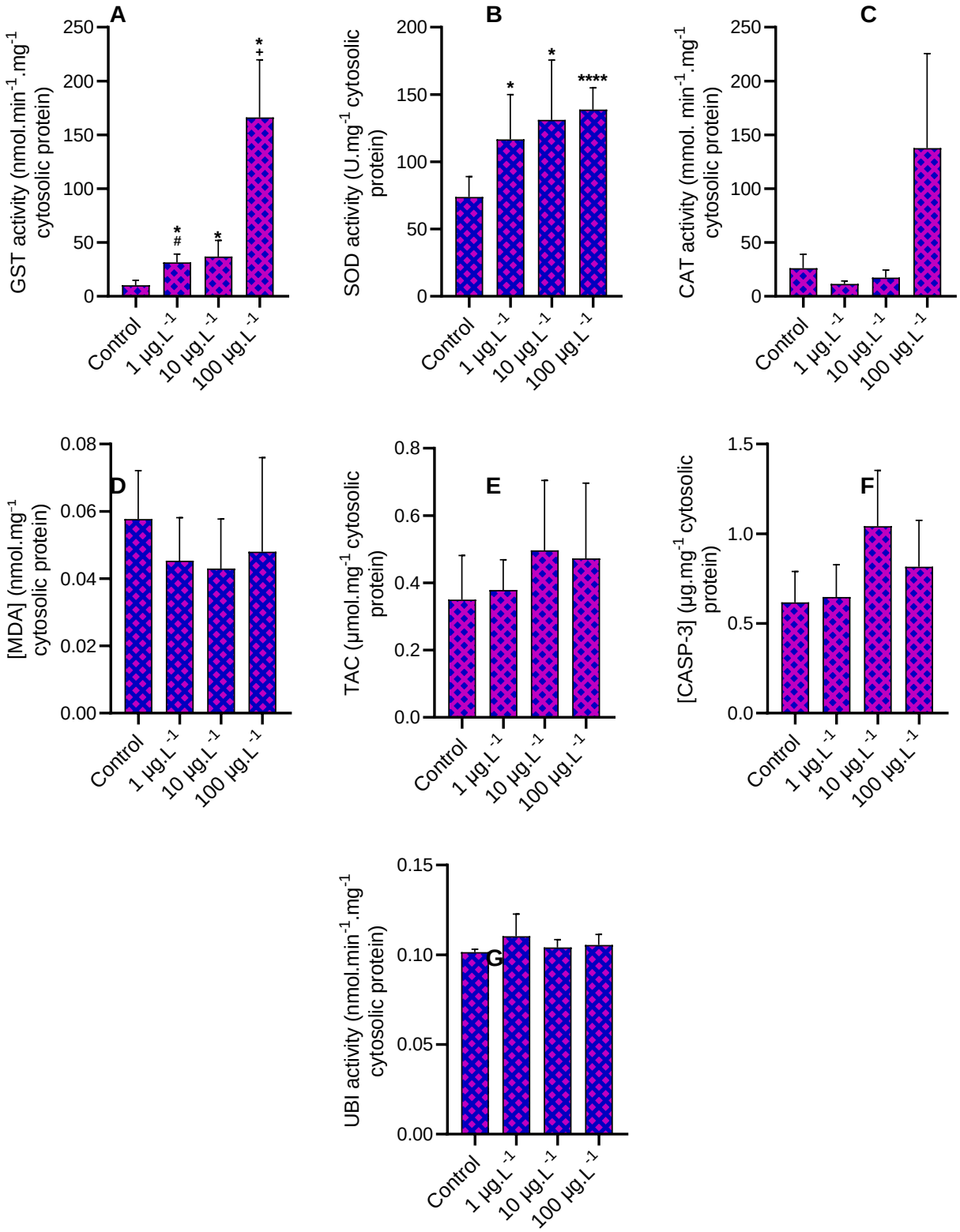

Figure 2-3 - (A) GST, (B) SOD and (C) CAT activity, (D) LPO, (E) TAC, (F) CASP and (G) UBI levels in mussels exposed to different concentrations (0,1,10 and 100µg. L^{-1}) of PFOA. All data are presented as mean ± s.d. * - p <0.05 and **** - p <0.0001, compared with control. # -p <0.05, compared with 1µg.L^{-1}, + -p <0.05, compared with 10 µg.L^{-1}

2.2.3.1.4 Lipid Peroxidation (LPO)

LPO is shown in **Figure 2-3D**, measured as the malondialdehyde (MDA) concentration, shows great variability and no discernible trend can be observed. Data suggests that the tested concentrations of the compound do not induce significant changes at this level.

2.2.3.1.5 Total Antioxidant Capacity (TAC)

Figure 2-3E shows a slight increase in total antioxidant capacity (TAC) according to the PFOA concentrations tested although no statistical differences were detected.

2.2.3.1.6 Caspase (CASP)

Figure 2-3F depicts caspase-3 concentrations across to the different PFOA concentrations, with higher average concentrations being determined in animals exposed to 10 $\mu g.L^{-1}$ and at 100 $\mu g.L^{-1}$ of PFOA, but no significant changes were detected.

2.2.3.1.7 Ubiquitin (UBI)

Figure 2-3G represents the ubiquitin (UBI) concentrations, which remained constant across the different PFOA concentrations tested. No significant changes were detected among the concentrations tested or in comparison to the control group.

2.2.3.1.8 Vitellogenin (VTG)

Figure 2-4 shows VTG values (mean ± standard deviation) for animals exposed to varying concentrations of PFOA. Although an increase in the VTG levels of the exposed females was observed according to the PFOA concentrations tested, this increase was not statistically significant. The VTG levels determined in the males remained similar for the PFOA concentrations tested.

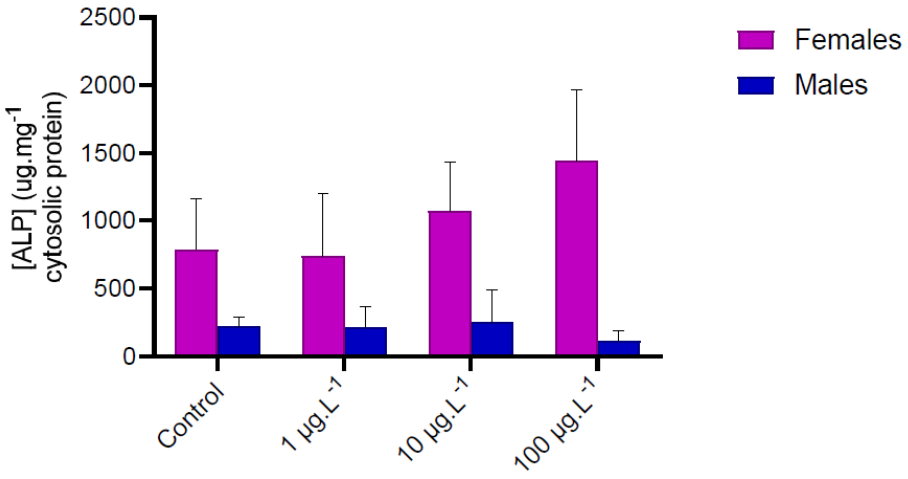

Figure 2-4 - Total alkali-labile phosphate (ALP) concentration, in mussels exposed to different concentrations (0,1,10 and 100µg. L^{-1}) of PFOA. All data are presented as mean ± s.d.

2.2.3.2 Correlation Analyses

The results of correlation analysis (Spearman) are presented in **Table 2.2-1**.

Table 2.2-1 - Spearman correlation matrix

	GST	SOD	CAT	LPO	TAC	UBI	CASP	VTG
GST	1.00							
SOD	0.66	1.00						
	p = 0.003							
CAT	0.22	0.06	1.00					
	p = 0.359	p = 0.780						
LPO	-0.29	0.04	0.00	1.00				
	p = 0.234	p = 0.856	p = 0.990					
TAC	0.33	0.48	0.10	0.09	1.00			
	p = 0.182	p = 0.020	p = 0.658	p = 0.695				
UBI	0.26	0.46	-0.13	-0.02	0.64	1.00		
	p = 0.308	p = 0.019	p = 0.571	p = 0.917	p = 0.003			
CASP-3	0.22	0.64	0.15	-0.01	0.82	0.58	1.00	
	p = 0.367	*p*< 0.0001	p = 0.470	p = 0.980	*p*< 0.0001	p = 0.004		
VTG	-0.14	0.26	0.31	0.31	0.38	0.14	0.44	1.00
	p = 0.540	p = 0.172	p = 0.119	p = 0.113	p = 0.058	p = 0.501	p = 0.019	

The correlation matrix presented in **Table 2.2-1** highlights significant relationships among several biochemical parameters related to oxidative stress and apoptotic activity, including GST, SOD, CAT, LPO, TAC, UBI, CASP-3, and VTG. A moderate positive correlation was observed between GST and SOD (r = 0.66, p = 0.003), indicating a potential link between glutathione transferase and superoxide dismutase activities. SOD also exhibited significant positive correlations with UBI (r = 0.46, p = 0.019) and CASP-3 (r = 0.64, p < 0.0001), suggesting that higher SOD activity is associated with moderate increased ubiquitin and caspase-3 levels. Furthermore, TAC showed a strong positive correlation with CASP-3 (r = 0.82, p < 0.0001), suggesting a close association between total antioxidant capacity and caspase-3 concentration, which may reflect an interaction between antioxidant defence mechanisms and apoptosis. UBI also showed a moderate significant correlation with TAC (r = 0.64, p = 0.003) and CASP-3 (r = 0.58, p = 0.004), further reinforcing an eventual connection between ubiquitin levels, antioxidant capacity, and apoptotic processes. In contrast, CAT and LPO did not show significant correlations with the other parameters, indicating that their roles may be more independent or complex within these interactions.

2.2.4 Discussion

It is well-known that PFASs induce oxidative stress (Lee et al., 2020b). Several studies indicate that PFAS exposure induces oxidative stress in aquatic organisms, affecting antioxidant enzyme activity and detoxification pathways (Amraoui et al., 2018; Arukwe & Mortensen, 2011; Jeong et al., 2016; Liu et al., 2007; Liu et al., 2008; Sant et al., 2018; Yang et al., 2014). The criteria of selecting 1µg.L^{-1} for the lowest tested concentration was based on various studies that documented levels of PFOA in surface waters ranging from 0.09 to 1270 ng.L^{-1} (Ahrens et al., 2009; Hansen et al., 2002; Jin et al., 2009; Loos et al., 2008; Quinete et al., 2009; Teng et al., 2009; Wei et al., 2007). This range reflects different contamination levels due to various factors such as proximity to industrial sources, urban runoff, and wastewater discharge. Higher concentrations were also tested to better understand their effects on the animals.

Recent studies have shown the effects of different PFASs on oxidative stress biomarkers in marine organisms, especially mussels (Geng et al., 2024).

Glutathione S-transferase (GST) is a key enzyme in phase II of biotransformation, facilitating detoxification by catalyzing glutathione conjugating with toxic substances. This makes harmful compounds less reactive and easier to eliminate, indirectly contributing to antioxidant defences by influencing glutathione availability (Vidal-Liñán et al., 2015). In our study, GST activity in mussels showed significant changes after 28 days of exposure to PFOA, with enzyme levels rising notably in comparison to controls. Similarly, varying responses in GST activity were recorded by different studies and species following exposure to PFAS. For instance, in the Australian rainbowfish (*Melanotaenia fluviatilis*), GST activity increased in the gills at the two highest concentrations of PFOA tested (1 and 10 $mg.L^{-1}$) during a 24-day exposure period. However, a decrease in activity was observed in the liver at the highest concentration (Miranda et al., 2020). Increased GST activity was also noted in the planarian *Dugesia japonica* after 10 days of exposure to PFOA across a range of concentrations (0.5, 5, 10, 20 $mg.L^{-1}$) (Yuan et al., 2017) and in the digestive gland of the Mediterranean mussel (*M. galloprovincialis*) exposed to PFOS for 21 days (2–10 $mg.L^{-1}$) (Gülsever & Parlak, 2018). Inhibition of GST activity was also observed in the hepatocytes of Nile tilapia (*Oreochromis niloticus*) exposed for 24 hours to PFOS or PFOA, particularly at the highest concentrations tested (1, 5, 15, and 30 $mg.L^{-1}$) (C. Liu et al., 2007).

These observations underscore the species-specific responses of GST activity to PFAS exposure, highlighting the intricate biochemical mechanisms in the detoxification and antioxidant defence systems of aquatic organisms, such as mussels. Elevated GST activity may protect against oxidative stress when antioxidant levels are diminished, as seen in previous studies where mussels transplanted to harbor areas exhibited increased GST levels to counteract oxidative damage (Vidal-Liñán et al., 2014). Moreover, GST is a key enzyme in the biotransformation process, and its enhanced activity in mussels exposed to higher concentrations of contaminants, such as PFOA, suggests an adaptive detoxification response (Vidal-Liñán et al., 2015). This enhanced GST activity could be linked to the organism's effort to mitigate the accumulation of harmful substances by facilitating their biotransformation and excretion.

Of the antioxidant enzymes analyzed, SOD is considered the first line of defence that converts superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), which is subsequently scavenged by CAT and/or GSH-Px (Zhang et al., 2016).

Our findings suggest that after 28 days of exposure, SOD activity in mussels exposed to PFOA increased significantly compared to the controls. In a previous study, SOD activity determined in the green mussel (*Perna viridis*) exposed to a mixture of perfluorinated compounds including PFOS, PFOA, perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) for 7 days (C. Liu et al., 2014), increased at lower concentrations (0–100 $\mu g.L^{-1}$) but declined at the highest concentrations (100–10,000 $\mu g.L^{-1}$). Similarly, in the freshwater mollusk (*Unio ravoisieri*) exposed to PFOS at concentrations between 2 and 10 $mg.L^{-1}$ for 7 days, SOD activity increased in those treated with 2 and 6 $mg.L^{-1}$ but decreased in those exposed to 10 $mg.L^{-1}$(Amraoui et al., 2018). An increase followed by a decrease in SOD activity was also observed in the amphipod (*Gammarus insensitives*) exposed to PFOS at concentrations of 1 $mg.L^{-1}$, 1.6 $mg.L^{-1}$, and 3.1 $mg.L^{-1}$ over 4 days (Touaylia et al., 2019). Conversely, in the zebrafish (*Danio rerio*), SOD activity in the liver increased after a 96-hour exposure to 200 $\mu g.L^{-1}$ of PFOS, while no changes were observed in the gills (Y. Li et al., 2017). In goldfish (*Carassius auratus*), no changes in SOD activity were detected in the liver after exposure to PFOA concentrations of 1.21 and 12.1 $\mu mol.L^{-1}$ over 4 days (Feng et al., 2015).

Other studies have shown a decrease in SOD activities following PFAS exposure, such as in the Chinese mitten crab (*Eriocheir sinensis*) exposed to high concentrations of PFOS (up to 10 $mg.L^{-1}$) for 21 days (F. Zhang et al., 2015) and in the freshwater cladoceran (*Daphnia magna*) exposed to concentrations of 0.008–5 $mg.L^{-1}$ of PFOS or PFNA, particularly at 0.2 $mg.L^{-1}$, over 7 days (Lu et al., 2015). Since SOD serves as a primary defence against oxygen toxicity by catalyzing the conversion of superoxide anions into oxygen and hydrogen peroxide, an increase in SOD levels in the group exposed to the highest concentration, this suggests that this enzyme plays a crucial role in protecting mussels from exposure-related oxidative stress. This protective response has been also observed in mussels exposed to environmental stressors, where elevated SOD activities in the gills acted as a biomarker for oxidative stress (Fernández et al., 2010).

Catalase (CAT), an enzyme within peroxisomes, is crucial for converting hydrogen peroxide (H_2O_2) into water and molecular oxygen. This study revealed that CAT activity in mussels significantly increased after 28 days of exposure to PFOA when compared to controls.

Previous research has shown varied effects on CAT activity due to exposure to perfluorinated compounds. For example, CAT activity decreased in the liver of medaka fish (*Oryzias latipes*) after being exposed to PFOA at concentrations of 10, 50, and 100 mg.L^{-1} for 7 days (J. H. Yang, 2010) and in water fleas (*Daphnia magna*) exposed to PFOS or PFNA at a concentration of 0.04 mg.L^{-1}(Lu et al., 2015). In contrast, increases in CAT activity were noted in both the digestive gland and gills of the freshwater mussel (*Unio ravoisieri*) exposed to PFOS ranging from 2–10 mg.L^{-1} (Amraoui et al., 2018). The Australian rainbowfish (*Melanotaenia fluviatilis*) showed increased CAT activity in the gills when exposed to 0.1 mg.L^{-1} of PFOA over 24 days. However, a decrease was observed in the liver at the highest concentrations tested (Miranda et al., 2020). Similarly, green mussels (*Perna viridis*) exhibited increased CAT activity after 7 days of exposure to PFOS, PFOA, PFNA, and PFDA up to a concentration of 100 µg.L^{-1}(C. Liu et al., 2014). Furthermore, a notable increase in CAT activity was recorded in the hepatocytes of Nile tilapia (*Oreochromis niloticus*) exposed for 24 hours to the highest concentrations of PFOS or PFOA tested (1, 5, 15, and 30 mg.L^{-1}) (Liu et al., 2007). Conversely, in the liver of rice fish, CAT activity was inhibited following exposure to PFOA, particularly at a concentration of 50 mg.L^{1} (Yang, 2010). These findings suggest that the perfluorinated compound-induced response in CAT activity is sensitive and varies across species, likely due to differing interactions within their antioxidant enzymatic defence systems.

In this study, the changes observed in VTG concentrations are not statistically significant, even though there was an increase in vitellogenin levels, namely in females exposed to 100 µg.L^{-1}. As referred by other studies, estrogenic effects were only observed at PFOA concentrations equal or higher than 500 µg.L^{-1}, suggesting that 100 µg.L^{-1} is unlikely to have an estrogenic effect in mussels. In effect, previous research, such as that carried out by Kim et al. (Kim et al., 2010), identified PFOA as an estrogenic substance effective in inducing VTG in male common carp (*Cyprinus carpio*) at concentrations ranging from 500 to 50,000 µg.L^{-1}.

In the ALP method, the presence of baseline VTG levels is common, and an estrogenic effect is only considered when these levels are higher than 100 µg.P. mg^{-1} protein (de los Ríos et al., 2012). The use of the indirect-ALP method as a biomarker of VTG is not consensual in the scientific community, and some constraints have been pointed out.

For example, a proteomic study by Sánchez-Marín et al. (2017) showed that the ALP method is not providing reliable information about Vtg levels. Thus, the methodology used may not be specific enough to distinguish between different phosphate sources, resulting in values that reflect a combination of factors, including natural reproductive biology and possible interference from other phosphorylated proteins. This lack of specificity can result in measurements that reflect multiple sources of phosphate, which are not necessarily related to vitellogenesis (Morthorst et al., 2014).

Additionally, PFOA has been shown to activate estrogen-responsive genes significantly, as evidenced by studies conducted by Wei et al. (Wei et al., 2007) in *Gobiocypris rarus*. However, Wei et al. (Wei et al., 2008) found PFOA to be a weaker estrogen inducer than 17β-estradiol (EE2), although its estrogen-like effects have been documented in various fish species. Molecular analysis in rainbow trout exposed to 200–1800 $mg.L^{-1}$ of PFOA indicated estrogenic gene activations strongly correlated with those triggered by EE2 exposure (Tilton et al., 2008).

