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Impact of emerging micropollutants on the reproductive health of the Mediterranean mussel *Mytilus galloprovincialis*

TESI DI DOTTORATO

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AChE	AcetylCholinEsterase
AChRs	Nicotinic Acetylcholine Receptors
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BPs	Bioplastics
°C	Degrees Celsius
µL	Microliter
µm	Micrometer
^{1}H NMR	Proton Nuclear Magnetic Resonance
act	Actin
ACTH	Adrenocorticotropin
ADG	Adipogranular cells
AMPK	5' AMP-activated protein Kinase
AP1	Activator Protein 1
AR	Androgen Receptors
ARDS	Acute Respiratory Distress Syndrome
Bax	Bcl-2-associated x protein
BCAA	Branched Chain Amino Acids
Bcl2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
cas3	Caspase 3
CAT	Catalase
cDNA	Complementary DNA
CDNB	1-chloro 2,4 dinitrobenvene
CEC	Contaminants of Emerging Concern
CNR-IRBIM	Institute for Marine Biological Resources and Biotechnology of the National Research Council
CRF	Hypothalamic Corticotropin-releasing Factor
Cu/Zn-SOD	Cooper/Zinc-SuperOxide Dismutase
Cx43	Connexin 43

Cyp450	Cytochrome P450
D_2O	Deuterated water
Da	Dalton
DDT	Dichloro-Diphenyl-Trichloroethane
DMPC	Dimyristoylphophatidylcholine
DNA	DeoxyriboNucleic Acid
DO	Dissolved Oxygen
dOo	Degenerated Oocytes
d-PAS	Diastase- Periodic Acid Schiff
DSS	Sodium 2,2-dimethyl-2-silapentane-5-sulfonate
DW	Distilled Water
E	Eosin
E2	Estradiol
EBR	ERα binding regions
EC	Emergent Contaminant
ECHA	European Chemicals Agency
ED	Endocrine Disrupting
EDC	Endocrine Disrupting Compound
EDTA	EthyleneDiamineTetraacetic Acid
EDTA AG	Advisory Group on Endocrine Disrupters Testing and Assessment
EFSA	European Food Safety Authority
elfα	Elongation factor α
EMPs	Emerging MicroPollutants
ERA	Environmental Risk Assessment
ERα	Estrogen Receptor α
Fas-L	Fibroblast-Associated cell-Surface-ligand
FAA	Free Amino Acids
FID	Free Induction Decay
FF	Female Follicle
FSH	Follicle Stimulating Hormone
FSW	Filter Sea Water
GC	Glucocorticoid

GC-MS	Gas Chromatography Mass Spectrometry
GJIC	Gap Junctional Intercellular Communication
GLTU5	GLUcose Transporter type 5
Gly	Glycogen
GPx	Glutathione Peroxidase
GR	Glucocorticoid Receptors
GRE	Glucocorticoid Response Elements
GSH	Reduced Glutathione
GST	Glutathione *S*-transferase
H	Hematoxylin
h	Hour
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic-Pituitary-adrenal gland Axis
HPG	Hypothalamic-Pituitary-Gonadal axis
IL-6	Interleukin-6
IMP	Inosine MonoPhosphate
ISS	Italian Higher Institute of Sanity
JNK	edc-Jun N-terminal Kinases
JRC	Joint Research Centre
K	Kelvin
KDa	KiloDalton
KH_2PO_4	Diidrogenofosfato di potassio
K_2HPO_4	Dipotassium phosphate
LH	Luteinizing Hormone
LPO	Lipid peroxidation
M	Molar
MAPK	Mitogen-Activated Protein Kinase
mbar	Millibar
MDA	Malondialdehyde
MDR1	Multi-Drug Resistance 1
MEC	Measured Environmental Concentration
MF	Male Follicle
mg	Milligrams

MHz	MegaHertz
min	Minutes
mL	Milliliters
mm	Millimeter
mM	MilliMolar
nmol	nanomol
MPs	Microplastics
mRNA	Messenger RiboNucleic Acid
MRP	Multidrug Resistance-associated Protein
ms	Milliseconds
mS/cm	MilliSiemens per cm
MTFs	Mouse Testicular Fragments
mV	MilliVolt
Na/K ATPase	Sodium/Potassium ATPase pump
NADH	Reduced Nicotinammine Adenine Dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor kappa-light-chain-enhancer of
nGRE	negative Glucocorticoid Response Elements
NLRP3	Nucleotide-binding domain, Leucine-Rich–containing family, Pyrin domain–containing-3
NOESY 1D	Nuclear Overhauser Effect SpectroscopY One Dimensional
NSAIDs	Non-Steroidal Anti-Inflammatories Drugs
O	Oocytes
OECD	Organization for Economic Co-operation and Development
Oo	Ovogonia
ORP	Oxidation and Reduction Potential
OvoA	5-histidylcysteine sulfoxide synthase
p	*p value*
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PCL	Poly-Caprolactone

PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PhACs	Pharmaceutically Active Compounds
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PKA	cAMP-dependent Protein Kinase
PLA	Acid polylactic
PNECaq	Predicted No-Effect Concentration of aquatic species
pO	Pear-shape Oocytes
POPs	Persistent Organic Pollutants
ppm	Parts per million
PS	Polystyrene
PVC	PolyVinyl Chloride
QSAR	Quantitative Structure-Activity Relationship
RAS	Rat Sarcoma Virus
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RF	Radio Frequency
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RQ	Risk Quotient
rRNA	Ribosomal RNA
RT	Room Temperature
RT-qPCR	Real Time-quantitative Polymerase Chain Reaction
SAIDs	Steroidal Anti-Inflammatories Drugs
Sal	Salinity
SCOTT	Succinyl-CoA transferase
SD	Standard Deviation
sec	Seconds
SPZ	Spermatozoa
SULT	Sulfotransferase

SVHC	Substance of Very High Concern
SW	Sea Water
TBA	Thio-barbituric Acid
TBARS	Thio-Barbituric Acid Reactive Substances
TCA	Tri-Chloroacetic Acid
tds ppt	Total Dissolved Solids parts per thousands
TEP	1,1,3,3-tetraethoxypropane
TNF	Tumor Necrosis Factor
Tris-HCL	Tris-Hydrochloride
UDP-Glucose	Uridine diphosphate glucose
UNEP	United Nations Environment Programme
VCT	Vesicular Connective Tissue cells
vOo	Vitellogenic oocytes
Vtg	Vitellogenin
WHO	World Health Organization
WoE	Weight of Evidence
WWTPs	WasteWater Treatment Plants
ΔΨ m	Relative value of the mitochondrial membrane potential

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CHAPTER 3

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CHAPTER 6

Biological responses of Mytilus galloprovincialis gonads exposed to polylactic acid microplastics (PLA MPs)

PREFACE

This thesis is aimed at evaluating the potential biological impact on the reproductive health of the Mediterranean mussel *Mytilus galloprovincialis* of different classes of emerging micropollutants (i.e. drugs, microplastics and bioplastics) that, mostly with the advent of the SARS CoV-2 pandemic, have become widely distributed in the aquatic ecosystems.

A detailed introduction to the concept of endocrine disrupting compounds (EDCs), with a focus to their general characteristics, mechanisms of action, and most abundant classes found in marine ecosystems was therefore described in the Chapter 1, together with an updated review of the potential threats of these emerging micropollutants on non-target organisms. The model species used for this thesis was also introduced, with a general description of its major characteristics and more specifically on the target organ, namely the gonads, with detailed sex-related peculiarities.

The overall aim of the thesis was therefore specified in the Chapter 2.

The fundamental physiological characteristics of the reproductive system of the two phenotypes (male and female) of the sentinel gonochorous species grown in a contaminant-free environment were therefore explored. To address this aim, a multi-biomarker approach including histological, histochemical and metabolomics investigation was applied on mussel gonads of both sexes in order to reveal sex-dependent peculiarities in terms of morphological tissue organization, presence of glycogen, and metabolome profiling (Chapter 3).

The potential biological impact induced by the glucocorticoid drug dexamethasone (Dex) were investigated on the male and female reproductive system of mussels, following a sub-chronic exposure (12 days) to different environmental concentrations of Dex. A multi-biomarker approach including conventional (i.e. chemical, histological, histochemical, immunohistochemical) and innovative (i.e. molecular and metabolomics) techniques was therefore applied on mussel gonads of both sexes in order to reveal sex-dependent responses induced by the anti-inflammatory drug tested (Chapter 4). In detail, alterations in energy metabolism and osmotic imbalance were recorded in both sexes, combined with the preservation of a proliferative activity at gonad level.

Moreover, the potential biological impact induced by two different doses of polystyrene microplastics (PS MPs) were also investigated on the male and female reproductive system of mussels, following an acute exposure of 48 hours. Histo-morphological, histochemical and metabolomics approaches were therefore applied on mussel gonads of both sexes in order to reveal sex-dependent responses induced by PS MPs (Chapter 5). In detail, differential compensatory mechanisms were observed between males and females to adapt to an altered cellular bioenergetic state, unbalanced osmoregulation and degenerative effects on gametic cells.

Additionally, the potential negative interferences of biological microplastics of polylactic acid (PLA MPs), tested at realistic concentrations, were evaluated on male and female gonads of the Mediterranean bivalve, challenged for 7 days of treatment. To address this aim, histological, histochemical and biochemical techniques were applied on mussel gonads of both sexes in order to reveal sex-dependent responses induced by PLA MPs (Chapter 6). In detail, the activation of anti-inflammatory strategies was observed in both sexes with a marked infiltration of haemocytes throughout the gonadal connective tissue, accumulation of glycogen reserves, and early compensatory strategies against the occurrence of oxidative stress induced by PLA MPs.

The general conclusions of this thesis, provided by a summary of the major findings reported by each experimental study conducted to explore the potential biological impact on the reproductive health of the Mediterranean mussel *Mytilus galloprovincialis* challenged by different classes of emerging micropollutants (i.e. drugs, microplastics and bioplastics), are reported in Chapter 7.

CHAPTER 1

Endocrine disrupting compounds as micropollutants

CHAPTER 1

Endocrine disrupting compounds as micropollutants

1.1 General introduction

In recent decades, it has been brought to light that the frequency of exposure to some classes of pollutants, called "Endocrine Disruptors" (ED), can give rise to biological alterations in adult and embryonic organisms that affect the health of the reproductive system and the physiology of many animals, including humans (Wei et al., 2024; Ghosh et al., 2022; Lu et al., 2022; Yilmaz et al., 2020).

The group of molecules identified as ED is highly heterogeneous and includes synthetic chemical compounds of various nature that are introduced into daily life with extreme ease, being drugs (Reis et al., 2024), personal care products (Darbre & Harvey, 2022), plasticizers (Xing et al., 2022), pesticides or their derivatives (Ji et al., 2020).

The pretentious nature of a satisfying state of well-being, typical of the most developed countries, leads to an increase in the "demand" on the market for these substances year after year. However, the problem does not arise so much because of the production of these compounds, but because of the spasmodic and inconsiderate use that is made of them, followed by a poor management in terms of removal from the environment.

Thus, inadequate disposal practices, improper infrastructures for waste treatment and not very innovative bioremediation plans determine that products such as drugs, metals, pesticides, plastic utensils and therefore plasticizers, created to improve and facilitate life, become a great burden for global health (Patel et al., 2020; Rosenfeld & Feng, 2011). The intensive use and indifference towards the potential consequences of these wastes leads to the accumulation of large quantities of these substances at environmental level (Afsa et al., 2020; Hahladakis, 2020), mainly close to coastal areas where wastewater treatment plant discharge usually occurs. Therefore, this has raised the attention of the scientific community towards the potential impact on non-target aquatic biota that is most frequently exposed to this class of micropollutants and their mixtures.

From what has been discussed, it appears evident that the experimental study focused on the exposure of model organisms to pollutants with endocrine disrupting value represents a crucial step for a greater understanding of the effects of EDs on biological systems that are not directly targeted. Thus, following the coordinates of the European Directive 2000/60 UE on water, in which “the body of water is considered as an ecosystem that must be safeguarded and that can be monitored through qualitative and quantitative elements deducible from the state of health of its fauna” (European Union, 2000), the use of experimental trials is attested as suitable to better identify and classify these types of substances.

The employment of marine invertebrates as model organisms is common and well suited to achieve this purpose. The sentinel species *Mytilus galloprovincialis*, due to its easy management, tendency to bioaccumulation and resilience towards various classes of pollutants, allows to obtain pertinent and detailed answers on the environmental risk assessment (Suárez-Ulloa et al., 2013). Furthermore, being an edible species and widely used within the Mediterranean diet, mussels as bioindicators in ecotoxicological studies raise more attention to the danger that substances such as ED entering the marine ecosystem may induce, becoming a threat to the reproductive health of resident biota and humans.

In the present work, conventional approaches such as chemical, histological, histochemical and immunohistochemical analysis have been combined and supported by the application of “-omics” techniques, such as metabolomics and transcriptomics, with the final aim to identify biomarkers that give a clearer and more comprehensive understanding of the effect of different classes of EDs, including drugs, microplastics and bioplastics, on male and female reproductive health of the Mediterranean mussel *M. galloprovincialis,* commonly used sentinel species in environmental biomonitoring.

1.2 Environmental monitoring

The European Marine Strategy Framework Directive MSFD-2008/56/CE, at article 3 paragraph 5, states "Good environmental status of marine waters means the ability to preserve ecological diversity, the vitality of seas and oceans so that

they are clean, healthy and productive by maintaining the use of the marine environment at a sustainable level and safeguarding the potential for uses and activities of present and future generations" (European Directive., 2008).

This Directive lays the foundations for a correct maritime policy applied to an ecosystem approach that aims to guarantee the protection of the sea system, proposing to commit to the protection of marine biodiversity, establishing a balance with the needs of eco-social development. However, the adaptive management that, by this Directive, has provided for constant monitoring over the last few years, has seen the balance tip towards an imbalance against the health of marine biota.

One of the five phases of the "Environmental Monitoring Program" is the assessment of the state of health of the marine environment that considers the human activities, the use of the marine environment and the costs of its degradation. To date, the impact of human activity in all its declinations has had severe repercussions on the state of health of marine ecosystems.

According to the Italian Civil Protection Department, marine pollution is a direct consequence of the release of contaminated wastewater from urban sewage, agricultural, industrial and sanitary drainage and from illegal discharge events (Legislative Decree, 1999) (Figure 1.1).

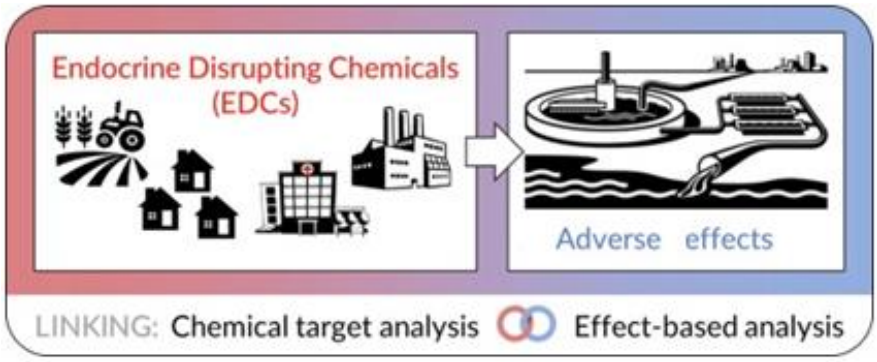

Figure 1.1 Summary overview of the release of EDCs into the aquatic environment.

These, not being appropriately treated at the level of wastewater treatment plants (WWTPs) not fully performing for the various types of compounds, convey the pollutants into the sea.

Among these, the “Emerging Contaminants” (EC) are the most commonly found chemical substances and are equivalent to those compounds of anthropogenic or even natural origin that are not yet precisely regulated and monitored at an environmental level, but which can lead to an adverse effect of a negative nature on the environment or on health of biota and humans, and are therefore labelled as Contaminants of Emerging Concern (CEC) for aquatic ecosystems (Schoenfuss, 2021). These CECs include active pharmaceutical compounds (PhACs), pesticides, industrial substances such as plasticizers and flame retardants, the well-known polycyclic aromatic hydrocarbons and also micro-materials such as microplastics (Figure 1.2).

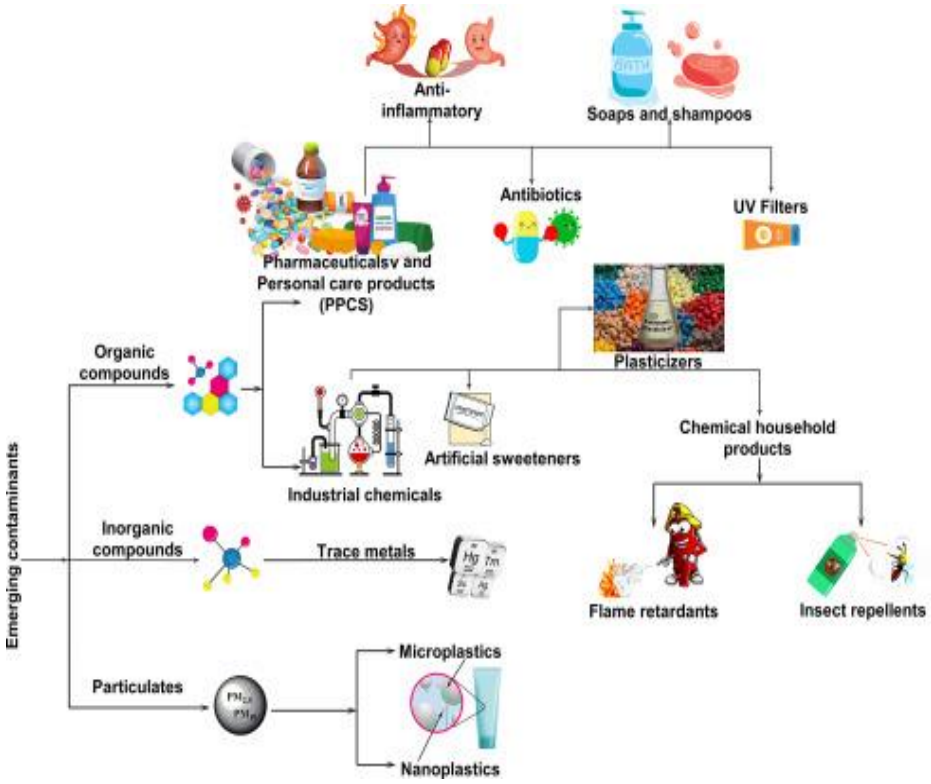

Figure 1.2 Overview of the Emergent Contaminants (ECs).

In recent years, greater concern has been directed at those substances that appear to demonstrate a tendency to interfere with the stability and functionality of the endocrine system, altering its normal activity and causing functional variations in organisms both in the adult (Thambirajah et al., 2022) and embryonic (Tang et al., 2020) state, in germ cells (sperm quality, ovarian failure) (Menezo et al., 2019), but also verifying a parental transmission of the effect from mother to foetus (Mallozzi, et al., 2016), thus threatening the normal functional state of hormonal

axes involved in reproduction, stress and cellular bioenergy and putting at risk the general state of health and future fertility.

The limited attention paid to these ECs to date, and in particular to the category of "Endocrine Disrupting Compounds" (EDCs), is due to the low concentration detected in the environment from sediments, surface and deep waters to biota (fish), in the range between parts per trillion (ppt) and parts per billion, i.e. between ng/L and μg/L (Figure 1.3)

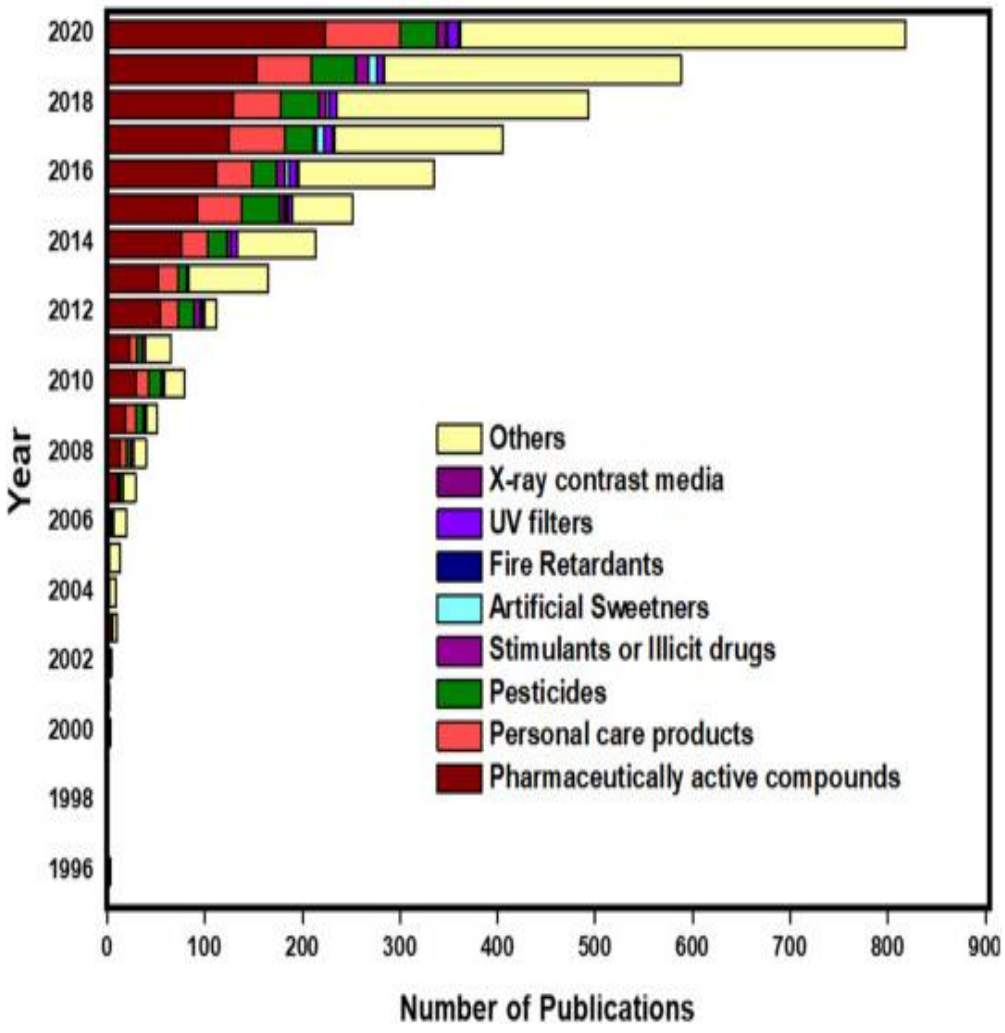

Figure 1.3 Overview of the most common and studied endocrine disrupting compounds (EDCs) according to the amount of publications.

However, the constant release of EDCs through WWTPs determines a greater frequency in the areas closest to the plants, such as coastal areas, and therefore a greater tendency to persistence of these chemicals in the environment (Köck-Schulmeyer et al., 2021; Rout et al., 2021).

For this reason, resident and non-directly targeted biota could be more exposed and subject to bioaccumulation, leading to short- and long-term negative effects on the health and sustainability of the ecosystem directly involved and not (Anand et al., 2022; Álvarez-Ruiz et al., 2021; Köck-Schulmeyer et al., 2021; Parida et al., 2021), since it could lead to biomagnification events in the higher species such as humans, correlated to the food web (Wang et al., 2021).

It has been proven that, when released into an aquatic ecosystem after partial or non-existent treatment, ECs can represent a significant risk (Parida et al., 2021). Many studies have considered the Risk Quotient (RQ) for different ECs classes, a parameter that is widely used for Environmental Risk Assessment (ERA), since it can be used as an effective parameter to estimate the potential effects of ECs on aquatic life. It is a ratio between the Measured Environmental Concentration (MEC) of a given compound and the Predicted No-Effect Concentration of aquatic species (PNECaq) (Nika et al., 2020).

Despite these efforts, the verification and evaluation of the effects of these CECs on the health of non-target biota is limited by analytical techniques that are sometimes not sufficiently sensitive.

Furthermore, it is difficult to investigate the existence of direct correlations between the pollutant, which represents the stress factor (xenobiotic), and the response at the level of the biological system. For this reason, different biomarkers are used with which to define a complete and clear picture representing the biological responses of biota to ECs. The variations in quality and quantity of biomarkers related to their specific action can be the validation of the link between the chemical and the effect of the stressor on the organism, thus determining its toxicity level (Khan et al., 2022; Diaz-Sosa et al., 2020).

It is known that there are four classes of biomarkers that establish a multilevel approach to understand what happens in the biological system. Briefly, these are: biochemical (enzymes); physiological (development, reproduction); toxicological (social behaviour, lethality, teratogenicity); and ecological (loss, acquisition, changes in ecosystem hierarchies) biomarkers (McCarty & Munkittrick, 2008). Using these biomarkers, it is possible to describe the toxicity of ECs in their complexity; for example, epigenic or genomic variations tend to be induced first

compared to changes in tissue organization, since they require a more complex and long-lasting architecture of modifications in terms of time.

The use of various classes of specific biomarkers for many categories of bioindicators, namely organisms that respond to the environment that surrounds them, provide information on the physical and chemical condition, thus helping to highlight not only the presence of xenobiotics, but also ensuring an early warning of alteration of the surrounding environment, contributing therefore to determine the origin of pollution (Lomartire et al., 2021).

Among the biological groups used in environmental biomonitoring, benthic macroinvertebrates, such as mussels, are widely used. They are predisposed to be excellent model organisms, because they are cheap and easy to manage in laboratory conditions, besides being able to bioaccumulate various classes of pollutants (Cappello et al., 2021).

In the ecotoxicological study, to evaluate both the state of health of the physical-chemical environment and that of the resident biota, it is necessary to use a bioindicator from which multilevel biomarkers can be measured, to obtain a complete picture of the health status. In this work, in addition to the biomarkers obtained with canonical techniques (morphological parameters, histochemical reactions and antigen-antibody reactions), innovative methodologies were also applied using the innovative "-omics" approaches, such as metabolomics based on Proton Nuclear Magnetic Resonance (^{1}H NMR), which nowadays are increasingly confirmed in environmental biomonitoring, presenting themselves as an additional contribution for a more complete and broad overview of the health status of the biological system (Sun et al., 2022; Zhang et al., 2021).

The integration of conventional and high-throughput approaches on a sentinel species allows to identify emerging pollutants as a worrying risk that has the potential to impact not only on the individual organism, but also on the species, causing repercussions on biodiversity, as well as on the stability of aquatic ecosystems.

1.3 Endocrine disrupting compounds (EDCs)

In 2017, the World Health Organization (WHO) updated the definition of endocrine disruptor dating back to “The Weybridge Report” of 1996, defining it as an “exogenous substance, or a mixture, that alters the functionality of the endocrine system, causing adverse effects on the health of the organism or of the progeny or of a (sub)population” (Solecki et al., 2017).

An endocrine disrupting compound (EDC) can act by simulating the mechanism of action of hormones or other cellular mediators, can compete with the ligands of specific extra or intracellular receptors deposited to transduce the signal or directly interfere in metabolic pathways by altering their normal development or, again in the transport of hormones or in their concentration and exert an epigenetic action or modulation of gene expression (Kirtana & Seetharaman, 2022; Yilmaz et al., 2020; Combarnous & Nguyen, 2019).

In nature there are compounds with endocrine disrupting activity. Among these, isoflavonoids, lignans and coumestans represent the class of phytoestrogens produced by plants and present in their seeds (soy, green tea, etc.). Furthermore, some types of molds also produce compounds with estrogenic activity such as resorcilic acid lactones known as mycoestrogens (Murkies et al., 1998).

However, almost all EDCs known to date are of synthetic nature and consequently of anthropogenic origin (Santos et al., 2007).

Pesticides, such as insecticides and fungicides, pharmaceutical compounds (PhACs) such as paracetamol, salicylic acid and dexamethasone (Zhao et al., 2024; Lalone et al., 2012), additives such as bisphenol A and phthalates, flame retardants such as polybrominated diphenyl ethers (ECHA, 2024; Cunha et al., 2022; Kloas et al., 2009), trace elements (mercury, cadmium, etc.) (Chakraborty et al., 2021), micro and nanoplastics both considered individually and for their adjuvant action of other EDCs (Lin et al., 2023; Ullah et al., 2023) represent only a part of this large class, which is constantly being updated, as demonstrated by some recent articles on polylactic acid (PLA), which appears to induce changes in the reproductive organs and hormone levels (Zhang et al., 2024; Chagas et al., 2021).

The Istituto Superiore di Sanità (ISS) reports that there are 320 active substances with endocrine disrupting characteristics for which there are already regulations in force for restriction and protection.

However, the identification of such substances is not entirely obvious, being a topic of recent scientific interest and constantly updated. At European level, EDCs are generically, but univocally, classified as compounds of worrying interest for having direct or indirect actions on the endocrine and reproductive health of organisms and for being persistent chemical substances or molecules and tending to bioaccumulation and biomagnification (Matovani & Balbi, 2022).

The identification of compounds with endocrine disrupting value is continuously developing. The bodies that have been charged with the task of carrying out these assessments are the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA) with the technical support of the Joint Research Centre (JRC), which have drawn up in 2018 the Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No. 528/2012 and (EC) No. 1107/2009, in which scientific opinions are reported that are taken on board by the risk analysis managers at the Commission and the European Parliament (ECHA, EFSA & JRC, 2018)

The criteria for designating a substance as a potential EDC (ECHA, EFSA& JRC, 2018) are the following:

1. "It causes an adverse effect in a healthy organism and/or on the progeny or on organisms that are not target. The effect may result in an alteration of morphology, physiology, growth, development, reproduction or lifespan, implying a damage to functionality and a negative involvement in sustaining or being more subject to other types of stress."

2. "Causes alteration of the normal endocrine system."

3. "The adverse effect is directly related to the mechanism of endocrine disruption."

The above parameters, as previously reported, apply to humans and non-target organisms (Figure 1.4).

Factors with respect to humans	Factors with respect to non-target organisms
'both positive and negative results'	*'both positive and negative results, discriminating between taxonomic groups (e.g. mammals, birds, fish, amphibians) where relevant'*
'the relevance of the study designs, for the assessment of adverse effects and of the endocrine mode of action' [c]	*'the relevance of the study design for the assessment of the adverse effects and its relevance at the (sub)population level, and for the assessment of the endocrine mode of action'* [c]
	'the adverse effects on reproduction, growth/development, and other relevant adverse effects which are likely to impact on (sub)populations. Adequate, reliable and representative field or monitoring data and/or results from population models shall as well be considered where available'
'the quality and consistency of the data, considering the pattern and coherence of the results within and between studies of a similar design and across different species'	*'the quality and consistency of the data, considering the pattern and coherence of the results within and between studies of a similar design and across different taxonomic groups'*
'the route of exposure, toxicokinetic and metabolism studies'	
'the concept of the limit dose, and international guidelines on maximum recommended doses and for assessing confounding effects of excessive toxicity'	*'the concept of the limit dose, and international guidelines on maximum recommended doses and for assessing confounding effects of excessive toxicity'*
'the biological plausibility of the link between the adverse effects and the endocrine mode of action' [c]	*'the biological plausibility of the link between the adverse effects and the endocrine mode of action'* [c]

Figure 1.4 Guideline for human and non-target organisms from ECHA, EFSA & JRC (2018).

The guideline states that, a priori, the potential risk should be interpreted considering it from two separate perspectives, since the conclusions on the criteria should be interpreted separately, but that the data necessary for the assessment should be filtered, considering possible overlaps and that information on non-target organisms can be useful for humans and vice versa.

The interpretation of the analytical results is based on the Weight of Evidence (WoE) approach in reference to Annex I of Regulation (EC) No. 1272/2008 (Regulation, EC No. 1272/2008, 2008) aimed at the classification and labelling of substances and mixtures (CLP Regulation) for which all data relating to the determination of the hazard must be considered as a whole, evaluating results obtained from *in vitro*, *in vivo* tests on animals and *in silico* as well as those obtained

from computer software such as Quantitative Structure-Activity Relationship (QSAR) (Andersson et al., 2018).

EDCs are an emerging reality in the field of toxicological and eco-toxicological research. In fact, they can be classified as Emerging Micropollutants (EMPs), falling within the descriptive framework of the European Directive 2013/39 since, although not all of them are included in environmental monitoring programs, they could prove to be such a risk as to consider their regulation appropriate, due to the potential ecotoxicological and/or toxicological effect, deriving from their presence in the aquatic environment (Directive 2013/39/EU, 2013).

In this regard, the Organization for Economic Co-operation and Development (OECD), within the Environment, Health and Safety Program, has developed guidelines to characterize and evaluate these compounds (Figure 1.5).

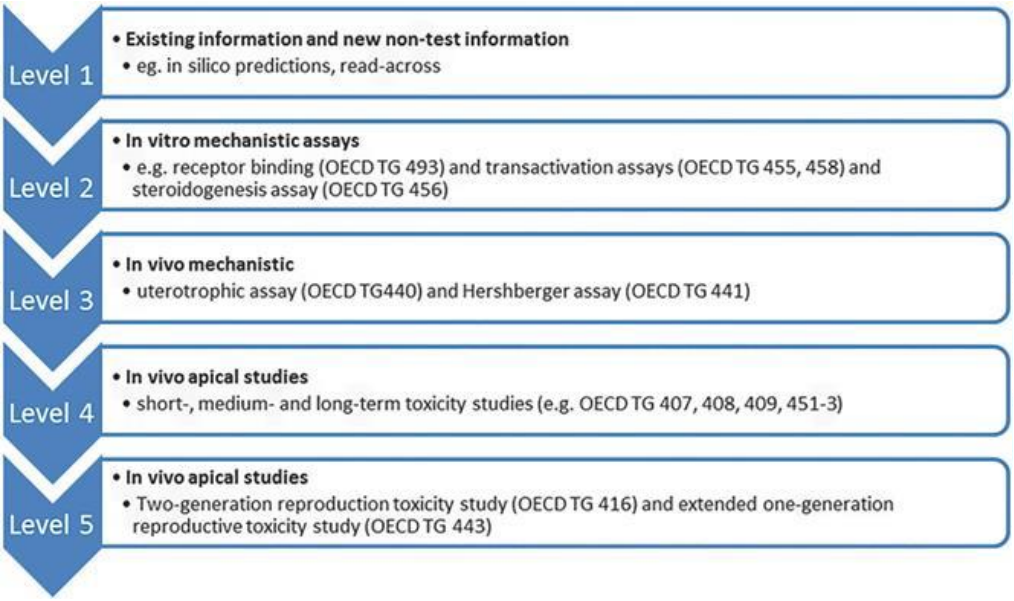

Figure 1.5 Conceptual framework of the OECD guidelines for the identification of the potential endocrine disrupting properties of chemicals.

The Advisory Group on Endocrine Disrupters Testing and Assessment (EDTA AG), among the results obtained, presents the Conceptual Framework for Testing and Assessment of Endocrine Disrupters that includes specifically standardized methods for the evaluation of structured EDCs, considering the complexity of the biological system under examination, and the Guidance Document No. 150, which is a guide on the interpretation of data obtained from single tests and suggestions

for further approaches to reduce the degree of uncertainty (Kucheryavenko et al., 2020).

The European Union has selected approximately 564 substances identified as potential endocrine disruptors, of which just under half have actually been attributed the role of EDC, while the effects of the others are still being evaluated. According to the European Regulation (EC) No. 1907/2006 of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), some of these have been classified (Legislative Decree, 2023; Ministry of Environment and Energy Security, 2023), as:

- Carcinogenic-Mutagenic-toxic for Reproduction
- Persistent, Bioaccumulative or Toxic
- Very Persistent, very Bioaccumulative

This is the rationale for an accurate investigation into the assessment of exposure risk of these compounds for both environment and human health.

1.3.1 Characteristics and mechanism of action of EDCs

Given the wide range of endocrine disrupting products, it is very difficult to find common chemical-physical characteristics for these compounds.

Their potential biological effect depends on the toxicokinetics of the compound itself, i.e. the ability to be absorbed, distributed and accumulated into the tissues, to be metabolized and excreted from the body, and on the toxicodynamics which refers to the interaction with specific sites inside and/or outside the cell (e.g. membrane/cytoplasmic/nuclear receptors, DNA responsive elements, transcription factors, enzymes) (Dutta et al., 2023; Noyes et al., 2014). Therefore, the biological effect of EDCs can vary depending on the type, but also on the level of exposure (Ghosh et al., 2022).

This context also includes the potential of exposure, which can be related to the growth status of the subject. In fact, in adult organisms it is more likely that a pathological state will develop, which may manifest itself over a longer period of time than the exposure time, while in an embryonic organism the effect could be

evident already in the early stages of life and persists or causes an imbalance that could induce death (Varjania & Sudha, 2020).

Some EDCs such as organochlorine pesticides (e.g. DDT), personal care products (e.g. triclosan) or industrial chemicals such as plasticizers (e.g. bisphenol A) can induce a dysregulation of Gap Junctional Intercellular Communication (GJIC) by either displacing connexin 43 (Cx43), a gap junctional protein, from the plasma membrane or by altering the phosphorylation pattern of its isoforms, resulting in the disruption of intercellular signalling between Leydig TM3 cells (Yawer et al., 2020).