PFOA also enhanced total aromatase activity, crucial for EE2 synthesis and regulation of internal levels (Du et al., 2013). Kang et al. (2019) reported that the gene expression patterns of VTG and choriogenin in Japanese medaka exposed to 10 $mg.L^{-1}$ of PFOA for 21 days mirrored those in fish exposed to estradiol (E2), leading to adverse reproductive outcomes, including reduced fecundity (Kang et al., 2019). Moreover, PFOA exposure was found to disrupt the thyroid T3/T4 ratio in the Australian rainbowfish (*Melanotaenia fluviatilis*), marking the first observation of its impact on circulating thyroid hormone ratios in fish. This disruption has implications for thyroid hormone homeostasis, an important indicator typically used to detect thyroid disturbances in laboratory and field studies (Brar et al., 2010; Chidakel et al., 2005; Yadav & Singh, 1987).

In our study, the PFOA concentrations tested did not resulted in clear LPO or caspase-3 and ubiquitin changes following exposure, suggesting that these concentrations are not sufficient to trigger significant changes or there are defence mechanisms acting to protect cells.

However, it should be emphasized that due to the current lack of studies on the effects of PFOA on mussels, it is only possible to make comparisons with effects on other species, such as fish and few bivalve species. Regarding VTG, the discussion is made with fish species exposed to PFOA due to the lack of data in bivalves.

On the other hand, it should be noted that in most of these studies the concentrations of PFOA tested were higher (in the $mg.L^{-1}$ range) than those tested in the present study. Therefore, up to 100 $\mu g.L^{-1}$, the detoxification and antioxidant mechanisms appear to respond to protect the organism effectively. This also highlights the urgent need for further research in other aquatic animals, as bivalves.

Furthermore, our results, supported by correlation analyses, suggest a network of interconnected biochemical pathways where oxidative stress markers and ubiquitin are closely linked to apoptotic processes. In effect, the strong correlations observed between SOD, TAC, and CASP-3, along with their associations with ubiquitin, underscore the significance of these pathways at the cellular and molecular level to protect animals. However, the absence of significant correlations involving CAT and LPO suggests that further investigation is necessary to understand their roles within this context fully.

2.2.5 Conclusions

This study provides compelling evidence of the impact of PFOA on the antioxidant defence and stress response systems in the mussel *M. galloprovincialis*. The differential responses of oxidative stress markers, such as superoxide dismutase (SOD) and catalase (CAT), across the various PFOA concentrations tested, underscore the complexity of PFOA-induced oxidative stress. These findings highlight the importance of assessing the ecological risks of PFOA and similar contaminants in the marine biota.

It is also important to note that the concentration used in this study are much lower than those tested by previous studies. The results suggest that up to 100 $\mu g.L^{-1}$, detoxification and antioxidant mechanisms can effectively protect the organism.

Moreover, the absence of observed mortality suggests that the mussels were able to manage their exposure to this level of contaminants successfully.

This research offers valuable insights for environmental management and policymaking, particularly in regulating and monitoring PFOA in marine ecosystems, especially in regions with high seafood consumption. Implementing stringent environmental policies to control PFOA levels in marine environments is crucial for mitigating potential risks to aquatic life and human health. Focusing on monitoring contaminant concentrations is essential for effective policy control. However, to achieve comprehensive contamination management, it must be complemented by preventive measures, remediation strategies, and health protection initiatives. By deepening our understanding of the biochemical effects of PFOA on marine organisms, this study contributes to the broader effort to address the challenges posed by persistent environmental contaminants.

Future research should continue to investigate the mechanisms underlying these responses and extend the scope to include other perfluorinated compounds and additional aquatic species. Such efforts are essential for developing comprehensive strategies for environmental protection and public health.

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2.3 Oxidative stress biomarker responses to 17α-ethinylestradiol (EE2) exposure in the mussel *Mytilus galloprovincialis*

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Abstract

The widespread occurrence of endocrine-disrupting compounds (EDCs) in aquatic environments has raised significant ecotoxicological concerns, particularly regarding their impact on marine invertebrates. Among these compounds, 17α-ethinylestradiol (EE2), a synthetic oestrogen widely used in oral contraceptives, is highly persistent and biologically active at very low concentrations. This study aimed to evaluate the effects of EE2 exposure on oxidative stress responses and endocrine disruption in the Mediterranean mussel *Mytilus galloprovincialis*. Mussels were exposed for 28 days to three EE2 concentrations (10, 30, and 300 $ng.L^{-1}$). Biomarkers of oxidative stress, including the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST), as well as Lipid Peroxidation (LPO) and total ubiquitin (UBI) and endocrine disruption, vitellogenin-like protein (VTG) were assessed.

Results showed a clear dose-dependent modulation of antioxidant defences, with significant increases in SOD and GST activities, particularly at 300 $ng.L^{-1}$ EE2, indicating enhanced oxidative challenge. CAT activity exhibited a biphasic response, elevated at low EE2 concentrations but suppressed at 300 $ng.L^{-1}$. However, LPO and UBI levels did not significantly increase. VTG-like protein levels increased according to EE2 concentrations tested, showing significant differences at 300 $ng.L^{-1}$, confirming the estrogenic mode of action of EE2 and its ability to disrupt endocrine regulation in bivalves.

These findings demonstrate that *M. galloprovincialis* exhibits a robust yet concentration-sensitive physiological response to EE2 exposure, marked by the activation of detoxification and antioxidant defence pathways, and induction of estrogen-responsive biomarkers. This study underscores the value of biomarker approaches in environmental risk assessment and supports the use of *M. galloprovincialis* as an effective bioindicator species for monitoring estrogenic pollutants in marine ecosystems.

Keywords: 17α-ethinylestradiol; *Mytilus galloprovincialis*; oxidative stress; antioxidant enzymes; vitellogenin; endocrine disruption

2.3.1 Introduction

Environmental pollution, particularly from domestic and industrial effluents, presents a significant challenge, with aquatic ecosystems being especially vulnerable, as these pollutants ultimately reach the ocean (Islam & Tanaka, 2004). Marine ecosystems exposed to various anthropogenic contaminants, are at high risk from industrial, agricultural, and domestic pollutants (Hamza-Chaffai, 2014). Therefore, investigating the impacts of these pollutants is essential for the protection of ecosystems. Coastal areas host several ecosystems, including estuaries, biogenic reefs, rocky areas, sandy beaches, salt marshes, mudflats, and seagrass beds (Cabral et al., 2019). The protection of these areas is critical, as they are threatened by human activities such as overfishing, maritime transport, dredging, and the discharge of chemicals (Bowen & Depledge, 2006).

These coastal regions, characterized by well-oxygenated environments, are preferred habitats for bivalves, making them important for ecotoxicological studies focused on food safety and the assessment of pollutant impacts (Casatta et al., 2015).

Mussels (*Mytilus spp.*) are commonly used as biological model organisms in research (Lopes-Lima et al., 2014). They are widely distributed geographically, sedentary, easily accessible, capable of tolerating various environmental conditions, and known for accumulating significant amounts of pollutants (Li et al., 2019; Viarengo & Canesi, 1991). Their filtration capacity, well-characterized physiology, morphology, and biochemistry make them effective "carriers" of pollutants through trophic chains, making them ideal organisms for studies on toxicology and bioavailability (Meador et al., 1995). Endocrine-disrupting compounds (EDCs) represent a significant threat due to their ability to disrupt the endocrine system, affecting hormone synthesis and enzyme activities (Grobin et al., 2024; Sukatis et al., 2024). Among EDCs, steroid hormones are commonly detected in wastewater. Natural oestrogens, including estrone (E1), 17β-oestradiol (E2), and estriol (E3), mainly originate from human and animal excretion, whereas the synthetic oestrogen 17α-ethinylestradiol (EE2), extensively used in oral contraceptives, is also frequently reported. These compounds interfere with cellular apoptotic processes, influence the epigenetic regulation of specific target cells, and disrupt the cell cycle (Sifakis et al., 2017). Despite their harmful effects, EDCs persist in the environment and are discharged through industrial, urban, and agricultural effluents.

These compounds infiltrate soil and eventually contaminate groundwater and drinking water, posing severe risks to human growth, development, and reproduction (Saravanakumar et al., 2022; Sukatis et al., 2024).

Steroid hormones are a group of molecules synthesized from cholesterol and characterized by a cyclopentanoperhydrophenanthrene ring structure (Ying et al., 2002). Natural oestrogens (E1, E2, and E3), together with the synthetic oestrogen EE2 commonly used in contraceptives, are among the compounds most frequently detected in wastewater. The primary distinction among these oestrogens is their configuration at carbon 17, which results in varying physicochemical properties (Dias et al., 2023).

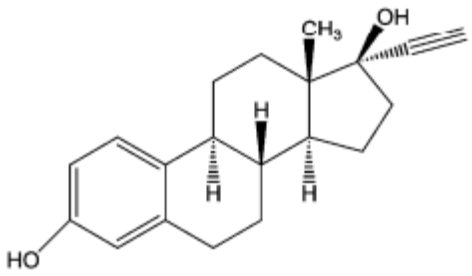

Figure 2-5 - Ethinylestradiol. Adapted from Ying et al. (2002)

EE2 has relatively low solubility in water (4.8 mg/L at 20 °C) and has a relatively high octanol-water partitioning coefficient (log Kow = 4.15), which causes it to be persistent and preferentially attach to the organic matter in the aquatic environment (Auriol et al., 2006). This organic compound is characterized by its non-polar, hydrophobic nature (**Figure 2-5**), low volatility, and resistance to biodegradation (Silva et al., 2012). Its lipophilic nature allows it to cross the plasma membrane and bind to intracellular receptors (Silva et al., 2012). Due to their lipophilicity, they undergo conjugation with sulphate groups and glucuronic acid in the liver during the first-pass effect in animals and humans, which reduces their estrogenic potency. However, environmental deconjugation by microorganisms can restore their estrogenic activity (Zayyat et al., 2024). Their lipophilicity also contributes to bioaccumulation and biomagnification within trophic chains, potentially reaching higher levels and impacting human health (Chiu et al., 2018). Commonly used in oral contraceptives and treatments for osteoporosis, cancer, and alopecia, EE2 is highly estrogenic and exhibits considerable resistance to degradation, making it a persistent environmental contaminant.

EE2 is incompletely removed by wastewater treatment (0.77% to 95% removal), leading to its widespread detection in aquatic environments.

Concentrations ranging from 18 to 83 $ng.L^{-1}$ have been reported in coastal worldwide habitats (e.g. Douro River estuary in Portugal, the Venice Lagoon in Italy, and the Gulf of Gdańsk in the Baltic Sea) (Almeida et al., 2020), with wastewater effluents being a significant source of contamination (Aris et al., 2014). In Portugal, Ribeiro et al. reported values below the detection limit in some samples, while concentrations as high as 83.1 $ng{\cdot}L^{-1}$ were documented in the Douro River Estuary (Ribeiro et al., 2009). Despite their harmful effects, EDCs are prevalent in the environment due to their continuous discharge through industrial, urban, and agricultural effluents. These compounds infiltrate soil, contaminating groundwater and drinking water, adversely affecting growth, development, and reproduction (Islam & Tanaka, 2004; Sukatis et al., 2024).

Oral contraceptives are estimated to release 700 kg per year of EE2 into the aquatic environment, whereas livestock practices may contribute up to 80,000 kg per year. Globally, human excretion accounts for 30,000 kg per year of natural estrogens and an additional 700 kg per year of synthetic EE2, while livestock in the United States and European Union alone discharge 83,000 kg per year, more than twice the human contribution. (Adeel et al., 2017).

The extensive use of pharmaceuticals presents significant risks to various aquatic ecosystems, including marine environments, where environmental concentrations of EE2 have been documented to range from 0.20 to 83.1 $ng.L^{-1}$ (Almeida et al., 2020). EE2 is highly toxic at concentrations as low as 0.1 $ng.L^{-1}$ and is recognised by the EU as an emerging aquatic pollutant (Zayyat et al., 2024). EE2 bioaccumulates in trophic chains, affecting various marine organisms, with bivalves being particularly vulnerable. Aquatic invertebrates are especially susceptible due to prolonged exposure from an early age, intensifying the toxic impact (Wojnarowski et al., 2021).

Exposure to EE2 negatively affects bivalves by causing the overproduction of reactive oxygen species (ROS), such as superoxide anion (O^{-}_{2}), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2).

These ROS activate antioxidant enzymes like catalase and superoxide dismutase, which work to reduce oxidative stress and prevent cell death due to membrane disintegration (Lopes, Coppola, Russo, et al., 2022). EE2 also disrupts lysosomal membrane stability and causes gonadal damage in *M. galloprovincialis*.

Additionally, it is known to induce vitellogenin gene expression in *Saccostrea glomerata*, affects metabolic pathways linked to anaerobic metabolism, osmotic stress, and reproduction in *Crassostrea virginica*, and elicits similar cellular and physiological effects in *Ruditapes philippinarum* (Brew et al., 2020; Fabrello et al., 2021; Lopes, Coppola, Russo, et al., 2022; Rodrigues et al., 2023). In seahorses (*Hippocampus guttulatus*), EE2 exposure reduces egg viability, delays sexual differentiation and maturation, decreases testosterone synthesis, and causes gestational disturbances (D'Alvise et al., 2020). However, recent studies have suggested a potential positive relationship between EE2 exposure and telomere length in *H. guttulatus*, with significant EE2 concentrations (21 $ng.L^{-1}$) promoting telomerase gene expression, possibly as a compensatory response to mitigate adverse effects (Prévot D'Alvise et al., 2024).

In fish, EE2 inhibits sexual development in young male western mosquitofish (*Gambusia affinis*), reduces DNA repair gene transcription in male zebrafish (*Danio rerio*), and increases vitellogenin levels in young rainbow trout (*Oncorhynchus mykiss*) (Wojnarowski et al., 2021). Additionally, EE2 exposure reduces the heart rate in snail embryos (*Marissa cornuarietis*) and disrupts the metamorphosis process in leopard frogs (*Rana pipiens*) (Hogan et al., 2008; Schirling et al., 2006). Overall, EE2 is known to reduce fertility rates and induce feminisation in males, leading to the production and release of female-associated hormones or proteins like vitellogenin. Biomarkers such as vitellogenin (VTG), total ubiquitin quantification, antioxidant enzymes, and lipid peroxidation are used to measure biological responses to contaminants.

Vitellogenin (VTG) is a phosphoprotein synthesized in the liver of female ovoviviparous organisms during yolk formation (vitellogenesis) in response to the hormone oestradiol (E2) (Souza et al., 2023). Usually, VTG is found in high concentrations in sexually mature females and low concentrations in young females. VTG is either absent or present in males at vestigial levels, depending on the species (Hara et al., 2016). However, after exposure to EDCs like EE2, VTG levels increase significantly in both males and young females, making it an effective biomarker for detecting endocrine disruption and xenoestrogen exposure (Jung et al., 2006).

Ubiquitination, a post-translational modification, signals target proteins for proteolytic degradation, DNA repair, autophagy, and endocytosis (Kandel et al., 2024). Thus, Ubiquitin is considered an excellent biomarker of oxidative stress, as oxidative stress leads to the oxidation of proteins, causing them to lose structure and function within the cell (López-Pedrouso et al., 2022). Specifically, in response to EE2, an increase in total ubiquitin concentration indicates cellular damage.

In the antioxidant defence system, glutathione S-transferase (GST) plays a fundamental role by catalysing the conjugation of xenobiotics with glutathione, thereby increasing their polarity and facilitating excretion. Additionally, GST contributes to cellular protection against oxidative stress induced by exogenous compounds. High concentrations of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) indicate the presence of reactive oxygen species (ROS) in the cell. Studies on organisms like *Danio rerio* and *Ruditapes philippinarum* have shown increased ROS production after EE2 exposure, highlighting the importance of these enzymes (M. G. Silva et al., 2022; Tamagno et al., 2022).

SOD converts the superoxide anion radical (O^{-2}) into hydrogen peroxide (H_2O_2) and molecular oxygen. Since H_2O_2 is still toxic to cells, CAT catalyses the reaction of two H_2O_2 molecules into two molecules of water and molecular oxygen (Dobal et al., 2022). GSTs, a multifunctional enzyme family, play a role in defending cells against oxidative stress induced by xenobiotics and are important in phase II detoxification through their conjugation activity. As these enzymes represent a biological adaptation for survival and protection against adverse environmental conditions, changes in their activity can indicate the presence of chemicals like EE2 disrupting cellular homeostasis (Park et al., 2020). Increased ROS formation from EE2 exposure can lead to cellular damage, including DNA oxidation, mitochondrial dysfunction, and lipid peroxidation (LPO), ultimately resulting in apoptosis or necrosis (Mattera et al., 2017).

This study aimed to expose *M. galloprovincialis* mussels to different concentrations of EE2 (0, 10 $ng.L^{-1}$, 30 $ng.L^{-1}$, and 300 $ng.L^{-1}$) over 28 days to analyze the effects of this exposure. Several oxidative stress biomarkers were assessed, including antioxidant enzyme activity (GST, CAT, and SOD), total ubiquitin quantification, lipid peroxidation (LPO) measured by MDA concentration, and vitellogenin expression.

This study can contribute to enhancing the scientific understanding of the risks posed by this chemical compound to the aquatic environment. In addition, this study aligns with the United Nations' 14th Sustainable Development Goal (SDG), which aims to conserve and sustainably utilize oceans, seas, and marine resources.

2.3.2 Materials and Methods

2.3.2.1 EE2 Stock Solution

To prepare the EE2 stock solution, 5 mg of EE2 (CAS No. 57-63-6, Sigma-Aldrich, USA) was weighed and dissolved in 100 mL of methanol (99.8% V/V, Honeywell, Seelze, Germany). The necessary volume was added to each aquarium to achieve the target concentrations of 10, 30, and 300 $ng.L^{-1}$. A separate aquarium containing only filtered seawater served as the control. Accounting for the dilution factor, the methanol concentration in each aquarium was determined to be ≤0.001%.

2.3.2.2 Experimental trial

M. galloprovincialis mussels (Lamarck, 1819) were manually collected from Guincho Beach (Cascais, Lisbon, Portugal) in March 2024. Mussels (n= 32) with an average length of 2.9 ± 0.3 cm and an average width of 1.4 ± 0.2 cm, were collected to minimize variations in bioaccumulation and biomarker responses that could result from differences in size or weight. The specimens were transported to the NOVA School of Science and Technology Laboratory in a thermal box to maintain a constant temperature and minimize additional stress. The experimental setup used to expose mussels (n=32) to EE2 was described previously (Copeto et al., 2024a; Copeto et al., 2024b). Briefly, upon arrival, the mussels were acclimated for 7 days in a 50 L saltwater aquarium filled with seawater from Guincho. The aquarium was equipped with filtration, recirculation, and aeration systems, and maintained under controlled conditions, including pH (8.1 ± 0.1), temperature (17.2 ± 0.1 °C), salinity (31 ± 1 g/L), and a 12-hour light/dark photoperiod. Mussels (n = 8) were distributed into four plastic tanks (35x27x18.5 cm) containing 8 L of salt water (31 ± 1 g/L) with the same salinity and physicochemical parameters as during the acclimation phase.

To reach the target concentrations for the exposure assay (10, 30, and 300 ng.L^{-1}), the required volume of EE2 from the stock solution (see section 2.1) was added to each 8-litre aquarium. These conditions were renewed every 48 hours. A separate aquarium with filtered seawater was used as the control.

The selected concentrations of 10 and 30 ng.L^{-1} were based on realistic environmental levels of EE2, which have been reported to range from 0.20 to 83.1 ng.L^{-1} (Almeida et al., 2020). A higher concentration of 300 ng.L^{-1} was also tested to understand its effects on mussels better. A separate aquarium with filtered seawater served as the control. Considering the dilution factor, the methanol concentration in each aquarium was estimated to remain below 0.001%. The mussels were fed daily with 1.5 mg/L of dried algae (*Chlorella sp*, Shine, Portugal).

2.3.2.3 Sample Treatment

At the end of the experimental period, the mussels were weighed in an analytical balance (Sartorius, Germany) and measured with a vernier calliper. The mussels were then opened, and the edible tissue from each specimen (16 males and 16 females) was carefully removed with the aid of scissors and tweezers. Gender identification was determined by visually inspecting the gonads, with males displaying a white-cream coloration and females showing an orange-pink hue. Each sample was individually homogenized using a Tissue Master 125 (USA) in 3 mL of phosphate-buffered saline (PBS; 140 mM NaCl, Panreac, Spain; 10 mM Na_2HPO_4, Sigma-Aldrich; 3 mM KCl, Merck, Germany; 2 mM KH_2PO_4, Sigma-Aldrich, Germany). The resulting lysate was transferred to 1.5 mL microtubes and centrifuged at 15,000 x g for 10 minutes at 4 ºC using a VWR centrifuge (Hitachi Koki Co., Ltd). The pellet from each sample was discarded, and the supernatant was transferred to new 1.5 mL microtubes (in duplicate) and stored at -45 ºC for subsequent biomarker analysis. Samples were thawed as required for each assay, with all procedures performed on ice to preserve sample integrity.

Total protein content

The total protein content of each sample was measured using the Bradford method (Bradford, 1976).

The Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 (BIO-RAD, USA) in 100 mL of ethanol (Honeywell, Germany) and 100 mL of phosphoric acid (H_3PO_4; Sigma-Aldrich, Germany) and then diluting the solution to a final volume of 1 L with distilled water. A stock solution of Bovine Serum Albumin (BSA; Nzytech, Portugal) was used to prepare standards with concentrations ranging from 0 to 4 mg/mL of BSA in PBS buffer (pH 7.2 ± 0.1) to generate a calibration curve. In a 96-well microplate (Greiner Bio-One GmbH, Austria), 20 µL of each standard and sample was added, followed by 180 µL of Bradford reagent to bring the total volume in each well to 200 µL. The absorbance was measured at 595 nm using a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA). The total cytosolic protein was determined as $mg \cdot L^{-1}$ for biomarker's normalization.

2.3.2.4 Biomarker Analysis

2.3.2.4.1 Glutathione-S-Transferase (GST) Activity

The protocol originally described by W. H. Habig et al.(Habig et al., 1974) was adapted and optimised for use with 96-well microplates(Copeto, Ganço, Ferreira, Silva, et al., 2024). GST activity was determined based on the conjugation reaction between the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and reduced L-glutathione (GSH), forming the GS-DNB complex, which interacts with GST in the sample. To prepare the substrate solution, 19.6 mL of PBS buffer (pH 7.2 ± 0.1) was mixed with 200 µL of 200 mM GSH (Sigma-Aldrich, St. Louis, MO, USA) and 200 µL of 100 mM CDNB (Sigma-Aldrich, St. Louis, MO, USA). In a 96-well microplate, 20 µL of each sample or blank (PBS or H_2O) was added in triplicate, and the volume in each well was adjusted to 200 µL with the substrate solution. Absorbance was measured at 340 nm using a microplate reader (Synergy HTX, BioTek, Winnoski, VT, USA) every minute for 6 minutes. The specific activity of GST was calculated using the molar extinction coefficient of CDNB (5.3 mM^{-1} cm^{-1}). GST activity results were normalized to the cytosolic protein content and expressed as nmol/min/mg protein.

2.3.2.4.2 Superoxide Dismutase (SOD) Activity

This assay was based on the indirect nitroblue tetrazolium (NBT) method previously described by Zhou et al. (Zhou & Prognon, 2006) and adapted and optimized for 96-well microplates (Copeto, Ganço, Ferreira, Silva, et al., 2024). In a 96-well microplate, each well received 200 µL of 50 mM potassium phosphate buffer (pH 8.0), 10 µL of 3 mM EDTA (Riedel-Haën, Seelze, Germany), 10 µL of 3 mM xanthine (Sigma-Aldrich, China), 10 µL of 0.75 mM NBT (Sigma-Aldrich, Germany), and 10 µL of the sample. The reaction was initiated by adding 10 µL of XOD (Sigma-Aldrich, Germany), and absorbance was measured every 2 minutes over 20 minutes using a microplate reader (Synergy HTX, BioTek, Winnoski, VT, USA). Results were normalized to cytosolic protein content and expressed as U/mg protein.

2.3.2.4.3 Catalase (CAT) Activity

This assay was conducted using a method previously described by Johansson & Borg (1988), adapted and optimized for 96-well microplates (Copeto et al., 2024a). A calibration curve was prepared using formaldehyde standards (0–75 µM) in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1% BSA. 50 µL of the sample, 30 µL of methanol (Honeywell, Germany), and 70 µL of potassium phosphate buffer (100 mM; pH 7.0; Sigma) were combined in each well. The reaction was initiated by adding 20 µL of 0.035 M hydrogen peroxide (Sigma-Aldrich, Germany), to each well, followed by a 20-minute incubation at room temperature on an orbital shaker (Optic Ivymen System, JP Selecta, Barcelona, Spain) in the dark. Next, 30 µL of 10 M potassium hydroxide (KOH; ChemLab, Zedelgem, Belgium) and 30 µL of purpald (32.4 mM in 0.5 M HCl; purpald; Aldrich, Germany) were added, followed by an additional 10-minute incubation under the same conditions. Finally, 10 µL of potassium periodate (65.2 mM in 0.5 M KOH) was added, and the mixture was incubated for 5 minutes in the dark. Absorbance was measured at 540 nm using a Synergy HTX microplate reader. Results were normalized to cytosolic protein content, and CAT activity was expressed as nmol/min/mg protein.

2.3.2.4.4 Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) was assessed by quantifying malondialdehyde (MDA), a by-product of LPO, using the Thiobarbituric Acid Reactive Substances (TBARS) assay (Ghani et al., 2017). A calibration curve was prepared with malondialdehyde (MDA; Merck, Germany) standards (0–0.1 µM) in ultrapure Milli-Q water. In 1.5 mL microtubes, 5 µL of each sample or standard was added to 295 µL of a solution consisting of 2.25 mL of PBS buffer (pH 7.2 ± 0.1), 0.625 mL of 8.1% (w/v) sodium dodecyl sulphate (SDS; Sigma-Aldrich, Germany), 4.675 mL of trichloroacetic acid (20% (w/v); TCA; Panreac, Spain), 4.675 mL of thiobarbituric acid (1% w/v; TBA; Sigma-Aldrich, Germany), and 2.525 mL of ultrapure Milli-Q water. The mixture was centrifuged at 3000 rpm for 1 minute, followed by incubation in a dry bath (Digital Thermoblock, Labnet) at 100ºC for 10 minutes. After cooling on ice, 62.5 µL of ultrapure Milli-Q water was added to each microtube, and 150 µL of each sample or standard was transferred to a microplate well. Absorbance was measured at 530 nm using a Synergy HTX microplate reader. Results were normalized to cytosolic protein content, with MDA concentrations expressed as nmol/min/mg protein.

2.3.2.4.5 Total Ubiquitin (UBI)

Ubiquitin quantification was conducted using an ELISA (Enzyme-Linked Immunosorbent Assay) method (Njemini et al., 2005). A calibration curve was generated using ubiquitin standards (UBPBIO-Ubiquitin-Proteasome Biotechnologies, USA) with concentrations ranging from 0 to 0.8 µg/mL. For each well, 50 µL of either sample or standard was added to the microplate, followed by overnight incubation at 4ºC. After incubation, the microplate was washed twice with a 0.05% Tween-20 solution prepared in PBS buffer (pH 7.2 ± 0.1). Subsequently, 100 µL of a blocking solution containing 1% BSA in PBS was added to each well, and the plate was incubated at 37ºC for 90 minutes. The plate was then washed three times with the Tween-20 solution. A primary ubiquitin antibody solution (P4D1; Sc-8017; Santa Cruz Biotechnology, Dallas, TX, USA) was prepared by diluting it in PBS buffer (pH 7.2 ± 0.1) containing 1% BSA to a final concentration of 1 µg/mL. Then, 50 µL of the primary antibody was added to each well, and the plate was incubated overnight at 4ºC.

Following this incubation, the microplate was washed three times, and 50 µL of secondary antibody (anti-mouse IgG Fc specific-alkaline phosphatase, Sigma-Aldrich, Germany) diluted in PBS buffer (pH 7.2 ± 0.1) containing 1% BSA to a final concentration of 1 µg/mL was added to each well. The plate was incubated at 37ºC for 90 minutes. Afterwards, the microplate was rewashed, and 50 µL of substrate solution (containing 157 mg of Tris-HCl [Sigma, USA], 58 mg of NaCl [Panreac], 50 µL of 5 mM $MgCl_2$ [Fluka, BioChemika, Buchs, Switzerland], and 10 mg of disodium 4-nitrophenyl phosphate hexahydrate [pNPP; Sigma-Aldrich, Gillingham, UK] prepared in 10 mL of distilled water at pH 9) was added to each well. The plate was incubated at room temperature for approximately 15 minutes. Finally, a stop solution (3M NaOH; Panreac, Spain) was added, and absorbance was measured at 405 nm using a microplate reader (Synergy HTX, BioTek, Winnoski, VT, USA). Results were adjusted to cytosolic protein content and expressed as U/mg protein.

2.3.2.4.6 Vitellogenin (VTG)

The quantity of vitellogenin (VTG) was determined by measuring the amount of inorganic phosphate, using an indirect method described by (Atkinson et al., 1973) adapted Standards with varying concentrations of inorganic phosphate (0 to 50 µM; Sigma-Aldrich, USA) were prepared to generate a calibration curve. The procedure began with extracting free phosphates from the sample using tertbutyl methyl ether (Sigma-Aldrich, USA). For this, 1 mL of the sample was mixed with 1 mL of the organic solvent and agitated for 30 minutes. The aqueous phase was then discarded. In the next step, 800 µL of the organic phase was combined with 200 µL of 1M NaOH and incubated at 50 ºC for 1 hour. For quantification, 25 µL of aqueous phase was transferred to a well of a microplate (Synergy HTX, BioTek, Winooski, VT, USA), in triplicates with 35 µL of vanadate molybdate reagent (Sigma-Aldrich, USA) diluted 1 for 2 and 140 µL of MilliQ water. Absorbance was measured at 410 nm using a microplate reader. Results were normalized to cytosolic protein content and expressed as µg/mg protein.

2.3.2.5 Water Quantification

2.3.2.6 Sample Preparation and Derivatization

Extraction was carried out using a volume of 10 mL of water sample (from the experimental aquaria) and placed into a 15 mL glass conical test tube and spiked with internal standard (Ethynylestradiol (20,21-$^{13}C_2$, 99%) ($EE2d_2$) from Cambridge Isotope Laboratories, Inc, MA, USA) (0.050 µg L^{-1}) final concentration.

The derivatization involved the addition of, 600 µL of potassium carbonate 5% (K_2CO_3, (99.0% purity) from Panreac Química SA.), 10 µL of pyridine (Sigma-Aldrich ≥99.5% purity), 200 µL of acetic anhydride was obtained from Sigma-Aldrich LiChropur (≥99% purity). The mixture was vortexed and allowed to react for 5 minutes at room temperature. After derivatization, the analytes were extracted by the dispersive liquid–liquid microextraction (DLLME) technique in which EE2 was extracted quick injection of 70 µL chloroform ($CHCl_3$ form Supelco EMSURE) (extraction solvent), resulting in a cloudy solution. This solution was vigorously shacked by hand. Finally, the conical tubes were centrifuged at 2000 rpm and 4 °C for 10 min. Dispersive particles were collected at the bottom of the centrifuge tube by a glass Pasteur pipette with latex bulb. The resulting organic phase was transferred to a microinsert of 100 µL and injected in gas chromatography–mass spectrometry (GC–MS) for analysis.

2.3.2.7 Gas Chromatography–Mass Spectrometry (GC/MS) Analysis

The gas chromatograph Shimadzu GCMS-QP2010 Gas Chromatograph Mass Spectrometer equipped with an autoinjector AOC-5000 was used to out the chromatographic analyses. Injections of 1 µL were made in the splitless mode with a 2.0 min purge-off time, and the injector temperature was set at 275 °C. Helium (99.9999%), at a constant flow rate of 2 mL min^{-1} was used as the carrier gas. Separation was performed using a fused-silica capillary column Zebron ZB-5MS W/Guardian (Phenomenex) coated with 5% polysilarylene and 95% of polydimethylsiloxane (30 m × 0.25 mm ID, 0.25 µm film thickness) with 10 m of Guardian capillary column but not with stationary phase.

The analysis was done following the oven program temperature: initial temperature 80 °C (held for 2 min), increased by 20 °C min^{-1} to 220 °C (held for 17 min), next increased by 20 °C min^{-1} to 250 °C and held at this temperature for 7 min, and increased again by 15 °C min^{-1} to 305 °C, and held at this temperature for 5 min. The transfer line was set at 275 °C and ion source at 200 °C with an electron impact ionization of 70 eV. The MS system was routinely set in selective ion monitoring (SIM) mode, and each analyte was quantified based on peak area using one target and two qualifier ion(s). For EE2 SIM ions m/z 296 for quantification and 338; 213 for qualifiers, in case of $EE2d_2$ SIM ions m/z 298 for quantification and; 340; 252 for qualifiers, with a retention time of 28.90 min and 28.88 min, respectively.

2.3.2.8 Calibration Curve and Quantification

Calibration curves and respective linearity parameters were obtained through matrix-matched calibration in water sample from the experimental aquaria. Appropriate volumes of the standard solution containing EE2 and $EE2d_2$ were added to blank samples (free of EE2 residues), spiked at six concentration levels (ranging from 10-500 $ng.L^{-1}$). Calibration curves were constructed by plotting the $EE2/EE2d_2$ ratio obtained against the concentration of the analyte. The limit of quantification (LOQ) was set at 10 $ng.L^{-1}$, corresponding to the lowest calibration standard. The calibration curve demonstrates a good linearity across the all range ($r^2 \geq 0.999$). Recovery tests were made by spiking samples with EE2 standard before extraction and derivatization. The experimental recovery was obtained from the difference between two measurements (sample and spiked samples). These samples were spiked with the target compounds at two concentration levels (250 $ng.L^{-1}$ and 50 $ng.L^{-1}$), recovery following the addition of 250 $ng.L^{-1}$ was 99.4% and Recovery following the addition of 50 $ng.L^{-1}$ was 103.6%.

2.3.2.9 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 9 software (version 9.5.1). The Kolmogorov-Smirnov test was employed to assess the normality and homogeneity of variances. Following this, ANOVA was performed, and Tukey's HSD test was subsequently applied to identify significant differences ($p < 0.05$) between the control and various exposure concentrations and among the different exposure concentrations.

2.3.3 Results

Mortality Rate

Exposure of mussels to different concentrations of EE2 did not result in significant mortality. A cumulative mortality of five deaths (16%) was recorded, corresponding to 12.5% in the control group, 12.5% at 10 ng·L^{-1}, 25% at 30 ng·L^{-1}, and 12.5% at 300 ng·L^{-1}.

2.3.3.1 Biomarkers analysis

2.3.3.1.1 Glutathione S-Transferase (GST)

GST activity is presented in **Figure 2-6**, showing a significant increase in the EE2-exposed groups, with the highest activity observed at 300 ng·L^{-1}. Statistical significance (p< 0.01) confirms a strong EE2-induced effect.

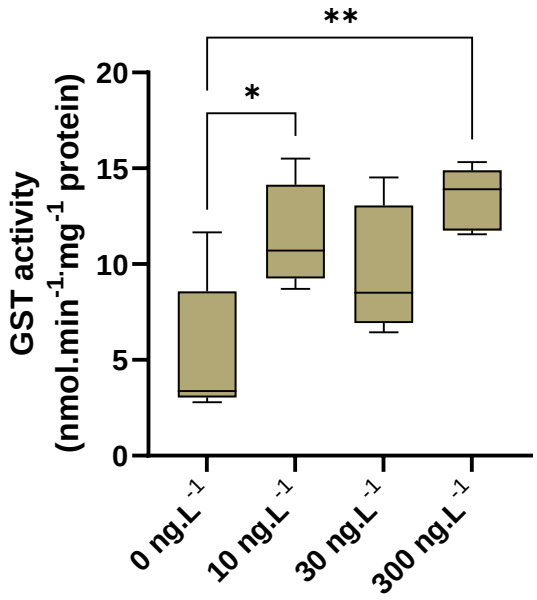

Figure 2-6 -GST activity in mussels exposed to different concentrations (0, 10, 30 and 300 ng·L^{-1}) of EE2.

2.3.3.1.2 Superoxide Dismutase (SOD)

The results of SOD activity (**Figure 2.7**) show an increase in response to EE2 exposure compared to the control group. The 300 ng·L^{-1} group exhibited the highest SOD activity (5688.4 U/mg protein), suggesting a dose-dependent response.

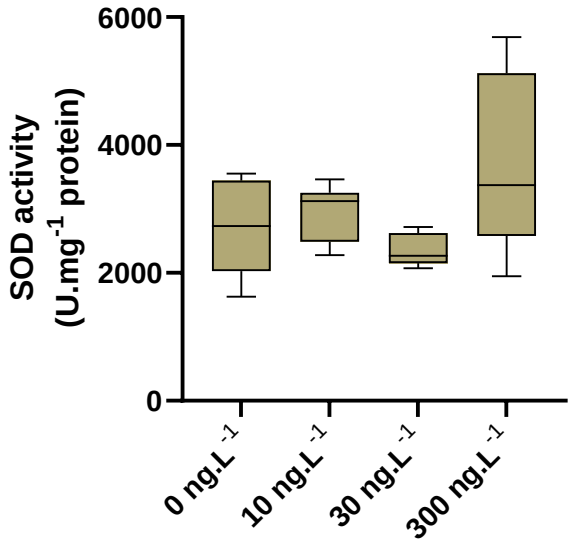

Figure 2-7 - SOD levels in mussels exposed to different concentrations (0, 10, 30 and 300 ng·L^{-1}) of EE2.

2.3.3.1.3 Catalase (CAT)

CAT activity is presented in **Figure 2-8**, showing a visible decrease from the control to 10 ng·L^{-1} (p< 0.05) and further to 30 ng·L^{-1} (p< 0.05). However, at 300 ng·L^{-1}, the value appears to remain similar to the control or even slightly increase, suggesting no decrease at the highest concentration.

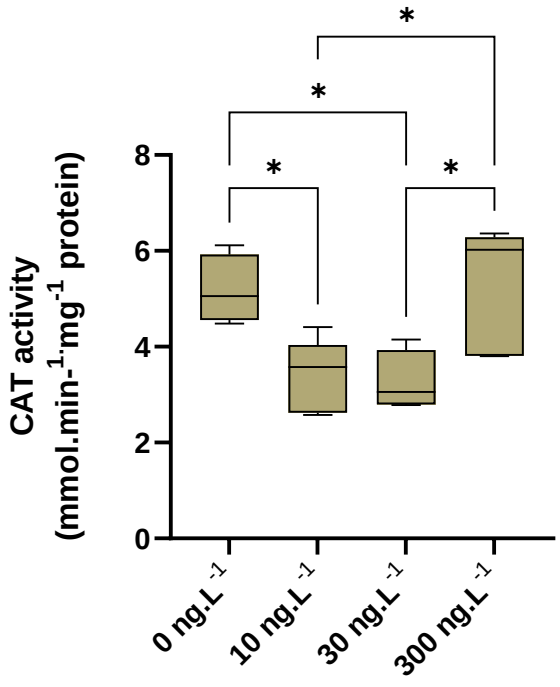

Figure 2-8 -- CAT activity in mussels exposed to different concentrations (0, 10, 30, and 300 ng·L^{-1}) of EE2.

2.3.3.1.4 Lipid Peroxidation (LPO)

The MDA results are presented in **Figure 2-9**, showing an increase in MDA levels with EE2 exposure concentrations, with the highest values observed in the 10 ng·L^{-1} group (0.0179 ng·L^{-1}).