Molecular docking analysis on the pesticide imidacloprid has shown that the neonicotinoid can bind to androgen receptors (AR) through the formation of hydrogen bonds in the active site of the receptor (Yuan et al., 2020).

Some PhACs that have a chemical structure like that of hormones, for example dexamethasone, a synthetic glucocorticoid-like compound, with a chemistry similar to cortisol, is able to bypass the plasma membrane, having hydrophobic chemical-physical properties, and to bind to glucocorticoid receptors (GR) at the cytoplasmic level (Noureddine et al., 2021; Yao et al., 2020).

Considering the potential role of agonist or antagonist of endogenous hormones, some have multiple actions. An example is given by bisphenol A (BPA), also known as diphenylolpropane, an organic compound consisting of two terminal phenolic radicals which, in addition to what was previously mentioned, can also interfere with the thyroid system as an antagonist of thyroid receptors on the plasma membrane and/or by altering the expression of genes related to the hormones themselves (Frenzilli et al., 2021).

Some flame retardants, such as bromophenols, a group of brominated compounds, can interact directly with protein structures with an enzymatic function, such as the sulfotransferase enzyme (SULT). In fact, the kinetics of inhibition by the compound on the SULT1A3 isoform has been demonstrated (Dai et al., 2022).

It has been suggested that exposure to persistent organic pollutants (POPs) during pregnancy is related to the perturbation of the thyroid system due to the epigenetic action of endocrine interference, i.e. the promotion of modifications on

DNA such as methylation or on histone proteins or non-coding RNAs (Kim et al., 2020; Pitto et al., 2020).

Recent studies have highlighted how different EDCs, or their mixtures, can induce alterations of the endocrine system without directly acting on the cellular targets previously mentioned, but still producing a result of equal importance. In fact, they can induce programmed cell death events affecting germ cells (De Angelis et al., 2017) and Sertoli cells (Zhang et al., 2021), causing fertility disorders in murine and porcine models, respectively. The concomitance of multiple substances, even in the case of EDCs, can produce additional effects (mixture/cocktail effects), so that simultaneous exposure can induce a negative effect at concentrations to which no effect has been linked when observed individually (Thurpp et al., 2018).

It should also be noted that for some of these compounds, such as micro and nanoparticles, and/or micro and nano-plastics, the mechanism of action as endocrine disruptors is not yet known.

Furthermore, it is possible to affirm that the effect deriving from the different EDCs depends both on their chemical-physical characteristics, and therefore on the way of interaction with biota, as well as on the dose and time of exposure.

Low concentrations can induce alterations of the endocrine and/or reproductive system, and this is due to exceeding threshold value for that substance, beyond which the toxic effect occurs and below which there are no disturbances (Sheehan et al., 1999).

It is also possible that events called “hormesis” may occur, whereby low doses may determine a greater effect than high doses. In this perspective, the dose-response curve follows a sinusoidal U- or inverted U-shaped trend (Diamanti-Kandarakis et al., 2009).

Instead, a study on the adrenal glands of the lizard *Podarcis sicula* exposed for 76 days to nonylphenol has highlighted how the effect of the well-known EDCs has stimulated in a time-dependent manner the hypothalamic-pituitary-adrenal gland axis (HPA), inducing increases in the levels of hypothalamic corticotropin-releasing factor (CRF), adrenocorticotropin (ACTH), and corticosterone (De Falco et al., 2014). Conversely, Park et al. (2020) found that exposure to nonylphenol resulted in a dose-dependent under-expression of spermatogenesis-related genes (Sycp3, Vasa, Dazl, Sohlh1, and Sohlh2) in cultures of mouse testicular fragments (MTFs),

derived from five-day postpartum neonatal murine testes and exposed for 30 to various concentrations of the compound.

Furthermore, the time factor also affects the potential accumulation of the substance. Many EDCs are lipophilic in nature, therefore tending to accumulate at the lipid level (Sousa et al., 2024), represented not only by adipose tissue, but also by lipophilic deposits present in various cells of the body districts, as an example the gonads. Therefore, chronic exposure could lead to persistence of the xenobiotic within the organism and a more intense effect of the EDC.

In addition, it is known that early exposures, such as at the embryonic level or in more susceptible developmental stages (Khorsandi et al., 2008), can alter the normal growth program and/or cause a toxic effect with consequent onset of a pathology.

Added to this is the sex factor. In fact, many biological responses to drugs or other exogenous substances can be sex dependent. This perspective could also be shared for EDCs, so much so that it has been reported that compounds that alter the endocrine system can interfere with glucocorticoid receptor signalling during gestation and cause asex-specific postnatal metabolic alterations disorders (Ruiz et al., 2020).

1.3.2 EDCs as environmental micropollutants

As it is known, endocrine disruptors represent a broad category of substances potentially capable of altering the function of the endocrine system and causing adverse effects on the reproductive health and that of the progeny (European Commission, 2018). Among these, pesticides, plasticizers (such as phthalates or bisphenol A), pharmaceutical products (Chen et al., 2022; Ríos et al., 2022; Kasonga et al., 2021;) are among the most widespread.

The pressing economic development of the last century has increasingly satisfied and accentuated the demands in various sectors (health, agriculture, industry, technology, etc.), allowing the production and placing on the market of large quantities of higher-performing synthetic substances such as polymers, biopolymers, plasticizers, drugs, pesticides, flame retardants, metals, metal alloys,

etc. (Maricanò et al., 2023; Tajik et al., 2020; Caliman & Gavrilescu, 2009), some of which, to date, appear to possess ED characteristics.

With the VII Environment Action Programme, the European Commission has outlined the international strategies to be followed with respect to endocrine disruptors until 2020, proposing to guarantee a high level of protection for citizens and the environment, while simultaneously preserving the European economy (Soloviov et al., 2020; Gruber & Obersteiner, 2016).

In this regard, according to REACH, the endocrine disrupting properties can lead to identifying the compound in question as a "Substance of Very High Concern" (SVHC). This classification, as reported in Annex XIV of REACH, makes the SVHC marketable only if the risk deriving from its use is adequately controlled (threshold value) or the socio-economic benefits outweigh the risk and there are no other alternatives (Regulation (EC) N. 1907/2006, 2006).

In the last decade, some progress has been made in categorizing the potential risk of EDs, as confirmed by scientific publications that have highlighted and consolidated the connection, for many types of compounds, between the endocrine disrupting nature, the causes, the consequences and the health of humans and wildlife (Solecki et al., 2017). A clear understanding, however, is still to be achieved about the potential impact of EDCs on non-target species that continue to be exposed to their ecotoxicological risk.

This is also the result of a controversy over the application of certain toxicological principles, such as the concept of "safety threshold", i.e. the dose below which no negative effects are expected (JRC, 2013). This is a very complex context since, to date, the concentrations of EDCs detected in the environment are in the range between ng-µg/L and ng-µg/dm^3, as they are subject to constant variations, dependent on the use of these compounds themselves (Finckh et al., 2022; Bojanowska-Czajka et al., 2021; Rogowska et al., 2020; Varjani & Sudha, 2020).

In general, the sources of exposure to EDCs are represented by air, water and food (Figure 1.6), the latter seems to be the most significant cause for humans, as it represents the account of overall exposures (Varjani & Sudha, 2020).

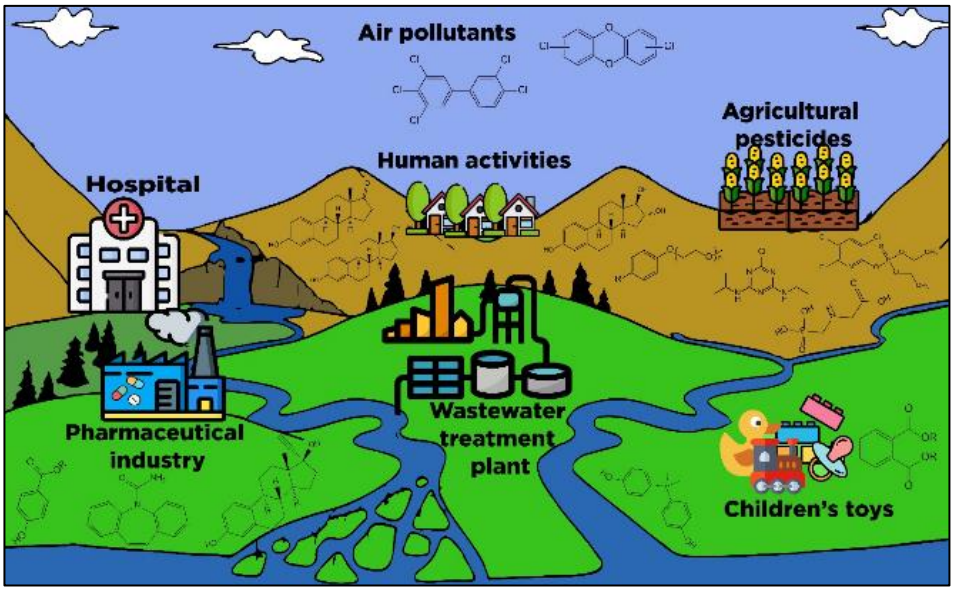

Figure 1.6 Presence of EDCs in various environmental matrices.

Many ED chemical compounds are present in the air in the form of particulate matter. These are combustion products, such as dioxins and polycyclic aromatic hydrocarbons, which are easily dispersible in the atmosphere and bioavailable by inhalation (Mohanty 2024; Novák et al., 2020).

However, the main condenser in which there is the greatest presence and persistence of EDCs is water, where the normal water cycle already determines the substantial accumulation of these compounds. Groundwater is the result of rainwater, landfill leaching and effluents that are industrial and agricultural waste sites (Castiglioni et al., 2020; Žižlavská & Hlavínek, 2020; Launay et al., 2016). Furthermore, this determines the dispersion of EDCs even in different environments distant from the production or discharge site, broadening their distribution in various ecosystems (Cunha et al., 2022; Varjani & Sudha, 2020; Porte et al., 2006).

To make the natural mechanism worse, there is the not entirely relevant capacity of wastewater treatment plants (WWTPs) towards all types of these compounds. The still inadequate management of industrial, sanitary and municipal wastewater, sludge disposal and accidental spills (Finckh et al., 2022; Afsa et al., 2020; Hashmi et al., 2020) makes it easy for these substances to enter the environmental matrices close to the WWTPs drainage channels. All water from domestic, sanitary, agricultural and industrial sources must be treated before being released into any surface water compartment (rivers, lakes, seas, oceans).

A European study, using analytical screening methods, revealed 366 compounds of emerging interest in 56 effluents of wastewater treatment plants, also assessing the biological risk based on acute toxicity for aquatic organisms (Finckh et al., 2022).

It has been observed that the concentration of emerging micropollutants is increased in the effluent water, compared to the inflow water in the purification plants. This is a phenomenon known as "negative removal", a problem whose nature depends on various factors (chemical-physical properties of the pollutant, absorption capacity, seasonal variations, etc.) that are common to all WWTPs, and which is aggravated by compounds whose action is not yet known and for which adequate legislative standards have not been established (Kumar et al., 2022). In this way, the water released from the sewage treatment plants is discharged into receiving basins such as rivers or lakes or, as happens for the plants close to coastal metropolises, into the sea (Rogowska et al., 2020). The monitoring of the chemical composition of the incoming and outgoing waters of wastewater treatment plants in small and large seaside resorts along the Baltic Sea coast has highlighted an increase in EDCs, mainly plasticizers, in relation to the intensification of human activities in these coastal areas (Jucyte-Cicine et al., 2024).

Furthermore, it should be considered that in an aquatic matrix, unlike a solid one (such as sediments), compounds can be found both individually and in the form of mixtures. Emerging contaminants, including EDCs, for which current treatment plants are not yet well equipped for chelation or catabolism, may also be able to produce complexes (Kumar et al., 2022).

Once released into the environment, the fate of these substances is dictated by their intrinsic chemical-physical characteristics and the type of environmental matrix in which they are found, such as deep and/or surface waters or sediments. The relative persistence and/or transformation into metabolic by-products, which could acquire a different ecotoxicological profile from the parent compounds (Darbre & Harvey, 2008), and their interaction with biological systems, may depend on this.

1.3.3 EDCs in marine ecosystems

As widely reported, the presence of EDCs has been found in various environmental matrices such as aquatic ones, especially marine and coastal ones (Cunha et al., 2022). The fauna whose habitat is the marine environment, represented by wild vertebrate and invertebrate species, is inevitably and directly exposed to these xenobiotics, but humans can also be subject to them, using water and/or eating compromised faunal products (Pironti et al., 2021).

Due to the numerous and most disparate human activities, a multiple number of Contaminants of Emerging Concern (CEC) have been introduced into the marine environment (Patel et al., 2020).

Many EDCs have been regulated over the years (organochlorine pesticides, etc.), while for others adequate safety parameters have not yet been defined. These include industrial chemical products such as plastics and active pharmaceutical compounds.

These are two classes of very bulky pollutants, mainly following the SARS CoV-2 pandemic during which broad-spectrum anti-inflammatory drugs and antibiotics and disposable plastic safety devices occupied a large space (Pashaei et al., 2022; Ravi & Golder, 2022; Shams et al., 2021).

1.3.3.1 Pharmaceutical active compounds (PhACs): effects on vertebrates and invertebrates

Since PHACs are biologically active compounds, they can easily interface and interfere with the canonical biological processes of non-target organisms even at the low doses detected in the environment (Fabbri & Franzellitti, 2016).

There are many classes of PhACs and among these, the steroidal (SAIDs) and non-steroidal (NSAIDs) anti-inflammatory drugs and the antibiotics are the most used and therefore, those whose concentrations are most represented in the aquatic matrix between ng/L and µg/L (Álvarez-Muñoz et al., 2015). Despite the low environmental detections, their ecotoxicological effect on non-target organisms has raised serious concerns (Duan et al., 2021).

A recent study found the presence of SAID dexamethasone (Dex) in the muscle (2.37 ng/g), liver (2.54-43.56 ng/g) and reproductive organ (2.54-37.23 ng/g) of six different species of marine vertebrates (*Trichinous blochii, Lutjanus campechanus, Lutjanus erythropterus, Lutjanus argentimaculatus, Carangoides armatu* and *Lates calcarifer*) (Ismail et al., 2021).

These are compounds that due to their organic nature tend to be easily internalized at the cellular level and to accumulate in various tissues, especially at the level of the digestive and reproductive system. However, few studies remain to date that investigated the effects of these compounds on marine vertebrates and invertebrates.

In fact, most of the investigations have focused on freshwater teleostes such as, for example, *Astyanax altiparanae* on which the acute effects of the NSAID diclofenac and caffeine have been considered, evaluating histological alterations of the reproductive tissue and liver of male specimens, besides the variations in the gene expression of the gene encoding *vitellogenin* (*Vtg*) (Godoi et al., 2020).

The environmental context can cause alterations in the normal sexual determination of lower vertebrates such as fish, causing altered endocrine functions and threatening fertility (Biswas et al., 2021; Celino-Brady et al., 2021). This can lead to metabolic alterations, therefore repercussions on growth, altered endocrine circuits such as the hypothalamic-pituitary-gonadal (HPG) axis, which can lead to a state of stress which in turn can determine immune responses and cellular damage (Balasch & Teles, 2021).

Although the interest of most studies has focused on the risk assessment in marine vertebrate species, it is also true that PhACs are able to bioaccumulate throughout the food chain, including invertebrates (Ruan et al., 2020). Indeed, given their high filtration rate, an experimental study has shown that only after 8 days of exposure, drugs such as diclofenac can easily accumulate in mussel *Mytilus trossulus* (Świacka et al., 2019). However, there are few investigations into the presence and effects of PhACs on marine invertebrates.

A broad-spectrum study by Mello et al. (2021) demonstrated the presence of drugs such as ibuprofen in mussel *Mytilus edulis* in Sepetiva Bay in quantities comparable to those detected in other parts of the globe, describing through an

ecological multi-analyte method the risk of the various trophic levels highlighting the indirect exposure to which humans are potentially subjected.

In species of socioeconomic interest such as *M. galloprovincialis* and *M. edulis*, significant presences of NSAIDs have already been found in the last decade (Capolupo et al., 2017), which have been shown to cause tissue-specific antioxidant responses such as the alteration of the activity of detoxifying enzymes of reactive oxygen species (ROS), including catalase (CAT) and superoxide dismutase (SOD), and induction of lipid peroxidation and DNA damage (Almeida et al., 2020).

A study on the species *M. galloprovincialis* highlighted how the NSAID ibuprofen, in addition to following the pattern of altered parameters common to other PhACs, such as the modification of the activity of key enzymes in the response to oxidative stress, including glutathione *S*-transferase (GST), reports how, more accentuated in males, the drug can have negative effects on the reproductive fitness (Gonzalez-Rey & Bebianno, 2012).

1.3.3.2 Polystyrene microplastics (PS MPs): effects on vertebrates and invertebrates

The use and subsequent production of plastic materials has drastically increased in recent years, related to the use of disposable materials during the SarsCoV-2 pandemic (Lee & Kim, 2022). In this regard, micro and nano-polymers present at various levels of the water column and deriving from a precious, but poorly managed, resource have caused damage to marine ecosystems. Their presence was found through Gas Chromatography Mass Spectrometry (GC-MS) analysis in solid (sediments), liquid (water) and organic (organisms) matrices (Ainali et al., 2021). The availability of microplastics (MPs) for marine biota has become no longer an emerging problem, but a real emergency. The problem exists not only for the entity itself, but also for the ability of these structures to adsorb on their surface, altered by chemical-physical agents, other organic compounds that can induce a combined effect (Guedes-Alonso et al., 2021).

There are not many data in the literature for which MPs are directly catalogued as EDCs. Some studies have focused their attention more on terrestrial vertebrate species such as mammals, in which polystyrene (PS) nanoplastics (NPs) are the

cause of morphological alterations at the level of the testis and in the expression of key genes in spermatogenesis (Amereh et al., 2020).

Few are those whose attention has focused on the marine environment, among these a study on the marine medaka *Oryzias melastigma*, that has highlighted how, after a 60-day exposure, 10 μm PS MPs cause sex dependent alterations of the reproductive system, with a delay in the maturation of the gonadal tissue and lowered fitness in female fish (Wang et al., 2019). For other types of MPs, scientific studies have also highlighted the negative influence induced on biological functions in some teleostes, underlining the fact that the effect may depend on the type of MPs that meets the biological system (Cormier et al., 2021).

"Reproductive toxicity" includes all those possible consequences that cause damage to the reproductive cycle (spermiogenesis and oogenesis), to the functional suitability of the gametes, and finally to the success of fertilization. In this regard, it has been reported in literature that PS MPs of a few micrometres in size can cause toxicity at the level of the reproductive system of cnidarians and echinoderms (Bilal et al., 2023).

Bivalves, which represent the class of filter feeders for excellence, are more predisposed to internalize MPs and accumulate them in the various body districts (De Marco et al., 2023; Cappello et al., 2021; Moreschi et al., 2020). As for marine vertebrates, also in bivalves the interfacing, accumulation and biological responses seem to be dictated by the characteristics of the plastic, but this is reducible to an alteration of the filtration rate, which has repercussions on the assimilation efficiency and therefore on the bioenergetics of the organism. To compensate for the energy imbalance induced by PS MPs, the impact involves the reproductive system, weighing on the normal development of the gonads (Sendra et al., 2021).

1.3.3.3 Polylactic acid (PLA) biomicroplastics: effects on vertebrates and invertebrates

To minimize the presence and environmental impact of plastic products of fossil origin, biodegradable materials have been designed, known as bioplastics, deriving from waste products of the food industry or in any case natural (Filiciotto

& Rothenberg, 2021). Since the biodegradability of this type of plastic requires suitable chemical-physical conditions, which in a natural environment are very difficult to establish, bioplastics can also generate micro-biopolymers (BMPs) which, like the original ones, enter the environment in the same way (Wang et al., 2021). To date, the information in the literature about the potential effect exerted by this type of emerging micropollutants such as EDC is not conspicuous, yet. However, some studies have been carried out on terrestrial vertebrates highlighting that polylactic acid (PLA) microplastics have determined an increase in weight, leading to oxidative stress in growing mice (Deng et al., 2023).

The study by Zhang et al. (2024) on the effects of PLA plastics on the adult zebrafish *Danio rerio* revealed how photo-degraded plastic tends to depolymerize and produce microscopic structures that increase its toxic effect, leading to histological alterations of the ovaries in adult female specimens and variations in the levels of sexual hormones, as well as a toxic transgenerational response.

If little is still known about vertebrates, knowledge about marine invertebrates is equally lacking.

An 8-day study on mussel *M. edulis* exposed to poly L-lactide (PLLA) microplastics at 10 µg/L and 100 µg/L demonstrated that in a short exposure period MPs can increase the activity and expression of detoxification enzymes, such as catalase (CAT) and glutathione *S*-transferase (GST) (Khalid et al., 2021).

1.4 Bivalve molluscs: *Mytilus galloprovincialis* as model organism

In this work, the marine mollusc *Mytilus galloprovincialis* was chosen as a model organism (Figure 1.7). The selection is based on many data in the literature describing the class of bivalves as excellent sentinel organisms in the *in situ* and *ex situ* environmental biomonitoring activity, as they have a key role in the hierarchy and functionality of marine ecosystems (De Marco et al., 2023; Tresnakova et al., 2023; Cappello et al., 2021; Marić et al., 2020; Suárez-Ulloa et al., 2013; Smolders et al., 2003) and represent an important commercial resource in aquaculture (Rios-Fuster et al., 2022; Babarro et al., 2019).

In particular, the species *M. galloprovincialis* has been widely used as an excellent resource in various research projects aimed at understanding the consequential link between marine pollution and the effect elicited in resident biota (De Marco et al., 2023; Cappello et al., 2021; Curpan et al., 2022; Taleb et al., 2009; Casas & Bacher, 2006).

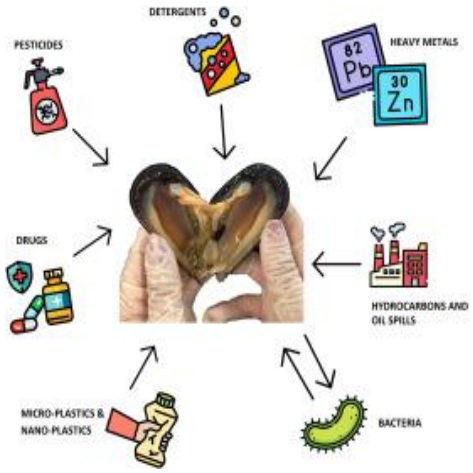

Figure 1.7 Overview of the Mediterranean mussel *Mytilus galloprovincialis* as a bioindicator model organism.

In the Monitoring Program for the Marine Strategy Art. 11, Legislative Decree 190/2010, now updated with the Ministerial Decree of 2 February 2021 and valid until 2026, the methodological sheets report the use of specimens of the species *M. galloprovincialis*, using the "Mussel watch protocol" reported within the ICRAM-MATTM Reference Analytical Methodologies, whose national and international use is aimed at monitoring the coastal marine environment with respect to emerging contaminants (ECs) (Barreira et al., 2024; Dessì et al., 2024; MASA, 2016).

The use of this organism as an experimental model arises from the ease with these specimens can be managed in a laboratory environment and in the field (De Marco et al., 2023; Romdhani et al., 2022; Milun et al., 2020). Mussels are filter-feeding animals that exploit the movement of a large ciliary area to take the contact with the aqueous medium in which the availability of oxygen is reduced and conveying food towards the digestive system (Curpan et al., 2022; Abidli et al., 2021). Being benthic and sessile organisms, they are more likely to interface with the particulate matter dispersed in the aqueous medium, and therefore with potential harmful substances present, which predisposes the species to be easily employed in

in situ environmental biomonitoring programs (Dessì et al., 2024; Bae et al., 2022; Parisi et al., 2022).

Hence, even if ECs concentrations are relatively low in the environment or in the aqueous matrix in the laboratory, the continuous and frequent uptake of these xenobiotics via filtration can lead to an assiduous contact with the animal. This may lead to the early onset of multilevel biological responses (at histological, biochemical, genomic, transcriptomic, proteomic and metabolomic level), and therefore to the availability of more biomarkers to investigate in order to understand the state of health of the biota.

This allows the tissues to more easily accumulate at the cellular level the substances transported inside the valves of the organism, which would act as protection. The bioaccumulation is strictly dependent on the quality of the components present in the marine matrix, but also on the physiological and biochemical state of the animal, as well as on the seasonal reproductive period (Chelyadina et al., 2022; Sussarellu et al., 2022; Kapranov et al., 2021; Esposito et al., 2021; Milun et al., 2020).

Therefore, different factors may be taken into account when studying the species *Mytilus galloprovincialis* as a highly suitable bioindicator for monitoring the contamination of marine environments.

1.4.1 General characteristics

The species *Mytilus galloprovincialis* was first described in 1819 by Jean-Baptiste de Lamarck. Its taxonomy follows the classification: phylum *Mollusca*, class *Bivalvia*, order *Mytiloida*, family *Mytilidae*, genus *Mytilus*, species *galloprovincialis* (Gardner, 1992; Seed, 1991).

It is a sessile and benthic mollusc typical of temperate zones and widely distributed in the Mediterranean area, therefore also known as the "Mediterranean mussel" (De Marco et al., 2023). However, its location covers larger territories, in fact it is also present along the European coasts bathed by the Atlantic Ocean and the south-eastern Pacific (del Rio-Lavín et al., 2022), also reaching north-western Ireland and south-eastern west of England (Gardner, 1992; Seed, 1974, 1969). The

species is also stationed in America (Oyarzún et al., 2024; Lins et al., 2021). The varied diffusion is mainly dependent on its delicious role in the food industry (FAO, 2024; Monfort 2014), and for this reason the commercialization, in this sector, has led to the birth of various aquaculture plants and the consequent naturalization in environments distant from the Mediterranean basin where the species it is indigenous, so much so that it even reaches Chinese bays such as Haizhou (Zhanfei et al., 2023).

The morphology of the species follows the canons of the bivalve class, undergoing phenotypic variations due to the influence of the habitat in which it lives (Gardner, 1992). Already from the early juvenile stages (veliger stage), the internal organs are protected by a bilateral shell, a three-layered biomineralized structure (nacre, fibrous prism and myostracum) with mechanical performance, made up of a protein matrix that envelops the carbonate mineral crystals of calcium (calcite, aragonite and vaterite) (Peharda et al., 2024; Gao et al., 2015). The shell is composed of two valves equivalent in shape and size, with a non-linear texture as they are the result of deposition processes (growth streaks) that originate from the umbo from which it develops in an inequilateral manner with a triangular shape. The most recent deposits are towards the umbo (the narrowest part) and can be more or less marked depending on the physiological state and external influences, while the older ones are at the opposite extreme, where the shell takes on a more roundish.

The anatomy follows a very distinct dorsal-ventral symmetry, in fact on the dorsal side the two valves are held together by a fibrous connective filament, known as the "zipper", and by two serrated structures that cooperate to hold the two parts together and keep them relaxed, while the closing mechanism is guaranteed by the contractile force of the parallel fibers of the posterior adductor muscle, firmly attached to the myostracus in the rounded ventral part (Trožić-Borovac et al., 2022). The external color of the valves is opaque black, which is why in some Italian regions the species is known as the "black date", while inside they take on a mother-of-pearl colour and a shiny, smooth texture.

Being a sessile organism, it is not capable of large movements but is equipped with a foot that can be everted and used as a lever for small movements towards currents richer in oxygen and nutrients (Tsvetanova et al., 2022). Above the base of the foot there is a small gland, called the "byssus gland", which secretes, during

byssogenesis, a gelatinous substance, made up of a highly hydrated and richly protein matrix which, in contact with the outside, tends to solidify into filaments which take the name of "byssus". These filaments are exploited by the benthic animal to form colonies and anchor themselves to rocky structures, in order to resist the mechanical action of the waves of the intertidal zone in which it has the habitat (Bortoletto et al., 2021).

As in the entire *Mytilus* genus, the internal organs are protected by the thin, soft connective tissue of the mantle that attaches to the inner edges of the shell along the pallial line. This delimits the pallial chamber and keeps the organs hydrated, helping the shell to retain the pallial fluid.

Further inside, an infrabranchial chamber (with inhaling purpose) and a suprabranchial one (with exhaling purpose) can be distinguished ventrally, compared to the gill filaments (Eggermont et al., 2020). With a symmetrical distribution, the gill lamellae are articulated along the entire length of the valve and are held together by the more fibrous gill arch placed towards the dorsal side with respect to the filaments bearing the lateral and frontal cilia which represent the interface for gaseous exchange and contribute to the osmoregulation and whose movement, being the organism a filter-feeding animal, allows feeding, through the mobilization of particulate matter suspended in the aqueous medium towards the gastrointestinal cavity (De Marco et al., 2023; Rossi et al., 2016).

The digestive system is made up of the mouth, esophagus, stomach and intestine (also known as the hepatopancreas or digestive gland). The latter is the main organ responsible for detoxification, in which the cells are organized into circular and tubular structures in the lumen of which lytic enzymes are released.

Only in recent years, a study by Eggermont et al. (2020) confirmed the presence of a well-articulated open cardiovascular system in which the heart develops in the anterior mid-dorsal area and is made up of a single ventricle and two atria that surround it bilaterally at the ventral level.

Not only does the heart act as a hydraulic pump, but also the posterior adductor muscle allows the circulation of hemolymph, a sort of blood made up of hemocytes (cells of the immune system) and plasma (Sun et al., 2023).

The *M. galloprovincialis* species is a physiologically gonochorous species, so individuals carry only male or female gametes, resulting in a different anatomical

organization of the reproductive system specific to sex. There are rare cases in which, in natural conditions, sexual hermaphroditism has been found for the species, at most, in laboratory conditions, through *in vitro* fertilization (Kenchington et al., 2019), and in conditions of eutrophication, environmental changes and pollution for which a sex reversal with male predominance occurred, potentially during the reorganization phase after spawning (Dalpé et al., 2022; Chelyadina et al., 2021). There is no sexual dimorphism in the species, therefore the distinction between the two sexes is not evident. Often, the gonadal tissues appear a different colour when fresh: yellow-milky white for males and orange-reddish for females (Mikhailov et al., 1996). However, this is a non-constant and univocal morphometric factor, as it depends on tissue maturation (Lopes et al., 2022).

1.4.2 General organization of the gonads in both sexes

The reproductive tissue of male and female organisms develops in a specialized area of the mantle, occupying almost the entire concave area of the valves.

It is made up of a reserve connective tissue which is not common to all molluscs, in fact, it is typical of the *Mytilidae* and *Ostreidae* families and is related to an adaptive specialization to obtain high reproductive fitness in extreme conditions such as intertidal ones (Mathieu & Lubet, 1993). The tissue is the main storage site of glycogen, in fact, it hosts the largest fraction compared to other tissues (gills, muscle, etc.) and plays a key role in the maturation process of germ cells (Pipe, 1987; De Zwaan & Zandee, 1972), representing the main energy source supporting gametogenesis in diploid bivalves (Osterheld et al., 2024). In a related species such as *Mytilus edulis*, glycogen metabolism is dependent on the concentration of tissue glucose (Gabbott et al., 1979), and it is therefore subjected to seasonal variations, also dependent on the presence of nutrients.

The cells of the vesicular connective tissue (VCT) are those that host the largest reserve of the polysaccharide in the gonad, while a smaller quantity is present in the adipogranular (ADG) cells, which host higher concentrations of proteins and lipids

(Pipe, 1987; Gabbott & Whittle, 1986; Gabbott, 1976), the former in greater numbers than the latter.

As previously reported, glycogen plays a fundamental role in supporting the maturation process. Its synthesis and degradation are subjected to short- and long-term seasonal control. In mussel *M. edulis* there is the highest concentration of glycogen during the summer (July-August) and the beginning of autumn (September-October), while at the end of autumn and beginning of winter (mid-January) there is a gradual decline which reaches its lowest levels in spring (mid-March), when the formation of mature gametes has concluded and their deposition occurs (De Zwaan & Zandee, 1972). These fluctuations coincide linearly with the reproductive cycle of *M. galloprovincialis* species and highlight the importance of this energy source for reproductive health and fitness.

This is evident by the fact that, during the gametogenic cycle, which is more or less synchronous in both sexes, an inversely proportional relationship is established between the connective tissue and the germinal tissue so that the earlier the maturation stage, the more abundant the connective tissue is, while the later it is, the lower the presence of VCT and ADG cells and the size of the follicle increases with the abundance of mature cells (Duinker et al., 2008; Suárez et al., 2005).

1.4.3 Male gonad organization and reproductive cycle

At the level of the male gonad, the germinal tissue represented by follicles containing the germ cells of the male lineage in different stages of maturation is found among the connective cells (Prisco et al., 2017).

As for other bivalve species, the basement membrane of the germinal epithelium extends towards the lumen, meeting various maturation stages of the male germ cells (Dei Tos et al., 2016), which tend to reduce in size, gradually as maturation progresses (Figure1.8 and Figure 1.9).

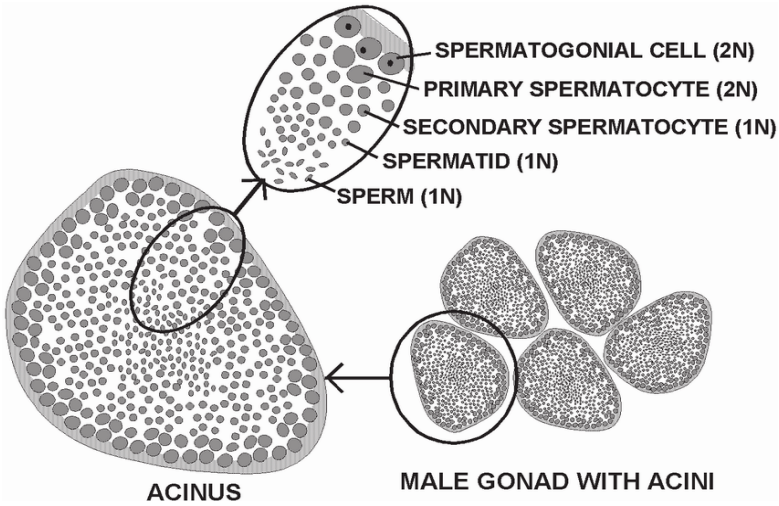

Figure 1.8 Representative drawing of the variation in germ cell size during gonad maturation in male gonads of *M. galloprovincialis.*

I. Spermatogonia: large diploid cells, presenting a spherical nucleus that occupies an abundant part of the cytoplasm and with a granular and acidophilic appearance. The basophilic nucleus has several nucleoli due to the intense transcriptional activity, in fact, these cells enter meiosis from which primary spermatocytes arise.

II. Primary spermatocytes: smaller in size than the previous ones, but still diploid (obtained from meiosis I). They stand out because they tend towards the lumen of the follicle and have a nucleus with very condensed chromatin.

III. Secondary spermatocytes: haploid cells, obtained from the completion of the first phases of meiosis, are evidently smaller with chromatin that takes on a picturesque "umbrella" shape. They divide very rapidly, producing two spermatids from each single cell.

IV. Spermatids: haploid spherical cells, deriving from meiosis II of the previous ones, with highly condensed chromatin which after spermiogenesis (cyto-differentiation process) change into spermatozoa.

V. Spermatozoa: very small haploid cells in which the umbrella-shaped nucleus takes on a more lanceolate shape. They are equipped with a driving tail and are positioned more centrally in the lumen of the follicle.

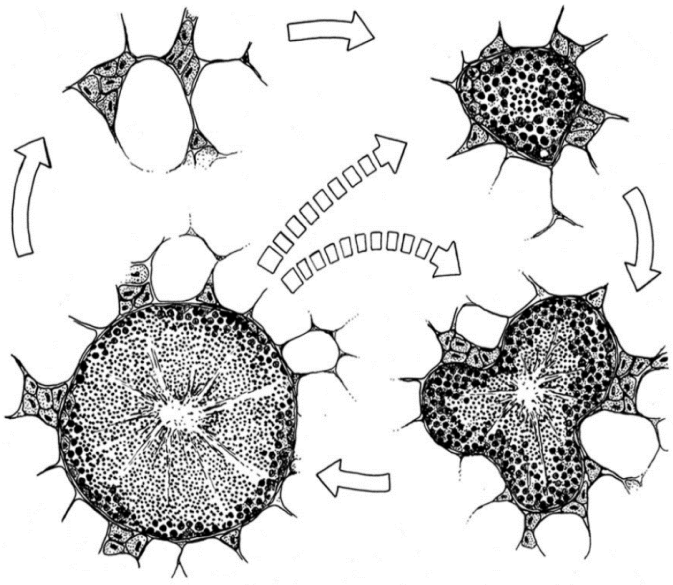

Figure 1.9 Representative drawing of the variation in size of the follicle and the connective tissue outside it in male gonads of *M. galloprovincialis.*

The reproductive cycle is divided into phases, defined as stages (Prisco et al., 2017; Duinker et al., 2008):

- Stage 0 (June), presence of abundant connective tissue represented by the VCT and groups of ADG cells dispersed between them. The follicles are small and few, sometimes with residues of sperm left over from previous emissions.
- Stage I (July-September), spermatogonia are distinguishable along the wall of the testicle.
- Stage II (late September-October-November), spermatogonia, I-II spermatocytes and spermatids have occupied part of the periphery of the follicular lumen. It is between this stage and the end of the previous one that the process of spermatogenesis occurs.
- Stage III A (December), in which the follicle is loaded with spermatozoa and germ cells in different stages of maturation, surrounded by an always abundant connective tissue.
- Stage III B (end of December-January), the first deposition of mature gametes occurs.
- Stage III C (February-March), in which there is the reorganization of the germinal tissue, therefore the more marked presence of immature cells

such as spermatogonia, spermatogonia, spermatocytes, spermatids and some residual spermatozoa from the previous emission. In this case the VCT cells are very scarce as the ADG cells, which are distributed to form a network around the filling follicles. At the end of the stage, after the rapid maturation phase, a new gamete deposition event occurs in March.

- Stage III D (April-June), the same condition as Stage 0 occurs, with the predominance of the connective tissue over the germinal tissue.

1.4.4 Female gonad organization and reproductive cycle

At the level of the female gonad, the germinal tissue represented by follicles containing the female germinal cells is found among the connective cells (Rosati et al., 2019).

As for males, also for females of *M. galloprovincialis* the germ cells are supported by the basal membrane of the epithelium which meets the various maturation stages of the germ cells of the female line (Dei Tos et al., 2016) which, unlike their male counterparts, tend to increase in size as they mature (Figures 1.10, 1.11 and 1.12).

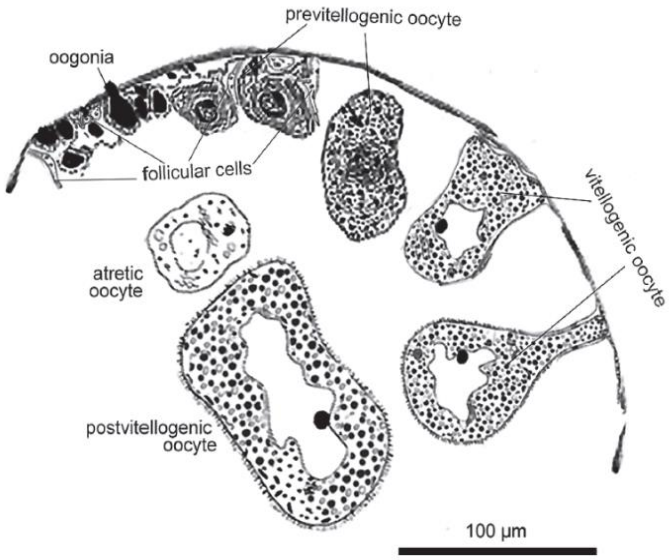

Figure 1.10 Stages of maturation of the germ cells in female gonads of *M. galloprovincialis.*

I. Ovogonia: diploid germ cells which organize themselves into nests close to the wall of the follicle and are slightly flattened and characterized by a slightly basophilic oval nucleus, which has an evident nucleolus in the centre, all immersed in a limited cytoplasm (ooplasm).

II. Prophase oocytes: diploid cells at the initial stages of prophase (leptotene, zygotene, pachytene), have a basophilic nucleus contained in a scant cytoplasm, delimited by an ovoid plasma membrane.

III. Previtellogenic oocytes: diploid cells larger in size than prophase oocytes, have a clear spherical nucleus containing one/two nucleolus(i), a sign of intense transcriptional activity, and a basophilic cytosol. The oocytes are larger than in the previous stage.

IV. Early vitellogenic oocytes: larger haploid cells with a spherical nucleus, which has one or more well-defined nucleoli inside towards the periphery. The ooplasm tends to lose basophilia, acquiring an acidophilic character due to the deposition of the yolk in the form of globules. The shape is typical of the stage, called "pear/club", since the cells are attached to the epithelium via a cytoplasmic extension that resembles a stem, while they project towards the lumen of the follicle.

V. Meddle vitellogenic oocytes: they are cells not very different from the previous ones, they are larger and spherical in shape. The ooplasm is now totally acidophilic, given the ever-increasing quantity of yolk accumulations, but they still remain anchored to the follicular epithelium.

VI. Late vitellogenic oocytes: they are haploid cells whose shape is spherical, but due to the high quantity inside the lumen of the follicle, they can be irregular in shape. The cytoplasm remains acidophilic, and the accumulation of yolk granules has ended, as they are ready to break contact with the follicle to channel themselves into the gonoduct and be emitted.

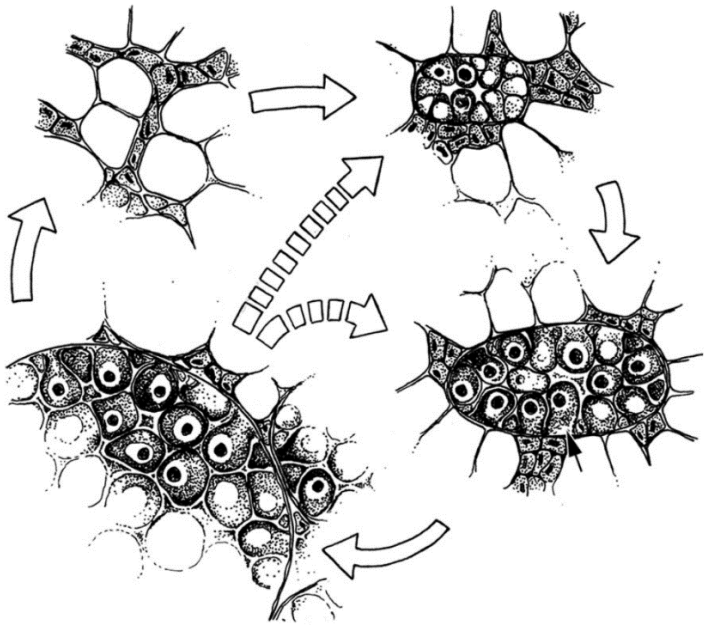

Figure 1.11 Representative drawing of the variation in size of the follicle and the connective tissue outside it in female gonad of *M. galloprovincialis*.

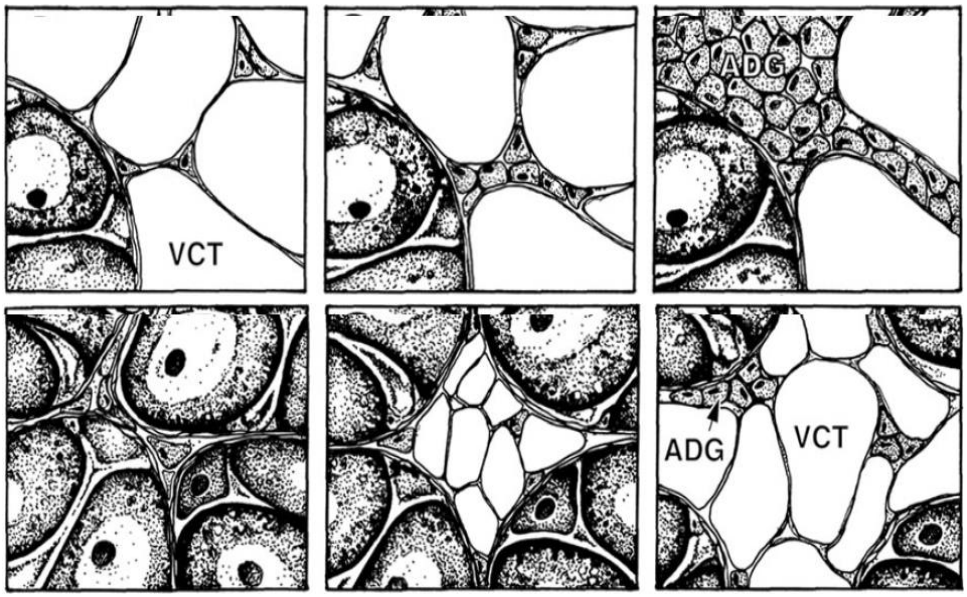

Figure 1.12 Representative drawing of the inversely proportional relationship between follicle maturation and connective tissue cells in female gonads of *M. galloprovincialis*. VCT (vesicular connective tissue cells), ADG (adipogranular cells).

The reproductive cycle is divided into phases, defined as stages (Figure 1.12) (Rosati et al., 2019; Duinker et al., 2008):

- Stage 0 (June-early September), presence of abundant connective tissue represented by the VCT and groups of ADG cells dispersed between them. The follicles are small and few, sometimes with residues of free oocytes in the lumen, left over from previous emissions. Nests of germ cells can be seen towards the wall of the follicle.
- Stage I (late September-October), early vitellogenesis is entered, multiple germinal nests are highlighted with oogonia also in the proliferative phase, early vitellogenic and meddle vitellogenic oocytes.
- Stage II (October-November), phase of late vitellogenesis in which different stages of maturation of the germ cells are evident: previtellogenic, meddle and late oocytes. Mature oocytes tend to settle towards the lumen of the follicles.
- Stage III (December) mature oocytes completely fill the follicles which, in addition to being increased in number, are also more capacious, while the VCT cells are decreased and the ADG cells organize themselves to form linear networks in the poor connective tissue. The first egg laying occurs between the end of December and the beginning of January.
- Stage III (February-March), for both females and males of the species, there is a reorganization of the germinal tissue similar to Stage II, with the greater presence of immature cells such as ovogonia, previtellogenic and medium oocytes within the follicles that are surrounded by connective cells. At the end of the stage, after the rapid maturation phase, a new gamete deposition event occurs in March, followed by a restoration of an undifferentiated condition typical of Stage 0.

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CHAPTER 2

Aims of the thesis

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The entire thesis project had the ambition of evaluating the reproductive health status of the Mediterranean mussel *Mytilus galloprovincialis* with a vision aimed at the "Systems Biology".

In detail, the aim of this PhD thesis was to understand and investigate the reproductive status of the model organism (biological system), considering it at multiple biological levels. To achieve this goal, a multi-biomarker approach was then employed, combining traditional biomarkers (i.e., chemical, histological, histochemical, immunohistochemical) with more innovative biomarkers (metabolomics and transcriptomics) widely used in the field of ecocytotoxicology in studies of environmental biomonitoring. Through the integration of the data obtained from canonical and more modern techniques, it was possible to validate new metabolic biomarkers that can be exploited to understand early the potential negative influences of new emerging pollutants, including different classes of EDCs, on the reproductive system of non-target marine biota.

There are four main pillars discussed within this thesis:

- The evaluation of the basal physiological state of the reproductive system of male and female mussels raised under uncontaminated conditions, highlighting possible divergences related to the phenotype and therefore dependent on sex, potentially due to the maturation state (seasonality) at the time of individuals collection (Chapter 3);
- The assessment of the biological effect on the reproductive system of both sexes after exposure of mussels to environmental concentrations of the glucocorticoid drug dexamethasone (Dex), a known EDC, verifying and highlighting potential alterations and sex responses at the level of the gonadal tissue (Chapter 4);
- The estimation of the biological risk on the reproductive health in male and female specimens after short-term exposure of mussels to polystyrene microplastics (PS MPs), known as emerging EDCs, investigating any potential sex-dependent biological responses (Chapter 5);

- The evaluation of the potential effect on the reproductive tissue in both sexes after exposure of mussels to biologically derived microplastics, specifically the polylactic acid (PLA) MPs, verifying the potential role of EDCs in non-target species such as mussel *M. galloprovincialis* (Chapter 6).

CHAPTER 3

Sexual differentiation
of the reproductive tissue
in the Mediterranean mussel
Mytilus galloprovincialis

CHAPTER 3

Sexual differentiation of the reproductive tissue in the Mediterranean mussel *Mytilus galloprovincialis*

3.1 Introduction

As described in the previous chapters, many molluscs from intertidal and epibenthic areas are well suited for the assessment of the ecological risk, and among these the Mediterranean mussel *Mytilus galloprovincialis* is a sentinel organism widely used in ecocytotoxicology studies for passive (*ex situ*) and active (*in situ*) environmental biomonitoring (Della Torre et al., 2024; Rusconi et al., 2024; Andrade et al., 2023; Azizi et al., 2023; Calisi et al., 2023; De Marco et al., 2023; Cappello et al., 2021; Parisi et al., 2019; Cappello et al., 2018) (Figure 3.1).

Figure 3.1 Uncontaminated area of the Messina coast (Messina, Italy).

Indeed, mussel *M. galloprovincialis* is a model organism that demonstrated to be able to highlight the relationships between different forms of pollution and the impact on biota. This is possible through the evaluation of biological responses, interpretable through a wide range of biomarkers (Boudjema et al. 2023; Cuccaro et al., 2023; Pizzurro et al., 2023; Wang et al., 2023, Cappello et al., 2021).

The organism, filtering about 5 L per hour and being sessile, is very predisposed to the accumulation of particulate matter dispersed in the aqueous medium. Therefore, it is more frequently subjected to the pressure exerted by potential contaminants (Smart et al., 2021).

Furthermore, the mussel *M. galloprovincialis* is an edible species, widely cultivated throughout the world. In fact, in 2019 it was classified as the fourth most farmed species, occupying the first place in the Italian ranking (Figueiredo et al., 2022). Particularly, in Sicily the production of mussels is fervent, mainly, in the area of the Tyrrhenian coast. In the area of Messina, most of the aquaculture plants based on the production of mussels are located in semi-closed brackish water basins (Bordignon et al., 2024; Afsa et al., 2023; Cappello et al., 2021) within the area of Torre Faro. These are protected systems that, however, like open systems (sea), can easily be susceptible to climate changes, human activities, and changes in nutrient availability (Figueiredo et al., 2022; Bayne, 1976).

According to what stated above, it is therefore crucial to assess the sexual differentiation of the reproductive tissue in the Mediterranean mussel *Mytilus galloprovincialis* at the specific period of collection of individuals to avoid any misunderstandings in the interpretation of the biological responses assessed within an ecotoxicological study. In this perspective, it is therefore needed an accurate evaluation of the maturation state of both sexes of mussels based on gonad tissue organization, glycogen availability, and metabolite profiling, with the aim to assess the baseline of the canonical reproductive state of the species taken as a model organism at the moment of sample collection for successive experimental trials.

Indeed, understanding the basic physiological condition of a specific organ is an essential step to validate the experimental data obtained since each organism, and mainly those whose habitats are subjected to strong environmental variations (Della Torre et al., 2024; Bastos et al., 2023), is susceptible to changes dependent on the ecosystem in which it lives. In this regard, the physiological reproductive status of both sexes of the Mediterranean mollusc was herein evaluated through a multi-biomarker approach, considering the potential implications of the seasonal period in which the specimens were sampled for the successive experimental trials, the synchrony of sexual maturation and the state of energy reserves supporting the gametogenic period.

3.2 Materials and methods

3.2.1 Collection of mussels from an uncontaminated area

In November 2022, adult specimens of the edible mollusc *Mytilus galloprovincialis* were collected from braids on which mussels are raised at the S.A.Co.M. farm, an uncontaminated area located in Messina (Sicily, Italy) (Figure 3.2). After measuring the physico-chemical parameters of the water were mussels grown at the farm, the animals were quickly transported to the laboratory at the University of Messina, located about 6 km from the sampling site, and kept during the journey in containers filled with water collected from the mussel farm, under continuous aeration.

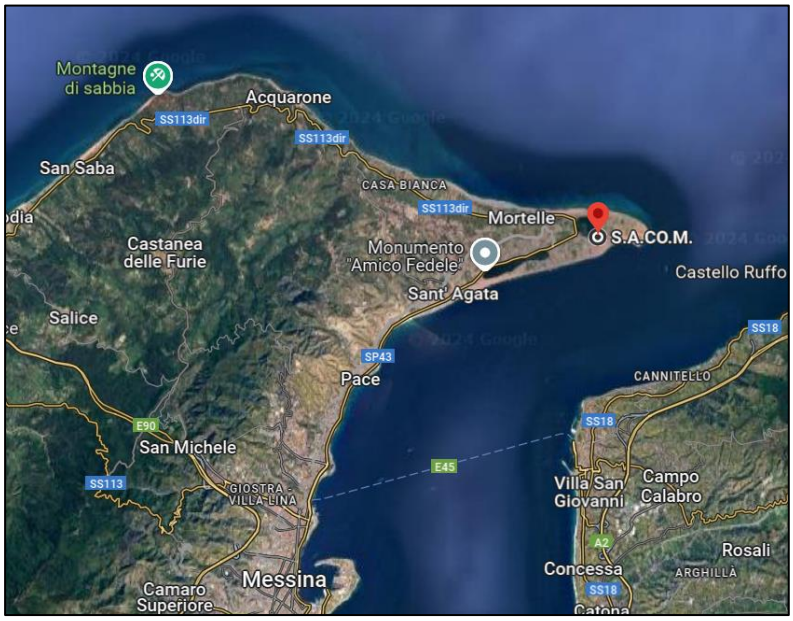

Figure 3.2 Satellite image of the sampling location.

Once in the laboratory, forty samples were randomly selected to allow the highest possible number of individuals of both sexes. Each organism was measured in length and weighed before being sacrificed. After cutting the adductor muscle to allow the valves to open, a predictive screening was performed to determine the sex approximately. Later, small fragments of gonads were crushed between the slide and the coverslip for the identification of the sex under the field of the optical

microscope (Zeiss) through the recognition of spermatozoa (for male mussels) and/or oocytes (for female mussels).Therefore, by using sterile tools, promptly part of the gonads was removed from a total of 10 animals per sex and placed in sterile tubes to be stored at -80 °C for metabolomics analysis. The remaining tissue was cut to obtain an area of less than 1 cm^2 to be stored in the refrigerator at +4 °C into histological tubes filled with 4% paraformaldehyde (PFA), made of 37% formaldehyde diluted with 1 M of phosphate buffered saline (PBS) at pH 7.4, or using directly the equivalent solution Immunofix, to be destined for the histological processing.

3.2.2 Physico-chemical analysis of the aqueous medium

Using a portable multiparameter probe (HI 9828, Hanna, Woonsocket, USA) equipped with microprocessor and amplifiers that convert the high impedance signals from the sensors into digital values, capable of automatically scaling the measurements by configuring each measured parameter, it was possible to evaluate *in situ* at the S.A.Co.M. farm, the physico-chemical state of the water in which the mussels were grown. Therefore, the following parameters were measured: dissolved oxygen (DO, mg/L), pH (pH, pHmV), temperature (°C), pressure (mbar), electrical conductivity (mS/cm), salinity (tds ppt, Sal), and electrons in transit (ORP).

3.2.3 Histological analysis

For histology slide preparation, it was necessary to completely cover the gonadal tissue fragments with a fixative solution, such as 4% PFA (37% formaldehyde diluted with 1 M PBS at pH 7.4), to block the activation of lytic processes that would have degraded the tissue, causing artifacts in the tissue observation, and therefore a not accurate interpretation of the data.

To avoid any interference, the protocol for histology slide preparation was performed entirely on the same day of sample collection. Therefore, after 4 hours

(h) in fixative, two rapid washes of samples of 10 minutes (min) each were performed in phosphate buffered saline (PBS) 1 M and pH 7.4.

As described by Afsa et al. (2023), to obtain a histological preparation that would last over time, it was necessary to eliminate all the water present within the tissue, otherwise this would have prevented the vision under the microscope field since it refracts the light. To this aim, an increasing series of alcohol (ethanol) (Mixetan; Cuneo, Italy) was therefore performed at room temperature, so that the new solvent completely replaced the water present within each single cell of the gonadal tissue, applying the following protocol: 1 h at alcohol 50°, 1 h at 70°, 30 minutes (min) at 80°, 30 min at 95°, 15 min at 100° I, and 15 min at 100° II.

Since alcohol is a polar solvent immiscible with paraffin (the embedding medium), an intermediate step was made by immersion of the samples in a mixture of 100° ethanol and xylene (Sigma-Aldrich, Darmstadt, Germany) (for 30 min), and then samples were moved to the pure solvent xylene (for 30 min). All these steps were performed in safety conditions under a hood at room temperature. At the end of this step, the successful dehydration of samples was confirmed by the fact that the tissues appeared completely diaphanous.

From here, a new mixture of the paraffin solvent (xylene, Sigma-Aldrich, Darmstadt, Germany) with the ultrapure Paraplast wax (Bio-Optica, Milan, Italy) was carried out for 1 h at 65 °C, so that the paraffin gradually replaced the xylene, soaking the tissue. The sample was then completely immersed exclusively in the embedding medium in two separate steps, including change and replacement of liquid paraffin, with each step lasting 1 h.

At the end of this step, the gonadal tissue was finally embedded in paraffin, and once the block was mounted, it was waited for it to solidify (Figure 3.3).

Figure 3.3 Refrigerated counter (left) and inclusion control unit (Leica, Milan, Italy).

With the aid of a rotating microtome (Leica Microsystems, Wetzlar, Germany), thin sections of 4 µm thickness were cut from each block and placed in series on a slide. All the slides were then left overnight in an oven at 26 °C, to let adhere them on the slide itself. The day after, the histological staining of gonadal tissues was performed (Figure 3.4).

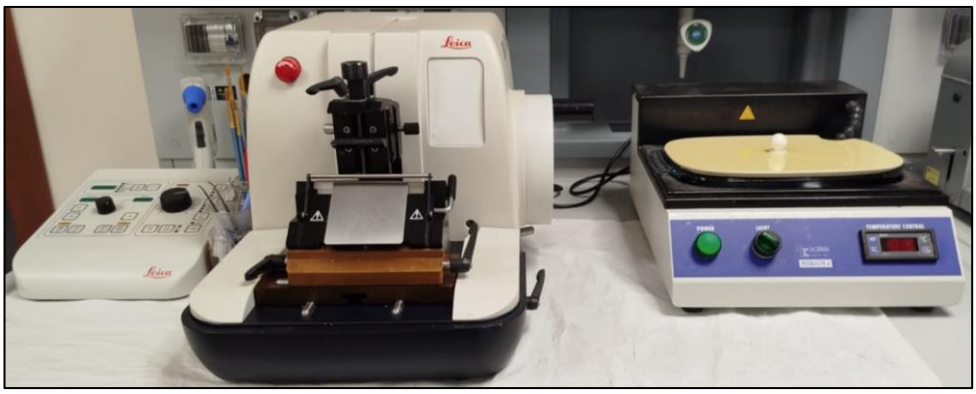

Figure 3.4 Automatic rotating microtome and thermostatic bath (right) (Leica Microsystems, Wetzlar, Germany).

First, the slides containing the sections were deparaffinized in two consecutive steps in xylene of 5 min each. Successively, a rehydration of samples was performed by immersion of the histological slides in a decreasing series of ethanol (100°, 95°, 80°, 70°, 50° and 35°) with each step of 5 min. The rehydration of tissues was necessary since the dyes used in the subsequent steps are prepared in aqueous solution. Indeed, the hematoxylin/eosin (H/E) colorimetric method was applied to all slides to ascertain the sex and histo-morphological organization of gonads of both male and female mussels.

Briefly, according to the protocol described previously (Cappello et al., 2021), first all the samples were rapidly stained into the indirect stain hematoxylin (15 sec), and then the second direct stain eosin (8 sec) was applied, followed by a rapid dehydration of slides through a brief increasing series of ethanol (95°, 100° I, 100° II) with each step of 5 min. Finally, two steps in xylene were performed to proceed with the last step including the use of the mounting resin (Eukitt® Quick-hardening mounting medium, Sigma-Aldrich, Darmstadt, Germany) to then finalize the histological preparation of mussel gonadal tissue. The H/E staining was performed to contrast and better visualize the morphology of the tissue, with H having a high

affinity for the acidic structures such as the nucleus that appeared in blue, and E showing affinity for the basic structures such as the cytoplasm that appeared in pink colour.

All the images were acquired using a Zeiss Axio Imager Z1 motorized microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany) (Maisano et al., 2017) (Figure 3.5) and reported with scale bar of 20 µm.

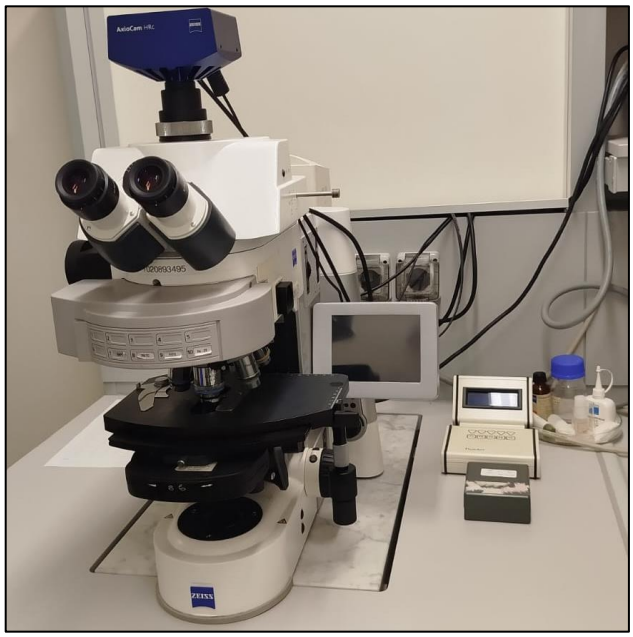

Figure 3.5 Zeiss Axio Imager Z1 motorized microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany).

3.2.4 Histochemical analysis

Generally, in bivalves, and specifically in mussels, glycogen is the main energy source supporting the gametogenic process of both sexes, and this undergoes seasonal variations depending on the reproductive cycle and the availability of nutrients (Li et al., 2024; Zhanfei et al., 2023). With the purpose to better understand the role of glycogen and its relationship with the maturation state of the gonads, its presence and quantity were detected and measured by a histochemical reaction with the use of periodic acid-Schiff (PAS) (Abcam, Cambridge, United Kingdom) combined with a d-PAS (diastasis-PAS), performed on two distinct, but serial,

histological sections of mussel gonads, following the protocol slightly modified of Meyerhold et al. (2018).

In brief, after treating the sections according to the histological protocol fully described previously (see section 3.2.3) up to the rehydration step (Afsa et al., 2023), only one of the two sections mounted on a slide was treated with d-PAS reaction, so that the treatment with the enzyme diastase/α-amylase (15 U/mg in PBS 1 M, pH 7.4) (Sigma-Aldrich, Darmstadt, Germany) hydrolysed the glycogen in one section, and a negative control was also obtained in the other section of the same slide (glycogen negativity).

After incubation at 37 °C for 20 minutes in oven, to reach the conditions of enzymatic activity, the section was treated with periodic acid for 5 min. Its excess was then removed by a wash in distilled water (DW), and the section was covered with Shiff solution for 10 min.

After 2 minutes in hot water (30 °C) and a further wash in DW to remove the salts, a normal hematoxylin (H) staining was performed for 30 sec, followed by a rinse, the rapid dehydration series and two steps in xylene prior to perform the mounting with a coverslip, as described in the previous section 3.2.3.

Also, for this analysis the images were all taken with a Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany) (Maisano et al., 2017) and exported as .jpeg files to be analysed with the free access software Image J (Image Processing and Analysis in Java) version 1.54i. By this software, using the colour threshold function, the number of pixels equivalent to the amount of polysaccharide was quantified and subsequently normalized.

Therefore, the presence of glycogen in gonads was assessed by examining the d-PAS area and the PAS positive area. The values of the PAS positive area in the sections treated with diastase (dPAS) were subtracted from those of the PAS positive area of the sections not previously treated with diastase (d-PAS) to eliminate the nonspecific responsiveness of other polysaccharides to periodic Shiff acid, so to attribute the positivity exclusively to the presence of the sugar reserve. The results were expressed in pixels (Mai et al., 2023; Chen et al., 2022; Jones et al., 2021).

3.2.5 Metabolomic analysis

3.2.5.1 Metabolite extraction from mussel gonads

The metabolomic analysis was performed on the gonads of both sexes of adult molluscs grown in the uncontaminated area of the S.A.Co.M. farm with the aim to evaluate the sexual differentiation of the reproductive tissue in the Mediterranean mussel *Mytilus galloprovincialis* at metabolite level at the specific period of collection, therefore within the autumn period since individual sampling occurred at the beginning of November 2022. Thus, the metabolic profile of gonads will be related to the reproductive cycle of mussels to establish the basic characteristics of the physiological state for both sexes and avoid any misunderstandings in the interpretation of the biological responses assessed within an ecotoxicological study.

For the extraction of polar metabolites, male and female gonads were treated with a "two-step" protocol (methanol/chloroform/water) (Cappello et al., 2018). Approximately 100 mg of male and female gonadal tissue were separately homogenized in sterile 2 mL tubes containing stainless steel beads (3.2 mm diameter) and a mixture of cold methanol (4 mL/g) and distilled water (0.85 mL/g) for 10 min at 50 oscillations/sec using the TissueLyser LT (Qiagen, Hilden, Germany) (Figure 3.6), so that the methanol could isolate all the polar components.

Afterwards, chloroform (4 mL/g) and distilled water (2 mL/g) were added to each sample and vortexed for 1 min with a vortex mixer (Falc, Perugia, Italy) (Figure 3.6) to obtain the separation of the apolar components.

After brief shaking, the samples were immersed in ice for 10 min and then centrifuged at 2000 g at 4 °C for 5 min (Centrifuge 5417R, Eppendorf, Milan, Italy) (Figure 3.6). After this step, a clear separation of three different phases is obtained, including the supernatant with the polar metabolites as obtained by methanol, intermediate phase made of protein, and a down phase containing the apolar metabolites as extracted by chloroform. Taking care not to mix the three phases, approximately 400-600 µL of the supernatant were collected and transferred in a second sterile 1.5 mL tube.

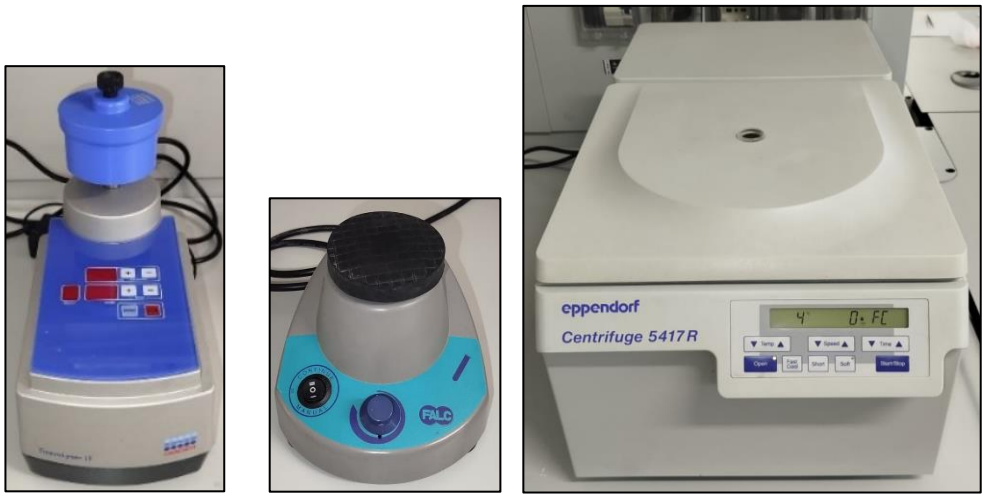

Figure 3.6 In order: TissueLyser LT (Qiagen, Hilden, Germany), vortex mixer (Falc, Perugia, Italy) and centrifuge 5417R (Eppendorf, Milan, Italy).

To obtain the polar metabolites to be identified at the successive analysis with the nuclear magnetic resonance (NMR) spectrometer, it was necessary to dry the samples to let evaporate the methanol. Samples were thus placed in a vacuum centrifugal concentrator (Eppendorf, Milan, Italy) (Figure 3.7) for the necessary time (about 3 or 4 h) since evaporation under vacuum prevents oxidation of samples during the drying process. At the end of this step, the pellets obtained from each sample were stored at -20 °C.

Figure 3.7 Concentrator plus with vacuum pump (Eppendorf, Milan, Italy).

Prior to the NMR analysis, all pellets were suspended in 0.1 M sodium phosphate buffer (660 µL; pH 7.0, 10% D_2O; Armar AG, Döttingen, Switzerland) and 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich, Darmstadt, Germany), used as the internal standard and representing the "Chemical Form Indicator" since it provides a chemical shift reference (δ = 0.0 ppm) for the NMR spectra. Moreover, the presence of D_2O provides instead the deuterium lock signal for the NMR spectrometer (Varian 500MHz Spectrometer (Varian Medical Systems, United states) (Figure 3.8). The solubilized polar fraction was thus introduced into 5 mm diameter NMR capillaries, taking care to obtain a solution as homogeneous as possible without the presence of bubbles that could alter the acquisition and resolution of the spectra.

Figure 3.8 Varian 500MHz Spectrometer (Varian Medical Systems, United states).

3.2.5.2 Metabolomics based on ^{1}H NMR and spectral pre-processing

Only polar metabolites with molecular weight not exceeding 1.5 KDa can be detected by the Varian-500 NMR spectrometer equipped with radio frequency (RF) channels with waveform generators working at a frequency of 499.74 MHz at 298

K. The acquisition of spectral data relating to each sample was performed with the NOESY 1D software (Nuclear Overhauser Effect SpectroscopY) with pre-saturation during a mixing time of 120 ms and proton relaxation delay of 2 sec.

With this acquisition mode it was possible to detect the resonance of protons very close to each other in space but not closely connected by chemical bonds, managing to better delineate even small molecules in which the proton nuclei are close together (nuclear Overhauser effect, NOE).

The ^{1}H NMR spectra were acquired at 25 °C with added FID (Free induction decay) and 32 dummy scans, each one in 3 min, using a wavelength of 11.6808 ppm with 32,768 complex data points, acquired with digital quadrature detection (Morgan et al., 2019). The use of NOESY 1D software allowed to increase the resolution of the acquisition, and consequently to better visualize the resonance of each functional group within the NMR spectra.

Using the Chenomx software (Chenomx NMR Suite version 10.0; Chenomx Inc., Edmonton, Canada), and specifically the Processor module, each spectrum belonging to the metabolic extracts of male and female mussel gonads was manually phased with baseline correction and calibrated by setting the DSS (the internal standard) to 0.0 ppm.

Metabolites were identified using the Profiler module within the Chenomx software by comparing the patterns of each peak based on their shape and chemical shift with those present in the Chenomx 500 Hz database and other public computer libraries. Also, metabolites were quantified relating the areas under each peak to that of the resonance of DSS, of which the concentration was known being the internal standard.

3.2.6 Statistical analysis

The data obtained from the histochemical analyses by d-PAS and PAS reaction and metabolomics were first processed with Excel to have the mean between the male and female samples separately and to calculate the respective standard deviation (± SD).

With the GraphPad software (Prism 10.0, San Diego, CA, USA) the percentage difference was obtained to verify the significance of each metabolite with respect to sex and between the two sexes, considering a $p < 0.05$.

For both analyses, a two-way analysis of variance was used, such as the two-way ANOVA test and the Turkey comparison test to establish the variance between the two independent variables, males and females.

3.3 Results

3.3.1 Physico-chemical data of the aqueous medium

As stated above, before collection of the specimens of *M. galloprovincialis* from the uncontaminated area within the S.A.Co.M. farm, the physico-chemical state of the water in which the mussels were grown were measured using a multiparametric instrument.

All the values describing the state of the aqueous medium referred only to the mussel sampling period, which was the beginning of the month of November 2022. With no significant variations, taking into account that each parameter was measured three times, the water of the mussel farming system presented the following characteristics:

- *Dissolved oxygen (DO)*: 8.61 mg/L
- *pH parameters*: 7.8±0.02 pH, -104.8 pHmV
- *Temperature*: 23.19 °C
- *Pressure*: 1016.1 mbar
- *Electrical conductivity*: 59.28-57.24 mS/cm
- *Salinity parameters*: 29.64 tds ppt, 39.70 Sal
- *Electrons in transit*: -108.2 ORP

3.3.2 Histological observations

The histological analysis carried out using the H/E colorimetric method allowed to describe the tissue organization of the male and female gonads of

mussels collected from the uncontaminated area of the S.A.Co.M. farm, confirming the distinction between the sexes and highlighting, based on the vesicular connective tissue (VCT) cell/germ cell ratio, the maturation state of the specimens under investigation.