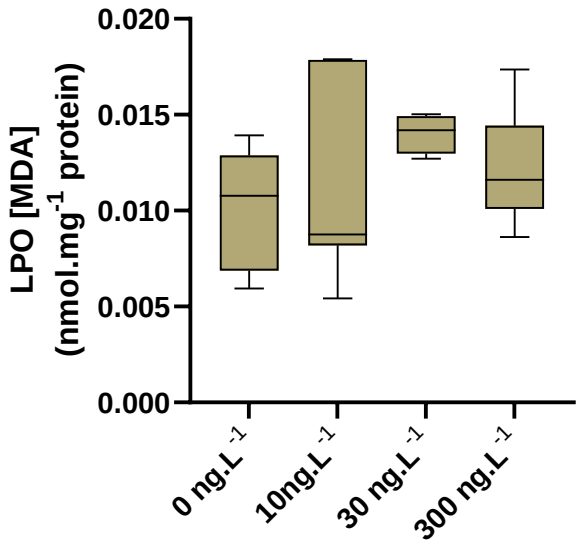

Figure 2-9 - MDA levels in mussels exposed to different concentrations (0, 10, 30, and 300 ng·L^{-1}) of EE2.

2.3.3.1.5 Ubiquitin (UBI)

The ubiquitin results are presented in **Figure 2-10**, showing a significant increase in response to the different EE2 exposure concentrations tested, with the highest levels observed at 300 ng·L^{-1} (0.109 µg/mg). The 10 ng·L^{-1} showed the lowest ubiquitin levels (0.0101 µg/mg).

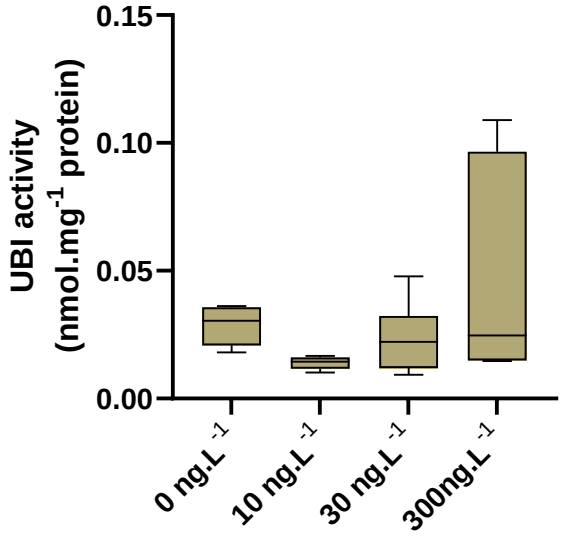

Figure 2-10 - UBI levels in mussels exposed to different concentrations (0, 10, 30, and 300 ng·L^{-1}) of EE2.

2.3.3.1.6 Vitellogenin (VTG)

In **Figure 2-11**, Vitellogenin (VTG) levels increased in a dose-dependent manner following EE2 exposure. These results demonstrate a clear estrogenic response, with VTG induction most pronounced at the highest EE2 concentration tested.

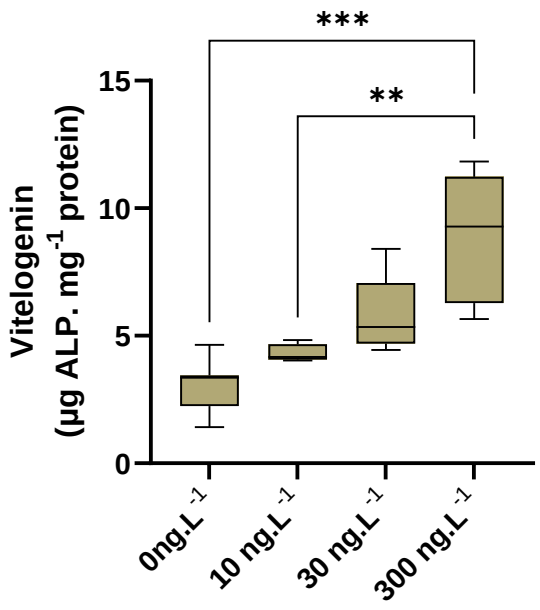

Figure 2-11 -VTG levels in mussels exposed to different concentrations (0, 10, 30, and 300 ng·L^{-1}) of EE2.

2.3.3.2 Water analyses

Table 2.3-1 - Results of EE2 determined in water samples.

Condition	T0h	24h	48 h
Control	- ND	-	-
10 ng·L^{-1} EE2	11.6±0.8	9.9±0.5	10.8±1.4
30 ng·L^{-1} EE2	-	23.6±2.6	25.4±3.9
300 ng·L^{-1} EE2	303.1±49.1	294.2±26.7	305.8±8.4

Table 2.3-1 summarizes the concentrations of EE2 measured in aquarium water at nominal exposure levels of 10, 30, and 300 ng.L^{-1} over 48 hours. EE2 levels were assessed at T0h, 24h, and 48 h to evaluate compound stability throughout the exposure. No EE2 was detected in control tanks at any time point, confirming analytical specificity and the absence of background contamination. At 10 ng·L^{-1}, concentrations remained stable (11.6 ± 0.8 ng·L^{-1} at T0h; 10.8 ± 1.4 ng·L^{-1} at 48 h), suggesting minimal degradation. In the 30 ng·L^{-1} group, values ranged from 23.6 ± 2.6 ng·L^{-1} initially to 25.4 ± 3.9 ng·L^{-1} at 48 h, indicating moderate stability despite slight underestimation of the nominal dose. The highest concentration (300 ng·L^{-1}) showed excellent stability, with consistent values across all time points (T0h: 303.1 ± 49.1 ng·L^{-1}; 48 h: 305.8 ± 8.4 ng·L^{-1}). Overall, the data confirms the persistence and chemical stability of EE2 under the tested conditions.

2.4 Discussion

Studying the effects of xenobiotics on marine biota is crucial for understanding their impact on aquatic ecosystems, as these environments often experience stress conditions that lead to cellular and molecular alterations, potentially causing oxidative stress and subsequent cellular damage. These changes can be effectively monitored by analyzing effect or response biomarkers. This study examined the impact of exposure to varying concentrations of EE2 on mussels (*M. galloprovincialis*). EE2, widely recognized in the literature as a potent endocrine disruptor, is prevalent in the marine environment due to its extensive use by humans, particularly as the active ingredient in female contraceptives (Dias et al., 2019; Katsiadaki et al., 2021).

Mortality is a commonly widely used endpoint to assess the severity of adverse effects of a given xenobiotic. This study selected EE2 concentrations (0, 10, 30, and 300 ng.L^{-1}) to reflect the range typically observed in aquatic environments, ensuring that the responses observed are representative of real-world exposure scenarios.

After 28 days of exposure to the different EE2 concentrations, no significant mortality was observed, indicating that the tested concentrations did not exhibit sufficient toxicity to cause death in the mussels. In total, one dead mussel was recorded in the control group and also in those exposed to 10 ng·L^{-1}, 300 ng·L^{-1}, and two in mussels exposed to 30 ng·L^{-1}.

Previous studies have also shown that these concentrations do not induce mortality in this species, even when combined with other compounds such as sodium lauryl sulphate (Lopes et al., 2022) and salicylic acid (Cunha et al., 2023). The lack of significant mortality at these concentrations is consistent with findings from other authors, including Islam et al. (2020), who reported no mortality at concentrations up to 1.25 x 10^6 ng.L^{-1} of EE2 in the oyster *Saccostrea glomera*ta (Islam et al., 2020). Similarly, Leonard et al. (2014) observed no mortality in *Lampsilis fasciola* mussels exposed to 1000 ng.L^{-1} of EE2, and Lopes et al. (2022) found absence of mortality in *M. galloprovincialis* exposed to concentrations up to 625 ng.L^{-1} of EE2 (Lopes et al., 2022).

Conversely, studies conducted on different species like zebrafish *Danio rerio* (Xu et al., 2008) and *Gobiocypris rarus* (Zha et al., 2008) have shown significant mortality at concentrations as low as 10 ng.L^{-1}. In zebrafish, exposure to 10 ng·L^{-1} resulted in >60% mortality over 90 days, whereas in rare minnows, exposure to 4 ng·L^{-1} caused 0% adult mortality but led to complete reproductive failure and severe gonadal alterations. This could be explained by the fact that bivalves have some protective mechanisms against exposure to this compound, such as reducing their filtration rate or keeping their shells closed for extended periods in the presence of xenobiotics, unlike other marine organisms (Lopes et al., 2022).

Furthermore, it is well known that bivalves of the genus *Mytilus* are used as sentinel organisms due to their resilience to contaminant exposure (Ben Cheikh et al., 2018). This adaptive capability may contribute to the reduced toxicity observed in mussels exposed to the different concentrations of EE2, potentially linked to an effective antioxidant defence response. However, previous studies have demonstrated that bivalves, can lower their metabolic rate, under mild stress conditions, thereby decreasing contaminant uptake and limiting adverse effects (Lopes et al., 2022).

Exposure to EE2 elicited clear changes in the antioxidant defence system of *M. galloprovincialis*. CAT, SOD, and GST activities were all altered relative to controls after 28 days, suggesting an oxidative stress response. The pattern of response was non-monotonic in several cases, CAT and GST showed a biphasic trend, with significant induction at the lowest (10 ng.L^{-1}) and highest (300 ng.L^{-1}) EE2 concentrations, but little change at 30 ng.L^{-1}. SOD activity increased mainly at the highest dose, suggesting a threshold effect for its activation.

Such non-linear dose–response behaviour is not uncommon for endocrine-active contaminants, which can elicit biphasic responses (Koutsogiannaki et al., 2014).

SOD is a crucial antioxidant enzyme that detoxifies superoxide radicals (O_2^-). The increased SOD activity suggests that EE2 induces oxidative stress, leading to an upregulation of the antioxidant defence system. The trend suggests that higher EE2 concentrations trigger a more pronounced oxidative stress response.

In our study, low EE2 levels appeared sufficient to trigger antioxidant defences, perhaps as an adaptive response, while an intermediate exposure (30 $ng.L^{-1}$) did not significantly deviate from baseline activity. At the highest EE2 concentration, a pronounced oxidative challenge re-engaged antioxidant enzyme, although CAT activity at 300 $ng.L^{-1}$ rose less than expected from the low-dose trend, potentially reflecting partial enzyme inhibition or saturation of the response under extreme stress.

CAT activity exhibited a significant increase at 10 $ng.L^{-1}$ EE2 and remained elevated, though not linearly at 300 $ng.L^{-1}$, with a dip in the 30 $ng.L^{-1}$ group. These results suggest an initial upregulation of hydrogen peroxide scavenging at a low EE2 dose, a plateau or slight suppression at a moderate dose, and a renewed induction under high-dose stress. One plausible mechanism is that low doses of EE2 generate mild reactive oxygen species (ROS) production or trigger signaling (e.g. via Nrf2 or estrogen-receptor related pathways) that enhances CAT expression. Similar activation of antioxidant enzymes in bivalves at sub-toxic pollutant levels has been documented; for example, chronic EE2 exposure in mussels was reported to elevate overall antioxidant enzyme activities compared to controls (Cunha et al., 2023).

The reduced CAT response observed at 30 $ng.L^{-1}$ compared to the lower dose could indicate that excessive ROS or direct toxic effects at high EE2 impair enzyme function. Indeed, prior studies have noted that under high pollutant stress, antioxidant enzymes can be inhibited or their induction is blunted (Cunha et al., 2023).

Lopes et al. (2022) observed inhibition of CAT and SOD in mussels at the highest concentrations of EE2, when in mixture with a surfactant, attributing this to overwhelming stress levels. Thus, the non-linear CAT activity in our experiment likely reflects a balance between inducive signaling at low exposure and enzyme saturation or damage at high exposure.

SOD activity remained near to control levels at 10 and 30 ng.L^{-1} but rose significantly at 300 ng.L^{-1}, indicating that only the highest EE2 dose generated sufficient superoxide stress to trigger a marked SOD response.

Cunha et al. (2023) similarly found that *M. galloprovincialis* exposed to EE2 (at ~100 ng.L^{-1} for 28 days) had enhanced SOD activity relative to controls reflecting activation of anti-oxidant defenses by estrogenic exposure In our case, the absence of a significant SOD change at 30 ng.L^{-1} could be due to the lower concentrations tested compared to those used in the studies conducted by Cunha et al.

The coordinated increase of SOD (at 300 ng.L^{-1}) with CAT and GST implies a concerted antioxidant defence deployment. Notably, SOD activity can be inhibited if stress is extreme, as observed in studies with mixtures exposures (Cunha et al., 2023), but in our single-compound exposure the mussels were able to mount a significant SOD response at the top dose. This indicates that 300 ng.L^{-1}, while high, was within the organism's capacity to respond adaptively rather than causing outright enzymatic inhibition. Such activation of SOD at high EE2 is in accordance with observations in other aquatic organisms (e.g. fish embryos), where EE2 induces oxidative stress at ≥100 ng.L^{-1} (Ramírez-Montero et al., 2022).

It underscores that EE2's pro-oxidant effects become apparent at elevated concentrations, potentially through mechanisms such as metabolic redox cycling or mitochondrial disruption. GST activity showed a pattern akin to CAT, with significant induction at 10 ng.L^{-1} and 300 ng.L^{-1}, but no notable change at 300 ng.L^{-1}.

GST is involved in detoxification, helping cells conjugate toxic compounds with glutathione (GSH) for elimination. The increase in GST activity suggests that EE2 induces detoxification mechanisms, likely due to oxidative stress and xenobiotic stress responses. The parallel increase in MDA and GST implies that cells attempt to mitigate oxidative damage through increased detoxification activity. Such induction of GST at low xenobiotic levels is a common adaptive response, enhancing the organism's capacity to eliminate the chemical before it causes damage (Belmokhtar et al., 2024).

Our findings are in line with other studies reporting increased GST activity in bivalves exposed to pharmaceuticals or estrogenic compounds. Cunha et al. (2023) found that mussels exposed to EE2 alone had significantly higher GST activity than controls. Interestingly, they

noted that when EE2 was combined with salicylic acid, GST induction was suppressed, suggesting an antagonistic interaction, whereas under single-compound exposure GST was a primary detoxification route.

In our single-compound scenario, the enhanced GST at 300 ng.L^{-1} implies an intensified detoxication response at high EE2 burden, likely conjugating EE2 metabolites or oxidative by-products to glutathione for excretion. The lack of GST upregulation at 30 ng.L^{-1} could indicate that this concentration was adequately managed by baseline detoxification capacity. It is also possible that at 30 ng.L^{-1}, mussels relied more heavily on alternative pathways, such as Phase I metabolism or glucuronidation, rather than GST-mediated conjugation. The dual peaks in GST activity (at 10 and 300 ng.L^{-1}) reinforce the notion of a biphasic response, a modest stress can prime the system, low-dose stimulation of GST, and a much larger stress demands maximal enzyme deployment, while intermediate stress may not sufficiently trigger additional GST production if baseline levels are already coping.

The observed pattern of CAT, SOD, and GST responses together demonstrates that *M. galloprovincialis* engages a multi-faceted antioxidant strategy against EE2. This agrees with the broader literature that pollutants like EE2 elicit integrated antioxidant and biotransformation defences in bivalves (Cunha et al., 2023).

Despite the activation of oxidative stress defences, indicators of cellular damage, like UBI levels and MDA levels, showed no statistically significant changes across EE2 treatments.

In the present study, concentrations of 10 to 300 ng.L^{-1} EE2 did not cause measurable lipid peroxidation in mussel tissues, nor an accumulation of ubiquitinated proteins. These findings suggest that the antioxidant and detoxification responses were largely successful in mitigating oxidative injury over the 28-day exposure.

The trend toward a slight UBI increase at 300 ng.L^{-1} suggests that some protein damage may have begun to occur under the highest EE2 exposure, though not enough to reach statistical significance. This subtle rise in ubiquitin-conjugated proteins could serve as an early warning of proteotoxic stress that might develop into significant damage if the exposure concentration or duration were increased.

Nonetheless, over the 28-day period, EE2 concentrations up to 300 ng.L^{-1} did not overwhelm the mussels' capacity to maintain proteome integrity and membrane stability.

Our findings align with studies showing that moderate, sub-lethal EE2 exposure can trigger antioxidant defences without causing immediate oxidative damage. Cunha et al. (2023) reported that mussels chronically exposed to EE2 exhibited elevated antioxidant enzyme activities (SOD, GPx, GST) but showed no significant increase in lipid peroxidation or protein carbonylation compared to controls. Only when EE2 was combined with another stressor (salicylic acid) did those authors observe marked lipid and protein oxidative damage, highlighting that EE2 by itself at environmentally relevant levels may not cause overt cellular injury .

This aligns well with our observation that single-compound exposure to EE2 did not elicit MDA or ubiquitin changes, whereas antioxidant enzymes were induced. It appears that *M. galloprovincialis* can effectively counteract the ROS produced by exposure to EE2 or estrogenic disturbance, thereby preventing the chain reactions that lead to lipid peroxidation and protein oxidation. In contrast, if the ROS production exceeds antioxidant capacity, one would expect to see increases in MDA and oxidatively damaged proteins. For example, Canesi et al. (2008) found a significant MDA rise in mussel digestive gland tissue upon exposure to a low-dose mixture of EDCs (including EE2), which coincided with a depression of catalase transcription.

In that short-term study, a 175% increase in MDA was observed at an intermediate mixture concentration, suggesting that under certain exposure scenarios, complex mixtures or possibly acute spikes, antioxidant defences can be insufficient and lipid peroxidation ensues. The negative correlation they reported between MDA and catalase activity indicates that when antioxidant defences are compromised, oxidative damage quickly becomes evident (Canesi et al., 2008).

In contrast, our 28-day exposure to single EE2, resulted in upregulation catalase and other enzymes, likely preventing such damage. These comparisons highlight the importance of context: mixture vs single pollutant, acute vs chronic, is critical in determining outcomes. *M. galloprovincialis* exposed to EE2 alone under controlled laboratory conditions managed to avoid oxidative injury, whereas in more complex or extreme conditions damage markers can became significantly elevated (Belmokhtar et al., 2024).

Mechanistically, the lack of elevated MDA and UBI levels in EE2 exposed mussels may reflect an effective adaptive or compensatory cellular response. The induced antioxidants (CAT,

SOD, GST) and possibly other protective systems (e.g. glutathione peroxidase, which often works alongside CAT, and molecular chaperones) likely quenched the ROS before they could damage lipids or proteins.

Moreover, mussels have efficient repair and removal mechanisms: mild oxidative damage to macromolecules can be repaired (e.g. via phospholipid hydroperoxide glutathione peroxidases repairing peroxidized lipids) or the damaged molecules degraded(Fernández et al., 2010). Ubiquitin tagging of damaged proteins followed by proteasomal degradation, is a key mechanism for maintaining cellular protein homeostasis.

In this study, the relatively stable ubiquitin levels across treatments suggest that the proteasome system was not markedly strained in mussels exposed to EE2. Even at the highest exposure concentration tested (300 $ng.L^{-1}$), oxidatively damaged proteins were likely degraded efficiently, keeping ubiquitin-protein conjugate levels steady. The slight, but non-significant increase in ubiquitin levels in mussels exposed to 300 $ng.L^{-1}$, may signal the early onset of proteome stress; however, the data suggests that the mussels' cellular machinery maintained an effective compensatory response, preventing any substantial accumulation of ubiquitinated proteins.

It is also worth noting that *M. galloprovincialis* is a relatively robust species that frequently endures fluctuations in oxygen levels, pollutants, and other stressors in coastal environments (Freitas et al., 2017; Gostyukhina et al., 2023; Pizzurro et al., 2024). They possess constitutively high antioxidant baseline levels, as an adaptation to intertidal oxidative challenges (Letendre et al., 2009), which might contribute to the resilience observed in the present study.