3.3.2.1 Histology of mussel male gonads

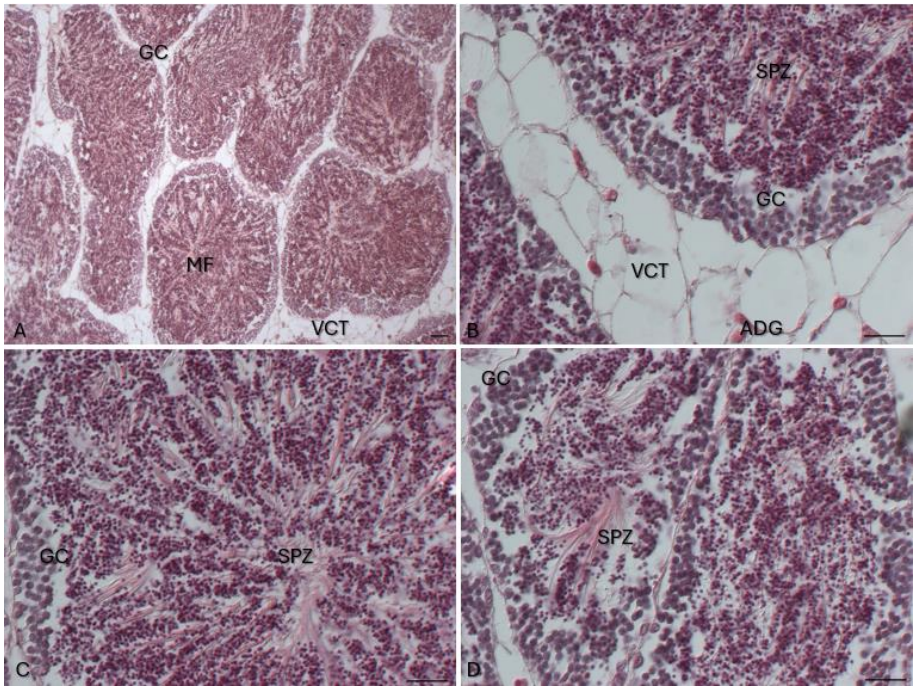

Figure 3.9 Representative histological sections of male gonads of *Mytilus galloprovincialis* collected in an uncontaminated area. (A) Abundant spermatozoa (SPZ)-filled male follicles (MF) are evident towards the lumen and maturing germ cells (GC) at the periphery (A, B, C and D). Abundant vesicular connective tissue cells (VCT) and few adipogranular cells (ADG) are evident between the follicles (A and B). Scale bar 20 µm.

A careful survey and evaluation of the organizational state of the male gonadal tissue did not reveal any obvious signs of alteration (Figure 3.9) since no disorganization was detected either in the connective tissue or in the male follicle.

The observation of the histology of mussel male gonads has demonstrated the correct coincidence between the state of maturation of the gonads and the reproductive period (autumn) of mussels, since the tissue was found to be transient between Stage II and IIIA, as expected (Prisco et al., 2017).

In detail, as it is evident from Figure 3.9: A, the connective tissue, represented mainly by the occurrence of VCT cells compared to the adipogranular (ADG) cells, less present and more adherent to the external walls of the follicles in a network fashion, is very reduced. Its cyclic presence is inversely proportional to that of the germ cells inside the follicles which, instead, appear numerous and in the maturation phase (some of them appeared as already mature). Therefore, this accredits what was reported for the genus *Mytilus*, *i.e.* the existence of an inversely proportional relationship between the cycle of the reserve tissue (VCT and ADG) and the gametogenic one (Duinker et al., 2008).

Inside them, as it is typical of the sexual differentiation phase that coincided with the sampling period, it is possible to observe several cells at different maturation state that, distributed starting from the basal membrane to above may be recognized as spermatogonia, spermatocytes I-II and spermatids that occupy a large part of the periphery of the follicular lumen. Moreover, the remaining central part is full of spermatozoa, whose acidophilic tails stand out in pink among the basophilic heads shown in blue-violet (Figure 3.9: B, C, D).

3.3.2.2 Histology of mussel female gonads

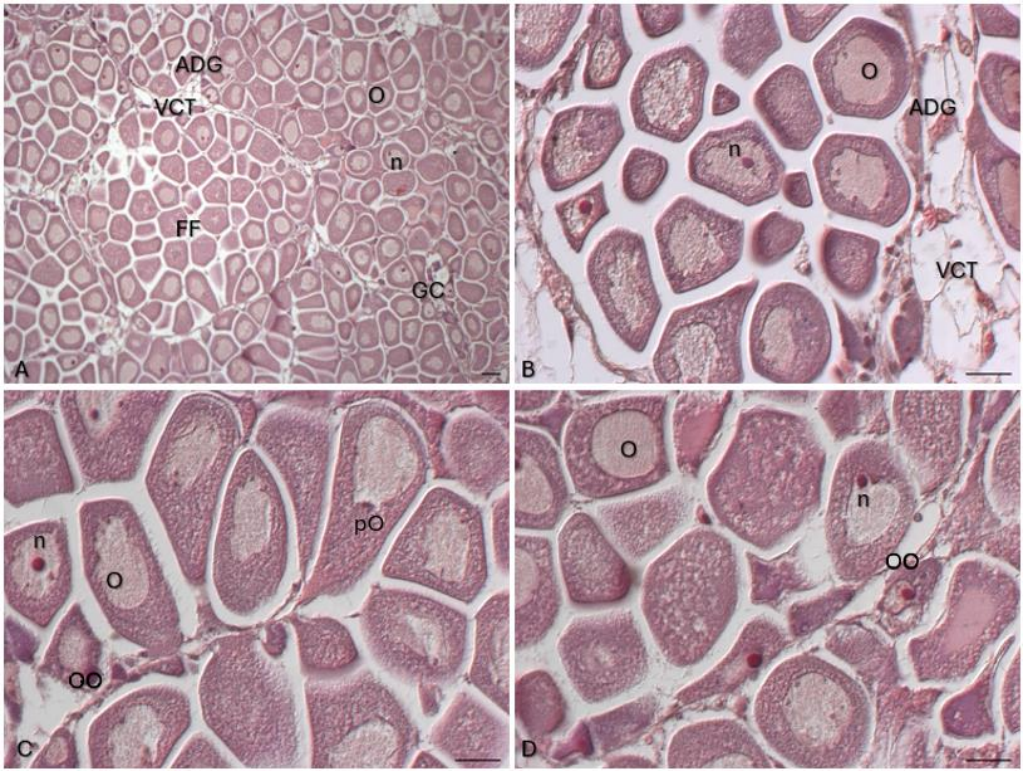

Figure 3.10 Representative histological sections of female gonads of *Mytilus galloprovincialis* collected in an uncontaminated area. (A) Abundant female follicles (FF) filled with mature oocytes (O) are evident, in some of which the nucleolus (n), few pear-shape oocytes (pO), ovogonia (OO) and some germinal cells (GC) are visible towards the walls (A, B, C, and D). (A) Between the follicles, abundant vesicular connective tissue cells (VCT) and few adipogranular cells (ADG) are evident. Scale bar 20 μm.

From the morphological analysis conducted by H/E staining on the female gonadal tissues, no changes in the germ cells and/or connective tissue emerged, as it is evident from the (Figure 3.10) that is representative of the physiological state of the gonadal tissue of female specimens taken from the mussel farm in November 2022.

Usually, the reproductive cycle of the two sexes of mussels is quite synchronous, allowing a good fertilization success and a high fitness of the population. This was confirmed by the observation of the histology of gonadal tissue, which showed that also the female specimens were in a transient maturation phase between the late vitellogenic phase (Stage II) in which different stages of differentiation are evident (Figure 3.10: A, B, C and D), and Stage III in which the mature oocytes completely fill the numerous follicles that settle in a reduced connective tissue (Figure 3.10: A, C and D) (Rosati et al., 2019).

As it is evident from Figure 3.10: A and B , even in female mussels the connective tissue, represented by the VCT cells more abundant than the ADG cells, is very reduced and its presence, as in males, is inversely related to that of the germ cells inside the follicles which, instead, are numerous and in the maturation phase (some cells are already mature). Next to oocytes still anchored to the follicular epithelium, reduced germinal nests crushed towards the wall were also found, a sign that underlines the disposition of the female gonad at the first egg deposition, which is followed by a rapid tissue reorganization to support a second one later (Dei Tos et al., 2016).

3.3.3 Histochemical results

The histochemical analyses performed to detect the neutral polysaccharide, such as the glycogen, were obtained by PAS reaction.

The red-magenta colour, dependent on the PAS reaction, was more marked in the sections positive for glycogen compared to those previously treated with diastase, therefore negative for the presence of the reserve sugar. This confirmed the applicability and validity of the protocol proposed by Meyerholz et al. (2018) for discriminating glycogen from other neutral polysaccharides present into the gonadal tissue.

3.3.3.1 Histochemistry of mussel male gonads

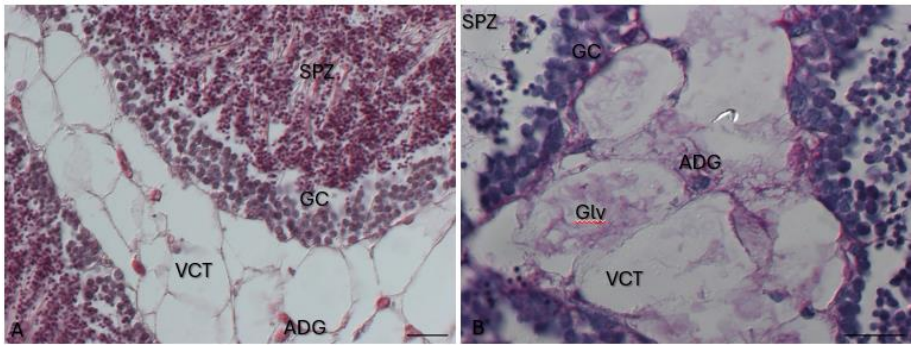

Figure 3.11 PAS reaction on the male gonads of *Mytilus galloprovincialis* from an uncontaminated area treated with the colorimetric method H/E (A) and with PAS reaction (B) in which glycogen (Gly) deposits within the cells of the vesicular connective tissue (VCT) are recognized by the red-magenta color. Adipogranular cells (ADG), spermatozoa (SPZ), germ cells (GC). Scale bars 20 μm.

The Figure 3.11 shows the male gonadal tissue treated with the H/E colorimetric method (Figure 3.11: A) and treated with PAS histochemical reaction (Figure 3.11: B). This comparison is necessary in order to better contextualize in detail the tissue organization of mussel male gonads, in which the large cells of the vesicular connective tissue (VCT) present weakly basophilic nuclei that settle on the periphery, as they are centripetally crushed by glycogen deposits evident in red-magenta (Alonso et al., 2019).

The detection of glycogen deposits not very abundant inside the VCT is in line with the crucial period of gametogenesis in which the animals were found, since the sugar reserve is of fundamental importance to guarantee a correct maturation process (Duinker et al., 2008). In fact, it is worthy to note that mostly of the follicles are full of maturing or already mature cells, whereas the presence of the

polysaccharide is low as it has been exploited to support the energy demands directed towards the gametogenic process.

3.3.3.2 Histochemistry of mussel female gonads

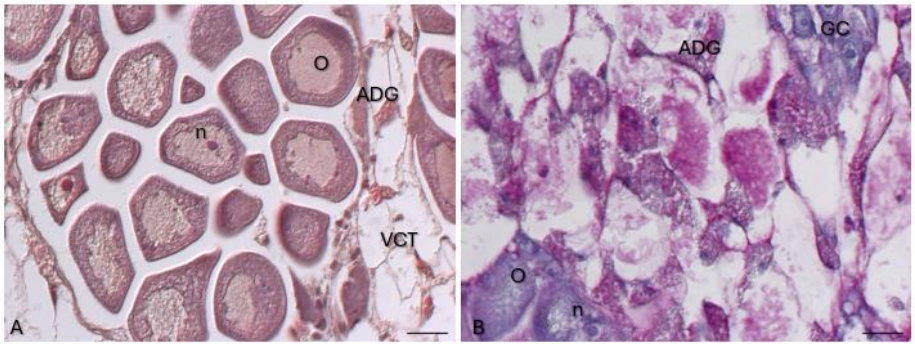

Figure 3.12 PAS reaction on the female gonads of *Mytilus galloprovincialis* from an uncontaminated area treated with the colorimetric method H/E (A) and with PAS reaction (B) in which glycogen (Gly) deposits within the cells of the vesicular connective tissue (VCT) are recognized by the red-magenta color. Adipogranular cells (ADG), oocytes (O), germ cells (GC). Scale bars 20 μm.

The PAS and d-PAS reaction used for the histochemical analysis showed a marked presence of glycogen compared to other polysaccharides at the level of the connective tissue of the mussel female gonad.

The Figure 3.12 shows the comparison between a histological slide of female gonads treated with the H/E colorimetric method (Figure 3.12: A) and another one treated with the PAS histochemical reaction (Figure 3.12: B), allowing to highlight and better understand the physiological condition of the female reproductive system at the time of sampling (autumn).

The Figure 3.12 describes the completeness of the tissue organization in which the large VCT cells present small and weakly basophilic nuclei that are crushed towards the cytoplasmic periphery by the glycogen deposits, which in this case seem more evident than what observed in male gonads, so much so that its presence is also attributable to the ADG cells that are dispersed among the VCT, and coloured more in magenta red.

As for males, also for females the inverse proportion between the cycle of connective tissue cells and germinal cells is confirmed. Particularly in this case,

given the maturation stage, it is evident how the ratio pushes more on the presence of a greater number of female gametes (oocytes) compared to VCT and ADG cells.

3.3.3.3 Histochemical comparison between mussel male and female gonads

The analysis of the histochemical images of male and female mussel gonads performed with the free access software Image J (Image Processing and Analysis in Java) version 1.54i, through the evaluation of the colour threshold, has allowed to quantify in pixels the quantity of glycogen inside the VCT and ADG cells.

From the analysis of the d-PAS area and the PAS positive area it was possible to measure the presence of glycogen in both sexes. The graph in Figure 3.13 shows the physiological difference in the presence of glycogen between the two sexes, respectively between males (blue) and females (pink) of the mussel *M. galloprovincialis* grown in the same farm under uncontaminated conditions and collected in autumn.

As it is demonstrated from the data, in a natural environment under the same physico-chemical conditions of the aqueous medium and considering that, in general, the sampled specimens were at the same maturation stage, the clear difference between the quantity of glycogen detected in males in respect to that found in females is evident. According to data, in male gonads the quantity of the polysaccharide is lower than that measured in females.

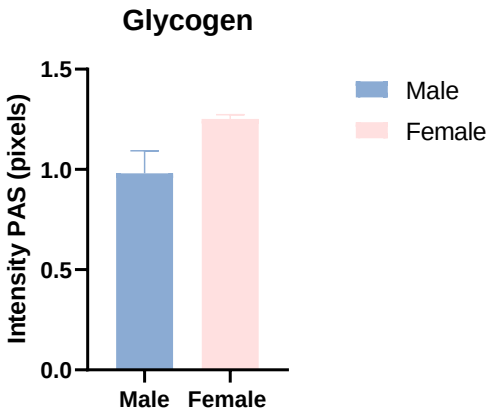

Figure 3.13 Representative histogram of the mean concentration (pixels) of glycogen in the male (blue) and female (pink) gonads of *Mytilus galloprovincialis* from an uncontaminated area.

3.3.4 Metabolomic comparison between mussel male and female gonads

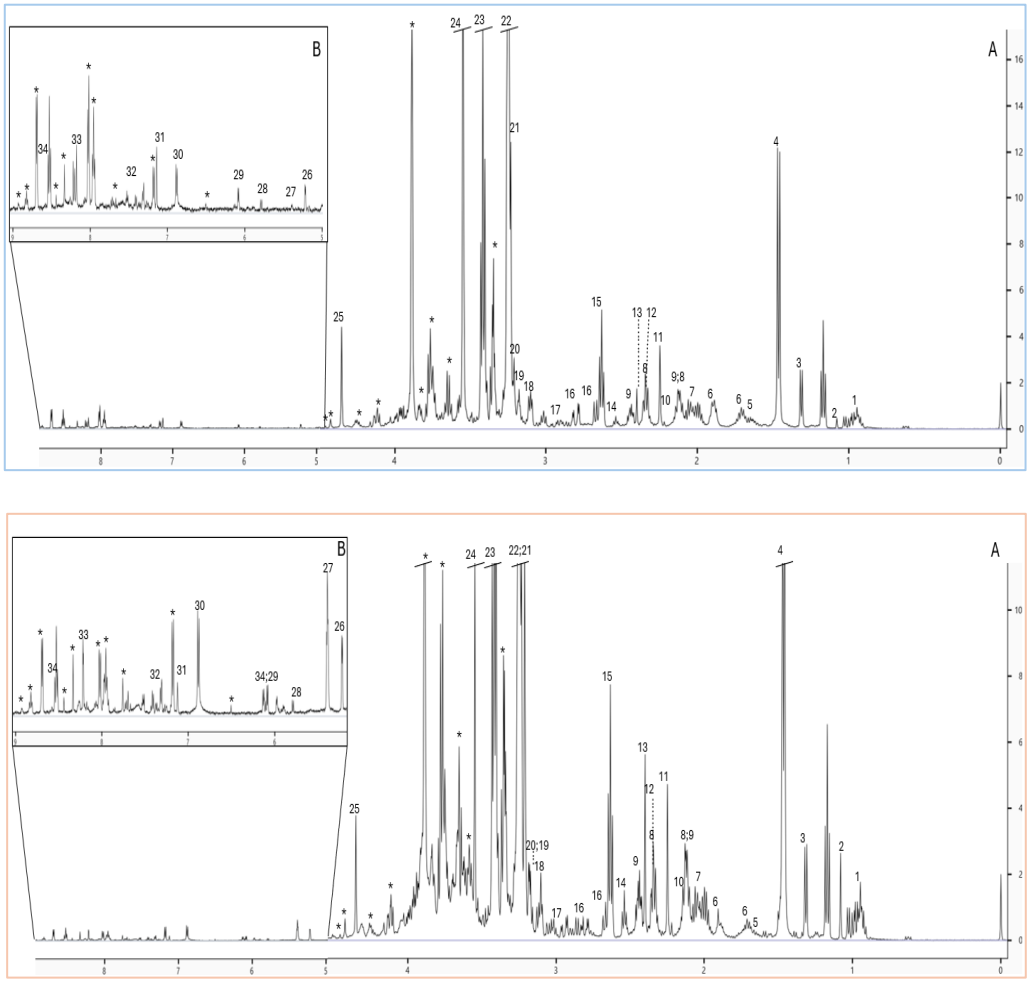

Figure 3.14 Representative 1H 1-D 500 MHz NMR spectrum of the male (in blue) and the female (in pink) gonads of *M. galloprovincialis* from an uncontaminated area with (A) showing the aliphatic region and (B) a vertical expansion of the aromatic region. Keys: 1-BCAA (leucine, Isoleucine, Valine), 2-Mytilitol, 3-Lactate, 4-Alanine, 5-Arginine, 6-Lysine, 7- Proline, 8-Glutamate, 9-Glutamine, 10-Acetate, 11-Acetoacetate, 12-Pyruvate, 13-Succinate, 14-βAlanine, 15-Methionine, 16-Aspartate, 17-Apsaragine, 18-Malonate, 19-Choline, 20-O-Phosphocholine, 21-Sn-Glycero-2-phosphocholine, 22-Betaine, 23-Taurine, 24-Glycine, 25-Homarine, 26-Glucose, 27-Glycogen, 28-Uracil, 29-ATP/ADP, 30-Tyrosine, 31-Histidine, 32- Phenylalanine, 33- Adenine, 34-IMP, *-Unknown.

The images reported (Figure 3.14) are respectively the NOESY 1D ^{1}H NMR spectra obtained from the extract of the male gonad tissue (in blue) and the female one (in pink) of adult mussels of the species *M. galloprovincialis* collected from the uncontaminated area within the S.A.Co.M. farm at the beginning of November 2022, therefore during the autumn season.

As it can be noted from the NMR spectra reported above, a similar metabolite profiling was detected in the gonads of both sexes. This implies that mussel gonads, not dependently from the sex, need the same pool of metabolites involved in different metabolic pathways for their physiological functions.

More in detail, as it was documented in other species of marine bivalves such as *M. coruscus* (Shang et al., 2021), metabolites involved in the aerobic respiration such as glucose and pyruvate, and the intermediates of the Krebs cycle such as succinate, fumarate, malonate and ATP/ADP, representatives of the primary energy pathways, were found in mussel gonads. These were accompanied by metabolites of secondary pathways such as those of the anaerobic metabolism including lactate, and those involved in lipid peroxidation such as acetoacetate (Cappello et al., 2021; Shang et al., 2021).

The metabolome of mussel gonads, as also documented in mussels *M. edulis* and *M. trossulus* (Sokolova et al., 2024), is characterized by the presence of organic osmolytes such as betaine and taurine and homarine, since in molluscs the control of osmoregulation is continuous and very fine.

Free amino acids (FAA) in bivalves are molecules that play a biologically important role, in fact, they can act as protein scaffolds and/or carbon substrates to obtain energy (Chen et al., 2021). As expected, the presence of FAA was detected in mussel gonads, represented for instance by the presence of branched chain amino acids BCAAs (leucine, isoleucine and valine) in both sexes.

Although the same compounds were detected in the metabolite profile of mussel gonads of both sexes, it is crucial to note from the overlapping of NMR spectra (Figure 3.15) depicted in blue for male and in red for female gonads, that the difference between the two phenotypes lies in the concentration in which metabolites were expressed.

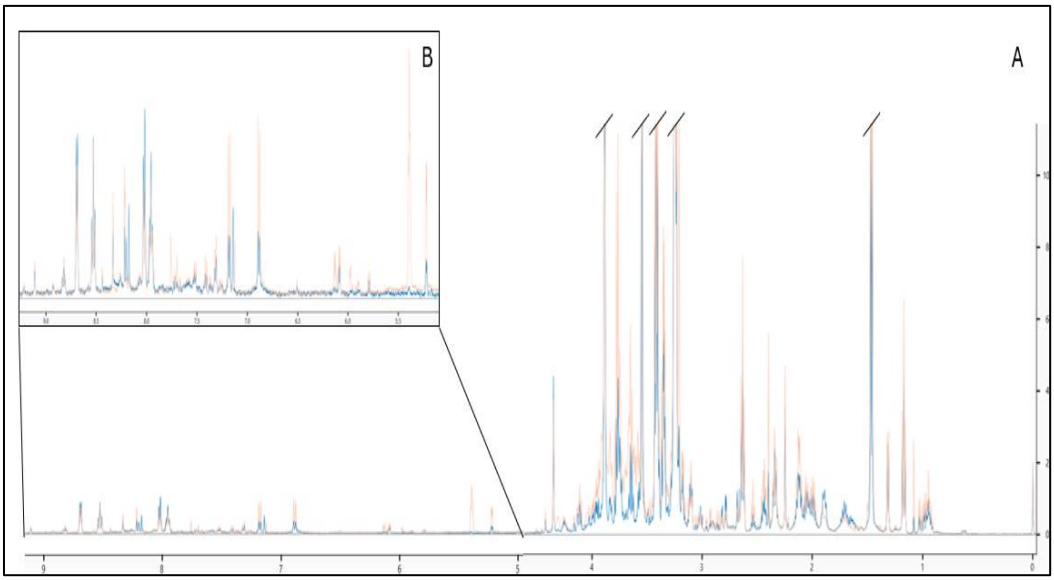

Figure 3.15 Overlay of representative 1-D 1H NMR spectrum at 500 MHz of male (blue) and female (pink) gonads of *M. galloprovincialis* from a uncontaminated area with (A) showing the aliphatic region and (B) a vertical expansion of the aromatic region, to better highlight the difference in metabolite concentration.

As it can be seen from the above image, the two NOESY 1D ^{1}H NMR spectra of mussel gonads of males and females are perfectly superimposable since they share the same metabolic profiling, a sign that the reproductive state in both sexes is supported by the same metabolic processes.

However, from the same image it is also evident the difference in the concentration of metabolites according to sex, which in some cases may be more abundant in males than females, while in other cases the opposite occurs.

To better clarify this issue, the most significant metabolites detected in mussel gonads were listed in the following table (Table 3.1) grouped according to the metabolism in which they are involved to, with description of their chemical shift and peak shape, the level at which they were found in male and female gonads, and then the percentage change relative to males.

Expressed in ppm, in the second column of the table (Table 3.1), the chemical shifts corresponding to the resonance point(s) of the metabolite within the ^{1}H NMR spectra are reported. In the same table there are also reported the concentration of the metabolites as detected in males (in blue) and females (in pink) referred to that of the Chemical Form Indicator (DSS) and expressed in mM with the relative SD,

and in the last column the percentage variation of the metabolite concentration reported for males compared to females.

As also confirmed by other authors, such as Ellis et al. (2014), FAA such as glycine appear to predominate in male gonadal tissues compared to female ones, as well as tyrosine is the most abundant metabolite in female gonads compared to males.

In addition, it has been shown that metabolites involved in the energy pathway were found at higher level in females than in their male counterparts, as evidenced by the decrease of about 77.79% of glucose and 96.15% of glycogen in males compared to females.

Similarly, also acetoacetate, a final product of lipid β-oxidation, was also found to be at a higher concentration in females than in males.

About osmolytes, the metabolites related to osmoregulation processes, they were also found at a higher concentration in the female gonad than in the male one, with a more moderate variation compared to other metabolic pathways.

In general, it appears that female gonads contain a higher concentration of some metabolites analysed than male gonads.

METABOLITES INVOLVED TO	CHEMICAL SHIFT AND PEAK SHAPE (ppm)	MALE GONAD	FEMALE GONAD	CHANGE %
AMINO ACID METABOLISM				
Alanine	3.8 (q), 1.5 (d)	30.78±0.54	95.66±1.09	↓69
β-Alanine	3.2 (t), 2.5 (t)	2.01±0.42	6.69±0.42	↓70
Arginine	3.8 (t), 3.2 (t), 1.9 (m), 1.7 (m)	19.58±0.69	13.41±1.14	↑46
Asparagine	6.9 (s), 4.0 (q), 2.9 (q), 2.8 (q)	4.90±0.26	9.07±0.75	↓46
Aspartate	3.9 (q), 2.8 (q), 2.7 (q)	12.44±1.98	7.09±0.78	↑75
Glycine	3.6 (s)	139.88±0.64	33.15±0.99	↑322
Glutamate	3.8 (q), 2.4 (m), 2.1 (m)	25.12±0.80	41.38±1.05	↓39
Glutamine	6.9 (s), 3.8 (t), 2.5 (m), 2.1 (m)	7.35±0.98	15.97±0.68	↓54
Histidine	4.0 (m), 3.2 (q), 3.1 (m)	2,46±0.45	2,19±0.17	↓11
Isoleucine	3.7 (d), 1.0 (d), 0.9 (t)	1.63±0.37	2.21±0.07	↓26
Leucine	3.7 (m), 1.7 (m), 1.0 (t)	1.43±0.05	2.94±0.16	↓51
Lysine	3.7 (t), 3.0 (t), 1.9 (m), 1.7 (m)	6.37±0.98	4.50±0.82	↑42
Phenylalanine	7.3 (m), 4.0 (q)	0.80±0.17	1.26±0.25	↓37
Proline	4.1 (m), 3.4 (m), 2.3 (m), 2.1 (m)	15.12±0.89	10.42±0.92	↑45
Tyrosine	7.2 (d), 6.9 (d), 3.0 (q)	1.09±0.21	3.93±0.60	↓62
Valine	3.6 (d), 1.0 (d)	1.48±0.17	2.25±0.27	↓52
ENERGY METABOLISM				
Acetate	1.9 (s)	1.93±0.4	1.42±0.04	↑36
Acetoacetate	3.4 (s), 2.3 (s)	5.74±0.51	7.74±0.69	↓26
ATP/ADP	8.5 (s), 8.2 (s), 6.1 (d), 4.6 (t), 4.2 (m)	0.46±0.05	2.03±0.08	↓78
Glycogen	5.4 (s), 3.8 (m), 3.6 (m), 3.4 (m)	0.48±0.08	12.35±0.84	↓96
Glucose	5.2 (d), 3.8 (m), 3.7 (m), 3.5 (m)	4.10±0.56	17.66±0.55	↓77
Lactate	4.1 (q), 1.3 (d)	5.72±0.41	6.02±0.79	↓5
Malonate	3.1 (s)	3.84±0.60	5.23±0.47	↓26
Mytilitol	1.0 (s)	1.12±0.12	7.18±0.18	↓84
Pyruvate	2.4 (s)	2.14±0.20	2.49±0.49	↓14
Succinate	2.4 (s)	1.35±0.35	4.86±0.59	↓72
OSMOREGULATION				
Betaine	3.9 (s), 3.3 (s)	121.12±0.76	166.42±1.29	↓27
Taurine	3.4 (t)	80.11±0.91	145.87±0.47	↓45
Homarine	4.4 (s)	0.41±0.06	0.59±0.09	↓29
NUCLEOTIDE AND THEIR DERIVATIVE METABOLISM				
Adenosine	8.3 (s), 4.4 (m)	0.26±0.06	0.33±0.16	↓20
Uracil	5.8 (d)	1.04±0.07	1.61±0.33	↓35
IMP	8.6 (s), 8.2 (s), 6.1 (d), 4.5 (t)	0.42±0.09	1.84±0.31	↓77
MEMBRANE COMPOUND				
Choline	4.1 (m), 3.2 (s)	0.73±0.13	1.09±0.11	↓33
O-phosphocholine	4.2 (m), 3.2 (s)	1.17±0.18	5.94±0.20	↓80
Sn-Glycero-3-phosphocholine	4.3 (m), 3.9 (m), 3.2 (s)	7.15±0.89	10.17±0.96	↓30
OTHER METABOLITES				
Methionine	2.6 (t), 2.2 (m), 2.1 (m)	21,99±2.96	32.63±2.17	↓33

Table 3.1 Summary table of metabolites detected from polar extracts of male (blue) and female (pink) gonads of *M. galloprovincialis* from an uncontaminated area with relative mean expressed with ±SD and percentage variation of metabolite concentration of males in respect to females.

3.4 Discussions

The aim of this study was to evaluate the sexual differentiation of the reproductive tissue in the Mediterranean mussel *Mytilus galloprovincialis* grown in an uncontaminated area at the specific period of collection of individuals, in this case autumn, to avoid any misunderstandings in the interpretation of the biological responses assessed within an ecotoxicological study. To fulfil this purpose, a multi-biomarker approach that incorporated histomorphological, histochemical and metabolomic investigations was adopted so that the state of gonadal maturation and the gametogenic process could be accurately interpreted, considering the bioenergetics and metabolic condition supporting it in a specific reproductive period, such as autumn.

Since individuals of both sexes were collected from the same area in the mussel farm, therefore with the same physico-chemical conditions in terms of oxygen availability, pH, temperature and salinity, and where the animals have been indiscriminately raised and fed with the same diet, suggests that the differences found at the metabolic level and in energy reserves are specifically due to sexual differences. Moreover, many studies confirm that the reproductive cycle follows a seasonal trend, being influenced by environmental conditions and nutrient availability (Bordignon et al., 2024; Kronberg et al., 2021), but here this is not the case since mussels were collected exclusively at the beginning of November 2022, therefore in autumn.

Histological analysis, showing the gonads of both sexes in an advanced maturation phase, highlighted the presence of more germ cells than connective cells in both sexes. Therefore, it is plausible to affirm that in a period in which gonads of both sexes are full of gametes, any sexual characteristics emerge more clearly and that therefore it is likely to hypothesize that within this specific scenario the two sexes have the potential to activate sex-dependent biological responses (Blanco-Rayón et al., 2020).

Glycogen in both sexes plays an important role in the bioenergetics of the tissue, being implicated as a primary energy source to support the gametogenic process (Li et al., 2024). However, both histochemical and metabolomic data showed a divergence between males and females, in fact, in the latter the reserve deposited inside the VCT and ADG cells appears to be more marked. This could be

explained as a sex-dependent difference since, in addition to energetically supporting the differentiation of gametes, glycogen in females serves also to form high-energy molecules such as the vitellogenin, a glycolipophosphoprotein that is catabolized to form yolk proteins including phosvitin and lipovitellin, which are future energy reserves for the development and growth of the embryo (Mincarelli et al., 2024; Xu et al., 2020; Li et al., 2017; Pipe, 1987).

Sex-dependent differences can be clarified by considering the different physiological needs that exist between males and females during the reproductive cycle, fertilization and the first sustenance of the embryo (James et al., 2023).

A marked difference, compared to other metabolites, has been left by osmolytes such as betaine, taurine. These are organic compounds that play a crucial role in maintaining the osmotic balance in many bivalve species such as *Crassostrea gigas* (Boulais et al., 2019), mainly in border environments such as the intertidal ones, where a successful fertilization depends on the mobility of spermatozoa. This latter is greatly influenced by the osmolarity of the gonadal fluid, very similar to that of sea water. In this study, the concentration of osmolytes was found to be higher in female than in male gonads.

In this perspective, finding a high concentration of glycine in male mussels fits well. In fact, this metabolite can act both as an osmolyte and also have a role within the energy pathway, entering the Krebs cycle and acting as a carbon base to produce energy (Tikunov et al., 2010), potentially available for sperm motility.

Surprisingly, unlike what reported by other authors (Ellis et al., 2014), glutamate was found to be present in males at a lower extent. However, this should not be interpreted as a contradiction since the metabolic profile can depend on different variabilities, including sex, maturation state, tissue, species and environment (Bordignon et al., 2024; Blanco-Rayón et al., 2020; Cappello et al., 2018).

3.5 Conclusions

The results obtained from this study have contributed to better understand the sexual differentiation under a physiological condition of adult male and female specimens of the Mediterranean species *Mytilus galloprovincialis* grown in a natural and uncontaminated habitat such as that of the mussel farm S.A.Co.M. (Messina, Italy), where mussels were collected. Furthermore, the combination of traditional and more modern techniques was able to specifically describe the sex-dependent divergences in mussel gonads during the period of autumn.

All the findings raised from this study should be accurately considered when carrying out an ecocytotoxicological investigation since the variability found into sex and reproductive stage of individuals can be reflected in the accumulation processes and biochemical responses triggered by the exposure to various xenobiotics, altering the interpretation of the data, and therefore misunderstanding the potential risk for the biota health exposed to pollution (Blanco-Rayón et al., 2020).

Therefore, environmental biomonitoring strategies should be consolidated in assessing the biological effect on any model system chosen, verifying the eco-toxic impact in both sexes, keeping in mind the state of the reproductive cycle and the degree of sexual maturity at the time of individuals collection.

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CHAPTER 4

Biological effects of environmental doses of dexamethasone in gonads of the mussel Mytilus galloprovincialis

CHAPTER 4

Biological effects of environmental doses of dexamethasone in gonads of the mussel *Mytilus galloprovincialis*

4.1 Introduction

Dexamethasone (Dex), also known as 9 α -fluoro-16 α-methylprednisolone or 16 α-methyl-9 α-fluoroprednisolone (Cohen, 1973), is a synthetic glucocorticoid (GC) with the molecular formula $C_{22}H_{29}FO_5$ and a molecular weight of 392.45 Da, that can be synthesized starting from 16-amethylhydrocortisone acetate, an intermediate derived from bile acids (Figure 4.1, 4.2).

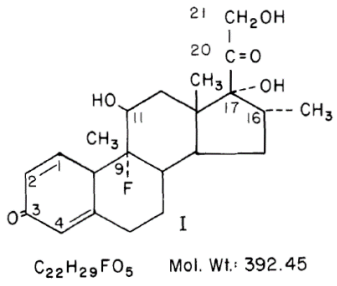

Figure 4.1 Chemical structure of dexamethasone.

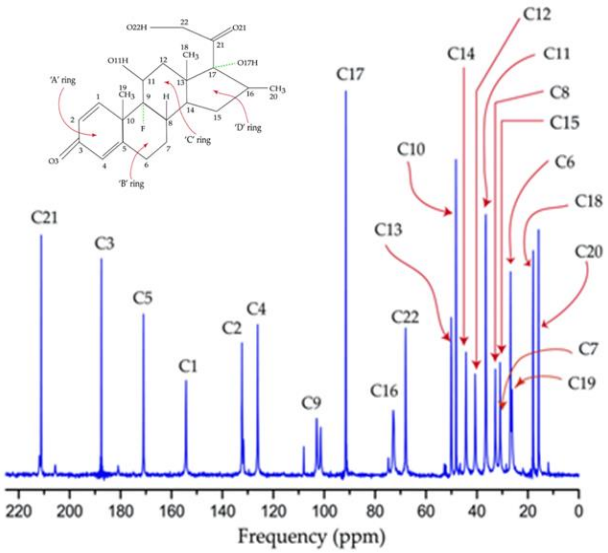

Figure 4.2 Structure with rings and 13C CP-MAS SSNMR spectrum of dexamethasone.

The glucocorticoid is found in the form of a white-yellowish powder, insoluble in water, stable in air, but being highly photosensitive. Indeed, it must be protected from direct light since in these circumstances Dex solutions lose half of the α-ketol side chain in 6/8 min (Cohen, 1973).

The WHO Essential Medicines document of 1977 already describes the characteristics of this drug, presenting it as a synthetic fluorinated adrenocortical steroid (Mehta et al., 2022) in which the 9-α-fluorine atom determines an increase in the anti-inflammatory activity (Soumya & Joe, 2021). In fact, Dex is 20-30 times more active than hydrocortisone and 25 times more active than cortisol (Quaresma et al., 2021; Dare et al., 2018), a property that has made it one of the most widespread synthetic steroids used against various inflammatory syndromes.

Because of its easy accessibility and low cost, it is used for acute or chronic manifestations of inflammation, in immunosuppression and autoimmune processes (psoriasis, arthritis, etc.), to combat allergies (McLaurin et al., 2021) and for the treatment of some types of cancer (for its antiemetic properties) and against nausea (Dey & Ghosh, 2021). With the advent of the SARS-CoV-2 pandemic, it has proven to be an excellent ally against acute respiratory distress syndrome (ARDS), reducing the mortality of infected patients by 46% (Tomazini et al., 2020; Wu et al., 2020).

It is a synthetic cortisol-like drug with low mineralocorticoid activity whose biological properties depend on the ability to interface with the plasma membrane thanks to the flexibility of the unsaturated nucleus and interhelical hydrogen bonds (Soumya & Joe, 2021).

Like other glucocorticoids, Dex can interact with the cell both at the plasma membrane and the cytosol, influencing various physiological processes regulating homeostasis, glucose, lipid and protein metabolism (Zhang et al., 2020). In fact, it can directly or indirectly influence the activity of kinases that underlie the lipid bilayer, and/or be found inside the cell such as Rat sarcoma (RAS) and edc-Jun N-terminal kinases (JNK), leading to the transduction of various signals (Liu et al., 2017), also depending on the interaction with other pathways (Figure 4.3).

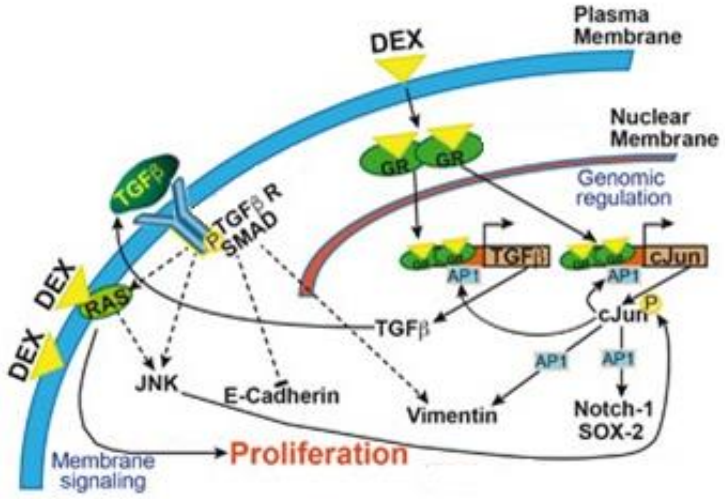

Figure 4.3 Cellular receptor pathways through which dexamethasone can interface with the biological system.

A mouse xenograft model has shown that the combined exposure of Dex and estradiol (E2) results in a protein-protein interaction with ERα, through the interaction between a C-terminal zinc finger domain of the glucocorticoid receptor (GR) on the ERα binding regions (EBR) of ERα and the help of Activator Protein 1 (AP1), resulting in the inhibition of the activity of the E2-ER complex and therefore the suppression of cancer cell proliferation (Karmakar et al., 2013).

In addition, by bypassing the semipermeable cellular barrier, thanks to the lipophilic properties intrinsic to the molecule, Dex interacts with the ligand-activated GR present in the cytoplasm. By dissociating them from the heat shock proteins, the GR dimerize promoting the mobilization of the complex towards the nuclear envelope. Once translocated into the nucleus, the complex influences transcription by binding either directly at the promoter region to trans-activation domains such as the GC-responsive element (GRE) or to trans-repression domains such as the negative GRE (nGRE), leading respectively to the induction or repression of the expression of several genes (Quatrini & Ugolini, 2021), or indirectly through transcription factors such as AP1 or Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Karmakar et al., 2013) (Figure 4.4).

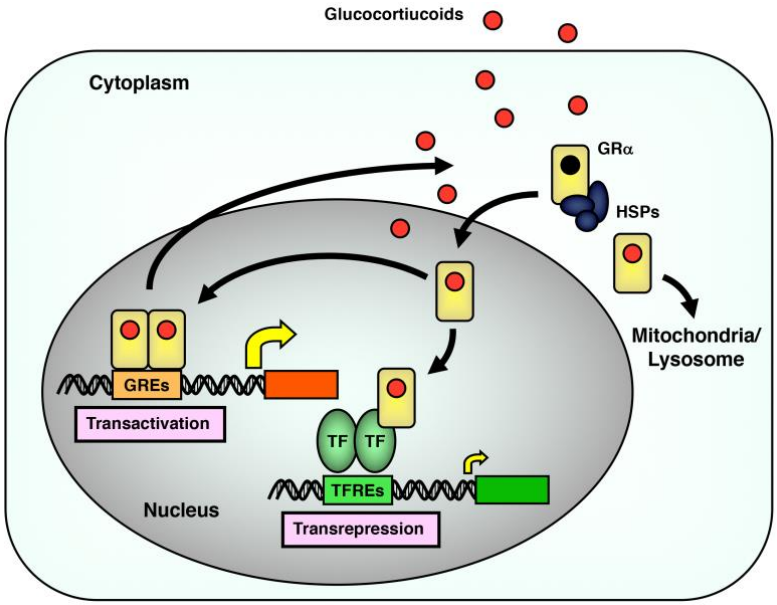

Figure 4.4 Representation of the mechanisms by which glucocorticoids, like dexamethasone, can influence transcriptional activity.

For example, Dex can activate the transcription of GLUcose Transporter type 5 (GLUT5), also exerting modifications on histones at the level of the GLUT5 promoter (Hwang et al., 2024), or promote the down-regulation of pro-inflammatory factors such as interleukin-6 (IL-6) (Yao et al., 2020) or induce the repression of the gene expression of pro-inflammatory cytokines such as the tumor necrosis factor (TNF), interleukins, cyclooxygenase-2 that are involved in the immune feedback as they can inhibit the working mechanism of cells such as neutrophils, macrophages and lymphocytes (Plush et al., 2021).

Therefore, dexamethasone has revealed to possess excellent anti-inflammatory properties, demonstrating to mimic the action of endogenous corticosteroids produced by the body. For this reason, it has been widely used to combat SARS-CoV-2 infection since many symptoms such as severe and fatal thrombotic events, were induced by acute inflammatory states caused by the hyperproduction of pro-inflammatory cytokines (Romanou et al., 2021).

The previous use of Dex as a common over-the-counter drug, sufficiently inexpensive and therefore widely distributed on a global scale given the known safety profile (Myles et al., 2021) compared to other PhACs, and the reduced capacity to keep it by the wastewater treatment systems (WWTP), approximately of 38.1% (Ramirez et al., 2024; Nguyen et al., 2021; Ammann et al., 2014; Arsand et al. 2013), have implied the increasingly significant release of the drug into many

environmental matrices. This has led to the worsening of the already present concentrations of Dex into the environment that, although they are in the range of ng/L-µg/L, are of major concern on a global scale (González-González et al., 2022; Charuaud et al., 2019; Creusot et al., 2014). The frequent and continuous release of Dex into the environment, further accentuated by the SARS-CoV-2 pandemic wave of the last three years, has led over time to the worsening of the presence of the synthetic glucocorticoid and its derivatives, especially in sites close to WWTP effluents, which usually flow close to estuaries and coastal areas (Castaño-Ortiz et al., 2024; Yang et al., 2024).

This increase in the environmental level of Dex raises the concern for the biota living into these habitats and draws attention to the potential biological risk for non-target organisms of the drug that reside in marine ecosystems nearby coastal areas (Topić Popović et al., 2023; Musee et al., 2021; DellaGreca et al., 2004). This situation is confirmed by various studies that attest the presence of PhACs, and in particular of dexamethasone, in aquatic environments at concentrations of up to 8.78 ng/L in surface water and 2.11 ng/L in drinking water (Quaresma et al., 2021).

Moreover, literature shows evidence of the bioaccumulation of synthetic glucocorticoid in a tissue-specific manner in various marine vertebrates and invertebrates (Cunha et al., 2022; Miossec et al., 2020), as also attested by Ismail et al. (2021) for mariculture fish at the level of different organs such as muscles, liver and gonads, in which the accumulation range of Dex is in the order of ng/g.

Despite the worrying environmental data, currently few studies have focused on evaluating the potential toxicity of realistic concentrations of Dex on non-target aquatic organisms. *In vitro* and *in vivo* studies, while not demonstrating a lethal effect on biota, have highlighted the potential of Dex to alter various aspects of the biological system such as reproduction, development and behaviour (Balasem et al., 2024; Sanchez-Aceves et al., 2024; Liang et al., 2023; Miao et al., 2022).

The attribution of Dex of having an EDC character in non-target terrestrial species is increasingly supported by several studies on murine models (Zhang et al., 2019; Colvin et al., 2013; Jux et al., 1998; Rabin et al., 1990). Indeed, these studies attest the influence of the drug on the hormonal system, and in particular on the regulation of the hormones of the hypothalamic-pituitary-adrenal (HPA) axis, having demonstrated its effect on the follicle-stimulating hormone (FSH), on

luteinizing hormone (LH), and on prolactin in female adult albino rats after a 30-day exposure to Dex, with consequent necrosis due to follicular degeneration events at the level of the Graafian follicles and vacuolization of the interstitial cells (Mohammed et al., 2023).

The ability of Dex to exert effects has been demonstrated to occur in both sexes. In fact, in six-week-old male mice exposed to 7 mg/kg per day of Dex for seven days, a decrease in the volume at the testicular level and a lowering of the quantity of maturing male germ cells were detected (Soleimani Mehranjani et al., 2022). Furthermore, from a biochemical point of view, an increase in malondialdehyde (MDA) was detected, as a sign of lipid peroxidation events occurring following exposure to Dex (Soleimani Mehranjani et al., 2022).

Some studies have verified that Dex can also have transgenerational effects. In fact, it has been ascertained that the exposure of rat fetuses to the drug causes gonadal dysfunction, interacting with prenatal GR at the level of the adrenal glands, thus confirming the possible heredity, and therefore the interference with the regulation of adrenal steroidogenesis (He et al., 2023).

Other scientific reports show that gestational exposures to dexamethasone can cause alterations in the folliculogenesis in the Cairo spiny mouse *Acomys cahirinus* with an increase in the number of ovarian follicles and corpora lutea, due to the suppression of apoptotic events, caused by an increased expression of anti-apoptosis factors (Hułas-Stasiak et al., 2018).

On the other hand, there are few studies conducted on aquatic species to evaluate the effects of Dex (Cuzziol Boccioni et al., 2020), most of which focus mainly on lower vertebrates with socio-economic relevance. Nonetheless, the effects of Dex in fish on the reproductive system and the influence on development have been known for a long time, as a study of Lethimonier et al. (2000) on the trout *Oncorhynchus mykiss* confirms. More recent experimental investigations have shown that the synthetic glucocorticoid can cause adverse effects on the female reproductive system and at the embryo-larval stage in the fish species *Pimephales promelas* after sub-chronic exposure to Dex at concentrations of 500 µg/L (LaLone et al., 2012).

As reported for higher vertebrates, also for lower ones it has been validated that dexamethasone can also have differential effects as EDC in males and females, as

documented by both *in vivo* and *in vitro* studies (Balasem et al., 2024; Liang et al., 2023; Zhong et al., 2021). Recent studies have in fact confirmed that the cytotoxic effect of Dex is somehow dependent on sex, as observed in adult females and males of the mosquitofish *Gambusia affinis*, a small freshwater fish that responded differently based on sex when exposed to Dex at concentrations between 0.5 μg/L and 50 μg/L for 60 days. Dex inhibited the transcription of the *vitellogenin* gene (*VTGC*) and downregulated sex hormone receptor genes, including *estrogen receptors* (*ERβ*) in the brain, describing a masculinizing effect on the female system, while in males it induced a positive regulation of the *vitellogenin* gene transcription with a potential impact on sex hormone levels, leading to alterations at the testicular level (Liang et al., 2023; Zhong et al., 2021).

Regarding the understanding of the biological impact of Dex on aquatic invertebrates, again the risk assessment is far from being widely documented despite many invertebrates belong to species of food interest. This gap is also due to the poor knowledge regarding the functioning of the endocrine system of many invertebrate species, which however represent approximately 95% of the known species, but on which the assessment of EDCs is still a problem today (Ford & LeBlanc, 2020; Porte et al., 2006). Overall, investigations on the impact of Dex are very limited to few studies conducted on crustaceans and molluscs (Jiang et al. 2023; Bal et al., 2017; Gervais et al., 2015; DellaGreca et al., 2004).

Therefore, based on what stated above, the aim of this work was to evaluate the impact and potential reproductive responses induced by Dex on male and female organisms of a non-target gonochorous species with socio-economic value such as the Mediterranean mussel *Mytilus galloprovincialis*, considering both organisms as biological systems and therefore investigating multiple levels of biological organization. In detail, through a multi-biomarker approach, the morphological, histochemical, metabolomic and molecular responses triggered by Dex were evaluated on gonads of both sexes of adult mussels exposed for 12 days to five experimental conditions, including four environmental concentrations of the glucocorticoid drug dexamethasone (C1: 0.004 μg/L; C2: 0.04 μg/L; C3: 0.0.4 μg/L; C4: 2 μg/L) and a negative control (C0: 0.0 μg/L). Additionally, temporal modulation of the responses provoked by Dex exposure were also evaluated during the experimental trial at fixed time-points, specifically at the beginning of the

exposure (T0), and after three (T3) and 6 (T6) days, and at the end of the experiment (T12).

Among the approaches applied to address the aim of this study, the innovative and sensitive metabolomics based on Proton Nuclear Magnetic Resonance (^{1}H NMR) was used to understand in detail the potential divergence at metabolite level in drug response between male and female mussels. Indeed, NMR-based metabolomics has proven to be an excellent and immediate investigation strategy that allowed to identify endogenous metabolite biomarkers whose modulation, induced by the treatment in both sexes, has provided a detailed description of the physiological-functional state of the gonads, directly related to the expressed phenotype (De Marco et al., 2023; Kronberg et al., 2021; Cappello et al., 2021, 2015; Ellis et al., 2014).

Finally, this work aims to provide a point of reflection, focusing attention on the negative influence that human activities are having on aquatic ecosystems and how these can have evident and worrying repercussions not only on the resident biota, but also on the entire food chain, and consequently also on human health. The adverse impact on the reproductive health of a species, such as the bivalve mollusc used in this investigation, which has a great value in the construction of intertidal ecosystems, can have adverse repercussions not only on the single organism, but in a broader perspective also at population level, within the ecological niche, and more globally on the entire ecosystem (Bal et al., 2017; Anderson & Wilde, 1994).

4.2 Materials and methods

4.2.1. Mussel acclimatization and experimental design

In November 2022, specimens of the marine Mediterranean mussel *Mytilus galloprovincialis* were collected from the S.A.Co.M. aquaculture farm, located in Messina (Sicily, Italy).

After a preliminary selection based on the average length of the valves of 4-5 ± 0.4 cm, in order to select adult organisms that had reached sexual maturity, the samples were transported to the scientific structure “Mesocosm Facility” of the

CNR-IRBIM of Messina (Sicily, Italy) and placed randomly, without distinction of sex, in glass aquaria filled with 15 L of filtered natural sea water (FSW) at 300 µm.

The specimens were acclimated for a total period of 15 days, constantly maintaining controlled laboratory conditions, namely temperature at 18 ± 1 °C, salinity 35‰, pH 7.8 ± 0.02, photoperiod light/dark (L/D) 12 h:12 h, and continuous aeration. During the acclimation period and for the entire duration of the experiment, FSW changes were performed three times a week, and the water change was combined with cleaning the tank and administration of a diet based on a commercial algae mix (Liquizell, Hobby).

Figure 4.5 Set up of the experimental plan in duplicate in which it is possible to see the glass tanks used for each condition (C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex), with the specimens of *M. galloprovincialis* allocated.

The experimental plan included five conditions: four groups of mussels were treated with different environmental doses of Dex, namely C1 (0.004 µg/L Dex), C2 (0.04 µg/L Dex), C3 (0.4 µg/L Dex), and C4 (2 µg/L Dex), whereas the negative control group was exposed only to FSW with no Dex (C0; 0 µg/L Dex), for a total exposure duration of 12 days (Figure 4.5). To evaluate the impact of realistic doses

of the synthetic glucocorticoid drug dexamethasone, the four concentrations of Dex were selected according to the level of Dex reported at environmental level, and after a careful evaluation of the data in the literature that accredit the presence of the drug in the range of ng-µg/L in different environmental matrices, including sewage water, fresh and marine waters (Chen et al., 2021; Ismail et al., 2021; Charuaud et al., 2019; Ammann et al., 2014; Creusot et al., 2014).

Starting from a stock solution of Dex (Adipogen, CAS 50-02-2, purity 129 98.8%) at concentration of 5 mg/L, the experimental concentrations tested were obtained by dilution directly in the medium (FSW).

The entire experimental plan was performed in duplicate, taking care to ensure optimal physico-chemical conditions for the entire duration of the sub-chronic exposure, as those maintained during the acclimation period of mussels. To evaluate the temporal trend of the biological responses induced by the exposure to the drug, samples were taken at fixed time-points, namely at the beginning of the exposure (T0), after three days (T3), after 6 days (T6) and after 12 days (T12) of the experimental trail, which coincided with the end of the exposure. At each fixed time-points, twelve specimens (n = 6 from each aquarium) were sampled from each experimental condition, to guarantee a number statistically significant of individuals for both sexes.

Using the appropriate instruments and maintaining sterility where necessary, after collection of mussels at the fixed time-points, a fresh evaluation of the sex was performed under light microscope by crushing a small fragment of the gonads between a glass slide and a coverslip, to distinguish male from female mussels, as explained in detail in the previous Chapter 3, section 3.1.2. Successively, a small piece of the gonads of each collected mussels was promptly introduced into 4% PFA fixative solution (Immunofix) at 4% (37% formaldehyde diluted with 1 M PBS at pH 7.4) for subsequent histological and histochemical analyses, while the rest of the tissue was stored in darkened and sterile test tubes in liquid nitrogen, until arrival at the laboratory at the University of Messina (Sicily, Italy), where they were stored in at -80°C for the successive chemical, metabolomic and molecular analyses.

4.2.2 Chemical analysis

The mussel samples were promptly transferred on dry ice to the Department of Medical, Surgical and Advanced Technologies "G.F. Ingrassia" of the University of Catania (Sicily, Italy) to perform chemical analyses aimed at measuring the bioaccumulation of Dex into the gonadal tissues. This analysis was conducted by High Performance Liquid Chromatography (HPLC), in detail by using the Agilent HPLC 1260 Infinity II (Agilent Technologies ©) present in the laboratory.

Sub-samples of mussel gonads, weighing about 100 mg and taken from each of the twelve organisms sacrificed for each experimental condition (C0, C1, C2, C3, C4) and at each time-point (T0, T3, T6, T12), were homogenized using 5 mL of high purity acetonitrile (Carlo Erba Reagents ©) for HPLC. This was followed by two sonication cycles of 10 minutes and a centrifuge at 2500 rpm for 5 minutes, to obtain the separation of samples into two phases. Subsequently, the supernatant was collected and subjected to filtration with 0.45 μm polytetrafluoroethylene (PTFE) filters, and then dried by a nitrogen flow. The extract was resuspended in 1 mL of acetonitrile for the determination of the bioaccumulation of dexamethasone using the HPLC Agilent 1260 Infinity II (Agilent Technologies ©) equipped with a UV detector.

The analysis was performed using a Waters© XTerra inverted phase (4.6 mm x 150 mm; 5 μm) and an isocratic elution in the mobile phase of acetonitrile and water (70/30 v/v; flow rate: 1 mL/min), to ensure that the influence of the retention time caused by the residence volume was negligible. A calibration curve of Dex (50-100-500-2500-5000 ng/L) was performed with measured linearity of R2: 0.9976. The limit of determination (LOD) for the drug was 25 ng/L and a negative control was also made.

Since large amounts of gonadal tissue were needed to proceed with the chemical analysis, it was necessary to perform a pool of samples to verify accurately the bioaccumulation of Dex in mussel gonads due to the lack of sufficient biological material.

4.2.3 Histological analysis

As previously described by Afsa et al. (2023), and more in the detail in the previous Chapter 3, section 3.2.3, the gonadal tissues collected for histological analysis were immediately fixed in 4% PFA (Immunofix) (37% formaldehyde diluted with 1 M PBS at pH 7.4) and kept at 4 °C for 4 hours.

After two quick washes in 1 M PBS at pH 7.4 for 10 minutes each, a slow dehydration was carried out, performing successive passages in an increasing series of ethanol, specifically 1 h at 50°, 1 h at 70°, 30 min at 80°, 30 min at 95°, 15 min at 100° I and 15 min at 100° II.

Afterwards, under a hood, the first passage was carried out in the clarifying agent xylene mixed at 50% with 100° ethanol for 30 min. Then, the mixture was replaced by only xylene for 30 min. After clearing, the samples were kept for 1 h in a mixture of xylene and ultrapure paraffin Paraplast (Bio-Optica, Milan, Italy), and then underwent two more passages of 1 h each in paraffin alone.

The blocks of paraffin with the gonadal tissues embedded into them were therefore manually cut in sections of 4 µm thickness with a rotating microtome (Leica Microsystems, Wetzlar, Germany), and mounted on slides. After a night in an oven at 26 °C, so that the sections would spread and adhere well to the glass slide, the histological sections of mussel gonads were deparaffinized with two sequential passages of 5 min each in xylene. Afterwards, the sections were rehydrated following a decreasing series of alcohols (100°, 95°, 80°, 70°, 50° and 35°), each step lasting 5 min, until reaching the distilled water (DW).

At this point, some slides were subjected to d-PAS/PAS reaction, as described in the following section 4.1.4, whereas the other slides were treated with the haematoxylin/eosin (H/E) colorimetric method. For the latter, each histological slide was immersed first in a H bath (basic dye) for 15 sec, the excessing dye removed by a quick passage in DW, and then a pause of 10 min in tap water at room temperature allowed a shift in the colour towards a more darked one, which confirms that the dye was bond to all the basophilic structures (such as the nucleus) within each cell of the treated tissue. After a further wash in distilled water, the protocol proceeds with the step in eosin (acid dye), in which the slide were immersed for only 8 sec, as a sufficient time to allow the dye to bind with the acidophilic structures of the cytoplasm, which will appeared in a pinkish colour.

This was followed, according to the validated protocol (Cappello et al., 2021), by a brief dehydration in a series of steps with increasing alcohols (95°, 100° I, 100° II) and then in a mixture of 100° alcohol and xylene for 5 min each, followed by two passages in xylene, used as a solvent for the mounting resin (Eukitt® Quick-hardening mounting medium) that allowed by a coverslip to finally mount the histological coloured sections in order to protect them for a long-lasting use. In this way, by an accurate histological observation under a light microscope, it was possible to determine more precisely the sex of mussels, the morphological organization of gonads and the potential alteration of the tissue following exposure to Dex.

All the images were acquired with a Zeiss Axio Imager Z1 motorized microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany) (Maisano et al., 2017) with immersion objectives.

4.2.4 Histochemical analysis

To identify the presence within mussel gonads of neutral glycopolysaccharide compounds, such as glycogen, a histochemical reaction with periodic acid Schiff (PAS) (Abcam, Cambridge, United Kingdom) combined with a d-PAS (diastase-PAS) was performed on two distinct but serial histological sections collected on the same slide, following the protocol of Meyerholz et al. (2018) with slight modifications, as also described previously in Chapter 3, sections 3.2.4. This allowed to detect the presence of glycogen in mussel gonads and to quantify it, comparing the positive control/PAS (glycogen present) with the negative control/d-PAS (glycogen absent). The d-PAS reaction was performed, only on one of the two sections of the slide and for all the samples collected and the experimental conditions and time-points taken into consideration. Thus, the section was previously delimited by the use of a PAP pen in order to draw a hydrophobic circle around the slide-mounted tissue, so that the 15 U/mg α-amylase solution in 1M PBS at pH 7.4 did not disperse and could not completely cover the slide, and therefore the two sections mounted on it. Subsequently, the slide was incubated at 37 °C for 20 min in an oven.

After a brief rinse in DW to remove the exceeding d-PAS, the section was dried well and softly with a filter paper. Periodic acid was thus applied to both sections (up and down) on the slide, and left to act for 5 min. After two quick washes in DW, Shiff Solution was used, covering both sections for 10 min.

At the end of this step, the slides were placed in a pathological tray with pre-determined spaces where to locate them and left for 2 min in a hot water bath (30 °C). After two rapid washes in DW, staining with hematoxylin took place and was left to act for 30 sec. The excess of the colour was removed by two washes in DW.

According to the canonical histological protocol, a rapid dehydration in alcohol (95°, 100° I, 100° II) followed by a step in a mixture of 100° alcohol and xylene and two passages in xylene was performed before sealing the sample with a glass coverslip by the use of the Eukitt resin.

The images obtained, all at the same 40X magnification, with a Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany) (Maisano et al., 2017) were exported in .jpeg format and processed using the Image J (Image Processing and Analysis in Java) software version 1.54i to quantify the amount of glycogen on mussel gonads, as highlighted by the PAS reaction. A full description of the measurement acquisition process was already reported in Chapter 3, section 3.2.4. The results were reported in pixels (Mai et al., 2023; Chen et al., 2022; Jones et al., 2021).

4.2.5 Immunohistochemical analysis

To assess the viability of germ cells, an indirect immunofluorescence analysis was performed exclusively on male gonad sections since haemocytes were detected inside the follicle that, according to Smolarz et al. (2017), are a sign of atresia and therefore of an ongoing apoptotic process.

The histological sections of mussel gonads were therefore treated with 1% Triton for 3 min to make the plasma membranes permeable to antibodies, followed by a brief rinse in 1M PBS at pH 7.4. The sections were then incubated with 10% bovine serum albumin (BSA, Sigma-Aldrich, Darmstadt, Germany) for 1 h in a

humid chamber at room temperature (RT), in order to bind all the possible unspecific sites within the tissue.

An indirect immunohistochemistry technique was therefore performed, that implies the use of unconjugated primary antibodies. In this study, the primary antibodies selected were all linked to the cell turnover, being the metabolic pathway worthy to be investigated in gonads following exposure to Dex. In detail, the anti-Caspase-3 antibody (anti-Cas3; mouse monoclonal antibody, Cambridge, UK) and the anti-Fas Ligand antibody (anti-FasL; rabbit polyclonal antibody, Cambridge, UK), were used in co-localization on the same histological section of gonads of mussels from each experimental group and time-point, and used with a dilution of 1 µg/mL in PBS 1X. Additionally, the anti-Proliferating Cell Nuclear Antigen antibody (anti-PCNA; rabbit polyclonal antibody, Abcam, Cambridge, UK) was used individually on gonadal section and diluted 1:200, always in 1M PBS at pH 7.4. The antibodies were incubated on histological sections overnight in a humid chamber at 4 °C.

The day after, after an accurate wash in 1M PBS for 10 min to remove the excess of antibodies, the sections were incubated in a humid chamber at RT for 2 h with goat anti-mouse IgG secondary antibody conjugated with termethyl rhodamine isothiocyanate (TRITC) (Jackson ImmunoResearch, Cambridge,UK) and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, Cambridge, UK), both diluted 1:200 in 1M PBS at pH 7.4. Buffered glycerin (glycerol dissolved in 1M PBS at pH 7.4) was then used for mounting the slides.

After a rapid drying, the slides were observed with a Zeiss Axio Imager Z1 epifluorescence microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam camera (Zeiss, Jena, Germany). The images were acquired by setting an appropriate filter for TRITC (515–590 nm) and FITC (495 nm) excitation.

4.2.6 Metabolomic analysis

4.2.6.1 Extraction of metabolites from mussel gonads

To assess the potential effects induced by Dex at metabolite level in gonads of male and female mussels, a "two-step" protocol (methanol/chloroform/water) was performed, according to Cappello et al. (2018), as fully described previously in Chapter 3, section 3.2.5.

Mussel gonads of both sexes, previously stored in sterile tubes at -80 °C after collection, were weighted to obtain sub-samples of about 100 mg. To homogenize the samples, stainless steel beads (3.2 mm in diameter) and a mixture of cold methanol and distilled water (methanol: water = 4 mL/g: 0.85 mL/g) were added to the tubes, that were placed for 10 min in a TissueLyser LT (Qiagen, Hilden, Germany) set at 50 oscillations/sec.

Successively, 4 mL/g of chloroform and 2 mL/g of distilled water were added to the samples, and then rapidly mixed for 1 min with a vortex. After shaking, the samples were kept on ice for 10 min and then centrifuged at 2000 g at 4 °C for 5 min, to obtain the separation between polar and apolar phases, with an intermediate protein phase. The supernatant, made of the aqueous phase containing hydrophilic metabolites, was collected from each sample, taking care not to touch the protein intermediate phase, and then transferred to new sterile tubes for a total volume of 600 µL. The samples were then dried using a centrifugal vacuum concentrator (Eppendorf 5301) to obtain a completely dry pellet to be stored at -20 °C until the analysis by NMR spectrometer.

Before being introduced into the spectrometer, the pellets were resuspended in 0.1 M sodium phosphate buffer (600 µL; pH 7.0, 10% D_2O; Armar AG, Döttingen, Switzerland) and 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich), used as an internal standard, which represents the "Indicator of the chemical form". The suspension containing the polar metabolites derived from the gonads of both sexes was transferred into very thin glass capillaries for NMR with a diameter of 5 mm, paying attention to avoid the formation of bubbles in order to obtain a homogeneous solution and ensure the success of the spectrophotometric reading.

4.2.6.2 ^{1}H NMR-based metabolomics and spectral pre-processing

The instrument used to detect the presence of polar metabolites with molecular weight not exceeding 1,500 Da was the Varian-500 NMR spectrometer equipped with RF channels with waveform generators working at a frequency of 499.74 MHz at 298 K.

The acquisition of the spectral data relating to each sample was the NOESY (Nuclear Overhauser Effect SpectroscopY) software with which, by exploiting the nuclear Overhauser effect, it was possible to determine the protons close in the chemical space, even if not directly connected, thus better detecting even small molecules in which the nuclei of the protons are closer. With this acquisition mode, innovative in respect to the previous PRE-SAT modality, it was possible to refine the resolution of the spectrum, reduce the water peak more finely to an almost imperceptible extent, and thus also identify metabolites in the region between 4.5 and 5.1 ppm.

Using the Chenomx Processor software (Chenomx NMR Suite version 10.0; Chenomx Inc., Edmonton, Canada), each spectrum belonging to the metabolic extracts of the gonads of both sexes was manually phased with baseline correction and calibrated by setting the DSS (standard) to 0.0 ppm. The identity of the metabolites was defined by comparing the peak patterns with the information provided by the Chenomx 500 Hz database and other public computer libraries.

Furthermore, always using the Chenomx Processor settings it was possible to establish the concentration of each detected metabolite, using the known dose of the DSS added previously in each step to serve as internal standard also for a quantitative purpose.

4.2.7 Molecular analysis

4.2.7.1 RNA extraction

Using sterile RNase-free tools, 50 mg of gonad sub-samples of both male and female mussels were dissected from the freshly sampled organism at the time of collection, and quickly placed in sterile 2 mL tubes in liquid nitrogen (-196 °C) for

transportation to the laboratory, where samples were stored at -80 °C to preserve the integrity of the tissue prior the subsequent molecular analyses.

At the time of analysis, 1 mL of TRIzol™ reagent (Invitrogen, Waltham, United States), a monophasic solution of phenol, guanidine isothiocyanate, and other components that facilitate the isolation of RNA, was added to each sample according to the manufacturer instructions, being a reagent necessary for the isolation of high-quality total nucleic acids. Tissue homogenization was thus performed in TissueLyser LT (Qiagen, Hilden, Germany) for 5 min, using 3.2 mm stainless steel beads at 50 oscillations/sec. To allow a better isolation of the molecules of interest (total RNA) from the rest of the cellular structures (proteins, lipids, etc.), each homogenate was incubated at room temperature (RT) on cold supports for 5 min.

To guarantee a clear separation of homogenates into the aqueous phase containing the ribonucleoprotein complex, the intermediate phase represented by denatured proteins, and the phenolic phase containing the remaining organic matters formed by lipids and proteins rich in hydrophobic amino acids, 0.2 mL of cold chloroform was added to each sample, which helps the complete denaturation of the proteins, also allowing to remove the lipids and stabilize the intermediate phase. After mixing manually the samples for a few seconds, and a rest of 3 min at RT during which the dissociation into two phases was already visible, then samples were centrifuged at 12000 rpm at 4 °C for 15 min and taking great care to extract the tube from the rotor in an oblique position, so to avoid to mix the three phases, only the supernatant containing the total RNA was then collected and introduced into sterile 1.5 mL tubes.

At this step, 0.5 mL of cold 100% isopropanol was added to each sample which was then incubated on ice for 10 min, to concentrate and recover the total RNA in solid form from the aqueous phase, after which a new centrifuge was performed at 12000 rpm at 4 °C for 10 min, to allow the total RNA to be collected in the form of a pellet at the bottom of the tube.

After removing the supernatant by inverting the tube and taking great care not to lose the pellet, cold 75% ethanol was added to the latter, which serves to further aggregate the RNA and allow its precipitation, obtained after a 5 min centrifuge at 4 °C for 7500 rpm.

After removing the ethanol by gently inverting the tubes, they were left to air dry for 10 min, before proceeding to suspend the pellet in approximately 100 µL of nuclease-free ultrapure water.

Finally, to quantify the total RNA obtained measuring the absorbance at 260 nm with NAnodrop®-2000 spectrophotometer (Qiagen, Hilden, Germany), which allowed to measure the amount of ribonucleic acid in 1 µL of sample, while the integrity was assessed with a 1.2% agarose gel electrophoresis. The remaining sample was then stored at -80 °C until it was possible to proceed to continue with the second part of the molecular analysis.

4.2.7.2 Removal of genomic DNA from RNA, cDNA synthesis and PCR reaction

To eliminate any possible residue of deoxyribonucleic acid (DNA), total RNA was treated with DNase I (Thermo Scientific, Waltham, United States). In detail, for 1 µg of RNA, 1 µL of 10X Reaction Buffer with $MgCl_2$ and 1 µL of DNase I were added, and the total volume of 10 µL was reached with RNase free water, as reported in the RevertAid RT Kit protocol (Thermo Scientific, Waltham, United States) used. The samples were incubated for 30 min at 37 °C, and lately 1 µL of 50 mM of ethylenediaminetetraacetic acid (EDTA) was added and incubated at 65 °C for 10 min.

After removing any DNA contamination, to reverse transcribe, 1 µL of Random Hexamer primer was added to the volume of the previous reaction. Then, 4 µL of 5X Reaction Buffer, 1 µL of Ribolock RNase Inhibitor (20U/µL), 2 µL of 10 mM d NTP Mix and 1 µL of RevertAid RT enzyme (200U/µL) were added in order, reaching a total volume of 20 µL.

After a slight agitation, the sample was placed in the thermocycler (Eppendorf, Milan, Italy) (Figure 4.6) with the following reaction settings: 25 °C for 5 min, 42 °C for 60 min, 70 °C for 5 min.

The obtained cDNA was used directly for the PCR reaction, which required a total number of 35 reaction cycles. To each 0.2 mL tube were therefore added: 10 µL of 5X Wonder Taq Hot Start Reaction buffer, 0.5 µL of cDNA, 1 µL of reverse primer (20 µM), 1 µL of forward primer (20 µM), 0.5 µL of Wonder Taq Hot Start

(Euroclone®, Milan Italy), and water for molecular use until reaching a final reaction volume of 50 µL.

Each reaction cycle included the following thermal steps: 95 °C for 1 min and 15 sec (denaturation), 60 °C for 15 sec (annealing), 70 °C for 20 sec (elongation).

The gene used for its expressive stability as housekeeping was the *elongation factor 1 α* (*ef1-α*). Before proceeding, each primer pair (reverse and forward) related to the single gene to be investigated was qualitatively evaluated with an electrophoretic run.

Figure 4.6 Thermocycler (Eppendorf, Milan, Italy) (left) and electrophoresis run tray (right).

4.2.7.3 Gene expression by real-time quantitative PCR (RT-qPCR)

To quantify the expression of key genes potentially altered in mussel gonads of both sexes following exposure to Dex, it was established to evaluate the genes involved in the redox balance, such as *copper/zinc superoxide dismutase* (*Cu/Zn-SOD*) and 5-*histidylcysteine sulfoxide synthase* (*OvoA*), those involved in the anti-apoptotic process, such as *B-cell lymphoma 2* (*Bcl2*) and those belonging to detoxification mechanism, such as *Multi-Drug Resistance 1* (ABCB/P-glycoprotein-like protein, *MDR1* gene) and *Multidrug Resistance-associated Protein* (ABCC/MRP-like, *MRP* gene). A quantitative polymerase chain reaction (qPCR) was therefore performed on each sample (n = 6 female gonads and n = 6 male gonads) for each experimental condition and time-point.

The sequences of each primer pair (reverse-Rv and forward-Fw primers) were designed on known complementary DNA (cDNA) sequences of the *Mytilus galloprovincialis* species received in the NCBI database (https://www.ncbi.nlm.nih.gov/), using dedicated software such as the Primer3Plus (https://www.primer3plus.com/index.html) and Bioinformatics-The Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/), whose obtained sequences are reported in the Table 4.1.