Although the EE2 concentrations tested are considered high from a contamination standpoint, they appear to fall within the adaptive range of the mussels' physiology over the 28-day exposure period. It is possible that prolonged exposure, especially during energetically demanding periods such as reproduction, could exceed the mussels' capacity for compensation and result in chronic damage. However, within the exposure period of this study, the lack of significant MDA and UBI changes is a reassuring indication that EE2 did not surpass the threshold necessary to cause measurable cellular damage in exposed mussels.

This finding suggests that biomarker responses, like enzyme induction, can be early warning signals occurring at exposures that are not yet causing harm at the tissue level.

Environmental monitoring programs often rely on such early indicators to act before lethal or sub-lethal damage occurs (Report, 2014). Our results exemplify this principle, with oxidative stress biomarkers activated in the absence of damage, highlighting a window of compensatory adaptation.

The study of EE2´s effects primarily focuses on its estrogenic potency, which can be evaluated by measuring the concentration of VTG (vitellogenin) in the samples, as numerous studies have shown a strong correlation between exposure to this xenobiotic and VTG expression (Andrew et al., 2008; Ciocan et al., 2010).

In our study, Vitellogenin-like (VTG-like) protein levels increased in a dose-dependent manner upon EE2 exposure, confirming the estrogenic mode of action of this contaminant. In control mussels, VTG-like protein levels remained at baseline, as expected for presumably male or non-reproductive individuals, whereas exposure to 10, 30, and especially 300 $ng.L^{-1}$ EE2 led to progressively elevated VTG-like concentrations.

The induction was statistically significant at 300 $ng.L^{-1}$, where VTG-like protein levels were markedly higher than in control group and significantly above those in the 10 $ng.L^{-1}$ group. At 10 and 30 $ng.L^{-1}$, VTG-like levels showed upward trends, although not significant ($p > 0.05$). This pattern suggests a threshold around or just below 100 $ng.L^{-1}$, beyond which mussels begin to express abnormally elevated levels of VTG-like proteins. At 300 $ng.L^{-1}$ EE2, the endocrine disruption is clear, with mussels synthesizing VTG-like proteins in response to the EE2 exposure. These findings mirror the well-known estrogenic effects of EE2 in other aquatic organisms, extending the evidence to bivalves.

While fish are typically more sensitive and show VTG induction at much lower concentrations, our results show that mussels also possess molecular machinery to respond to xenoestrogens by upregulating vitellogenin or its analogues. The observed dose-dependent response, with 300 $ng.L^{-1} \gg 30\ ng.L^{-1} > 10\ ng.L^{-1} \approx$ control is consistent with EE2 acting via oestrogen receptor-mediated pathways. Higher EE2 concentrations likely result in greater receptor occupancy or activation, driving more VTG gene expression and protein production.

Notably, even the lowest tested dose (10 $ng.L^{-1}$) elicited a near-significant rise in VTG-like proteins, suggesting that *M. galloprovincialis* can respond to EE2 at environmentally relevant concentrations on the order of tens of $ng.L^{-1}$.

Although 10 ng.L^{-1} did not cause a significant induction in the present study, the slight increase suggests that a subtle effect may be present. Prolonging exposure or increasing replication might reveal a significant response even at 10 ng.L^{-1}.

These results agree with field and laboratory studies indicating that bivalves produce VTG-like proteins when exposed to estrogenic substances. In male mussels, which normally have the VTG gene silenced, exposure to exogenous oestrogen or oestrogen mimics can induce the expression of this female-specific protein. For instance, males of *M. galloprovincialis* collected from polluted canals in Venice were found to have significantly elevated VTG-like protein in their haemolymph compared to those from reference sites, indicating exposure to environmental xenoestrogens. Interestingly, females at those sites did not show a comparable increase since they naturally undergo vitellogenesis during that period (Gagné et al., 2001).

This sex-specific difference observed in the field study (males accumulating abnormal VTG, while females showed normal levels) underscores that the VTG induction is a contaminant-driven effect rather than just seasonal variation, essentially showing a feminization effect in males. Our laboratory findings replicate the core aspect of such observations under controlled conditions with a known estrogenic compound. Likewise, long-term *in situ* exposures of mussels to treated sewage effluents have shown significant increases in vitellogenin-like proteins in both sexes.

Quinn(2004) exposed zebra mussels (*Dreissena polymorpha*) to tertiary-treated municipal effluent for 112 days and observed a clear increase in VTG, measured as ALP, in exposed mussels compared to those upstream of the outfall (Quinn et al., 2004). They confirmed by electrophoresis that the ALP signal corresponded to vitellin-like proteins, solidifying the evidence of endocrine disruption in those mussels. Notably, the effluent in that study contained a mixture of estrogenic chemicals, with 17β-oestradiol, EE2, and bisphenol A identified as major contributors to its estrogenicity.

The agreement between Quinn (2004) field study and our findings are noteworthy, as it suggests that EE2 alone can account for a significant portion of the estrogenic effect observed in effluent-exposed mussels. In both cases, after roughly 1 to 4 months of exposure, mussels showed elevated vitellogenin-like proteins, confirming that their endocrine system is sensitive to estrogenic stimulation.

However, discrepancies exist regarding how readily molluscs elicit a VTG response to oestrogens, often due to methodological differences. Earlier studies frequently relied on indirect assays like ALP to infer VTG levels. These studies commonly reported increases in response to estrogen or wastewater effluent exposure (Gagné et al., 2001; Quinn et al., 2004).

In our study, we used a vitellogenin-like protein assay, where a clear dose- dependent induction was observed. In contrast, more recent work by Morthorst (2014) raised concerns about false positives in molluscan VTG measurement. They exposed freshwater mussels (*Unio tumidus*) to 17β-oestradiol for seven weeks and measured yolk protein levels using both ALP and a species-specific ELISA. The ELISA, which directly detects actual VTG protein, found no oestrogen-induced increase, even though baseline differences between females and males were detectable (Morthorst et al., 2014). Conversely, the ALP assay failed to differentiate between sexes and indicated elevated "VTG-like" levels regardless of treatment. This led the authors to conclude that the ALP based methods can be confounded by other phosphorylated proteins (Morthorst et al., 2014).

Our findings of significant VTG-like elevation at 300 $ng.L^{-1}$ EE2 are supported by the bulk of ecotoxicological evidence (especially from field studies) indicating that mussels and other bivalves exhibit vitellogenin responses to estrogenic EDCs. However, the lack of significant responses at 10–30 $ng.L^{-1}$ in our data, (despite a trend to increase) also resonates with the notion that mollusks are generally less sensitive than fish to these compounds.

For comparison, in fish (e.g. male rainbow trout or zebrafish), EE2 at 10–100 $ng.L^{-1}$ would cause massive induction of vitellogenin – often a several-fold or orders-of-magnitude increase (Quinn et al., 2004). In contrast, mussels exhibit only modest, though still biologically relevant, VTG responses at comparable concentrations. This reduced sensitivity may be attributed to a lower affinity of molluscan estrogen receptors for EE2, variations in endocrine signaling pathways, or the presence of limiting co-factors involved in vitellogenin synthesis.

Additionally, molluscan vitellogenesis is not solely under endocrine (oestrogen) control as it is in vertebrates; other signaling systems may modulate VTG production, possibly requiring a stronger or more prolonged stimulus to trigger a pronounced response.

Our results, in conjunction with prior studies, suggest that there is indeed a threshold concentration of estrogenic exposure below which VTG induction in bivalves is minimal and

above which it becomes detectable. In our mussels this threshold lies between 30 and 300 ng.L^{-1} for a 28-day exposure, whereas in more prolonged field exposures (3/4 months) even lower ambient levels (tens of ng.L^{-1} or less) have led to VTG elevation (Gagné et al., 2001; Quinn et al., 2004). It is possible that with longer exposure, the 30 ng.L^{-1} group in our study might have eventually shown significant VTG induction as well.

The biological implications of VTG-like protein induction in mussels are significant. Vitellogenin is an egg yolk precursor normally expressed in females during oogenesis, its abnormal presence in males or in females out of season indicates endocrine disruption and feminization. In mussels, such feminization could affect reproductive output. For example, Blaise *et al.* (2003) observed that caged freshwater mussels downstream of a sewage effluent had skewed sex ratios with a higher proportion of females, suggesting that chronic estrogenic exposure might impair male viability or alter sex determination (Blaise et al., 2003).

In our experiment, we did not assess reproductive endpoints or histopathology, but the induction of VTG-like proteins at high EE2 indicates that these mussels were experiencing an activation of the vitellogenic pathway. If sustained, this could divert energy from other physiological processes and potentially interfere with normal gametogenesis (e.g. intersex gonadal development or reduced sperm production in males). It's noteworthy that vitellogenin induction is a reversible biomarker, if the estrogenic pressure is removed, the organism can cease production (F. Ghanem, 2021) However, prolonged exposure throughout a reproductive cycle could lead to cumulative effects such as altered gamete quality or spawn timing. Thus, the VTG response we measured is an early sign of endocrine disturbance that could presage more profound reproductive effects if EE2 exposure were to continue or intensify.

Our study demonstrated that exposure of *M. galloprovincialis* to environmentally relevant concentrations of 17α-ethinylestradiol (EE2) led to significant activation of antioxidant enzymes (CAT, SOD, GST) without corresponding increases in lipid peroxidation (MDA) or ubiquitin (UBI), suggesting a compensatory mechanism that mitigated oxidative damage.
This aligns with evidence that moderate ROS levels are physiologically important, for example, in sperm capacitation (Philibert et al., 2023), whereas excessive ROS induced by endocrine disruptors like EE2 can impair sperm function (Aitken et al., 2012; Alahmar, 2019).

The concentrations of EE2 in the water across the tested exposure levels (10, 30, and 300 ng.L^{-1}) remained stable over the 48-hour period, demonstrating the compound's high persistence in the aquatic environment. This stability is essential for ecotoxicological studies, as it ensures that the biological responses observed are the result of consistent exposure to the intended dose.

The minimal variation observed at 10 ng.L^{-1} (from 11.6 ± 0.8 ng.L^{-1} at T0 to 10.8 ± 1.4 ng.L^{-1} at 48 h) suggests that EE2 maintains its integrity in low-concentration scenarios. Similarly, the 300 ng.L^{-1} condition demonstrated excellent stability across all time points, with only minor fluctuations well within the analytical margin of error. At the intermediate concentration of 30 ng.L^{-1}, although the initial measurement was slightly below the nominal value (23.6 ± 2.6 ng.L^{-1}), the slight increase to 25.4 ± 3.9 ng.L^{-1} after 48 hours suggests re-equilibration within the system rather than degradation.

Overall, the data confirm that EE2 is chemically stable over short-term exposures under controlled conditions.

2.5 Conclusions

This study shows that *M. galloprovincialis* responds to 17α-ethinylestradiol (EE2) exposure with significant biochemical alterations that reflect both oxidative stress and endocrine disruption, even at concentrations within the range previously reported in contaminated aquatic environments.

After 28 days of exposure, mussels exhibited complex and concentration-dependent modulation of antioxidant defences, particularly in the activities of CAT, SOD, and GST. These enzymes are key components of the cellular redox system, and their upregulation indicates that EE2 induces the formation of ROS, challenging the redox homeostasis of exposed organisms. While SOD activity increased significantly only at the highest EE2 concentration (300 ng·L^{-1}), CAT and GST showed biphasic responses, with higher activities at both low (10 ng·L^{-1}) and high (300 ng·L^{-1}) concentrations, but a decline or plateau at intermediate levels (30 ng·L^{-1}).

Despite the oxidative challenge, no statistically significant increase was observed in MDA or UBI levels, suggesting that oxidative damage to lipids and proteins was either absent

or successfully mitigated by the antioxidant systems. The most striking effect observed was the dose-dependent increase in vitellogenin-like (VTG-like) protein levels, particularly significant at 300 ng·L^{-1}. These findings reinforce the use of *M. galloprovincialis* as a sentinel species for monitoring xenobiotics, including EDCs in coastal environments.

As final remarks EE2, even at low ng·L^{-1} levels, can interfere with key physiological processes in marine mussels, particularly those linked to oxidative balance and reproductive regulation. This has potential implications not only for organism's health but also for population-level dynamics, especially if exposure occurs during sensitive developmental or reproductive windows.

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3

CHAPTER 3 - TRANSCRIPTOMIC ANALYSIS

3.1 Transcriptomic analysis of the mussel *Mytilus galloprovincialis* exposed to environmental contaminants

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Abstract

Per- and polyfluoroalkyl substances (PFASs), brominated flame retardants (BFRs), and synthetic estrogens are widespread marine contaminants, yet their molecular effects in non-model invertebrates remain poorly understood. This study assessed the transcriptomic responses of the mussel *Mytilus galloprovincialis* after 28-day laboratory exposure to environmentally realistic concentrations of perfluorooctanoic acid (PFOA; 1 and 100 µg·L^{-1}), tetrabromobisphenol A (TBBPA; 1–100 µg·L^{-1}), and 17α-ethinylestradiol (EE2; 10 and 1000 ng·L^{-1}). Whole soft tissues from male and female mussels were analysed using strand-specific RNA sequencing.

High-quality RNA-Seq data were obtained (mean 24 million reads per sample; >87% bases with≥Q30 quality), but mapping efficiency varied substantially across references and pipelines. Alignment to available *M. galloprovincialis* genomes yielded low mapping rates (7–35%), while higher rates were achieved using an older genome (48–76%) or pseudoalignment against a curated transcriptome (up to ~65%). Exploratory analyses showed that sample clustering was driven mainly by mapping efficiency and genetic background rather than by treatment, indicating strong population-structure effects within the *Mytilus* complex.

Differential expression analysis identified 20 significantly regulated transcripts (padj < 0.05, $|\log_2 FC| > 1$, |Wald| > 4), downregulated genes related to muscle contraction, energy metabolism, and intracellular signaling. Toll-like receptor 4 (TLR4) was the only upregulated gene, suggesting selective activation of innate immune pathways.

Overall, extreme genomic heterozygosity, presence–absence variation, and population stratification limit the resolution of reference-based RNA-Seq in *M. galloprovincialis*. These findings highlight the need for pan-genome resources and population-aware, integrative multi-omics approaches to identify molecular biomarkers in marine ecotoxicology reliably.

Keywords: RNA-Seq; *Mytilus galloprovincialis*; PFOA; TBBPA; EE2; population stratification

3.1.1 Introduction

The increasing detection of emerging contaminants such as per- and polyfluoroalkyl substances (PFASs) and brominated flame retardants (BFRs) in marine environments has raised significant ecological and regulatory concerns. These compounds are environmentally persistent, bioaccumulative, and capable of inducing a range of sublethal effects on aquatic species. Among them, perfluorooctanoic acid (PFOA) and tetrabromobisphenol A (TBBPA) stand out due to their extensive use in industrial and consumer products, such as non-stick coatings, textiles, electronics, and flame-retardant (Geng et al., 2024; Li et al., 2021; Miglioli et al., 2021).

Marine bivalves, including *Mytilus edulis*, *Mytilus galloprovincialis*, and *Ruditapes philippinarum*, are widely recognized as effective sentinel organisms for ecotoxicological monitoring. These filter-feeders exhibit high sensitivity to environmental contaminants and are suitable for integrating multiple levels of biological response, ranging from biochemical alterations to molecular and transcriptomic changes (Blalock et al., 2018; Lopes et al., 2022).

Exposure to PFOA in *M. edulis* has been shown to disrupt oxidative balance and metabolic function, affecting antioxidant enzymes such as SOD and CAT, increasing lipid peroxidation (MDA), and altering the expression of detoxification-related genes (Li et al., 2021; Zhou et al., 2024). Multi-omics integration revealed significant modulation of the MAPK, PPARγ, and JNK signaling pathways, affecting amino acid, lipid, and carbohydrate metabolism. Even after 7 days of depuration, recovery was incomplete, suggesting prolonged physiological disruption (Li et al., 2021).

In *R. philippinarum*, PFOA-induced immunotoxicity was confirmed through transcriptomic and metabolomic profiling, coupled with intestinal microbiota shifts. PFOA exposure altered the hemolymph metabolome and favored pro-inflammatory bacterial genera (*Mycoplasma*, *Bacteroidetes*), while transcriptomic data indicated activation of immune-related pathways such as TLR/MyD88/NF-κB, PI3K-Akt-mTOR, and apoptosis/autophagy cascades (Li et al., 2024).

These results suggest a close interplay between immune regulation and gut microbiome disruption in mollusks under PFAS stress.

When combined with the PBDE BDE-47, PFOA elicited synergistic toxic effects in *M. galloprovincialis*. Co-exposure led to enhanced tissue damage, oxidative stress, and disruption

of purine metabolism, amino acid metabolism, and the Nrf2-Keap1 antioxidant defence system (Geng et al., 2024). Such interactions illustrate the complexity of pollutant mixtures and the necessity of considering combined exposures in environmental assessments.

TBBPA, a widely used brominated flame retardant, has been shown to exert endocrine-disrupting effects in bivalves. In *M. galloprovincialis*, TBBPA exposure led to increased gametogenesis and altered sex hormone levels. Gene expression analysis revealed upregulation of SULT1E1 and STS, involved in steroid sulfonation and desulfation, respectively, indicating interference with estrogen metabolism (Wang et al., 2021). Additionally, proteomic and metabolomic analyses demonstrated sex-specific responses, with males showing greater modulation of energy metabolism, cytoskeleton remodeling, apoptosis, and stress response proteins (Ji et al., 2014, 2016).

At the larval stage, TBBPA also impaired shell formation and neurodevelopment. Environmentally relevant concentrations altered the expression of genes involved in shell biogenesis (e.g., tyrosinase) and interfered with key neurotransmitter systems, serotoninergic, dopaminergic, and GABAergic, suggesting neuroendocrine disruption during early development (Miglioli et al., 2021). These effects can translate into impaired survival, development, and recruitment success, potentially affecting population stability.

Furthermore, decabromodiphenyl ethane (DBDPE), a TBBPA alternative, has been shown to act as an endocrine disruptor by modulating lipid and cholesterol metabolism and interfering with reproductive signaling pathways. Transcriptomic analyses in *M. galloprovincialis* revealed significant disruption of genes involved in cholesterol transport, sex steroid biosynthesis, and energy homeostasis (Wang et al., 2023).

In addition to industrial contaminants, synthetic estrogens such as 17α-ethinylestradiol (EE2), commonly found in wastewater effluents, have been reported to induce estrogenic responses in marine mussels.

Although the canonical vertebrate vitellogenin gene (VTG) is not consistently expressed at the transcript level in mussels (Ciocan et al., 2010), studies show EE2 exposure leads to gametogenic alterations, increased lipid accumulation, and oxidative stress (Lopes et al., 2022; Pretti et al., 2023). Transcriptomic analyses have proposed alternative biomarkers such as ESR-like genes, FABP, and HSD17β (Blalock et al., 2018; Lopes et al., 2022).

Contrary to vertebrates, where estrogens act through nuclear estrogen receptors (ERs), bivalve ERs often lack ligand-binding domains, indicating that estrogens and EDCs may act through non-genomic signaling pathways, such as PI3K/Akt or MAPK, or via modulation of other transcription factors (Blalock et al., 2018; Lopes et al., 2022).

While each study provides mechanistic insights into how specific contaminants affect marine invertebrates, holistic approaches integrating physiological, histological, transcriptomics, proteomics, metabolomics, and microbiomics endpoints are still scarce. Given the complex nature of environmental mixtures and species-specific modes of action, such integrated approaches are essential to identify robust biomarkers of exposure and effect, characterize adverse outcome pathways (AOPs), and inform regulatory frameworks aimed at protecting aquatic ecosystems from EDCs and persistent pollutants (Blalock et al., 2018; Lopes et al., 2022; Pretti et al., 2023; Wang et al., 2023).

This study aims to investigate the transcriptomic responses of marine bivalves exposed to realistic and sublethal concentrations of TBBPA, PFOA, and EE2 under controlled laboratory conditions.

3.1.2 Materials and Methods

3.1.2.1 TBBPA, PFOA and EE2 Stock Solution

The TBBPA stock solution (CAS No. 79-94-7; ≈99.3%; ref. 330396, Sigma-Aldrich, Shanghai, China) was prepared by dissolving 0.1 g of TBBPA in 100 mL of methanol (99.8% v/v, Honeywell, Seelze, Germany). For PFOA (CAS No. 335-67-1, Sigma-Aldrich, Shanghai, China), 0.1 g of the compound was dissolved in 100 mL of methanol (99.8% v/v, Honeywell, Seelze, Germany) to obtain a stock solution. For EE2 (CAS No. 57-63-6, Sigma-Aldrich, USA), 5 mg of the compound was dissolved in 100 mL of methanol (99.8% v/v, Honeywell, Germany).

3.1.2.2 Experimental trials

The experimental exposure of *Mytilus galloprovincialis* to TBBPA, PFOA, and EE2 was conducted following a previously established protocol (Copeto et al., 2024a; Copeto et al., 2024b). Mussels were manually collected from "Guincho" coast (Cascais, Lisbon, Portugal) in

February 2024. Individuals of similar size were selected to minimize variability in bioaccumulation and biomarker responses.

Following collection, mussels were transported to the NOVA School of Science and Technology laboratory facilities in a thermal container to maintain a stable temperature and minimize physiological stress. Upon arrival, they were acclimatized for five days in a 50 L aquarium filled with natural seawater collected from "Guincho". The aquariums were equipped with filtration, recirculation, and aeration systems to maintain optimal water quality. The environmental conditions during acclimatization were carefully controlled, with a pH of 8.1 ± 0.2, temperature of 20.0 ± 1.0°C, and salinity of 33 ± 1 $g \cdot L^{-1}$. The photoperiod was set to 12 h light/12 h dark, and dissolved oxygen levels were maintained above 6 $mg \cdot L^{-1}$.

After acclimatization, mussels (0.835 ± 0.219 g) were randomly distributed into separate 8-L aquariums under static conditions. Each tank contained eight individuals and was maintained in isolation to prevent cross-contamination. The water parameters (temperature, pH, and salinity) were monitored daily to ensure stability.

Mussels were exposed to specific concentrations of TBBPA (1, 10, and 100 $\mu g \cdot L^{-1}$), PFOA (1 and 100 $\mu g \cdot L^{-1}$), and EE2 (10 and 1000 $ng \cdot L^{-1}$), prepared from stock solutions and added to the aquariums. A control group was maintained in filtered seawater under identical conditions. Water renewal was performed every 48 hours to sustain exposure, and methanol concentrations in the aquariums were maintained at <0.01% to avoid solvent-related toxicity. The mussels were fed daily with 1.5 $mg \cdot L^{-1}$ of Chlorella sp. (Shine Superfood, Portugal), which was predissolved in seawater before being added to the aquariums.

3.1.2.3 Sample Treatment

At the end of the experiment, each mussel was weighed using an analytical balance (Sartorius, Germany) and measured with a micrometer. The entire soft body of each mussel (16 males and 16 females) was carefully separated from the shell using a scalpel and tweezers, then collected and stored in microtubes at −80°C for further analysis. Gender identification was performed based on gonad coloration, with males displaying a white-cream color and females

exhibiting an orange-pink hue. Four independent biological replicates were collected per compound for each concentration level studied, consisting of two female and two male specimens.

3.1.2.3.1 RNA Extraction

Total RNA was extracted from marine bivalve tissues using the NucleoSpin® RNA Kit (Macherey-Nagel), following the manufacturer's protocol with modifications to optimize RNA yield and purity.

Before processing, tissues (~30 mg) were homogenized in 350 µL of Lysis Buffer RA1, supplemented with 3.5 µL β-mercaptoethanol to inactivate RNases. The homogenized lysate was cleared via centrifugation (11,000 × g, 1 min) using NucleoSpin® Filters to remove debris. Ethanol (70%) was added (350 µL) to optimize RNA binding conditions before loading onto NucleoSpin® RNA Columns. To eliminate genomic DNA contamination, an on-column DNase treatment was performed with 95 µL DNase reaction mixture, incubated for 15 min at room temperature. The column-bound RNA underwent three sequential washes, 200 µL RAW2 buffer (to inactivate DNase), 600 µL RA3 buffer (to remove salts and protein residues),250 µL RA3 buffer, followed by centrifugation at 11,000 × g, 2 min to ensure complete membrane drying. RNA was eluted in 60 µL of RNase-free water and stored at -80°C until further analysis.

3.1.2.3.2 RNA Quality Assessment

RNA concentration and purity were assessed using a NanoDrop One Microvalume UV-Vis spectrophotometer (Thermo Fisher Scientific), measuring absorbance at 260 nm (A260), 280 nm (A280), and 230 nm (A260/A230).

RNA concentration: Mean 166.34 ng/µL, A260/A280 ratio: Mean 2.11 (optimal range 2.0–2.2), indicating high RNA purity with minimal protein contamination, A260/A230 ratio: Mean 2.23 (acceptable range 2.0–2.4), confirming low salt and phenol contamination, Baseline absorbance: Mean -0.036, with stable readings at 340 nm. Samples with A260/A280 <2.0 or A260/A230 <2.0 were flagged for potential contamination and subjected to additional

purification when necessary. Extracted RNA with an RNA Integrity Number (RIN) >9.0 was deemed suitable for downstream RNA-Seq applications (**Appendix B1**).

3.1.2.3.3 cDNA synthesis and Library Preparation

Libraries were generated using the TruSeq Stranded mRNA Library Prep Kit (Illumina, Part #1000000040498, v00, October 2017), following the manufacturer's protocol. Each library was constructed starting from 1 µg of total RNA, with poly(A) mRNA enrichment and strand-specific cDNA synthesis. Library quality and concentration were assessed using the Fragment Analyzer (Advanced Analytical Technologies) with the DNF-474 High Sensitivity NGS Fragment Analysis Kit (1 bp – 6,000 bp). The molarity of each library was determined using PROSize software (Advanced Analytical Technologies). A peak around 140 bp, likely corresponding to adapter dimers, was detected in the initial assessment. To remove these dimers, an additional purification step was performed using AMPure XP beads (0.8× ratio, Beckman Coulter). A second quality control was conducted post-purification, confirming the effective removal of adapter dimers. Final libraries were normalized to a concentration of 2 nM based on quantification results and further validated using fluorometric quantification (Qubit, Thermo Fisher Scientific).

3.1.2.4 Library Pooling and Sequencing

Equal volumes (5 µL) of each library were pooled to create the final sequencing library pool. The pooled libraries were diluted from 2 nM to 750 pM, following the recommendations in the NextSeq 1000 and 2000 Product Documentation (Illumina, Doc #200027171, v03.1, March 2024). The denaturation of the pooled libraries was performed directly in the sequencing instrument. Sequencing was conducted on an Illumina NextSeq 2000 instrument using a P2 flow cell with a paired-end 2 × 100 bp read length. The run included dual-indexing, and raw sequencing data were stored locally.

3.1.2.5 Primary Data Processing

Raw sequence data underwent primary analysis using the Illumina onboard analysis pipeline, including base calling and quality control. The sequencing run generated a total yield of 85.63 Gbp, with an average Q30 percentage of 84.39% and a loading concentration of 99.58%, all within the expected quality range.

3.1.2.6 Transcriptomic Data Analysis Pipeline

Raw sequencing data (FASTQ files) were processed using a standardized RNA-Seq bioinformatics pipeline, encompassing steps from quality control to functional interpretation.

3.1.2.6.1 Quality Control (QC)

The 32 FASTQ files were subjected to quality control using FastQC (v0.11.8) and InterOp (v3.0.35). FastQC processes FASTQ sequence files and generates a quality control report composed of several modules, each addressing a different potential issue in the sequencing data (Andrews, 2010). The Illumina InterOp libraries, on the other hand, provide routines for reading and writing InterOp metric files, which are binary files produced during sequencing runs and contain detailed run statistics. To facilitate visualization and interpretation, the individual FastQC reports and InterOp statistics from the 32 samples were aggregated into a single, comprehensive report using MultiQC (v1.11) (Ewels et al., 2016).

3.1.2.6.2 Read alignment

Mapping RNA-Seq reads to the genome poses additional challenges compared to DNA sequencing, as many reads span splice junctions and therefore require gapped or split-read alignments. A common strategy is the use of splicing-aware aligners, such as STAR (Dobin et al., 2013) and DRAGEN (Illumina). Alternatively, alignment-free tools (e.g., Sailfish, Salmon, and Kallisto) implement pseudoalignment approaches, where reads are decomposed into k-mers before being assigned to transcripts, without performing base-by-base alignments. This results in a substantial gain in speed while maintaining accurate transcript quantification (Everaert et al., 2017; Zhang et al., 2017).

In your study, we used STAR (v2.7.10b) to align raw sequencing reads from the 32 samples against the NCBI *M. galloprovincialis* reference genome GCA_037788925.1 (2024). This version was selected for its high assembly quality, although functional annotations were not yet available. In parallel, we employed DRAGEN (v4.2.7) to align the reads against the older NCBI reference genome GCA_001676915.1 (2017), which, despite being more fragmented and of lower quality, contained limited structural gene annotations provided by the original authors (not curated by NCBI).

Additionally, transcript quantification was performed using Kallisto (v0.48.0) against the reference genome GCF_965363235.1 (2025), incorporating available genome-based functional annotations to ensure accurate gene-level assignment and biological interpretation.

3.1.2.7 Exploratory analysis – Global read count patterns

Before conducting differential gene expression (DGE) analysis, it is essential to explore global expression patterns to ensure consistency across replicates and to verify that major sources of variation correspond to the experimental conditions (Dündar F, Skrabanek L, 2019). To this end, we performed both hierarchical clustering and principal component analysis (PCA) using transcript quantifications obtained with Kallisto.

3.1.2.7.1 Hierarchical clustering

Sample-to-sample similarity was assessed using the Pearson correlation coefficient (r) calculated from rlog-transformed and normalised counts. Distances (d) between samples were defined as d = 1 – r, and agglomerative hierarchical clustering was performed using the R function hclust. The resulting dendrograms provided a visual representation of sample grouping (Liang, 2004).

3.1.2.7.2 Principal component analysis (PCA)

As a complementary approach, PCA was applied to the same rlog-transformed data to determine whether variability was greater between experimental conditions than within replicates. PCA reduces dimensionality by projecting the original high-dimensional data (genes/transcript counts) onto a smaller set of orthogonal components that capture the

maximum variance (Dündar F, Skrabanek L, 2019; Liang, 2004). PCA was performed using the base R function prcomp and the plotPCA function from DESeq2, which employs ggplot2 for graphical output (Wickham, 2016).

3.1.2.8 Differential gene expression analysis

Differential gene expression (DGE) was assessed using DESeq2, applied exclusively to transcript quantifications obtained with Kallisto. DGE tools generally aim to estimate the effect size of expression changes between experimental conditions based on read counts from biological replicates, while also testing statistical significance and correcting for multiple comparisons. In our analysis, the DESeq function was applied to the raw read counts after filtering out lowly expressed genes.

3.1.3 Results

3.1.3.1 Quality control

FastQC – The sequencing depth averaged 24 million reads per sample (range: 18–32 M), with an average GC content of ~41%. Both the mean Phred quality score across base positions **(Fig. 3-1)** and the distribution of reads per mean Phred score **(Fig. 3-2)** indicated consistently high-quality data across all samples. InterOp – The proportion of bases with a quality score ≥ Q30 was above 87% for all samples.

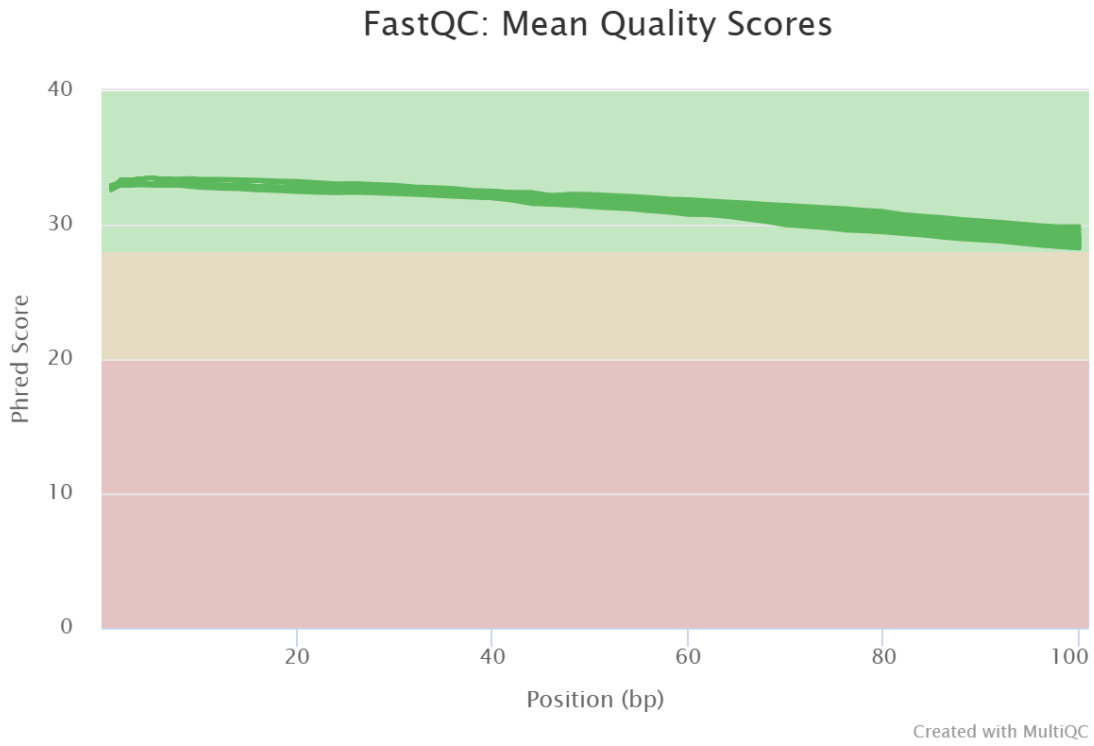

Figure 3-1 - FastQC plot of mean Phred quality scores across read positions. Mean Phred quality scores for all 32 samples remained within the green area (≥ Q28) across the entire read length, indicating consistently high sequencing quality.

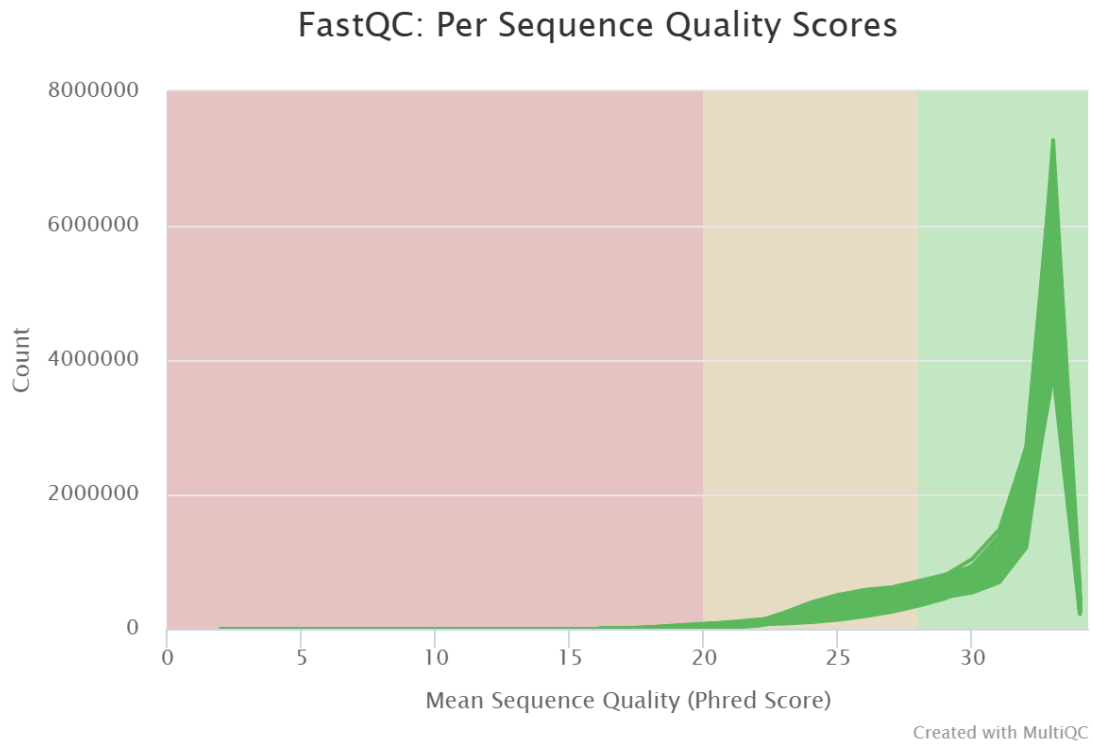

Figure 3-2 - FastQC plot of the number of reads per mean quality Phred score in the 32 samples.

3.1.3.1.1 Read alignment

Using STAR against the *M. galloprovincialis* reference genome GCA_037788925.1 (2024), the overall mapping rates were very low, ranging from ~7% in sample SMC23-TBBPA-1-3 to a maximum of ~35% in SMC1-Control-1. For comparison, in model organisms such as *Homo sapiens*, mapping rates above 80% are generally considered good and above 90% very good.

With DRAGEN, applied to the older reference genome GCA_001676915.1 (2017), mapping rates were higher, ranging from 48% (SMC31-TBBPA-100-3) to 76% (SMC3-Control-3). However, the main limitation was the poor annotation: more than 98% of aligned reads mapped to intergenic regions, and coverage ≥1× was achieved for only just over 1,000 genes.

Finally, using Kallisto against the reference genome *M. galloprovincialis* transcriptome GCF_965363235.1 (2025), mapping rates varied between ~9% and 65%, consistent with the species' high genomic heterozygosity and pan-genomic structure, the use of the curated reference sequence annotation ensured accurate gene assignment and reliable biological interpretation.

3.1.3.1.2 Exploratory analysis – Global read count patterns

After normalizing and transforming the read counts, we evaluated global expression patterns prior to differential gene expression analysis. Contrary to expectations, the global expression profile did not exhibit greater similarity among replicates within the same condition. Sample similarity was assessed using hierarchical clustering (**Fig. 3-3**) and principal component analysis (PCA) (**Fig. 3-4**).