To interpret the analytical data, the target genes (*Cu/Zn-SOD, OvoA*, *Bcl2, MDR1* and *MRP*) were normalized in respect to those of the reference gene *ef1-α* (GenBank ID: AB162021.1; Giannetto et al., 2017), whose sequences were also reported in the Table 4.1.

Figure 4.7 Rotor-Gene Q thermocycler (QIAGEN, Hilden, Germany).

For quantitative analysis, the 1X DyNAmo HS SYBR Green qPCR Kit (Thermo Scientific, Waltham, United States) was used, proceeding with the use of the reaction mixture with 1X DyNAmo HS SYBR Green, 2 µL of cDNA template (dilution 1:20) and 0.5 µM of each primer. Amplification was performed on a Rotor-Gene Q thermocycler (QIAGEN, Hilden, Germany) (Figure 4.7) using the following thermal steps: 95 °C for 15 min (hold), 35 cycles of 94 °C for 10 sec

(cycling), 60 °C for 20 sec (annealing) and a final melting curve analysis cycle (from 72 to 95 °C) to confirm the presence of a single product. Triplicate analyses were performed for all reactions and three negative controls (without cDNA) for each oligo pair were used.

Using QIAquant 96 Real-Time PCR software (QIAGEN), relative mRNA expression levels were calculated and quantified using the comparative Ct method ($2^{-\Delta\Delta Ct}$), which is based on the mean Ct of the chosen reference gene and the Ct values of each target gene. By assigning a value of "1" to the control condition, the measurement of the expression levels of the target genes was expressed as fold changes compared to the control.

Gene name	Forward (5'-3') Reverse (5'-3')	Size (bp)	GeneBank #Accession
copper/zinc superoxide dismutase (*Cu/Zn-SOD*)	5'-AGGCGCAATCCATTTGTTAC-3' 5'-CATGCCTTGTGTGAGCATCT-3'	212	JN863296.1
5-histidylcysteine sulfoxide synthase (*OvoA*)	5'-ATGTCCAGATCGCCTACG-3' 5'-CAGTGTCATCCCACGACATC-3'	169	BK012004.1
B-cell lymphoma 2 (*Bcl2*)	5'-TTGGTGGGTCTTTGTCAGTG-3' 5'-CCATTGCGCCTATTACACCT-3'	234	KC545829.1
ABCB/ P-glycoprotein-like protein (*MDR1* gene)	5'-TAGTAGTCCCAGGTCCGAGC-3' 5'- CACACACGTAGCATAGCGGA-3'	199	FM999809.2
ABCC/ MRP-like (*MRP* gene)	5'- CAGACGGGACAAACGATGGA-3' 5'- ACAAAAAGTGGAGAGGTGTCCC-3'	145	FM999810.2
elongation factor 1 alpha (*ef1-α*)	5'-CCTCCCACCATCAAGACCCA-3' 5'-GGCTGGAGCAAAGGTAACAAC-3'	145	AB162021.1

Table 4.1 Gene name, sequences primer forward and reverse, size amplificated, #accession in GeneBank software of genes: *copper/zinc superoxide dismutase* (*Cu/Zn-SOD*) and *5-histidylcysteine sulfoxide synthase* (*OvoA*), *B-cell lymphoma 2* (*Bcl2*), *ABCB/P-glycoprotein-like protein* (*MDR1*), *ABCC/MRP-like* (*MRP*) and *elongation factor 1 alpha* (*ef1-α*).

4.2.8 Statistical analysis

The data obtained from the chemical, histochemical, metabolomic and molecular analyses were processed with Excel, and expressed as a measure of the mean and standard deviation (± SD) for the chemical, histochemical and metabolomic data, and with the $2^{-\Delta\Delta Ct}$ method (fold change method) for the molecular data.

With the GraphPad software (Prism 10.0, San Diego, CA, USA) the percentage difference in data between the two sexes was measured to verify its significance, considering a p value <0.05.

For the data obtained from the histochemical analysis focused on the measurement of glycogen reserves, from metabolomics focused on the quantification of polar metabolites involved in a variety of cell pathways, and from the molecular analyses used to evaluate the relative gene expression, the two-way ANOVA was applied to establish the variance among the different tested concentrations of Dex and the various fixed time-points, and for the same concentration of Dex at different time-points and for the same concentration of Dex between the two sexes (male and female mussels).

4.3 Results

4.3.1 Chemical data

The chemical analysis performed on a pool of gonadal tissues for each experimental condition and time-point was conducted at the Department of Medical, Surgical and Advanced Technologies "G.F. Ingrassia" of the University of Catania (Sicily, Italy) by using HPLC, and confirmed as expected the absence of the glucocorticoid drug dexamethasone in the specimens not subjected to treatment (C0) and sampled at the beginning of the experiment (T0), while significant quantities of Dex were found in all the exposed organisms (Figure 4.8).

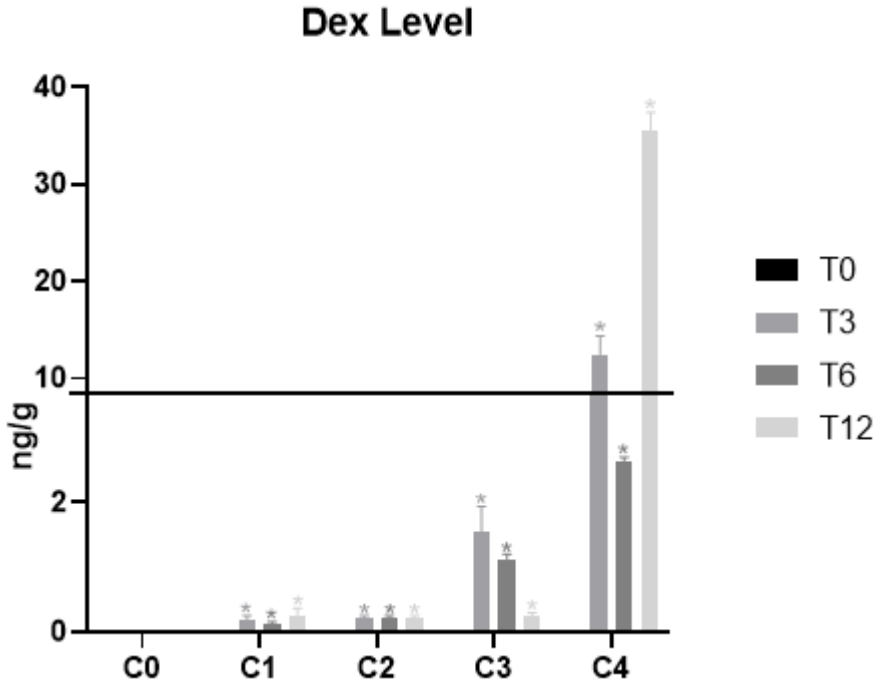

Figure 4.8 Dexamethasone concentration (ng/g) at level of the reproductive tissue of mussel *M. galloprovincialis* from control group (C0) and exposed to different concentration of C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Results are shown as means ± SD. Asterisks (*) indicate significant differences between treated group and control group subjects ($p < 0.05$).

For all the treatment conditions and for each time-point, except for T0, a statistical significance was revealed with a p <0.05. In summary, at the lowest concentrations, such as C1 (4 ng/L) and C2 (40 ng/L), the bioaccumulation of the drug was more moderate than at the two higher doses C3 (0.4 µg/L) and C4 (2 µg/L) at all the fixed sampling times (T3, T6, T12). It was evident that as the dose of Dex increased, there was a general accumulation of the drug at the level of the reproductive tissue. Specifically, for the organisms treated with the two lowest concentrations (C1 and C2), the data showed a slight bioaccumulation of the drug, while for the concentration C3 a marked accumulation occurred at times T3 and T6. However, it was less intense than the level of Dex found at C4, at which the highest bioaccumulation of Dex was detected, with the maximum dose measured at the initial time T3 and at the final time T12.

4.3.2 Histological observation

The histomorphological investigation performed on thin sections of gonads highlighted, through the affinity of basic (hematoxylin) and acid (eosin) dyes, all the cell types constituting the male and female mussel gonadal tissue.

From these histological observations, it was evident that in both sexes the organization of the gonadal tissue did not undergo appreciable perturbations in the samples challenged with Dex compared to the control during the entire exposure. In fact, an intact and well-compacted connective tissue was evident in gonads of both sexes, consisting of a few ADG cells dispersed in abundant VCT cells (Figure 4.9 and Figure 4.10).

In males the follicles had well-defined walls to which were juxtaposed, stained more markedly in blue-violet, rounded and larger germ cells corresponding to spermatogonia. Further inside, spermatocytes and spermatids had occupied a large part of the periphery, while in the lumen of the follicle spermatozoa were abundantly present whose tails stood out in pink, and this was in line with the maturation period between stage II and IIIA (Prisco et al., 2017), corresponding to the autumn season (month of November) during which the experiment took place (Figure 4.9).

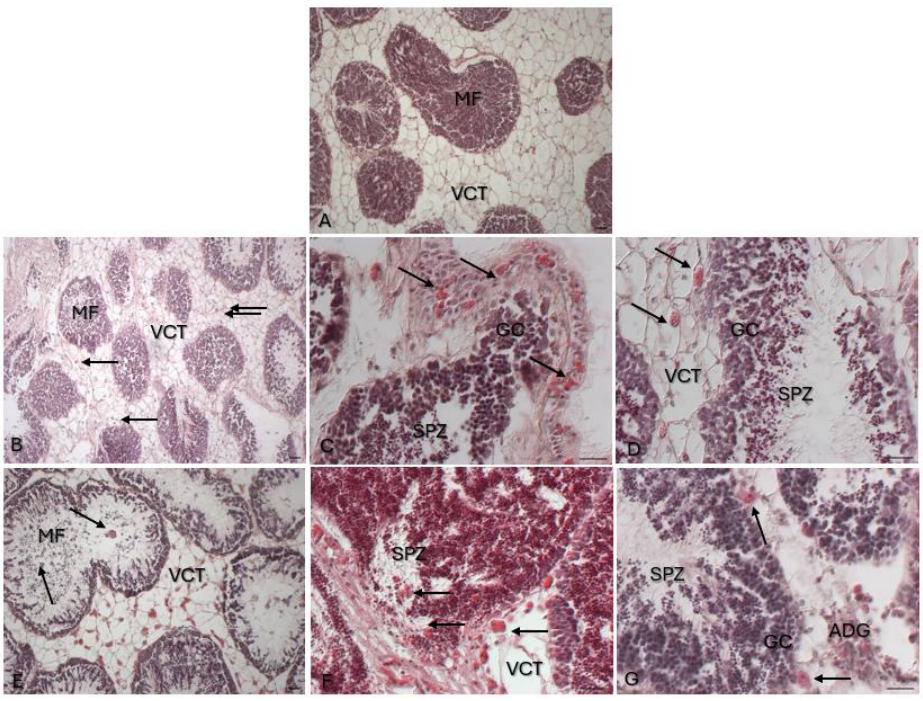

Figure 4.9 Representative histological sections of male gonads of *M. galloprovincialis* from the control group (A) and the exposed group (B) C2 at T3 hemocytes (arrows) covering the entire connective tissue, (C) C3 at T3 hemocytes moving in clusters from the periphery of the tissue towards the follicles (arrows), (D) C4 to T3 hemocytes spreading singly along the connective tissue around the male follicles, (E) C1 at T6 hemocytes (arrows) spreading throughout the connective tissue and within the tissue. F) C3 at T6 with clusters of hemocytes infiltrating (arrows) within the follicle, (G) C3 at T12 hemocytes present only at the level of the connective tissue infiltration (arrows). Male follicle (MF), vesicular connective tissue cells (VCT), adipogranular cells (ADG), spermatozoa (SPZ), germ cells (GC). Scale bars 20 μm.

Overall, it was possible to highlight that both in males (Figure 4.9: B, C, D) and females (Figure 4.10: B and C) occurred an early response to the treatment with dexamethasone since a strong presence of hemocytes was detected in both gonads starting from the T3 sampling time. Two classes of hemocytes were distinguished: hyalinocytes (hemocytes with a smooth shape) and granulocytes (hemocytes with a more granular appearance).

In the male gonad, starting from the concentration C2 at T3 (Figure 4.9: B), groups of hemocytes settle from the periphery and then moved and were found along the entire connective tissue in a dose-dependent manner (Figure 4.9: B, C, D, E and F). This remains constant at all sampling times, to undergo a slight attenuation at T12 (Figure 4.9: G). Respectively, at C3 and C4 for T3 (Figure 4.9: C and D) and at C3 for T6 (Figure 4.9: F), small groups of hemocytes tended to penetrate the follicular wall and settle in the lumen of the follicle causing a disorganization of the germ cells present.

The female counterpart showed an organization typical of the maturation period corresponding to stage II and III (Rosati et al., 2019), for which it was easy to find germinal cells in the maturation phase (such as previtellogenic, medium and late oocytes) and mature oocytes (Figure 4.10). Furthermore, the presence of pear-shaped oocytes stood out in the female gonadal tissue (Figure 4.10: B and C). As in the male gonad at T12 a more moderate distribution of hemocytes was detected (Figure 4.10: E).

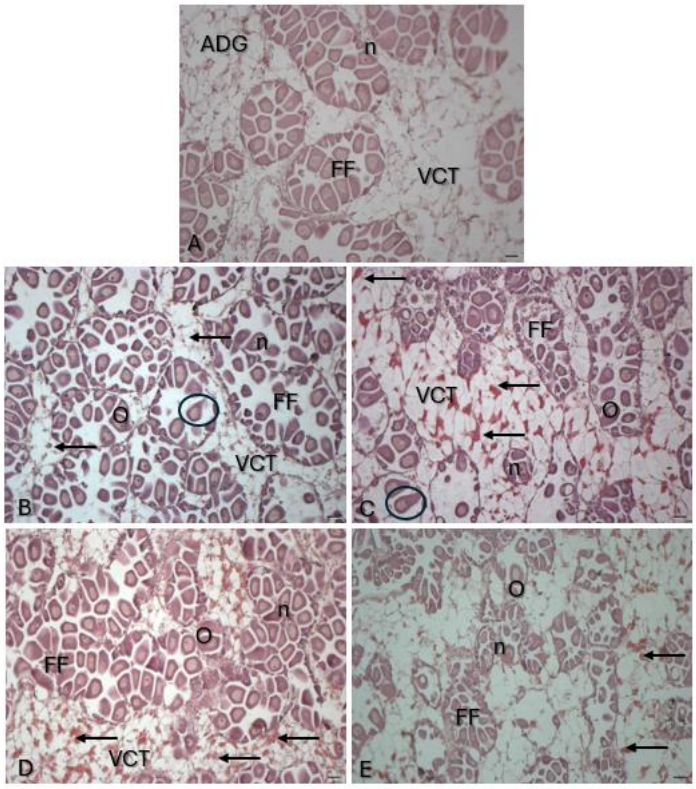

Figure 4.10 Representative histological sections of female gonads of *M. galloprovincialis* from the control group (A) and the exposed group (B) C2 at T3 where the follicles are sparsely filled with oocytes and a moderate presence of hemocytes is found on the connective tissue (arrows), (C) C4 at T3 the connective tissue is covered by abundant solitary and grouped hemocytes (arrows), (D) C3 at T6 the hemocytic infiltration (arrows) at the connective tissue level increases between the follicles filled with oocytes, (E) C4 at T12 a more moderate distribution of hemocytes is evident with disorganization of the connective tissue and follicles sparsely filled with oocytes. Female follicle (FF), vesicular cells of the connective tissue (VCT), adipogranular cells (ADG), germ cells (GC), oocytes (O), pear-shaped oocytes (circle) Scale bars 20 µm.

4.3.3 Histochemical results

Considering that dexamethasone is a glucocorticoid that influences various metabolic processes such as the glucose metabolism (Zhang et al., 2020), and taking into account that glucose and its reserves (glycogen) are of fundamental importance during gametogenesis for the maturation of germ cells (Duinker et al., 2008; Zandee et al., 1980; De Zwaan & Zandee, 1972), through a histochemical analysis by PAS reaction combined with a d-PAS the presence of glycogen in the gonads of both sexes was herein investigated (Meyerholz et al., 2018).

Glycogen, being a neutral polysaccharide, when present in gonadal tissue was highlighted in red-magenta since the periodic acid selectively oxidizes glycosidic bonds making available closely contiguous aldehyde groups that react with the Shiff reagent determining the detection of the homopolymer (Alonso et al., 2019). Also, the sections treated only with the periodic acid of Shiff (PAS) presented a more intense red-magenta colour than those previously subjected to diastase (α-amylase), and this confirmed the applicability of the method to discriminate glycogen from other polysaccharides present in the gonadal tissue (Meyerholz et al., 2018).

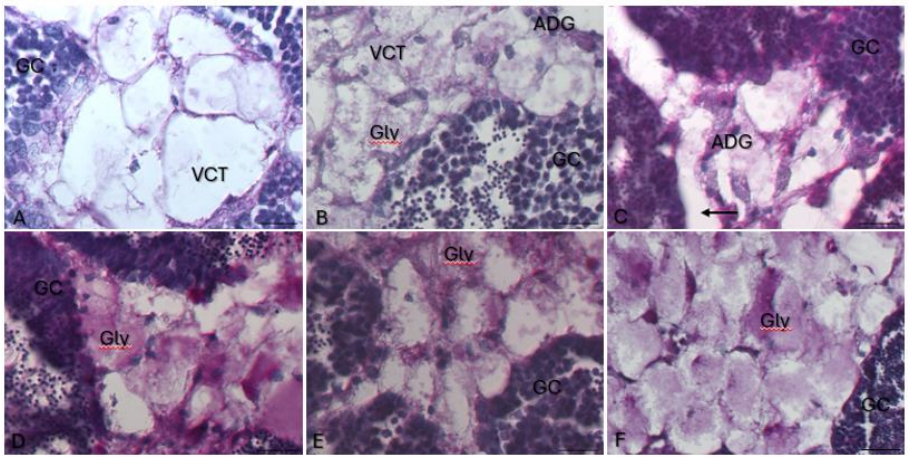

Figure 4.11 PAS reaction on male gonads of *M. galloprovincialis* of (A) the control negative d-PAS (B) C3 at T3 low PAS positivity in VCT, (C) C1 at T6 VCT almost completely translucent, with evident white spaces (arrows), (D) C4 at T6 VCT rich in glycogen, (E) C1 at T12 VCT poorly PAS positive, (F) C3 at T12 VCT with increased PAS positivity. Glycogen (Gly), vesicular connective tissue cells (VCT), adipogranular cells (ADG), germ cells (GC). Scale bars 20 μm.

As it can be seen from Figure 4.11, in male gonads the histochemical analysis showed at time T3 only at the level of VCT cells, after a decrease (not shown in Figure 4.11) at C1 (Figure 4.12), an increase in the presence of glycogen at C2 (not shown in Figure 4.11, but present in Figure 4.12) and C3 (Figure 4.11: B) which, however, is not maintained at C4 (Figure 4.12). At T6 (Figure 4.11: C) a decrease in the presence of the polysaccharide is recorded for C1 (4 ng/L) so much so that the image appears similar to the negative control (d-PAS) (Figure 4.11: A), while for higher concentrations an increase in glycogen is observed, albeit variable (Figure 4.11: D). At T12 for almost all the treatment conditions an increase in glycogen is observed, more evident at C3 (Figure 4.11: F), which undergoes a slight

decrease (not shown in Figure 4.11) at C4 (Figure 4.12), in which the presence of glycogen is like that of the control.

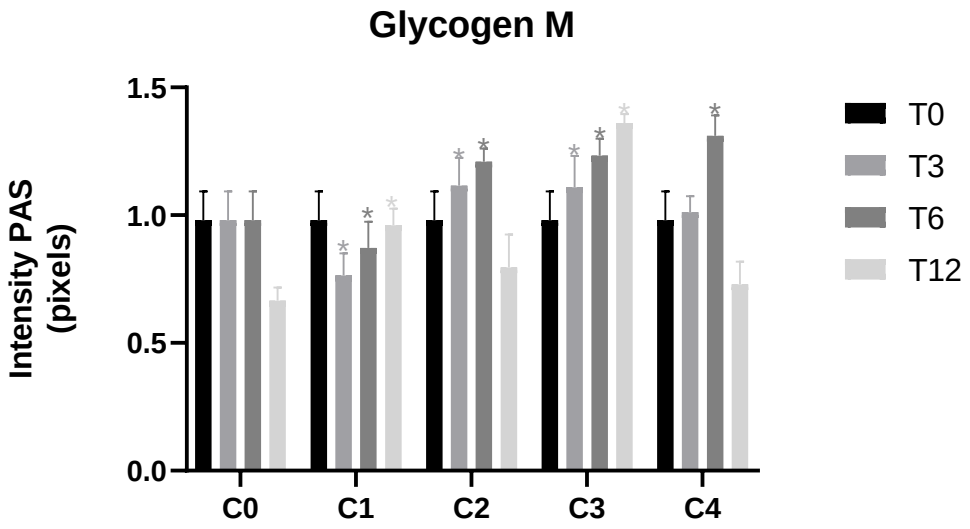

Figure 4.12 Representative histogram of the mean concentration (pixels) of glycogen concentration in the male gonads of *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Instead, at the level of female gonads, PAS positivity was found both inside the VCT cells and in the ADG cells which, as it can be seen from the image Figure 4.13: C, are grouped and marked in red-magenta. At T3, as it can be noted from the empty spaces inside the VCT cells, there is a significant loss (p <0.05) of glycogen compared to the control for all the concentrations tested (Figure 4.13: B and Figure 4.14), while at T6 a significant increase occurred, almost for all the conditions so much so that positivity was also reported at the level of the ADG cells (Figure 4.13: C). At T12, at C1 a slight decrease in glycogen was instead highlighted (Figure 4.13: D), which has been maintained at low levels until the highest concentrations where a slight increase is observed (Figure 4.14).

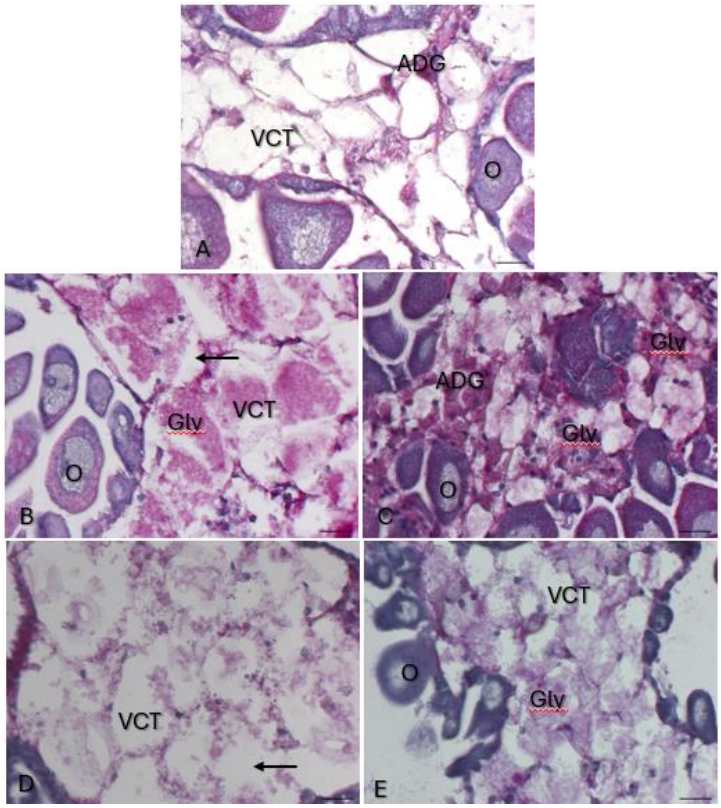

Figure 4.13 PAS reaction on female gonads of *M. galloprovincialis* of (A) the control negative d-PAS, (B) C4 at T3 PAS positive VCT, but with evident white empty spaces (arrows), (C) C3 at T6 presence also of ADG PAS positive, (D) C1 at T12 VCT semi-empty of glycogen with evident white spaces (arrows), (E) C4 at T12 with increase of the number of PAS positive VCT. vesicular connective tissue cells (VCT), adipogranular cells (ADG), germ cells (GC). Scale bars 20 µm.

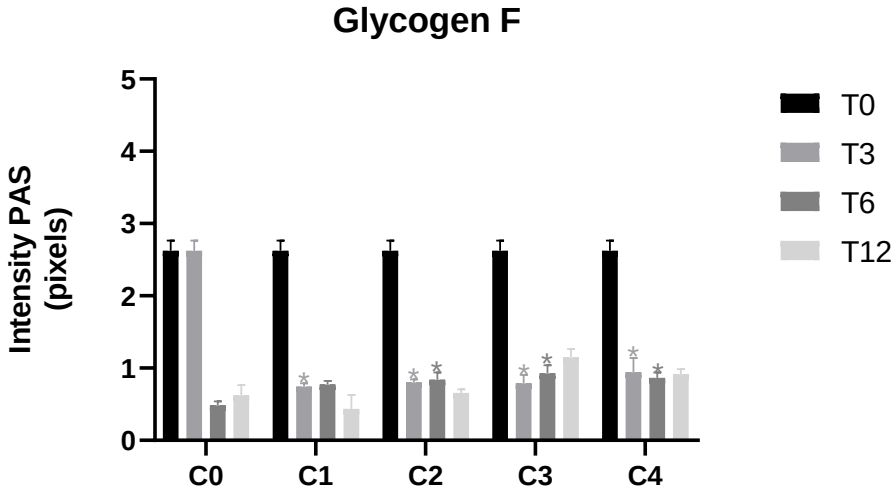

Figure 4.14 Representative histogram of the mean concentration (pixels) of glycogen concentration in the male gonads of *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex.

Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

4.3.4 Immunofluorescence data

Considering the results obtained from the morphological analysis of mussel gonads that revealed an intense immune response mainly in males induced by Dex, an immunofluorescence analysis was performed on samples that had demonstrated hemocytes infiltration within the tissue.

It is known that in mussel *Mytilus trossulus* the presence of melanized hemocytes within the male follicle is a sign of atresia induced by apoptosis. Smolarz et al. (2017) stated that this phenomenon can be considered as a biomarker of endocrine interference, and for this reason on tissue sections cut in series, a co-localization technique was applied using the anti-cas3 antibody, labeled in green with FITC fluorophore, in combination with the anti-FasL antibody, labeled in red with TRICT (Figure 4.15: C, D and E) as well as independently on other 4 μm sections the anti-PCNA antibody, labeled in red with TRICT (Figure 4.15: F, G and H) was also investigated to verify the progress of cell turnover.

Surprisingly, for all the experimental conditions no immunopositivity of the anti-cas3 antibody was detected (Figure 4.15: C, D and E), while it was evident that at T3 there was a greater immunointensity of the anti-FasL antibody (Figure 4.15: D), a situation that was attenuated for the samples collected at time T6 (Figure 4.15: E).

For the anti-PCNA antibody able to detect the agent responsible for promoting DNA synthesis and cell proliferation, there was a high immunopositivity on the spermatogonia located on the walls of the follicles at T0 (Figure 4.15: F) that tended to decrease in the treated samples at T3 (Figure 4.15: G), but that intensified further at T6 (Figure 4.15: H).

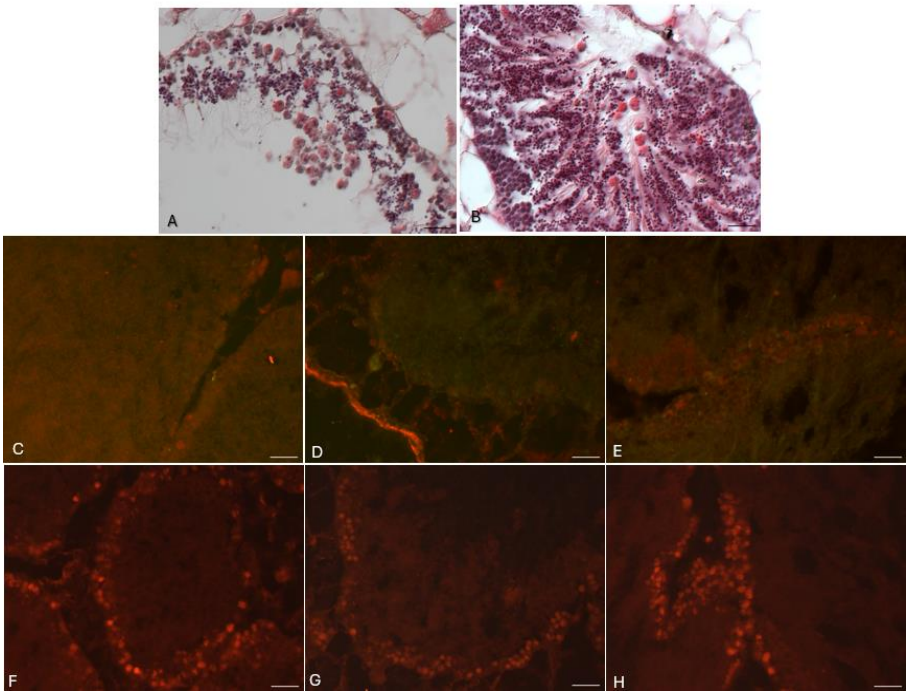

Figure 4.15. Sections of male gonads of *M. galloprovincialis* PAS histochemical reaction. (A) C4 at T3 H/E stained section, (B) C3 T6: H/E stained section, (C) T0 with colocalization of anti-cas3, anti-FasL antibodies, evident negativity to anti-cas3 and anti-FasL, (D) C4 at T3 with colocalization of anti-cas3, anti-FasL antibodies, positivity to anti-FasL antibody on germ cells, no responsiveness to anti-cas3 antibody, (E) C3 at T6 with colocalization of anti-cas3, anti-Fas L antibodies, no responsiveness to anti-cas3 antibody, poor positivity to anti-FasL antibody, (F) T0: positivity to anti-PCNA antibody on germ cells, (G) C4 at T3with evident reduction of anti-PCNA antibody positivity, (H) C3 at T6 with increase of anti-PCNA antibody positive germ cells. Green: conjugated-FITC. Red: conjugated-TRICT. Scale bars 20 μm.

4.3.5 Metabolomic data

The ^{1}H NMR-1D spectra obtained from the metabolomic analysis of gonad tissues from both sexes and subjected to the treatment conditions previously reported, have highlighted how the glucocorticoid drug dexamethasone can induce changes in male and female specimens of *Mytilus galloprovincialis* at metabolite level concerning, above all, the energy pathway (with changes in glucose, glycogen, lactate, acetoacetate), the osmotic pathway (with changes in betaine, taurine) and protein turnover (with changes in leucine, isoleucine, valine).

Specifically, at the level of male gonadal tissue, glucose showed an inverted bell-shaped temporal trend, highlighting a significant increase at T3 for C1, C2 and C3, with a decrease at C4 that was not significant (p <0.05). Time T6 described the bell bowl point, for which a general decrease occurred, while at T12 there was an overall increase in the metabolite, with a maximum peak for the highest Dex concentration (C4) (Figure 4.16: M).

The behaviour of female organisms appeared to be different from the male counterpart. In fact, at T3 a significant decrease in glucose was recorded for all the tested Dex doses compared to the control. At T6 an increase occurred at all concentrations tested with a slight depletion, significant compared to the control, at C4. At T12 a trend similar to that found at T3 was observed, which confirmed a significant sugar depletion at C1 with a further increase at C4 (Figure 4.16: F).

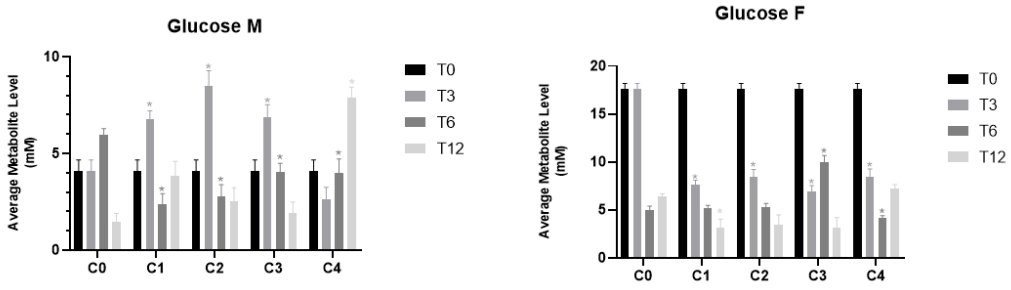

Figure 4.16 Graphs of concentrations of glucose (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; p < 0.05) are indicated with asterisks (*).

Glycogen in male organisms, after a significant decrease at the lowest concentration (C1) at T3 and T6, fitting with the data obtained from histochemical analysis, followed a general temporal increase that was time-dependent for C3 and that reached the maximum value for C4 at T6 (Figure 4.17: M).

Instead, in the female gonad it was evident how glycogen underwent an early and significant (p <0.05) depletion due to the treatment undergone at T3. At T6, compared to the control, a general increase in the metabolite was noted, significant at C2 and C4. Conversely at T12, there was a significant decrease for the lower

concentrations of Dex that reversed into an increase at the higher ones (Figure 4.17: F).

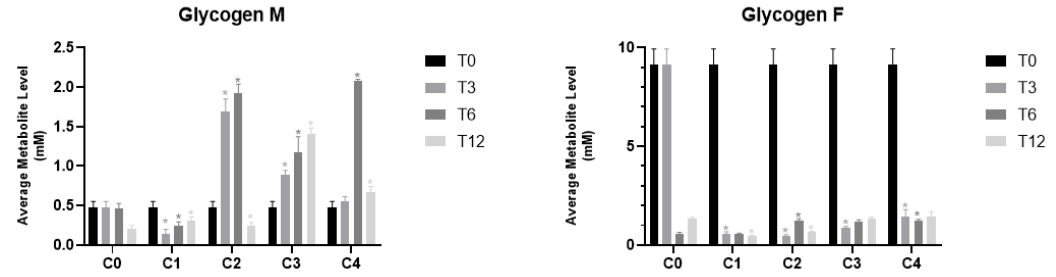

Figure 4.17 Graphs of concentrations of glycogen (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

In regard to lactate, involved in the energy production through anaerobic pathways, a convergent trend was found for both sexes at time T3 and T12. In fact, at the gonad level in both males and females at T3 there was a decrease in lactate for C1 and C2, and an increase for the higher doses (C3 and C4), while at T12 a general increase was detected compared to the control. Nonetheless, at T6 the two sexes demonstrated a divergent response with in males a general increase in lactate significant for almost all the treatment conditions, while in females the opposite trend was recorded with a significant decrease in lactate compared to the control. In the male gonad lactate tends to undergo a significant decrease at lower doses at T3 and then increase at the higher ones, remaining high at T6 and T12 (Figure 4.18).

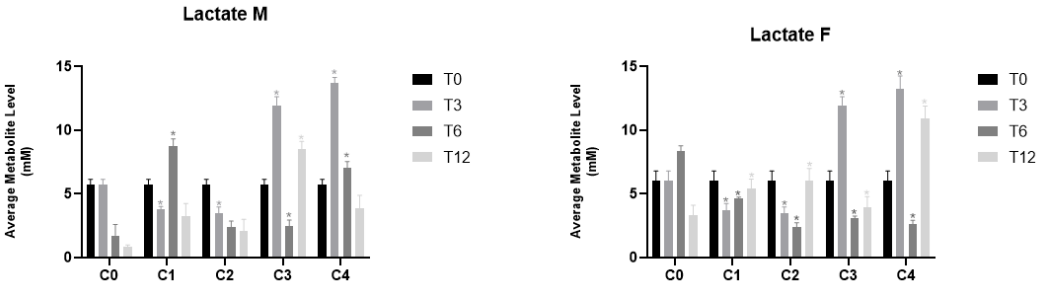

Figure 4.18 Graphs of concentrations of lactate (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

To support the fact that the anaerobic energy pathway was also altered following exposure to the synthetic glucocorticoid, in male gonads acetoacetate, the final product of lipid catabolism, underwent an early and significant increase for all the experimental conditions at T3, which was maintained and confirmed also for the subsequent sampling times (T6 and T12). In detail, at T3 there was a significant (p <0.05) increase starting from C2 and at T6 it remained constant as well as at T12, apart from C2 where a slight but not significant decrease occurred in acetoacetate level (Figure 4.19: M).

Female organisms also reacted in a similar way, but not as early as males since at the lower Dex doses (C1 and C2) of T3 a decrease in acetoacetate level is observed, which increased only at C3 and C4. This behaviour was also maintained at T6, except for C4 where a significant decrease was recorded. At T12 the female situation tended to coincide with that of males since an increase was observed that appeared significant (p <0.05) at all the concentrations tested, except for C2 where a decrease was recorded (Figure 4.19: F).

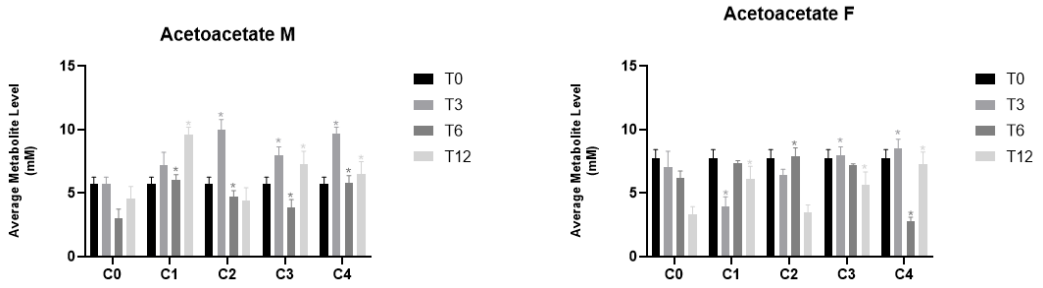

Figure 4.19 Graphs of concentrations of acetoacetate (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

As previously mentioned, alterations attributable to protein turnover were also found in mussel gonads challenged with Dex. Branched-chain amino acids (BCAA), namely leucine, isoleucine and valine, showed a similar trend in both sexes.

In detail, at T3 an increase in leucine availability was detected in both sexes, with an earlier increase in males compared to females who showed a significant increase limited, however, to the higher concentrations (C3 and C4) (Figure 4.20: F), while males responded earlier already to C1 and C2 (Figure 4.20: M). At T6, however, the behaviour of the essential amino acid seemed the same in the two sexes since a negative variation was recorded at C1, C2 and C3, while at C4 an increase occurred, significant only in males. At T12 in a variable way, but both for males and females, a significant increase in leucine was highlighted at C1 and C4, with a slight decrease at C3 in the female gonads (Figure 4.20).

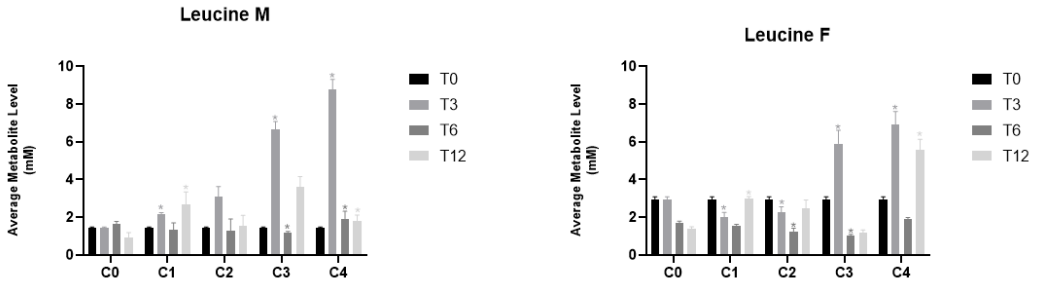

Figure 4.20 Graphs of concentrations of leucine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Also, the other BCAAs, namely isoleucine and valine, followed the same trend during the exposure to Dex in both sexes as described for leucine. In fact, at T3 in males, isoleucine showed a dose-dependent increase which, however, was significant only at the highest concentration (C4) (Figure 4.21: M), while in females it underwent a significant decrease at the lower concentrations (C1 and C2), to then show a significant increase at C3 and C4 (Figure 4.21: F). At T6, as for leucine, the concentration of the gluconeogenic amino acid in both sexes underwent a decrease at C1, C2 and C3, significant only for the female gonads at C1 and C2, which increased at C4. At T12 for both males and females, isoleucine underwent a general increase that only in females for C3 resulted into a significant decrease (Figure 4.21).

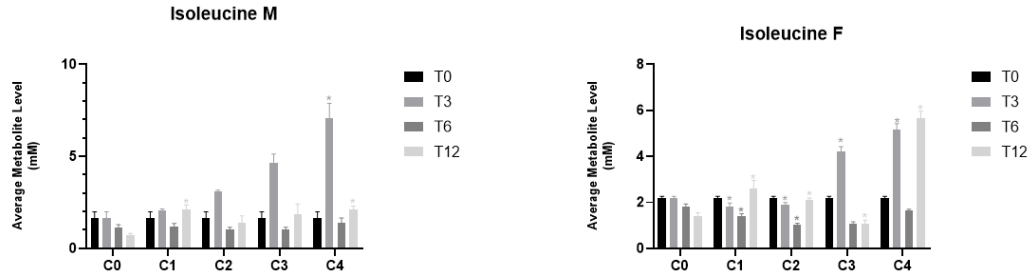

Figure 4.21 Graphs of concentrations of isoleucine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Valine, another essential amino acid, which has a carbon chain that constitutes the supporting structure, behaves like the previous BCAAs, having demonstrated at T3 in males a significant dose-dependent increase (p <0.05) for all the doses tested (Figure 4.22: M), a behaviour that in females was manifested significantly, as for the other BCAAs, at C3 and C4 but that showed a slight decrease at the lowest Dex doses (C1 and C2) (Figure 4.22: F). At T6 there was a general decrease in both sexes that in females remains constant at all the Dex doses tested and significant (p <0.05) at C2 and C3 (Figure 4.22: F), while in males a slight increase was recorded at C1 and C4. At T12, males showed a general increase in valine availability, significant in all the tested conditions (Figure 4.22: M), a situation that was also significantly manifested by the female gonad for C2 and C4, but which at C3 underwent a significant (p <0.05) decrease (Figure 4.22: F).

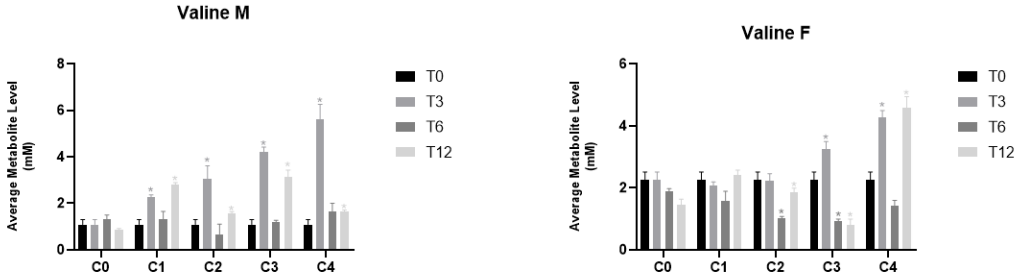

Figure 4.22 Graphs of concentrations of valine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

In regard to osmolytes, betaine in males at T3 underwent a significant decrease at the lower concentrations (C1 and C2), except for C1, while it assumed an increasing trend at the higher Dex doses (C3 and C4). At T6, except for the increase recorded at the lower and higher doses, the trend of the metabolite was depleted, a behaviour that occurred only at C2 of T12, while the response to the remaining treatments was of increase compared to the control (Figure 4.23: M). In the female gonad, betaine levels at T3 tended to significantly decrease compared to the control, while at T6 an increase was observed, except for a significant decrease at the highest dose (C4). At T12 a variable but overall increasing trend occurred in respect to control (Figure 4.23: F).

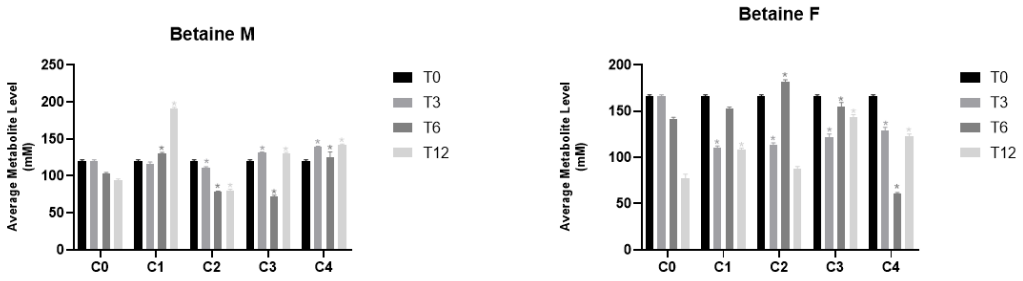

Figure 4.23 Graphs of concentrations of betaine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 μg/L, C1: 0.004 μg/L Dex, C2: 0.04 μg/L Dex, C3: 0.4 μg/L Dex and C4: 2 μg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Another metabolite that plays an osmoregulation role is taurine, which in males at T3 underwent a significant increase as well as at T6, but only for the concentrations C1 and C4, while at C2 and C3 the values reported a decrease inside the gonad. At T12, except for a depletion at C2, the trend of the metabolite was of increase with the maximum level recorded at C1 (Figure 4.24: M). In female organisms at T3 a significant decrease in taurine was observed for all the treatments, a trend that was reversed at T6 for the doses C1, C2 and C3, but which reappeared at C4. At T12 an overall increase in taurine was recorded, except for the depletion found at C2 (Figure 4.24: F).

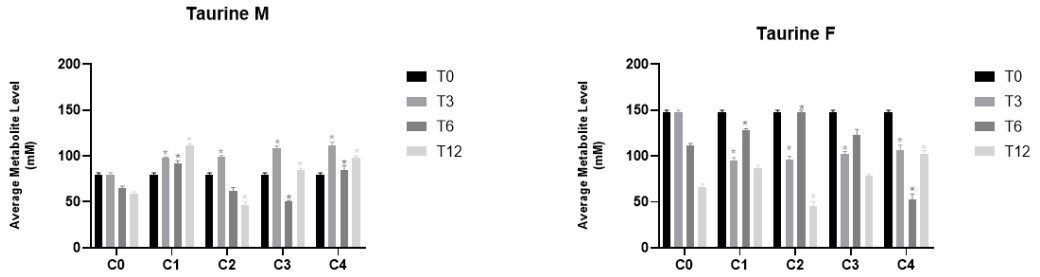

Figure 4.24 Graphs of concentrations of taurine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Glycine, an amino acid involved in different metabolic pathways such as detoxification and osmoregulation, showed a significant decrease in male gonads at T3, particularly marked at the two highest concentrations (C3 and C4). A picture that was maintained at T6, but that was reversed at T12 as demonstrated by a significant increase at all the doses used (Figure 4.25: M). Females showed an opposite response, reporting a general increase in the metabolite at T3, more evident at C1 and C2. Instead at T6, with the exception of C3, a significant depletion was recorded. At T12, as occurred at the initial sampling time, the gonadal concentration of the metabolite tended to a new increase, more evident at the two highest concentrations (Figure 4.25: F).

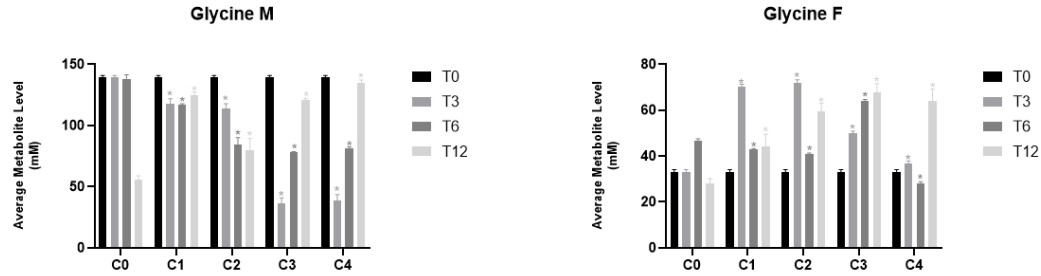

Figure 4.25 Graphs of concentrations of glycine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* at different temporal endpoint (T0, T3, T6, T12) and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

METABOLITES INVOLVED TO	CHEMICAL SHIFT AND PEAK SHAPE (ppm)	CHANGE IN %																					
		C1						C2						C3						C4			
AMINO ACID METABOLISM		T3		T6		T12		T3		T6		T12		T3		T6		T12		T3		T6		T12	
Glycine	3.6 (s)	↓14	↑3613	↓17	↓8	↑114	↑55	↓17	↑117	↓39	↓99	↑4	↑117	↓72	↑51	↓44	↑37	↑108	↑146	↓70	↑11	↓42	↓40	↑133	↑130
Isoleucine	3.7 (d), 1.0 (d), 0.9 (t)	↑28	↓137	↑5	↓22	↑215	↑73	↑89	↓14	↓12	↓99	↑86	↑46	↑187	↑92	↓9	↓44	↑179	↓29	↑336	↑134	↓22	↓10	↑205	↑281
Leucine	3.7 (m), 1.7 (m), 1.0 (t)	↑52	↓191	↓18	↓6	↑209	↑115	↑117	↓23	↓35	↓99	↑76	↑61	↑365	↑101	↓29	↓39	↑295	↓20	↑512	↑136	↑25	↑12	↑108	↑320
Valine	3.6 (d), 1.0 (d)	↑110	↓117	↑13	↓16	↑222	↑67	↑182	↑1	↓48	↓99	↑83	↑30	↑286	↑45	↓10	↓52	↑255	↓45	↑418	↑89	↑26	↓25	↑88	↑210
ENERGY METABOLISM																									
Acetoacetate	3.4 (s), 2.3 (s)	↑25	↓477	↑103	↑19	↑117	↑52	↑74	↓17	↑58	↓99	↓3	↓21	↑38	↑3	↑29	↑16	↑61	↑41	↑69	↑11	↑94	↓55	↑43	↑80
Glycogen	5.4 (s), 3.8 (m), 3.6 (m), 3.4 (m)	↓78	↓1277	↓47	↓104	↓62	↓36	↑199	↓93	↓31	↓97	↑21	↓77	↑89	↓93	↑156	↑339	↑603	↑19	↓40	↓88	↑309	↑558	↑193	↑54
Glucose	5.2 (d), 3.8 (m), 3.7 (m), 3.5 (m)	↑65	↓1103	↓60	↑5	↑127	↓50	↑142	↓52	↓51	↓99	↑34	↓46	↑68	↓61	↓32	↑100	↑19	↓50	↓36	↓52	↓33	↓16	↑335	↑8
Lactate	4.1 (q), 1.3 (d)	↓34	↓333	↑300	↓45	↑275	↑58	↓39	↓42	↑10	↓100	↑141	↑70	↑109	↑9	↑12	↓63	↑924	↑13	↑140	↑120	↑221	↓68	↑352	↑210
OSMO-REGULATION																									
Betaine	3.9 (s), 3.3 (s)	↓8	↓5619	↑26	↑7	↑102	↑36	↑1	↓31	↓24	↓99	↓15	↑7	↑9	↓26	↓29	↑7	↑38	↑82	↑15	↓21	↑2	↓57	↑50	↑56
Taurine	3.4 (t)	↑21	↓5006	↑38	↑13	↑87	↑26	↑22	↓33	↑1	↓99	↓19	↓29	↑33	↓29	↓23	↑6	↑40	↑14	↑37	↓25	↑24	↓50	↑65	↑48

Table 4.2 Summary table of the percentage changes (%) in response at C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex at different temporal endpoint (T0, T3, T6, T12) of the concentrations of the most significant metabolites in male (blue) and female (pink) gonads of *M. galloprovincialis* after a 48-h exposure, relative to the sex control. (Two ONOVA test, $p < 0.05$) s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet.

4.3.6 Molecular investigation

Given the presence of a marked long-lasting hemocyte recall mainly affecting the connective tissue in both the male and female gonads, suggesting a clear sign of a pro-inflammatory state, the gene expression of the *Cu/Zn-SOD* enzyme was investigated at the end of the exposure period (T12). The data relating to male gonad revealed, except for a down regulation at C1, a general increase significant at the other concentrations with $p < 0.05$, demonstrating the influence of Dex (Figure 4.26). The same attitude was observed in the female counterpart with a significant up-regulation at all doses tested ($p < 0.05$), except for C2 (Figure 4.27).

Confirmation of a perturbation of the redox balance within the reproductive tissue of both sexes was given by the increase significant in the transcriptions of the *OvoA* gene for females (Figure 4.27) and males, except for C4 (Figure 4.26).

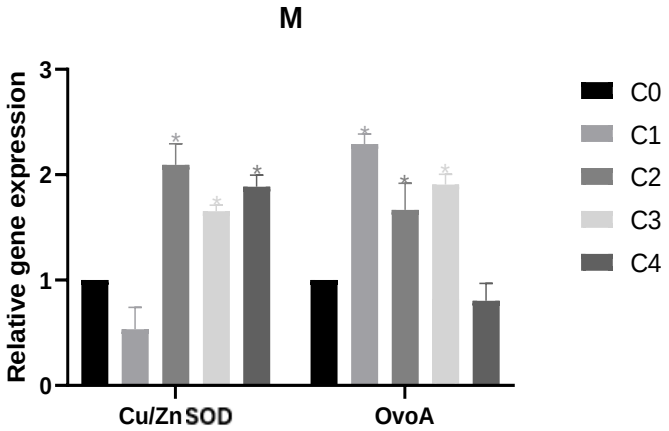

Figure 4.26 Gene expression analysis of *copper/zinc superoxide dismutase* (*Cu/Zn-SOD*) and *5-histidylcysteine sulfoxide synthase* (*OvoA*) of male gonads of the mussel *M. galloprovincialis* at T12 and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

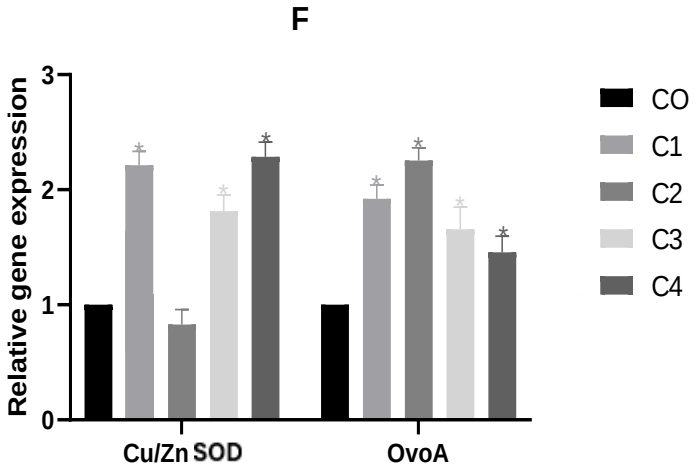

Figure 4.27 Gene expression analysis of *copper/zinc superoxide dismutase* (*Cu/Zn-SOD*) and *5-histidylcysteine sulfoxide synthase* (*OvoA*) of female gonads of the mussel *M. galloprovincialis* at T12 and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

The possibility that the proinflammatory state was accompanied by apoptotic processes was also considered. Therefore, the expression of the *Bcl2* gene was evaluated and both sexes detected increases in gene expression with values with $p < 0.05$ at almost all concentrations in males and for females, except for C2 (Figure 4.28).

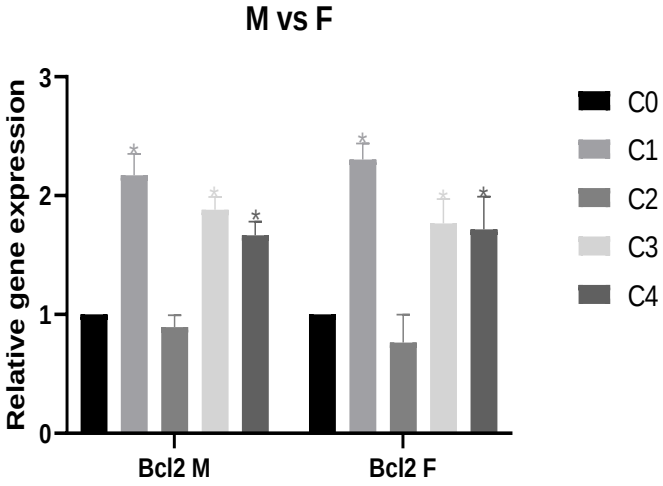

Figure 4.28 Gene expression analysis of *B-cell lymphoma 2* (*Bcl2*) of male and female gonads of the mussel *M. galloprovincialis* at T12 and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex,

C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Further analysis was conducted, investigating the molecules involved in the detoxification mechanism, such as *MDR1* and *MRP*. In the males, for both genes a significant dose-dependent increase was highlighted for *MDR1* transcripts and for *MRP*, except in this case for C1 (Figure 4.29). Instead, in the female counterpart the trend was reversed, demonstrating a general and significant down-regulation for *MDR1* and *MRP* for the doses tested (Figure 4.30).

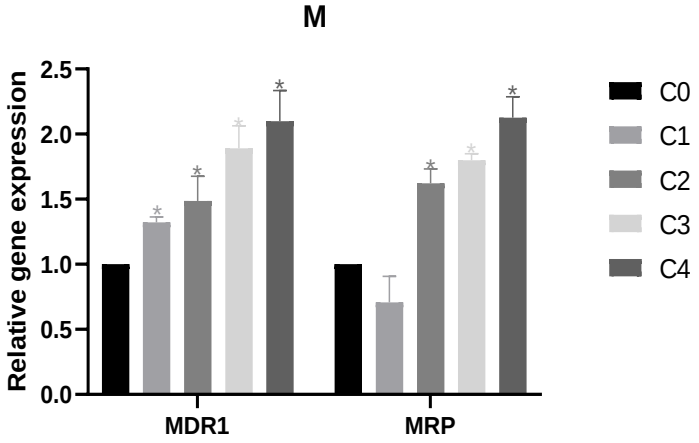

Figure 4.29 Gene expression analysis of *ABCB/P-glycoprotein-like protein* (*MDR1*), *ABCC/MRP-like* (*MRP*) of male gonads of the mussel *M. galloprovincialis* at T12 and at C0: 0 µg/L, C1: 0.004 µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

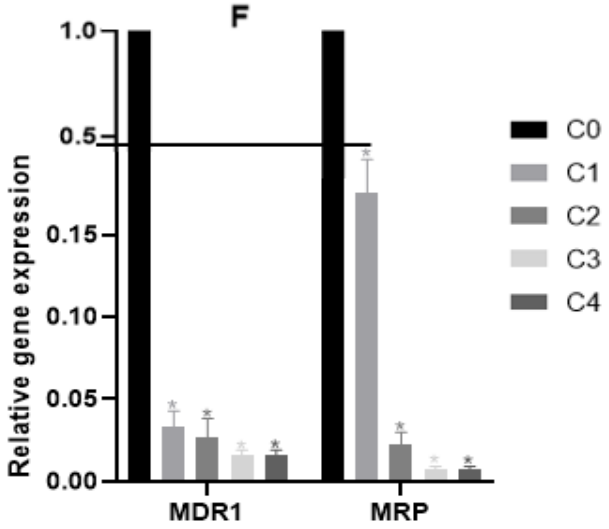

Figure 4.30 Gene expression analysis of *ABCB/P-glycoprotein-like protein* (*MDR1*), *ABCC/MRP-like* (*MRP*) of female gonads of the mussel *M. galloprovincialis* at T12 and at C0: 0 µg/L, C1: 0.004

µg/L Dex, C2: 0.04 µg/L Dex, C3: 0.4 µg/L Dex and C4: 2 µg/L Dex. Significant differences compared to the control (Two ANOVA test; $p < 0.05$) are indicated with asterisks (*).

4.4 Discussions

Due to the ever-increasing anthropogenic pressure exerted on the environment, the state of many ecosystems, mainly marine ones, is constantly put at risk. Those that are characteristics of primary importance such as biodiversity, vitality and productivity are currently difficult to safeguard also due to inappropriate waste disposal practices and the non-optimal capacity of WWTPs to retain and/or inactivate all types of pollutants present in urban, sanitary, industrial and agricultural wastewater (Köck-Schulmeyer et al., 2021; Rout et al., 2021).

This determines the greater exposure to certain substances that are capable, due to their intrinsic characteristics, of persisting in the aqueous matrix and can therefore interact more frequently with the resident biota.

With the advent of the SARS CoV-2 pandemic, this condition has worsened, so much so that many PhACs, such as dexamethasone (Dex), used for the treatment of acute inflammatory states have been widely found in the environment, showing their potential bioaccumulation at the level of various biological tissues of non-target aquatic species (Desgens-Martin & Keller, 2021; Ismail et al., 2021).

Among these, Dex, widely used in acute respiratory distress syndrome (ARDS), has raised particular concern as it is a synthetic glucocorticoid capable of interfering at multiple levels with the endocrine system. Indeed, it has a chemistry like that of cortisol, a lipophilic nature that allows it to bypass the plasma membrane, having hydrophobic physico-chemical properties and the capacity to bind to glucocorticoid receptors (GR) (Noureddine et al., 2021; Yao et al., 2020).

Therefore, the aim of this work was to evaluate the potential biological responses of the exposure to Dex at the level of the reproductive system of male and female organisms of a gonochorous species with important ecological and socio-economic value, such as the Mediterranean mussel *Mytilus galloprovincialis*, through a multi-biomarker approach to have a complete overview of the biological risk to which marine biota can be exposed.

The approach used, which considered organisms of both sexes as a biological system consisting of multiple levels of biological organization to be investigated, demonstrated how males and females can respond to the same stressor by implementing different physiological strategies based on their phenotype. This supports the intent from which this work was born, that is to highlight how a contaminant can exert differential effects based not only on the evolution or developmental state of the model organism but more simply in relation to sex.

As demonstrated by the chemical analysis, the tested drug, like other PhACs, is internalized and bioaccumulated in biota in a short period of exposure. Indeed, Dex possesses lipophilic properties that make it able to bypass the plasma membrane (Cámara et al., 2023; del Carmen Gómez-Regalado et al., 2023) and accumulate at the level of the gonads, an organ rich in energy reserves such as lipids, glycogen and proteins (Kronberg et al., 2021; Duinker et al., 2008), already after only three days of treatment and at relatively low environmental concentrations (4 ng/L). With increasing time and doses, the glucocorticoid tended to accumulate, but it was noted that for the lower tested Dex concentration (C1) and the highest one (C4) at time T6 a decrease in the tissue presence of Dex occurred. This could be due to the activation of detoxifying strategies that would mediate the elimination of the xenobiotics. For this mechanism, the ABCB family proteins including P-glycoprotein (also known as Multidrug Resistance Protein 1, MDR1), may be involved since they mediate the transport of xenobiotics by mobilizing hydrophobic and/or amphipathic and low molecular weight substances that have not undergone any modification in the extracellular environment. Otherwise, also the ABCC transporters may have a role in this context, since it is known that the Multidrug Resistance-associated Protein (MRP) cooperates in the elimination of substrates coming from phase I and II of the detoxification process (Balbi et al., 2023; Franzellitti et al., 2019). It is possible that, by the molecular approach, this hypothesis was confirmed, since in the male gonad the expression of the genes related to the biotransformation pathway, namely MDR1 and MRP, suffered a dose-dependent up-regulation at time T12. This suggests that the organism at the male gonad level attempts to increase tolerance to the stress represented by Dex, implementing this adaptation strategy common in molluscs, as reported for other types of PhACs (Queirós et al., 2023). Instead, in the female gonads an opposite

behaviour occurs, in fact, significant down-regulation of the genes involved in the detoxification mechanism has been found. A similar attitude towards dexamethasone has also been found in a study conducted on the same species at the level of the digestive gland, in which the author underlined the possibility that the response could be depended on the dose (Pes et al., 2021), this idea correlates well with the possibility of a hormetic event dependent on drug in function of the sex (Agathokleous et al., 2022). In additions, it possible that the females can also develop detoxification mechanisms not necessarily dependent on ATP-dependent transporters, in fact, in the female gonad levels of detoxification further upstream could be more involved, such as Cyp450 (phase I) whose gene expression can be regulated by glucocorticoid receptors (GR) (Burkina et al., 2015) that respond to Dex, for which it is possible to deduce that the direct interaction with the drug could have stimulated one mechanism over another.

The early influence of Dex in both sexes is demonstrated by the presence of hemocyte populations distributed throughout the connective tissue of the gonads. The greater presence in males and their distribution among the maturing germ cells inside the follicle, underlines a more intense inflammatory activity compared to the female counterpart. Experiments with dimyristoylphophatidylcholine (DMPC) membranes, used to simulate plasma membranes, have recognized that Dex is very easily adsorbed by DMPC monolayers, having a high affinity for the lipid monolayer due to its hydrophobic character. This fact indicates that Dex is capable of spontaneously inserting itself into a condition of lipid density like the biological membrane, and in this way it can promote changes in the membrane packing and form three-dimensional aggregates and thanks to the flexible nucleus (Dey & Ghosh, 2020) access the interior of the cell (Cámara et al., 2023). In fact, the intense hemocytic recall of the classes of hyalinocytes (smooth hemocytes) and granulocytes (haemocytes with a granular appearance), initially arranged in small groups and then individually, indicates how already after only three days of exposure the mussel gonad of both sexes perceives and collects the action exerted by the tested glucocorticoid.

The activation of immune cells in response to a pro-inflammatory state is a typical behaviour implemented in the genus *Mytilus* when subjected to pollutants such as drugs that can induce a pro-oxidative state, even at the level of the gonads

(Afsa et al., 2023; Koagouw et al., 2021; Parisi et al., 2021) and in this case it seems to be the evidence of the endocrine interference exerted by Dex.

The presence of a prolonged inflammatory condition is also confirmed by the data reported by the molecular analysis relating to the time T12. The activation of the antioxidant system is typical of the response to the drug also in fish species (Guiloski et al., 2015; Costantini et al., 2011), as Dex leads to the production of reactive oxygen species (ROS). This is supported by the fact that in differentiated human rhabdomyosarcoma cell cultures it was discovered that treatment with Dex determines a decrease in the relative value of the mitochondrial membrane potential ($\Delta\Psi$ m), with a consequent increase in the generation of ROS, which leads to an alteration of the mitochondrial morphology, therefore dysfunction and apoptosis (Oshima et al., 2004). Realistic Dex concentrations resulted in an oxidative condition in the common carp *Cyprinus carpio* embryos that determined a dose-dependent up-regulation of enzymes such as *SOD* and *CAT*, as well as a high lipid peroxidation at a dose of 40 ng/L (Gutiérrez-Noya et al., 2023). Instead, Pes et al. (2021) found that in the species *M. galloprovincialis* the drug had an impact on the detoxification system, altering the expression of the enzyme glutathione peroxidase (*GPx*). This differential result could be due to the target organ chosen for the investigation, such as the digestive gland, since a divergent tissue-dependent response is possible (Guiloski et al., 2015), or to the high doses tested in the order of mg/L that could have caused an imbalance in the expression of *SOD*.

In the work presented here, it was chosen to focus on the cytoplasmic enzyme *superoxide dismutase* (*Cu/Zn-SOD*) and *ovothiol synthase A* (*OvoA*) as modulators of the antioxidant response, since both enzymes have an important role in detoxification from ROS. Specifically, *Cu/Zn-SOD* (copper/zinc dependent superoxide dismutase) converts superoxide into hydrogen peroxide (H_2O_2), which is further converted into water and oxygen (Vasquez et al., 2022), while *OvoA* (*iron II-dependent sulfoxide synthase*) is the first enzyme that mediates the formation of ovothiol, involving the conjugation of cysteine and histidine and leading to the formation of 5-L-histidyl-L-cysteine sulfoxide, a non-enzymatic molecule that acts as a scavenger of H_2O_2 (Diaz de Cerio et al., 2020). At the gonad level of both sexes of *M. galloprovincialis*, although following a non-monotonic trend, as it is typical of the response to many EDCs (Wang et al., 2023), the *SOD* enzyme tended

to a general increase, a sign of an induction by ROS. An increase in this enzyme after an exposure period of almost two weeks underlines how Dex, for both sexes, promoted a long-lasting pro-oxidant action in this marine invertebrate, a condition that has not been reported in studies on testicles and ovaries of mammals that, although highlighting the dysfunctional action at mitochondrial level that leads to apoptosis, recorded a decrease in antioxidant enzymes such as *SOD* and *CAT* after prolonged exposure (Patel et al., 2024; Jeje et al., 2021). It is however possible that being a non-target organism the drug may have determined a response of this type, as it has been demonstrated for other types of PhACs on aquatic organisms (Afsa et al., 2023; Ács et al., 2022). This is supported by the increase, albeit variable, of *OvoA* enzyme.

Ovothiol, produced by the action of this last enzyme aforementioned, plays a very important role in the gonads of many marine invertebrates, like glutathione (Murano et al., 2022) and in the species during gametogenesis, mainly during the maturation phase of both oocytes and spermatozoa, serving as protection during the maturation process (Diaz de Cerio et al., 2020). Furthermore, since the fertilization of bivalves is external, the gametes must protect themselves from what is dispersed in the aqueous medium (Castellano & Seebeck, 2018) as they must face the oxidative explosion that is triggered at the time of fertilization (Wong et al., 2004). Therefore, since the species is in the crucial period of gametogenesis, it was not surprising to find an increase in *ovothiol synthase A* transcripts compared to the control, mainly after a prolonged period of exposure to the drug, as the greater expression of the enzyme may have been determined by the persistence of the pro-oxidant state promoted by Dex.

The implementation of detoxification strategies is also supported by the trend of glycine, as revealed by metabolomics. Indeed, in male gonads was found a general decrease in the amino acid at T3 and T6. Glycine is a fundamental constituent of the tripeptide glutathione (GSH), which could underlie a more accentuated metabolism of the detoxifying molecule (Ramirez et al., 2022; Cappello et al., 2021; Jones et al., 2008) and therefore a greater need to capture glycine to form new GSH molecules and cope with stress. However, this amino acid could be also involved in osmoregulation processes, and therefore behave differently depending on the phenotype. In the literature, the potential osmotic

effect of Dex on aquatic organisms is not well documented. Despite this, it is known that in marine vertebrates the synthetic glucocorticoid can upregulate the Na/K ATPase isoform promoting the re-uptake of Na^+ ions (Hamilton et al., 2022). This could cause the *de novo* synthesis or absorption of organic osmolytes and not, in order to compensate the altered osmolarity as also supported by studies on freshwater invertebrates such as the bivalve *Ligumia subrostmta* in which Dex injections promoted Na^+ uptake within two hours of administration compared to controls (Saintsing & Dietz., 1983). However, other studies describe a different behaviour according to which Dex, like cortisol, would compromise the activity of the Na/K ATPase pump in the *Spaurus aurata* species, in which the reduction of the activity of this pump may lead to an increase in plasma ammonia (Jerez-Cepa et al., 2019). This case would however produce an increase in osmolarity that could be buffered by an increase in the concentration of osmolytes, a condition that finds credence in the female gonad in which a general increase in glycine was observed throughout the exposure, and in the male gonad with the continuation of the treatment. The divergence in clarifying the mode of action of cortisol-like compounds, such as the drug under analysis, could depend on the interaction of these compounds with other hormones, such as prolactin or growth hormone, as described in studies on some teleosts (McCormick, 2001; Mancera et al., 1994).

The influence of Dex on osmotic balance is also demonstrated by the variation of betaine in female gonads, where there was a decrease at T3 followed by an increase at T6 (except at the highest concentration of Dex, where a decrease occurs) and at T12 again a new increase. As reported for another species phylogenetically close to *Mytilus*, such as *Crassostrea gigas*, osmolarity plays an important role in the gonads (Boulais et al., 2019). A hypo-osmotic condition could explain the decrease of osmolytes such as betaine and taurine at T3 for all Dex concentrations, as observed in gill and mantle tissues of *M. edulis* larvae subjected to low salinity (May et al., 2017), in agreement with what can occur at T6 for the higher concentration at which the greatest deposition of dexamethasone was recorded with respect to time. In aquatic invertebrates such as bivalves, there is a close relationship between FAAs and osmoregulation (Gilles, 1987), particularly significant in species and sub-species of *Mytilus* such as *Macoma balthica*, in which betaine is used to balance seasonal fluctuations of salinity (Kube et al., 2007).

The trend of other osmolytes accredits an action on osmotic regulation promoted by Dex. In fact, in males there was an increase in betaine at T3 except for the lowest concentration, followed at T6 by a decrease at C2 and C3 and an increase at C1 and C4, while at T12 (except for C2) there was an increase similar to that of females. Instead, taurine underwent an increase at T3, while at T6 it had an increase at C1 and C4 and a decrease at C2 and C3, at T12 (apart from a depletion at C2 as in females), there was a constant increase. The trend just summarized in the male gonad has demonstrated how betaine assumed a variable trend after three days of exposure with an increase at the highest Dex dosage and a depletion at the lowest ones, perhaps due as well as in female specimens to a hypo-osmotic state that would also seem to apply at T6. If the decrease in betaine and other osmolytes is related to a hypo-osmotic state, it is plausible that their increase in male is the result of a hyper-osmotic stress, as reported for another species phylogenetically close to *Mytilus*, such as *Crassostrea gigas*, in which osmolarity plays a fundamental role in the functioning of germ cells. In fact, in the testicular fluid of the bivalve a high osmolality was recorded due to the high concentration of Na^+, since the onset of sperm motility depends on both Na^+ and K^+ ions (Boulais et al., 2018). Taurine seems to play a relevant role, maintaining a constant increase at all concentrations even after twelve days of exposure, a behavior that could reflect a sex-dependent phenotypic response, as observed in *Perna viridis* exposed to triazophos (Zhang et al., 2017).

However, data in literature clearly show how Dex, through binding to the glucocorticoid receptor (GR), is involved in osmosensing mechanisms that, depending on the tissue, species and sex, can activate different pathways even at the molecular level (Chow et al., 2013; Shrimpton & McCormick, 1999).