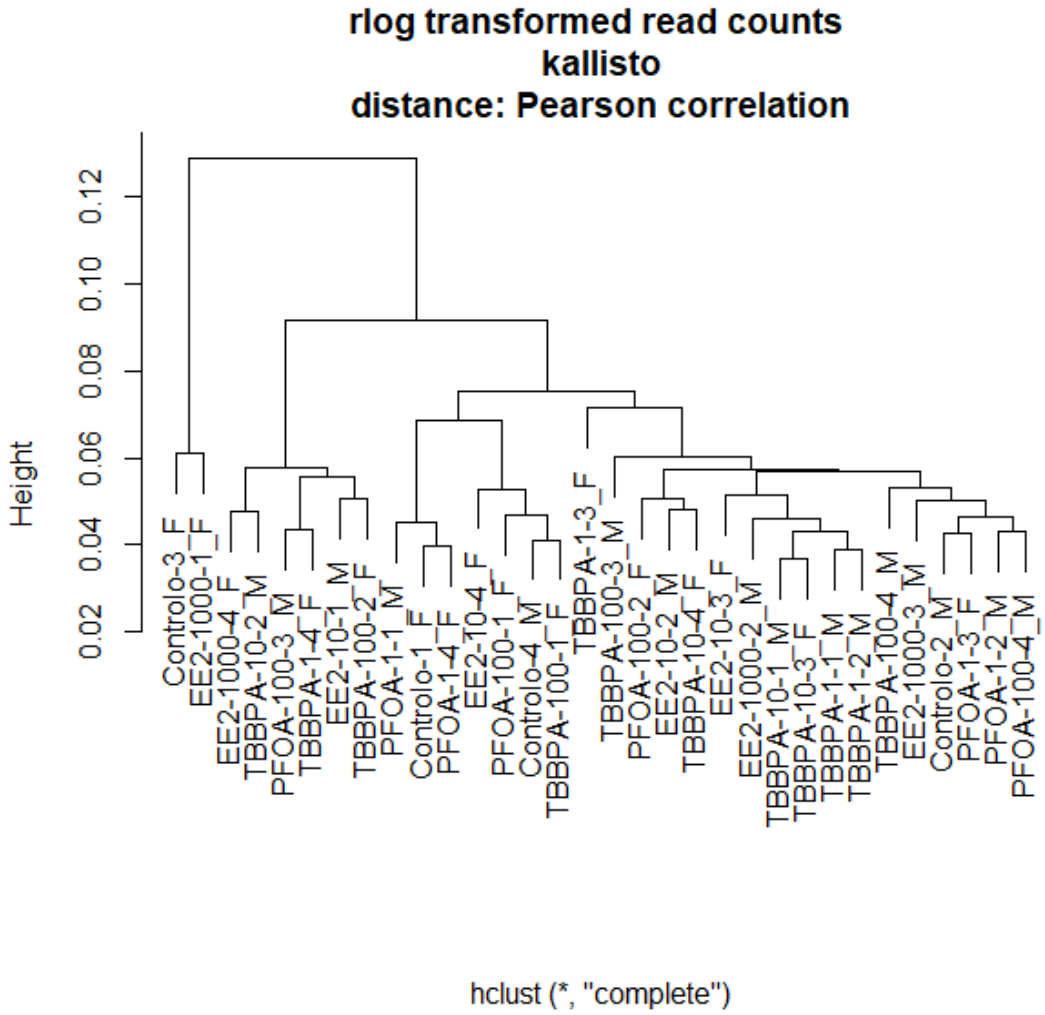

Figure 3-3 - Hierarchical clustering of RNA-Seq samples. Dendrograms representing the hierarchical clustering of the normalized, regularized log_2-transformed read counts across all samples.

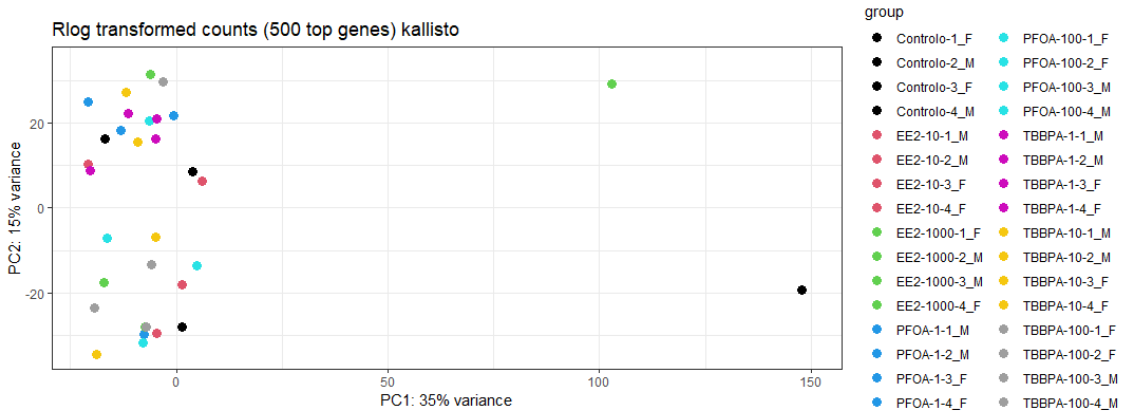

Figure 3-4 - Principal component analysis of RNA-Seq samples. PCA plots of the normalised, regularised log_2-transformed read counts, generated with the DESeq2 function plotPCA using the 500 genes with the highest variance (default option).

3.1.3.1.3 Differential gene expression analysis

Volcano plots (**Fig 3-5 and 3-6**) illustrate the distribution of differential gene expression based on log2 fold change (log2FC) and |Wald statistic|. Vertical dashed lines represent the log2FC threshold (|log2FC| > 1), and the horizontal dashed line marks the Wald statistic cutoff (|Wald| > 4). Highlighted points correspond to significantly downregulated genes (blue) and upregulated genes (gold), while grey points represent non-significant transcripts.

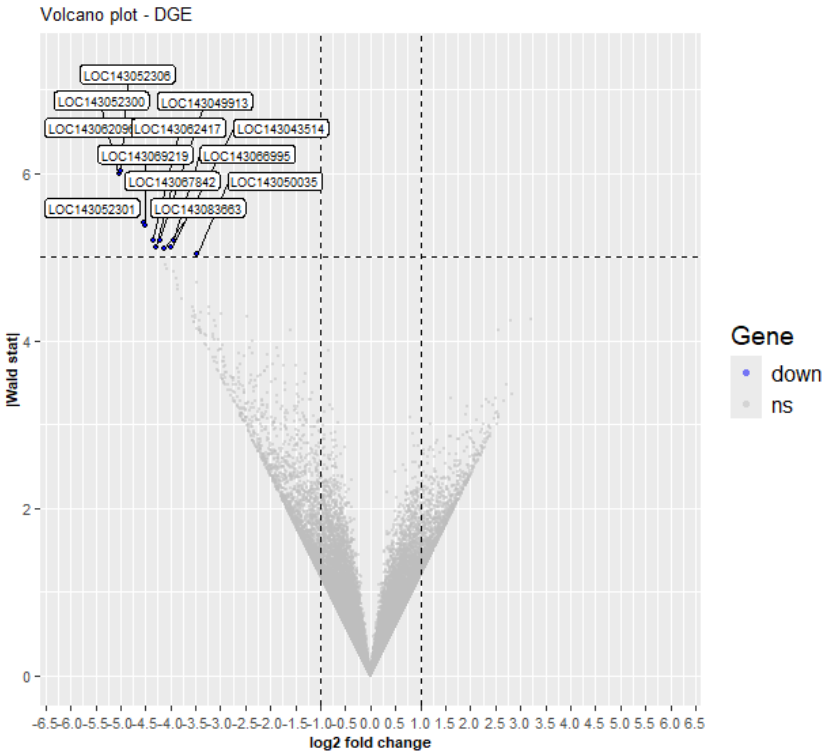

Figure 3-5 - Volcano plot of DEGs between treatment (1 μg.L^{-1} PFOA) and control groups (down-regulated genes labelled).

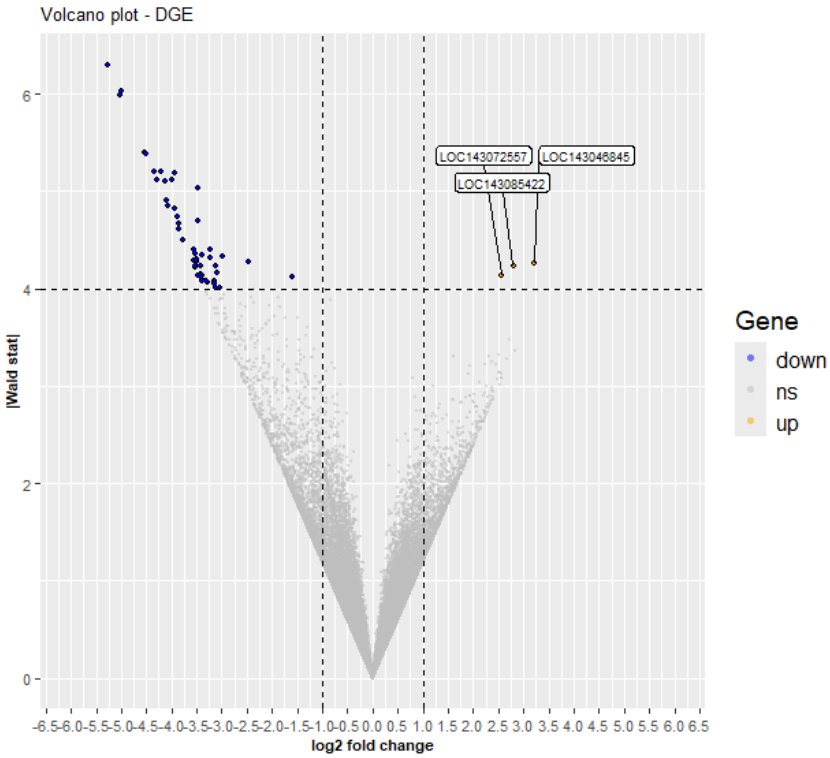

Figure 3-6 - Volcano plot of DEGs between treatment (1 µg.L^{-1} PFOA) and control groups (up-regulated genes labelled).

Twenty transcripts **(Appendix B2)** met the significance criteria (padj < 0.05, |log2FC| > 1, |Wald| > 4), with 17 downregulated and 3 upregulated. Most annotated DEGs were downregulated, including genes related to muscle contraction and energy metabolism (e.g., myosin heavy chain, myosin essential light chain, SLC2A3-like), together with signaling-associated transcripts (e.g., HHIP-like, MTMR9-like, cyclic nucleotide-binding domain protein-like). Toll-like receptor 4 (TLR4) was the only annotated gene upregulated, suggesting selective activation of innate immune pathways.

3.1.4 Discussion

The genomic landscape of *M. galloprovincialis* presents unique challenges for transcriptomic analyses. With a genome size estimated between 1.3–1.4 Gb and an average heterozygosity of ~1.7%, this species exhibits levels of allelic diversity that far exceed those of most model organisms, including humans (Gerdol et al., 2020) . The consequence of this extreme polymorphism is a dense distribution of SNPs and extensive hemizygous regions enriched in repetitive and mobile elements. Such features not only complicate genome assembly but also affect downstream analyses by creating alignment ambiguities, reference biases, and reduced mapping efficiencies.

The pangenomic architecture of *M. galloprovincialis* further contributes to these difficulties. Approximately 45,000 genes are considered core, but an additional ~20,000 dispensable genes are subject to presence–absence variation (PAV), representing one of the first large-scale examples of this phenomenon in animals (Gerdol et al., 2020).

Subsequent work has shown that dispensable genes account for nearly 38% of the mussel genome and are widely distributed across chromosomes, often forming “hotspots” of variability (Saco et al., 2023). Many of these genes are enriched in stress-response and immune-defense functions, suggesting that they contribute directly to adaptation and resilience under

environmental stressors. However, from an analytical perspective, transcripts encoded by dispensable or highly divergent genes may be entirely absent from a given reference genome, making them unmappable. This inevitably results in false negatives and an underestimation of biological responses when relying on single reference-based approaches.

Our alignment results exemplify this problem. When reads were aligned using STAR against the most recent *M. galloprovincialis* assembly (GCA_037788925.1, 2024), mapping rates were strikingly low (7–35%). This is far below the >80% typically achieved in model organisms and highlights both the incompleteness of current references and the influence of genome complexity. DRAGEN alignment against the older genome (GCA_001676915.1, 2017) improved mapping rates (48–76%) but was limited by poor annotation, with most reads mapping to intergenic regions. Salmon, which uses pseudoalignment and probabilistic transcript assignment, achieved intermediate mapping rates (45–73%) and provided the best compromise between efficiency and functional annotation (Sun et al., 2025).

To overcome these constraints at the transcript level, we quantified reads using Kallisto, which uses pseudoalignment and probabilistic transcript assignment, against the reference sequence *M. galloprovincialis* transcriptome (GCF_965363235.1, 2025), mapping rates varied between ~9% and 65%. Among the differentially expressed genes, a clear pattern emerged reflecting modulation of muscular, metabolic, and signaling pathways. Key structural and contractile transcripts, including myosin heavy chain, striated muscle-like and myosin essential light chain, striated adductor muscle-like, together with the solute carrier family 2, facilitated glucose transporter member 3-like gene, were downregulated, suggesting a reduction in muscular contractile capacity and glucose-dependent energy supply. Additionally, genes associated with intracellular signaling and cellular homeostasis, namely cyclic nucleotide-binding domain-containing protein 2-like, HHIP-like protein 2, and myotubularin-related protein 9-like, also exhibited downregulation, indicative of suppressed regulatory and stress-response pathways. In contrast, Toll-like receptor 4 (TLR4) was the only transcript upregulated, highlighting the activation of innate immune mechanisms and the likely engagement of pathogen- or damage-associated molecular pattern recognition processes.

These findings confirm that pseudoalignment methods are advantageous for non-model, polymorphic genomes, although they cannot fully overcome the limitations imposed by incomplete references and PAV.

Another major finding was the effect of population structure. Exploratory analyses revealed two clearly distinct clusters in PCA, which accounted for 50% of the total variance. Surprisingly, replicates from the same experimental condition did not consistently group together; instead, clustering corresponded more closely to mapping percentages. Samples in the right-hand PCA cluster showed mapping efficiencies of ~54%, whereas those in the left-hand cluster averaged ~70%, with two intermediate replicates at ~65%. This pattern strongly suggests that our dataset included individuals from at least two genetically distinct backgrounds, potentially representing *M. galloprovincialis, M. edulis,* and hybrids. The presence of such cryptic diversity is plausible given that these species often overlap geographically, and hybridization has been well documented (Diz & Skibinski, 2024).

Population stratification has been recognized as a critical confounder in both genomic and transcriptomic analyses. If not accounted for, background genetic variation can generate spurious differences that mimic treatment effects (Brown et al., 2014; Price et al., 2006). In humans and other model organisms, this issue is commonly addressed through inclusion of ancestry covariates derived from PCA, surrogate variable analysis (SVA), or mixed-effect models (Tung et al., 2015).

In our study, the failure of replicates to cluster by treatment indicates that population effects outweighed contaminant-induced expression changes. This has profound implications for environmental transcriptomics, without careful control of genetic background, it becomes difficult to disentangle true contaminant responses from variation driven by ancestry or hybrid status. Integrating genotyping or molecular barcoding into experimental design will therefore be essential for future studies, particularly in sentinel species with complex population structures.

The biological implications of these findings are significant. The contaminants studied, PFOA, TBBPA, and EE2 are well established as endocrine disruptors and immunotoxicants in marine bivalves (Li et al., 2021; Lopes et al., 2022; Wang et al., 2021).

Our results show that genomic complexity and hidden stratification may obscure or even mask their transcriptional signatures. For example, dispensable immune-related genes might be absent in certain individuals, leading to apparent variability in response that reflects genomic architecture rather than contaminant exposure. Similarly, the clustering of samples by genetic background rather than treatment suggests that ecotoxicological outcomes cannot be fully interpreted without accounting for host genomic diversity. This highlights an urgent need to integrate population genomics with ecotoxicology to produce reliable biomarkers of exposure and effect.

Finally, the technical lessons from this work are broadly applicable. First, reliance on a single reference genome is insufficient for species with extreme heterozygosity and PAV. Pan-genome resources and hybrid assembly approaches should become the standard in mussel transcriptomics.

Second, pseudoalignment tools such as Salmon and Kallisto provide useful alternatives to alignment-based pipelines, but they still require curated annotations to achieve their full potential. Third, population stratification must be treated as a biological variable, not a nuisance factor, as it can dominate expression variance. Future studies should combine RNA-Seq with genotyping-by-sequencing or SNP arrays to control ancestry.

3.1.5 Conclusion

This study highlights both the opportunities and challenges of applying RNA-Seq to highly polymorphic, non-model organisms such as *M. galloprovincialis*. Despite the limitations encountered in alignment performance and the inability to perform a robust differential expression analysis, the results presented here provide several important contributions.

First, we confirmed that high heterozygosity and the presence of genes subject to presence–absence variation (PAV) are major factors underlying low mapping rates and analytical complexity. This finding reinforces the need for pan-genome approaches or population-specific references to ensure reliable analyses in marine bivalves.

Second, the unexpected clustering patterns observed among biological replicates, likely related to the presence of *M. edulis* individuals or hybrids, demonstrate the importance of accounting for genetic stratification in environmental transcriptomics. This underscores the need to integrate genotyping or barcoding strategies in future experiments to ensure population homogeneity.

Finally, this work provides a valuable transcriptomic dataset and a transparent description of both the methodological approaches and the technical pitfalls encountered. Such information will help guide future research, avoid methodological biases, and promote strategies better adapted to the genomic complexity of non-model species.

Our results illustrate both the promise and the limitations of transcriptomics in *M. galloprovincialis*. While detailed differential gene expression analysis was not feasible under current conditions, the study provides a transparent account of the methodological hurdles and biological complexities inherent to non-model species.

By acknowledging these challenges and proposing solutions pan-genome resources, population-aware designs, and integrative multi-omics we lay the groundwork for future ecotoxicological studies that can more accurately capture the interplay between contaminant exposure and genomic variability in marine ecosystems

Altogether, while detailed biological interpretation remains limited, this study offers a solid foundation for future multi-omics analyses and the development of molecular biomarkers to support environmental monitoring and risk assessment.

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4

Conclusions and future perspectives

This thesis provides novel insights into the ecotoxicological effects of three major groups of emerging contaminants —synthetic estrogens (EE2), brominated flame retardants (TBBPA), and perfluoroalkyl substances (PFOA) —on the Mediterranean mussel *Mytilus galloprovincialis*. By combining biomarker-based approaches with transcriptomic analyses, it was possible to assess toxicological responses at biochemical, molecular, and physiological levels, thus strengthening the understanding of mechanisms underlying the impacts of endocrine-disrupting chemicals (EDCs) and persistent organic pollutants (POPs) on marine invertebrates.

Mussels exposed to tetrabromobisphenol A showed that low concentrations (1–10 $\mu g \cdot L^{-1}$) were efficiently detoxified, as evidenced by GST and other enzymatic activities. However, at 100 $\mu g \cdot L^{-1}$, significant oxidative stress was observed, with strong correlations between total antioxidant capacity, lipid peroxidation, and enzymatic biomarkers. In addition to oxidative damage, apoptosis (as indicated by caspase-3 activity) and neurotoxicity (as evidenced by AChE inhibition) were triggered, highlighting the multi-level toxicity of TBBPA. These findings highlight the potential ecological risk posed by brominated flame retardants, particularly in coastal environments where sediments serve as long-term reservoirs.

Even environmentally relevant concentrations of perfluorooctanoic acid (1–10 $\mu g \cdot L^{-1}$) induced significant alterations in antioxidant defences, with increased SOD and GST activities and variable modulation of CAT and LPO. Evidence of cellular stress, protein degradation (via ubiquitin), and apoptotic activation (as indicated by caspase-3) was detected, reinforcing the potential for sublethal toxicity. Nevertheless, preliminary results suggested sex-dependent responses, which may have important implications for reproduction and population dynamics. Given the persistence and bioaccumulative properties of PFAS, these findings highlight serious concerns for both ecosystem health and seafood safety.

Exposure to 17α-ethinylestradiol elicited a clear and concentration-dependent oxidative stress response. Antioxidant enzymes, particularly SOD and GST, were upregulated at higher concentrations (300 $ng \cdot L^{-1}$), indicating enhanced reactive oxygen species (ROS) production and cellular stress. Catalase exhibited a biphasic pattern, indicating an adaptive response at low exposure levels, followed by enzyme inhibition at higher doses. Notably, the induction of vitellogenin-like proteins confirmed EE2's estrogenic mode of action in mussels, validating VTG as a reliable biomarker of endocrine disruption in bivalves.

This study demonstrates both the potential and the limits of applying RNA-Seq to a highly polymorphic, non-model bivalve. Low mapping rates and analytical complexity were driven by high heterozygosity and presence–absence variation (PAV), underscoring the need for pan-genome resources or population-specific references in marine bivalves. Unexpected clustering among biological replicates—likely reflecting cryptic species structure (*M. edulis* or hybrids)—highlights the importance of genetic stratification control (e.g., genotyping/barcoding) in environmental transcriptomics. Although a robust differential expression analysis was not feasible, the work delivers a transparent methodological account and a valuable transcriptomic dataset, clarifying technical pitfalls and offering practical guidance for future studies. By advocating population-aware designs, pan-genome alignment strategies, and integrative multi-omics, this thesis lays the groundwork for ecotoxicological research that better captures the interplay between contaminant exposure and genomic variability. In sum, despite limited biological interpretation at the gene level, the study provides a solid foundation for developing molecular biomarkers and advancing environmental monitoring and risk assessment in complex, non-model marine species.

Collectively, these results confirm that *M. galloprovincialis* is a robust sentinel species for monitoring endocrine disruptors and persistent contaminants. The integration of multi-biomarker and transcriptomic data highlights both immediate biochemical stress responses and longer-term molecular reprogramming, pointing to potentially profound consequences for individual fitness, population viability, and ecosystem stability.

While this work combines biochemical and transcriptomic endpoints, the next step is to integrate proteomics, metabolomics, and microbiome data to provide a holistic view of contaminant-induced stress. Such integrative approaches will enable the construction of adverse outcome pathways (AOPs), linking molecular initiating events to organismal and ecological effects.

Most current studies, including this thesis, are restricted to 28-day exposures. Chronic and multigenerational experiments are necessary to determine whether observed molecular and biochemical alterations translate into reproductive impairment, developmental abnormalities, and reduced population recruitment. Epigenetic mechanisms should also be explored as potential drivers of transgenerational effects.

Marine organisms are rarely exposed to single contaminants. Future work must focus on contaminant mixtures, including PFAS, BFRs, pharmaceuticals, and microplastics, to understand interactive effects. Co-exposure studies have already demonstrated synergistic effects (e.g., PFOA + PBDEs), underscoring the urgency of addressing chemical cocktails that reflect real-world conditions.

Future research should bridge the gap between laboratory findings and ecological consequences by combining biomarker data with population modelling, field studies, and mesocosm experiments, enabling a more accurate assessment of how contaminant exposure affects growth, reproduction, survival, and ultimately, the provision of ecosystem services.

The data from environmentally realistic concentrations should be directly incorporated into regulatory frameworks such as the EU Water Framework Directive, the REACH regulation, and EFSA risk assessments, to strengthen evidence-based policymaking and support the establishment of threshold levels for contaminants of emerging concern in marine ecosystems.

Expanding biomonitoring programs using *M. galloprovincialis* and other filter-feeding bivalves would provide valuable spatial and temporal data on contaminant levels and biological responses. These networks could serve as early warning systems for ecosystem health and seafood safety, helping identify pollution hotspots and guiding remediation efforts.

Given the high consumption of shellfish in Portugal and across Europe, the transfer of contaminants from marine ecosystems to humans via seafood requires urgent attention. Future studies should adopt a One Health framework, linking environmental contamination, bioaccumulation in marine organisms, and human health outcomes. This integrated perspective will be critical for ensuring both ecological sustainability and public health protection.

A

Appendix (Biomarker Analysis)

A.1 The effects of Tetrabromobisphenol A (TBBPA) on the mussel *Mytilus galloprovincialis*: a multi-biomarker approach

Condition	Bradford ($mg \cdot mL^{-1}$ protein)	GST ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein)	SOD ($U \cdot mg^{-1}$ protein)	CAT ($mmol \cdot min^{-1} \cdot mg^{-1}$ protein)	LPO ($nmol \cdot mg^{-1}$ protein)	AchE ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein)	TAC ($\mu mol \cdot mg^{-1}$ protein)	Casp-3 ($\mu g \cdot mg^{-1}$ protein)	UBI ($ng \cdot mg^{-1}$ protein)
Control	3.9	3.5	-	1.9	-	0.043	0.25	0.024	4.9
	3.0	1.1	28.7	0.6	-	0.041	0.32	0.022	7.6
	4.4	6.6	15.5	4.4	0.016	0.032	-	0.027	3.6
	4.2	2.0	22.0	1.4	-	0.034	0.26	0.021	5.8
	2.4	2.6	19.6	7.3	0.019	0.04	-	0.035	-
	1.7	-	44.4	-	0.018	0.032	-	-	-
	1.3	6.5	64.9	-	-	-	-	-	-
	1.8	0.2	44.6	-	0.010	0.021	-	0.050	-
	4.2	2.8	11.0	6.6	0.014	0.027	0.26	0.046	-
	3.0	3.7	-	3.4	0.012	0.026	0.32	0.080	-
	5.7	1.6	10.0	10.6	0.014	0.008	0.19	0.036	5.7
1 $\mu g.L^{-1}$	1.8	1.4	21.2	8.5	-	0.032	-	0.014	15.9
	4.0	0.9	18.2	2.6	0.012	0.013	0.27	0.036	7.7
	6.3	4.5	8.7	3.1	0.015	0.017	0.18	0.016	5.8
	4.5	1.9	17.7	1.1	-	0.018	0.24	0.058	15.1
	3.8	1.3	16.6	-	-	0.020	0.28	-	12.9
	2.5	-	-	8.0	0.029	0.023	0.43	-	8.4

10 µg.L^{-1}	3.0	3.2	20.5	8.5	0.017	0.014	0.31	-	9.3
	3.6	2.9	26.7	3.9	0.025	0.008	0.27	0.058	13.4
	3.3	3.0	20.4	5.6	0.018	0.008	0.33	0.068	11.0
	3.0	3.3	22.5	3.2	-	0.013	0.36	0.027	19.4
	2.1	2.6	36.6	5.6	-	0.017	0.53	0.026	5.0
	1.7	-	-	-	0.022	0.009	-	0.048	19.1
	1.2	2.8	-	-	0.021	0.018	-	-	13.1
	1.9	4.5	26.6	30.1	0.032	0.017	0.53	0.058	9.4
	0.6	-	58.8	7.4	0.079	0.069	1.8	0.105	28.0
100 µg.L^{-1}	1.7	5.5	18.7	11.2	0.033	0.051	0.62	0.051	12.6
	1.1	22.1	33.4	-	0.057	0.045	1.06	0.073	9.6
	0.7	6.9	79.1	-	0.063	0.071	1.57	0.131	43.4
	0.5	19.2	119.9	17.2	0.042	-	1.91	0.193	55.0
	1.0	-	20.2	-	0.030	0.052	1.03	0.091	23.7
	2.5	21.6	-	-	-	-	0.43	0.090	8.7

A.2 The impact of Perfluorooctanoic acid (PFOA) on the mussel *Mytilus galloprovincialis*: a multi-biomarker evaluation

Condition	Bradford ($mg \cdot mL^{-1}$ protein)	GST ($nmol \cdot min^{-1} \cdot mg$-1 cytosolic protein)	SOD ($U \cdot mg^{-1}$ cytosolic protein)	[MDA] ($nmol \cdot mg^{-1}$ protein)	[UBI] ($ng \cdot mg^{-1}$ cytosolic protein)	CAT (mmol. min-1·mg^{-1} cytosolic protein)	Casp-3 ($\mu g \cdot mg^{-1}$ cytosolic protein)	TAC ($\mu mol \cdot mg^{-1}$ cytosolic protein)
control	0.711	11.21	-	0.062	-	47.95	0.865	0.333
	0.730	4.76	-	-	0.10	12.55	-	-
	0.550	13.55	98.02	-	0.09	35.69	0.638	0.203
	0.602	17.51	64.46	0.076	0.10	33.85	0.405	-
	0.615	6.84	84.61	0.031	0.10	15.77	0.721	0.414
	0.650	6.05	74.82	0.068	-	19.13	0.583	0.353
	0.854	11.93	60.33	0.056	0.10	12.90	0.393	0.231
	0.731	-	61.09	0.050	0.10	31.61	0.716	0.565
1 $\mu g.L^{-1}$	0.534	30.13	138.67	0.067	0.12	16.03	0.833	0.489
	0.562	22.74	78.49	0.030	0.10	10.85	0.480	0.288
	0.487	31.66	131.41	0.039	0.12	8.26	0.706	0.380
	0.545	-	91.08	-	-	12.99	0.490	0.285
	0.533	-	114.97	0.041	0.10	10.86	0.593	0.295
	0.603	41.37	174.47	0.052	-	14.49	0.834	0.442
	0.662	-	126.45	0.043	0.11	9.17	0.844	0.472
	0.594	-	78.31	-	0.09	10.23	0.400	-
10 $\mu g.L^{-1}$	0.615	32.96	85.69	0.026	0.10	8.60	-	0.263

100 µg/L^{-1}	0.538	-	152.80	0.047	0.10	9.96	0.765	0.422
	0.511	54.49	177.90	0.048	-	22.86	0.912	0.395
	0.518	50.24	184.91	0.060	0.10	13.58	1.128	0.701
	0.727	-	79.85	0.053	0.10	-	0.673	0.268
	0.531	27.67	94.05	0.024	0.11	17.90	1.455	0.747
	0.549	19.497	169.97	-	0.11	23.91	1.321	0.679
	0.565	-	104.43	-	0.09	25.54	-	-
	0.469	167.76	151.47	0.039	0.11	60.79	0.670	0.378
	0.849	246.08	113.34	0.019	0.11	190.80	0.581	0.377
	0.595	180.99	143.00	0.049	0.10	-	0.916	0.490
	0.690	-	134.52	0.048	0.09	-	0.612	0.270
	0.686	129.29	151.47	0.038	0.10	65.59	0.670	-
	0.472	106.92	-	0.092	-	-	1.293	0.848
	0.544	-	121.81	0.014	0.10	233.51	0.969	-
	0.595	-	155.71	0.085	0.12	-	-	-

A.3 Oxidative stress biomarker responses to 17α-ethinylestradiol (EE2) exposure in the mussel *Mytilus galloprovincialis*

Condition	Bradford ($mg \cdot mL^{-1}$ protein)	GST ($nmol \cdot min^{-1} \cdot mg$-1 cytosolic protein)	SOD ($U \cdot mg^{-1}$ cytosolic protein)	[MDA] ($nmol \cdot mg^{-1}$ protein)	[UBI] ($ng \cdot mg^{-1}$ cytosolic protein)	CAT (mmol. min-1·mg^{-1} cytosolic protein)	Vitelogenin (µg ALP/ mg protein)
control	2.812	2.78	1628.213	-	-	4.485	-
	1.975	5.49	2291.128	0.011	0.022	-	2.239
	1.983	3.28	1937.819	0.011	0.018	4.780	3.442
	1.378	-	2367.669	0.014	-	-	1.416
	0.848	3.37	3537.691	0.006	0.027		3.387
	1.148	11.67	3162.637	-	0.036	-	4.641
	1.139	-	3096.537	0.013	0.034	5.329	3.352
	1.215	-	3553.114	0.007	0.036	6.118	2.680
10 $ng.L^{-1}$	1.382	8.71	3122.483	0.005	-	3.667	4.105
	0.857	-	3253.058	0.009	0.014	-	4.834
	1.545	15.51	2486.895	0.016	0.012	2.574	-
	1.912	11.20	-	-	-	4.411	4.033
	1.351	13.69	3156.588	0.018	0.016	-	-
	1.216	9.44	3461.949	0.008	0.010	2.672	4.172
	1.529	-	2717.707	0.009	0.015	-	4.497
	1.965	10.22	2276.323	0.018	0.017	3.573	-
30 $ng.L^{-1}$	2.832	-	-	-	0.048	-	4.439
	1.779		2071.141	0.015	0.026	4.152	4.951

300 ng.L^{-1}	1.317	-	2718.363	0.013	0.009	2.848	8.403
	2.051	14.53	2232.127	-	-	2.781	5.724
	1.998	8.36	2265.725	-	0.018	3.265	-
	1.768	8.66	2530.581	0.015	0.013	-	5.339
	1.214	6.45	-	0.014	0.027	-	-
	1.305	-	2662.152	-	-	3.814	5.650
	1.243	-	3598.170	0.012	0.092	-	10.636
	1.489	11.97	3145.040	0.011	0.019	-	7.230
	1.755	15.32	2549.257	0.011	0.015	6.255	8.266
	0.879	-	5150.167	0.013	-	6.232	10.303
	2.082	11.56	1946.587	0.009	0.015	6.364	5.971
	0.786	13.91	5688.345	0.017	0.030	5.809	11.832
	0.867	14.47	5037.649	-	0.109	3.800	11.434

B

Appendix (Transcriptomic Analysis)

B.1 Transcriptomic analysis of the mussel *Mytilus galloprovincialis* exposed to environmental contaminants

Sample	Height/ Width/Thick-ness)	Gender	Weight (mg)	RNA ID	Sample No.	Nucleic Acid ($ng.\mu L^{-1}$)	A260/A280	A260/A230	A260	A280	Nucleic Acid Factor	Baseline Correc-tion (nm)	Baseline Absorb-ance
Control	39/21/14	F	1086	C1F	1	109.966	2.102	2.232	2.749	1.308	40.0	340	0.000
	32/10/18	M	395	C5M	2	200.015	2.130	2.283	5.000	2.348	40.0	340	0.004
	34/18/12	F	1231	C6F	3	23.134	2.107	1.62	0.578	0.274	40.0	340	0.014
	32/11/19	M	592	C8M	4	93.644	2.119	2.389	2.341	1.105	40.0	340	0.011
PFOA 1 $\mu g.L^{-1}$	31/17/14	M	629	P12M	5	230.728	2.108	2.459	5.768	2.737	40.0	340	-0.264
	34/16/13	M	593	P13M	6	184.193	2.119	2.375	4.605	2.173	40.0	340	-0.261
	43/22/16	F	1198	P16F	7	184.182	2.111	2.473	4.605	2.181	40.0	340	-0.255
	44/25/16	F	1139	P18F	8	206.900	2.111	2.431	5.172	2.45	40.0	340	-0.251

PFOA 100µg.L^{-1}	32/16/14	F	545	P1001F	9	62.68	2.136	1.518	1.567	0.734	40.0	340	-0.258
	33/16/12	F	635	P1002F	10	221.59	2.112	2.389	5.54	2.623	40.0	340	-0.240
	32/18/12	M	492	P1004M	11	228.945	2.102	2.526	5.724	2.723	40.0	340	-0.240
	36/19/12	M	563	P1005M	12	167.851	2.116	2.418	4.196	1.983	40.0	340	-0.246
EE2 10ng.L-1	35/19/13	M	503	E101M	13	395.166	2.12	2.377	9.879	4.659	40.0	340	0.04
	35/19/14	M	567	E102M	14	100.273	2.363	2.054	2.507	1.061	40.0	340	0.053
	34/19/14	F	904	E107F	15	168.674	2.390	2.041	4.217	1.764	40.0	340	0.186
	36/21/16	F	890	E108F	16	125.89	2.234	2.51	3.147	1.409	40.0	340	0.275
EE2 1000ng.L^{-1}	35/18/13	F	475	E10003F	17	145.432	2.260	2.315	3.636	1.609	40.0	340	0.012
	36/16/15	M	353	E10006M	18	161.169	2.111	2.016	4.029	1.909	40.0	340	0.045
	31/15/10	M	583	E10007M	19	155.797	1.661	1.74	3.895	2.345	40.0	340	-0.246
	35/22/13	F	464	E10008F	20	149.05	2.119	2.217	3.726	1.759	40.0	340	0.118

TBBPA 1 µg.L^{-1}	32/16/11	M	446	T12M	21	172.857	2.105	1.952	4.321	2.053	40.0	340	0.002
	34/20/13	M	462	T14M	22	153.504	2.123	2.39	3.838	1.808	40.0	340	0.023
	31/19/15	F	638	T16F	23	64.628	2.178	2.283	1.616	0.742	40.0	340	0.025
	39/20/14	F	510	T17F	24	317.466	2.127	2.488	7.937	3.732	40.0	340	0.029
TBBPA 10 µg.L^{-1}	33/18/14	M	425	T102M	25	64.635	2.057	2.373	1.616	0.786	40.0	340	0.026
	33/20/12	M	369	T104M	26	197.148	2.088	2.442	4.929	2.361	40.0	340	0.024
	36/19/13	F	636	T107F	27	47.544	1.924	1.436	1.189	0.618	40.0	340	0.023
	36/18/14	F	660	T108F	28	170.118	1.945	2.38	4.253	2.186	40.0	340	0.038
TBBPA 100 µg.L^{-1}	36/19/17	F	1275	T1001F	29	130.207	2.116	2.207	3.255	1.538	40.0	340	0.054
	33/20/12	F	710	T1002F	30	451.292	2.137	2.447	11.282	5.278	40.0	340	0.029
	30/17/13	M	371	T1006M	31	119.247	2.080	2.493	2.981	1.433	40.0	340	0.032
	35/21/12	M	575	T1008M	32	119.033	2.127	2.402	2.976	1.399	40.0	340	0.032

B.2 List of 20 differentially expressed genes (padj < 0.05), 17 down-regulated and 3 up-regulated transcripts, with 7 genes assigned putative functional annotation.

Gene LOC	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Gene description	Gene type
LOC143083663	107.72645	-4.12084	0.80764	-5.10232	3.35508E-07	0.00868	uncharacterized	down
LOC143083612	39.88345	-3.47615	0.74089	-4.69184	2.70760E-06	0.03504	uncharacterized	down
LOC143043219	55.65847	-3.51639	0.83380	-4.21730	2.47247E-05	0.04266	uncharacterized	down
LOC143045910	897.76734	-3.51685	0.83168	-4.22863	2.35114E-05	0.04266	myosin heavy chain. striated muscle-like	down
LOC143046845	30.99217	3.20593	0.75270	4.25925	2.05115E-05	0.04266	uncharacterized	up
LOC143057723	61.97510	-2.46133	0.57490	-4.28130	1.85806E-05	0.04266	uncharacterized	down
LOC143059680	17.18295	-2.98137	0.68909	-4.32655	1.51463E-05	0.04266	solute carrier family 2. facilitated glucose transporter member 3 -like	down
LOC143065479	9.00582	-3.76801	0.83728	-4.50028	6.78653E-06	0.04266	Cyclic nucleotide-binding domian-containing protein 2 like	down
LOC143067102	23.38427	-3.54650	0.82596	-4.29380	1.75637E-05	0.04266	HHIP-like protein 2	down
LOC143067701	956.63634	-3.13022	0.73879	-4.23698	2.26544E-05	0.04266	myosin essential light chain. strated adductor muscle-like	down
LOC143072083	145.17917	-3.41811	0.80699	-4.23561	2.27934E-05	0.04266	uncharacterized	down
LOC143072566	18.71416	-3.50610	0.82045	-4.27340	1.92515E-05	0.04266	uncharacterized	down
LOC143080716	215.41530	-3.23914	0.73520	-4.40582	1.05384E-05	0.04266	uncharacterized	down
LOC143083666	1194.87791	-3.40667	0.78474	-4.34116	1.41730E-05	0.04266	uncharacterized	down
LOC143085422	32.48100	2.79241	0.65992	4.23147	2.32172E-05	0.04266	Toll-like receptor 4 (TLR4)	up
LOC143042138	65.46283	-3.42575	0.82490	-4.15295	3.28212E-05	0.04954	uncharacterized	down
LOC143054894	39.82201	-1.60328	0.38858	-4.12601	3.69111E-05	0.04954	myotubularin-related protein 9 -like	down

LOC143064592	5.04272	-3.41492	0.82934	-4.11763	3.82793E-05	0.04954	uncharacterized	down
LOC143072557	43.51511	2.55787	0.61929	4.13035	3.62209E-05	0.04954	uncharacterized	up
LOC143072576	19.53911	-3.46222	0.83679	-4.13748	3.51146E-05	0.04954	uncharacterized	down

Sandra Copeto

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⟨2026⟩