Dexamethasone, like many glucocorticoids, affects the metabolism of carbohydrates, lipids and proteins (Zhang et al., 2020, 2021), which are fundamental in a tissue such as the gonads especially during the period of sexual maturation. For this reason, the trend of metabolites that could clarify the divergent behaviour found in the gonads of the two sexes was evaluated. In fact, males underwent a general and early alteration of glucose levels, showing a temporal trend inverted bell typical of the action of EDCs (Diamanti-Kandarakis et al., 2009), characterized by an initial increase followed by a depletion at T6 and a new increase

at T12. An early response of this type is typical in *M. galloprovincialis*, when stimulated by xenobiotics, as occurred after 48 h of exposure to microplastics for which an increase in both glucose and glycogen occurred in response to an early energy need to cope with the toxicity induced by the stressor (Cappello et al., 2021). The phenotype, in regards to the energy pathway, seems to have had a certain influence since in female gonads a high level of glucose pre-exposure was highlighted, which remains constant after three days in untreated organisms but which undergoes an inversion already at the lowest concentration (C1) for which a strong initial depletion occurred at T3 followed by an increase at T6 and a general decrease at T12, which described an inverted U trend (Diamanti-Kandarakis et al., 2009) further sign of the action of Dex as an endocrine disruptor.

The different attitude of the two phenotypes to the glucocorticoid drug has also been highlighted with regard to glycogen, so much so that females preferred to implement a faster strategy to obtain energy, exploiting the primary substrate, such as glucose (Wang et al. 2022), and also drawing from the glycogen stored in the VCT cells which, in the PAS reaction, are rich in empty white spaces, demonstrating a decrease in the reserve of the branched monosaccharide. In females, at T12 for the lower concentrations, a significant reduction in glycogen levels occurred. Baqué et al. (1996) suggested that at relatively low doses (10 nM) the drug acts on primary cultures of rat hepatocytes promoting glycogenolysis processes, an event that has also been recorded for μM concentrations, attributing the response to the activation of the enzyme glycogen phosphorylase and to the duration of the treatment period (Gomez-Muñoz et al., 1989). However, at the highest dose (C4) an increase in glycogen occurs, indicating a dualism that has also been highlighted in reserve tissues such as the liver of mammals and dependent on the different concentration of Dex (Divari et al., 2020), a hypothesis confirmed in this case by the greater bioaccumulation of the compound at the highest concentrations of the three exposure times, with greater relevance for the dose of 2 μg/L (C4).

The initial impact of stress was demonstrated by the early response to Dex followed by the activation of compensatory strategies by the organism to recover an initial physiological state potentially attributable to the phenotypic plasticity of the species (Atasaral-Şahin, et al., 2015), glucose being a crucial support in gametogenesis processes (Gabbott et al., 1979). It has been reported that, when the

needs of the organism, viewed as a whole, are greater and must counterbalance an adverse condition, the energy reserves contained in the gonad are redirected to other compartments to support the survival of the organism (Danton et al., 1996). However, the deprivation of energy resources at such an important time as the reproductive period, during which the exposure was carried out, plays a negative role on the reproductive health status of the species. Glucose and glycogen are two fundamental metabolites during Stage II (late vitellogenesis) and Stage III, where large amounts of energy must be made available to allow the maturation of female gametogonia (oogonium) and late vitellogenic oocytes for the growth of future embryos and for the synthesis of vitellogenin (auto/heterosynthesis), a glycolipophosphoprotein, at its own expense (Rosati et al., 2019; Prisco et al., 2017; Agnese et al., 2013). It is also possible that the low glucose levels, mainly with prolonged exposure, were the direct result of the drug which, as verified in a parallel study on gills (data not yet published), involves hypermetabolism at mitochondrial level (Desquiret et al., 2008).

In males at the initial sampling time, unlike females, no decrease in the presence of glycogen was demonstrated except at the lowest concentration (only at T3 and T6), a sign that indicates that the cause of the increase in simple sugar does not seem to be related to the use of connective tissue carbohydrate stores. As supported by Irshad et al. (2021), in an organ in which glycogen plays an important role such as in the liver it is possible that this is due, after an exposure of about two weeks, to the increase in the activity of the enzyme glycogen synthase (GS), whose task is to bind a UDP-glucose residue to the non-reducing end of a glycogen molecule forming an α-1,4 glycosidic bond, thus lengthening the linear chain (Roach et al., 2012). A study on myocardial tissue in adult male Wistar rats subjected to acute administration of glucocorticoids reported the occurrence of glycogen accumulation mediated, however, by 5' AMP-activated protein kinase (AMPK) and correlated with the activation of GS and a lower oxidation of glucose that would thus be directed to become an energy reserve (Puthanveetil & Rodrigues, 2008). What has been reported could also be consistent for the gonad, in which glycogen, as in the previously considered organs, has a key role in supporting the metabolic activities, in this case those supporting gametogenesis (Qin et al., 2023; Gabbot et al.,1979).

Although in a different way, the perspective just described would have suggested that both sexes were affected by Dex interference and advanced differential responses to preserve the production of healthy gametes suitable for the formation of the embryo (Tran et al., 2019). The divergence in gonadal responses between the two sexes could not only reflect a modulation in the activity of proteins and enzymes, but also be due to an intrinsic genetic difference. In this regard, the concept of doubly uniparental inheritance (DUI) has been proposed for another species of the family *Mytilidae*, such as *Modiolus modiolus* (Robicheau et al., 2017). In confirmation of this, a study revealed a 20% nucleotide variation in DUI in the blue mussel *M. trossulus* and sub-species (Rawson et al., 2005). The possibility of this type of variation dependent on traits such as species and sex could induce a different attitude towards Dex as it was highlighted for the mussel *Crassotrea gigas* exosed to heavy metals (Qin et al., 2023).

Another possibility that could explain the increase in glucose could be provided by a study on rheumatological patients, in which glucocorticoids induce hyperglycemia mainly in males also due to other factors, such as age (Shah et al., 2022; Nakamura et al., 2021; Tamez-Pérez et al., 2015). The increase in glucose could also be due to secondary events such as the activation of the glucogenosynthesis pathway as discovered by Divari et al. (2020) in the liver of cattle exposed to low doses of dexamethasone and prendnisolone, in which changes in the regulation of genes involved in the gluconeogenic pathway (pyruvate carboxylase, phosphofructokinase 2/fructose biphosphatase 2) occur. All these hypotheses are supported by the fact that the reproductive tissue can also be receptive to glucocorticoid drugs such as Dex (Omairi et al., 2022; Leatherland & Barkataki, 2010).

For the gonad, the implementation of proteolytic events to obtain energy has been hypothesized. This idea has been confirmed by the detection of the alteration of BCAAs from the metabolomic analysis, some of which, as it is known, can be gluconeogenic amino acids. At T3, already from the C1 concentration, an increase in BCAAs was recorded in male gonads, a condition that in the female counterpart was detected only in response to higher concentrations of Dex, which would indicate a greater resistance of the phenotype to catabolic events. This could be the result of a further role of the drug, as also recorded by Dahabiyeh et al. (2020) and

by Schakman et al. (2009), according to which in various tissues of small rodents (liver, brain and muscles) treatment with Dex could activate proteolytic events (Hong & Forsberg, 1995). Metabolic behaviour in both phenotypes at T6 seemed to be independent of sex and the overall result of genetic and physiological compensatory strategies common to the *Mytilus* genus for better adaptation to stress conditions (Guo et al., 2023; Lassoued et al., 2021; Lacroix et al., 2015).

In this regard, the lower availability of FAAs could have been due to their use to build new protein structures that had previously been catabolized for tissue needs or to be converted into carbon bodies to satisfy energy demands. It is known that Dex, through interaction with cytosolic receptors, can transduce the signal by binding to glucocorticoid response elements (GRE) (García et al., 2023), but it can also exert its action through receptors located on the plasma membrane and associated with G proteins which, through a signal cascade mediated by specific kinases belonging to the cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinase (MAPK) classes, mediate a faster response. In hepatocarcinoma cells, it seems that p38MAPK could decrease oxidative phosphorylation in mitochondria, also causing changes in the respiratory chain. In fact, it has been shown that Dex induces a drastic reduction in the activities of complex I (-60%) and complex II (-75%) and an enhancement of complex III as compensation (Desquiret et al., 2008). This could explain the contextual implementation of the anaerobic pathway for energy production, as the data led to suppose.

In fact, in both sexes, especially in the male gonad, already at the lowest concentration of Dex tested, an increase in the ketone body synthesized from acetyl-coenzyme A (acetyl-CoA) as a final product of fatty acid metabolism, such as acetoacetate, occurred (Laffel, 1999), which would suggest that the energy demand was immediately pronounced. As it was reported, exposures to environmental doses of the order of ng/L (20-40 ng/L) compromise the redox balance in aquatic vertebrates (Gutiérrez-Noya et al., 2023; Pes et al., 2021), so the timely increase has validated the initial idea of a strong pro-oxidant state (Musee et al., 2021; Jerez-Cepa et al., 2019) that seems to have a more pronounced effect in males than in females. Such a sign of lipid peroxidation is typical of the action of different classes of pollutants (Martin-Folgar et al., 2022; Elizalde-Velázquez & Gómez-Oliván,

2021; Tresnakova et al., 2021) in which PhACs (De Marco et al., 2022) such as Dex become strong. In addition to this, in the interpretative perspective of the data obtained, at the level of a tissue that requires energy to carry out the normal process of gamete maturation, the ketone body could also be employed to produce carbon intermediates that can enter the Krebs cycle, through the transfer of a portion of CoA from succinyl-CoA to acetoacetate by the enzyme succinyl-CoA transferase (SCOT), an enzyme whose presence has been demonstrated in the mitochondria of germ cells and in mouse spermatozoa (Tanaka et al., 2004), and that could also play a role in mussels that show many homologous genes. Thus, as in other invertebrates and vertebrates, also in *M. galloprovincialis*, acetoacetate can be reduced to β-hydroxybutyrate by the enzyme β-hydroxybutyrate dehydrogenase in a reaction that requires NADH, related to the oxidation of fatty acids. Furthermore, it is possible that as observed in *Perca fluviatilis*, being a cortisol-like substance Dex may also modulate some enzymes of the Krebs cycle (Milla et al., 2018), leading to the activation of a secondary metabolism.

This idea is supported by the alteration in lactate. In fact, both in the male and female gonad the spectra showed an early increase of the metabolite, this could be justified by a direct action of dexamethasone on the production of lactic acid (Vernon & Taylor, 1988), in line with the considerations made so far, that the drug has the ability to induce in both sexes and in two different ways a greater energy demand since lactate would act as a substrate on which the enzyme lactate dehydrogenase acts to feed the cytoplasmic energy, in this case mainly put at the service of the maturation of the germ cells. As demonstrated by the general picture, also in this case, the female specimens demonstrate greater resilience since at T6, they implement compensatory and adaptive strategies to restore the original physiological condition, an effort that, however, with the continuation of the treatment was not able to be sustained. The use of lactate as an energy substrate has been shown to be important in the reproductive context of the species, for the correct maturation of spermatocytes and spermatids, as it has been shown to be a protection strategy against apoptotic events that the drug could induce, like other pollutants such as metals (Lettieri et al., 2023). An idea that finds support in Mahmoud et al. (2009), who confirms that dexamethasone is able to promote degeneration in mouse testes after seven days of treatment and follicular

degeneration with necrosis in the rat adult (Mohammed et a., 2023). Since no evident alteration was found at the level of male and female germ cells, it was hypothesized that, in both sexes, strategies were implemented to protect the potential apoptotic process induced by Dex. Thus, a molecular analysis was performed to evaluate the expression of the anti-apoptotic gene *Bcl2*. Unlike what was reported by Chen et al. (2020), on skeletal muscle cells in which Dex treatment induced both the under-expression of *Bcl2* and the over-expression of *caspase 3* and *Bax* (pro-apoptotic protein) diverting the outcome towards the apoptotic process (Feidantsis et al., 2021), in the present study an opposite phenomenon was reported whereby a greater expression of the *Bcl2* gene occurred. A study on a transgenic line of *Danio rerio* demonstrated how the over-expression of *Bcl-2* can suppress Dex-induced cell death (Langenau et al., 2005) in a non-target species.

The hemocytes found inside the male follicle would be indicative, as reported by Smolarz et al. (2017), of atresia in the species *M. trossulus* and, therefore, the result of a clear endocrine disruption event. Thus, an immunofluorescence analysis was performed on male gonads, which demonstrated the failure or postponement of the implementation of a death program induced by Dex (Xu et al., 2020; Walsh, et al., 2002), having not found immunopositivity to the anti-cas3 antibody, a proteolytic enzyme effector of apoptosis, further proof of the ability to adapt and resist of this marine species. It was evident that after the initial impact that caused a strong perturbation at the level of gonadal balance, demonstrated both by an increase in immunopositivity to the anti-FasL antibody that promotes the extrinsic pathway of apoptosis (Zhu et al., 2022), and by a lower positivity to the anti-PCNA antibody (cell proliferation nuclear antigen), as also reported on mouse testes exposed to Dex (Annie et al., 2019), at the level of the spermatogonia an increase in fluorescence was found as the exposure continued, which suggested a possible signal of recovery of the male reproductive tissue, in agreement with the absence of immunopositivity to the anti-cas3 antibody (Romero et al., 2011). It is possible that the biological effect induced by Dex depended on the stage of maturation of the gonad. In fact, the results of a study on the seminiferous epithelium at stages VII and VIII of mice exposed to Dex for seven days confirmed that the expression of FasL depends on the spermatogenic stage, and that the VII is the most sensitive to the glucocorticoid (Mahmoud et al., 2008). However, the evidence of a decrease

in the immunopositivity of the anti-FasL antibody and an increase in the immunopositivity of the anti-PCNA antibody by increasing the time of treatment with Dex, demonstrated that despite the endocrine disruption exerted by the synthetic glucocorticoid, the organism retains its proliferative capacity and therefore the possibility of reorganizing the gonadal tissue.

4.5 Conclusions

Overall, these results suggest that organisms of both sexes of the Mediterranean mussel *Mytilus galloprovincialis*, after a sub-chronic exposure of twelve days, activate early biological responses to dexamethasone. This was evident from the mobilization of different classes of hemocytes already at the lowest concentrations and after only three days of exposure, a sign of a pro-inflammatory state with greater effect in male gonads. The alteration of key metabolites of the aerobic pathway (glucose and glycogen) suggests that in both organisms the drug stimulated the energy demand to compensate the need with the implementation of secondary and anaerobic pathways (BCAAs, lactate and acetoacetate).

Furthermore, the osmotic imbalance found compared to control organisms for both sexes, seems to confirm the negative influence of Dex on the gonads in which metabolic adaptations are implemented on multiple fronts to restore the original physiological condition to preserve the production of healthy oocytes and spermatocytes suitable, intended for external fertilization for the formation of offspring. Therefore, it is possible to confirm, even in this non-target species, the exercise of endocrine interference induced by the synthetic glucocorticoid Dex.

However, the evidence of a decrease in the immunopositivity of the anti-FasL antibody and a preservation of the proliferative activity, demonstrated by the positivity of the anti-PCNA antibody with continued exposure to the drug, have shown that despite the endocrine interference exerted by Dex, the organism retains the ability to reorganize and therefore to resist to the stress. Nevertheless, there could be repercussions on the normal reproductive activity of these bivalves, on the maturation and suitability of gametes for fertilization and on reproductive fitness.

What has been described is evidence of how in a relatively short time, environmental doses of dexamethasone can negatively affect the reproductive health of a species that plays a key role in the ecological niches of the intertidal ecosystems and within the Mediterranean food pyramid.

Therefore, this study suggests to paying more attention to the consequences of human activity and implementing environmental biomonitoring plans that should include ecocytotoxicological studies on both phenotypes, since biological responses can be sex-dependent, and therefore influence the overall ecotoxicological risk.

4.6 References

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CHAPTER 5

Biological responses of Mytilus galloprovincialis gonads exposed to polystyrene microplastics (PS MPs)

CHAPTER 5

Biological responses of *Mytilus galloprovincialis* gonads exposed to polystyrene microplastics (PS MPs)

5.1 Introduction

Today's world is a plasticized reality, made of objects that accompany the daily routine and make life easy and fast. As versatile as plastic is, today its image has taken on a new interpretation due to the poor management that has been made of it.

Man's initial intent was to create an easy-to-use material that would last over time, to obtain the maximum benefit from it. Paradoxically, plastic, in its most generalist sense, has become the basis of the disposable consumption chain and therefore, the largest anthropogenic waste found in the environment, especially in marine ecosystems (Nanayakkara et al., 2024). Furthermore, with the advent of the SARS CoV-2 pandemic, normal lifestyle has undergone changes, making it necessary to use disposable materials for personal protection, disposable plastics for diagnostic tests, etc., all of which has led to a greater flow of waste, exerting a greater load on management systems that have demonstrated inefficiency in dealing with the problem due to excessive and frequent pressure (Sahni et al., 2024). Some studies have shown that in the world about 40% of waste was sent to landfills, 25% to incineration, 16% to recycling, but 19% ended up in the environment (Ganguly & Chakraborty, 2024).

Each plastic product is nothing more than the result of the union of small bricks, identical or different monomers, which constitute homopolymers or copolymers that have different compositions, densities, dimensions and colours. Plasticisers can be added to this picture, which cooperate to make the plastic material more ductile and flexible. However, due to its structure, the plastic can, under appropriate physico-chemical, mechanical and biological conditions, be subjected to depolymerisation, producing smaller-sized polymers such as microplastics (MPs) (PlasticsEurope, 2021; Zhang et al., 2021a; Jang et al., 2018).

The intrinsic properties of the polymer determine the characteristics of the microplastic derived from it and therefore, the behaviour of the particle in the

environmental matrix, the potential adsorption of any contaminants or microorganisms, the availability towards the biota, the bioaccumulation in the tissues, and therefore the possible degree of toxicity (Le Bihanic et al., 2020; Zhu et al., 2020; Xu et al., 2020; Hantoro et al., 2019).

Due to the variability of weight, shape and density, MPs are distributed along the entire water column from surface to deep waters to rocky sediments, and for this reason their distribution in the marine ecosystem is variable and ubiquitous and provides frequent opportunities for the biota to encounter this type of pollutant (Ainali et al., 2021).

The Mediterranean Sea has been assessed as one of the most productive basins of MPs, partly due to the improper waste management activities of the countries bordering its coasts and partly to the rate of plastic debris comparable to that of the convergence zone of the ocean gyres, defining the Mediterranean one of the areas with the highest rate of pollution in the world (Simon-Sánchez et al., 2022).

The abundance of these MPs in such a limited area as the Mediterranean basin is easily subjected to the influence of various environmental factors (wind, rain, tides, etc.) and ocean dynamics that lead to frequent accumulation in the coastal zone, where they can persist longer over time because they are confined by wave mechanics (Zhang et al., 2017). This determines for intertidal habitats a frequent and persistent exposure to this type of pollutant (Hantoro et al., 2019). Their presence has been found in various marine organisms sampled on site, among these vertebrates (seabirds, fish) and invertebrates, mainly benthic (crustaceans and bivalves) (Sfriso et al., 2024; Parolini et al., 2023; Ding et al., 2021; McIlwraith et al., 2021; Wang et al., 2019b). Data in the literature show evidence of bioaccumulation within the trophic levels constituting the marine food pyramid (Miller et al., 2020), as MPs can be ingested by the biota, internalized in the tissues and be translocated between one level and another through ecological relationships (prey-predator) (Ogunola et al., 2023).

Microplastic ingestion has been reported for various bivalve species, such as the native Mediterranean species *Mytilus galloprovincialis* (Dambrosio et al., 2023) which occupies a prominent place in the Mediterranean diet. Microplastic pollution is widespread worldwide, the study by Ding et al. (2021) demonstrated how in different seasonal periods and in species sampled in two different sites, close to the

coastal-tourist area of Qingdao (China), where mariculture activities were carried out, an abundance of MPs was found in scallop *Chlamys farreri,* mussel *Mytilus galloprovincialis,* oyster *Crassostrea gigas*, and clam *Ruditapes philippinarum*. This research field further underlined the validity of bivalves as bioindicators of environmental pollution, mainly by MPs.

The choice of polystyrene (PS) as a microplastic grade was because most plastic materials present this type of low-density polymer either as a homopolymer or in combination with other grades as a copolymer. In fact, due to its properties, including good flexural strength, chemical stability and economy, polystyrene is an excellent ally of the plastic industry (Sfriso et al., 2024; Siddiqui et al., 2023).

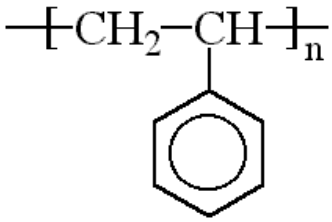

Figure 5.1 Chemical structure of styrene, monomer of the polymer polystyrene.

It is an aromatic polymer made up of styrene monomers (Figure 5.1), produced from ethylene and benzene. It is a thermoplastic and durable homopolymer used to produce packaging (also because it is translucent), toys, expanded polystyrene, etc.

Due to the tendency to photodegradation, accredited by the presence of phenolic rings in the chain that are excited when subjected to UV rays, the polymer tends to transfer bond energy that involves the formation of radical species (such as the polystyryl radical) that imply a greater reactivity of the compound and a tendency to scission of the chain with the formation of secondary products (such as cabonyl compounds and styrene monomers). This is the main cause for its increased occurrence in various environmental matrices in the form of micro and nanoparticles (Nugnes et al., 2022; Kik et al., 2020), and its ability to interact with other substances and with the biological systems (Wei et al., 2023; Masiá et al., 2021).

Studies report how the action of polystyrene microplastics (PS MPs) on non-target aquatic biota can lead to adverse effects at multiple levels of the biological system. In fact, a recent study by Tamura et al. (2024) highlighted how the exposure to 2 μm PS MPs promoted changes in brain-gut communication in the medaka

Oryzias latipes, and how these were translated into behavioural abnormalities, as also demonstrated by other studies conducted on different aquatic species (Santos et al., 2021). Meanwhile, a metabolomic analysis on a related species, such as *Oryzias melastigmas*, highlighted perturbations at the hepatic level with a negative influence on aerobic pathways as revealed by a decrease in monosaccharides and intermediates of the Krebs cycle and increase in the synthesis of fatty acids and methyl esters (Ye et al., 2021). Other authors have confirmed the presence of cellular damage following a 21-day treatment with PS MPs with an average diameter of 3-8 μm on the liver and gills of aquatic vertebrates, finding the induction of oxidative stress combined with the alteration of the stability of the plasma membranes (increase of malondialdehyde) and damage to proteins and DNA (Kaloyianni et al., 2021). Furthermore, the study revealed how the biological response of different fish species (*Perca fluviatilis* and *Danio rerio*) can be more or less susceptible to the pollutant (Kaloyianni et al., 2021). Furthermore, another problem developed by MPs is their ability to act as vectors for other types of organic and inorganic pollutants, ensuring their transport, possible contact with biota and a possible worsening of the toxic effect (de Souza et al., 2022; Katsumiti et al., 2021).

Just as the reactivity of the compound can vary according to the species, so also the size and/or the developmental stage can affect the assessment of the damage induced by PS MPs. In fact, a study on zebrafish embryos has highlighted how the size can affect gene expression and the quantity of neurotransmitters (Jeong et al., 2022). In line with the data found in the literature, it is evident that PS MPs of various sizes can cause structural and cellular anomalies due, for example, to the steric hindrance exerted by the polymer and to the imbalance of the redox state, inducing an increase in genes such as *SOD*, *CAT* and *GST*, whose products are responsible for detoxification from reactive oxygen species (ROS) and toxic products that would otherwise perturb the organism (De Marco et al., 2022).

Some studies have highlighted how PS MPs can lead to alterations in the reproductive cycle, causing toxicity at the level of the reproductive system of cnidarians (Bilal et al., 2023; Eom et al., 2022). Other studies carried out on the male vertebrate *Oryzias melastigma* have highlighted how, following chronic exposure to environmental doses of PS MPs of 10 μm, sex-dependent alterations of the hypothalamic–pituitary–gonadal (HPG) axis and of steroidogenesis occurred,

causing anomalies in the correct maturation process of the gonads and a transgenerational effect (Wang et al., 2019a).

There is little information about the potential effect of PS MPs like EDC on marine invertebrates. A multigenerational analysis by Sussarellu et al. (2016) would seem to demonstrate that bivalves are also subjected to the influence of the contaminant during the reproductive cycle. In fact, the authors reported that, after exposure to PS MPs of 2-6 μm, in female oysters there was a decrease in both the number and size of oocytes, while in males a decrease in sperm motility. As for the impact on the offsprings, a lower yield of D-larvae compared to the control was highlighted.

Against this background, in this study a gonochorous species in which the sexes are separated was chosen to evaluate the impact of PS MPs, and to specifically understand the potential sex-dependent biological responses following an acute exposure of 48 h. The Mediterranean mussel *Mytilus galloprovincialis* was then selected as model organism due to its filter-feeding lifestyle and resilience (De Marco et al., 2023; Lopez et al., 2023; Masiá et al., 2021), to evaluate the impact of two different concentrations (0.5 μg/mL and 1 μg/mL) of polystyrene MPs on the gonadal tissue of males and females, during the final phase of the reproductive cycle, preceding the emission of gametes.

Through a multi-biomarker approach, it was possible to interpret the potential biological impact of PS MPs by considering organisms of both sexes as a system consisting of multiple biological levels. In detail, histomorphological analyses were performed using colorimetric (H/E) and histochemical (PAS) methods, to verify the presence of structural anomalies and the carbohydrate profile, respectively, in the tissue organization of mussel gonads, that could compromise the successful emission of gametes. Also, metabolomic analyses using Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy were performed to have a complete view of the potential cellular pathways altered by PS MPs. Overall, the results obtained from exposure to PS MPs could influence the phenotype of mussel gonads, and therefore the physiological-functional state of males and females (Cappello et al., 2021; Kronberg et al., 2021; Ellis et al., 2014).

5.2 Materials and methods

5.2.1 Mussel acclimatization and experimental design

In collaboration and with the great support of the group of Prof. Luigi Rosati from the University of Naples Federico II (Naples, Italy), a research project was conducted to evaluate the ecocytotoxicological impact of PS MPs on the intertidal marine species *Mytilus galloprovincialis*.

In detail, specimens that were considered to have reached sexual maturity, considering the size of the valves, were sampled from a breeding area near Bacoli (Naples, Italy) in the period between December and January (Figure 5.2).

The animals were quickly and under appropriate conditions transferred to the Zoological Station Anton Dohrn (Naples, Italy), where they were placed in 3 aquaria randomly without having previously investigated the sex.

Figure 5.2 Sampling area of Bacoli site (Naples, Italy).

Thus, to have a statistically significant number of male and female samples, 20 specimens were housed in each container, filled with sea water (SW), so that the needs of each animal could be satisfied with 500 mL of SW. The animals were kept in appropriate conditions, designed to guarantee their well-being for the entire duration of the experimental plan, before which an acclimatization period of 15 days was carried out, during which a temperature of 18 ± 1 °C was maintained, as well as a photoperiod L/D 12 h:12 h and constant ventilation.

The parameters of the experimental treatment were established considering what is reported in the literature data regarding the micro-polymer examined.

The experimental plan, conducted in triplicates, involved the use of PS MPs with a diameter of 5 μm (PS-R-5.0, MP-pristine-solution 10% w/v, MicroParticles GmbH, Berlin, Germany), and considered three conditions: a negative control (Ctrl: 0 μg/mL), a low concentration of PS MPs (0.5 μg/mL), and a high one (1 μg/mL). To evaluate the short-term biological responses potentially given at the level of the gonadal tissue of both male and female organisms, six specimens per sex were sampled for each experimental condition after two days of exposure (48 h).

By using the appropriate instruments and maintaining sterility where necessary, all samples were sacrificed and for all of them sub-samples of gonads of both sexes were sampled, part of which were destined for histological analysis, then preserved in Bouin's fixative, and the rest was frozen at -80 °C for metabolomic analysis.

These last samples were transported on dry ice and destined for the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences of the University of Messina (Messina, Italy), where a Varian-500 NMR spectrometer is available to conduct ^{1}H NMR analysis (Figure 5.3).

Figure 5.3 Panoramic views of (left) the Zoological Station Anton Dohrn (Naples, Italy) and (right) the University of Messina, Department of Chemical, Biological, Pharmaceutical and Environmental Sciences (ChiBioFarAm; Messina, Italy)

5.2.2 Histological and histochemical analysis

As previously described by Agnese et al. (2019), the collected fresh gonad sub-samples were completely immersed for 24 h in Bouin's solution, so that the fixative could preserve the integrity of the tissue and allow the analysis to be successful. As reported in the previous chapters (Chapter 3 and Chapter 4), the subsequent steps of dehydration in an increasing series of alcohols, clearing and paraffinization were carried out until the paraffin-embedded sample was obtained. Thin sections of 5 μm were manually cut with a rotating microtome and mounted on a slide, deparaffinized and hydrated with a series of decreasing alcohols until reaching DW.

At this point, some slides were treated with the hematoxylin/eosin (H/E) colorimetric method according to Rosati et al. (2019), in order to be able to discriminate the sex, highlight the organization of the gonadal tissue of male and female mussels, and possible structural alterations. The remaining slides were treated with the PAS reaction, then oxidized for 10 min with 0.5% periodic acid, rinsed in double-distilled water and treated for 45 min with Schiff reagent (step carried out in the dark). To block the reactive process and prevent the oxidation from continuing, several washes were carried out in a solution of 2.5% sodium bisulfite dissolved in 0.05 N hydrochloric acid. The PAS reaction was used to highlight the presence of neutral polysaccharides, such as glycogen.

With a Zeiss Axioskop and the Axiovision 4.7 software (Zeiss, Jena, Germany) for the acquisition, all the morphological evaluations deriving from H/E and PAS were carried out.

5.2.3 Metabolomic analysis

5.2.3.1 Extraction of metabolites from mussel gonads

To obtain a complete picture of the reproductive health status of male and female mussels, a metabolomic analysis was performed as reported by Cappello et al. (2018). By carrying out a “two-phase” protocol (methanol/chloroform/water), sub-samples of about 100 mg of gonads of both sexes were treated as reported in the previous chapters (Chapter 3 and Chapter 4). To summarize briefly, after

homogenization with stainless steel beads (3.2 mm diameter) and a mixture of cold methanol and distilled water (methanol:water = 4 mL/g : 0.85 mL/g), in TissueLyser LT (Qiagen, Hilden, Germany) for 10 min, chloroform (4 mL/g) and DW (2 mL/g) were added to each sample. After shaking samples, and a short step on ice, they were centrifuged (2000 g at 4 °C) to obtain the separation between polar and apolar phases. Thus, for all male and female samples, 600 µL of supernatant (aqueous phase containing hydrophilic metabolites) were collected, taking great care not to touch the protein interphase, and immediately transferred in a clean tube and then placed in a vacuum centrifugal concentrator (Eppendorf 5301, Milan, Italy) to obtain a completely dry pellet to be stored at -20 °C until analysis by NMR.

Prior to the analysis, each sample was resuspended in 0.1 M sodium phosphate buffer (600 µL; pH 7.0, 10% D_2O; Armar AG, Döttingen, Switzerland) and 1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Sigma-Aldrich), used as an internal standard, or indicator of the chemical form. The suspension containing the polar metabolites derived from the gonads of both sexes were transferred into very thin glass capillaries for NMR, introducing the liquid very slowly so as not to create bubbles inside that would make the sample inhomogeneous, and to ensure the success of the spectrophotometric reading.

5.2.3.2 Metabolomics based on ^{1}H NMR and spectral pre-processing

Using the NOESY (Nuclear Overhauser Effect SpectroscopY) software associated with the Varian-500 NMR spectrometer equipped with RF channels with waveform generators working at a frequency of 499.74 MHz at 298 K, it was possible to detect polar metabolites with molecular weights not exceeding 1.500 Da. Thanks to this new acquisition adaptation, more detailed spectra were obtained, and it was possible to minimize water interference.

Manual phasing for each acquired spectrum was done using the Chenomx Processor software (Chenomx NMR Suite version 10.0; Chenomx Inc., Edmonton, Canada), correcting the baseline and setting the DSS (standard) to 0.0 ppm.

Using the Chenomx 500 Hz database and other public computer libraries, it was possible to identify and calculate the concentration of most metabolites present

into the sample, and with the Chenomx NMR Suite package (Chenomx Profiler) all the spectra were divided into Chemical Shift buckets from 0.005 ppm (0.8 ppm to 8.8 ppm). The data were normalized according to Cappello et al. (2018).

5.2.4 Statistical analysis

The processing of data obtained from metabolomic analyses was done with Excel, as a measure of the mean and standard deviation (± SD), and then through the GraphPad software (Prism 10.0, San Diego, CA, USA) the percentage difference between sex was obtained to verify its significance, considering a *p value* <0.05. The one-way ANOVA computer module was therefore applied to verify the variance between the two concentrations of PS MPs (0.5 μg/mL and 1 μg/mL) compared to the control for each sex (male and female).

5.3 Results

5.3.1 Histological data

The data of the histomorphological analysis of 5 μm thin sections, obtained by affinity of basic (hematoxylin) and acid (eosin) dyes that stained the nuclei and cell cytoplasm respectively, highlighted the clear difference in the organization of the gonadal tissue between the control organisms (0 μg/mL) not exposed to the micro-contaminant and the treated ones (0.5 μg/mL and 1 μg/mL), in fact, the absence of hemocyte cells was noted in the male and female control groups (Figure 5.4: A and Figure 5.5: A), while an immune response was found to occur in the groups treated with PS MPs in both sexes (Figure 5.4:B,C and Figure 5.5:B,C).

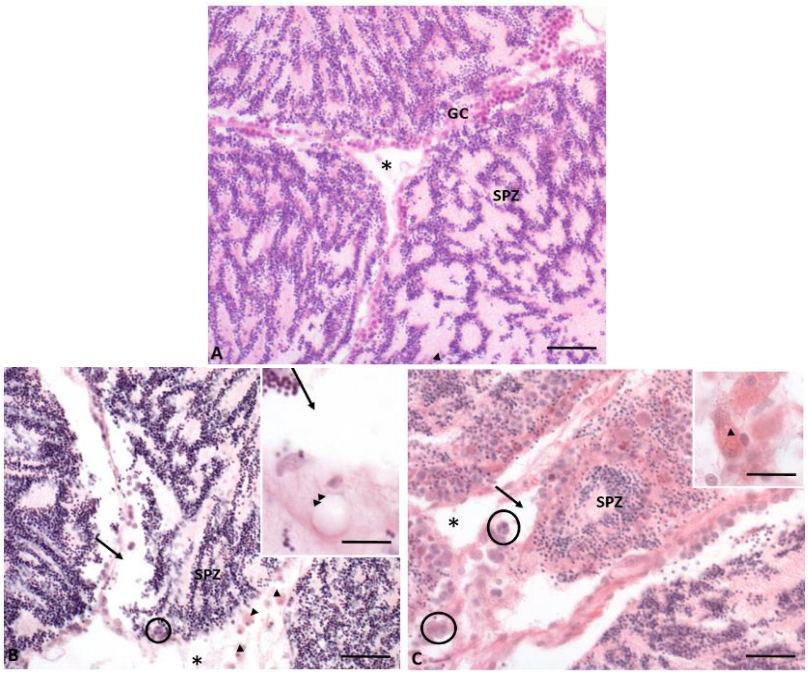

Figure 5.4 Sections of male gonads of *M. galloprovincialis* stained with the colorimetric method hematoxylin-eosin (H/E). (A) Control: canonical organization with intact follicles rich in spermatozoa (SPZ) towards the lumen and germ cells (GC) at the periphery. (B) Samples exposed to 0.5 µg/mL PS MPs: evidence of less stained zones arranged between the germ cells (arrow). (C) Samples exposed to 1 µg/mL PS MPs: disorganized follicles in higher number, with evident interruptions between the germ cells (arrow). Male follicle (MF), connective cells (*), hemocytes (arrowhead), rosette-like structures (circle), vacuolizations (double arrowhead). Scale bars: A-B-C: 30 µm, B and C insets: 10 µm.

The specimens of all the three conditions appear in line with the maturation stage that coincides with the seasonal period during which the specimens were sampled (December-January). In fact, male gonads showed a typical organization between the end of stage III A and stage III B (Prisco et al., 2017), in which the follicles appear with few germ cells towards the periphery and loaded with spermatozoa towards the lumen, ready to be deposited. In line with this, the presence of a reduced connective tissue represented by a few VCT cells and almost imperceptible ADG cells (Duinker et al., 2008) (Figure 5.4: A).

It was evident how male gonads were influenced by both concentrations of PS MPs, demonstrating dose-dependent effects. In fact, male specimens exposed to the lower concentration (0.5 µg/mL) of PS MPs showed a disorganization of the follicle (Figure 5.4: B), represented by empty spaces that were created between the sperm cells towards the lumen and a disconnection between the germ cells and the basal

membrane of the epithelium. The situation tended to worsen in the samples taken from the treatments at the higher PS MPs concentration (1 µg/mL) so much so that the disorganization between the sperm cells inside the lumen intensified, with an evident widening of the empty spaces, while the germ cells re-grouped together completely losing contact with the wall of the male follicle (Figure 5.4: C).

Regardless of the dose tested, at the level of the reduced connective tissue the presence of hemocytes was noticed, that highlighted an early innate immune response to PS microplastics.

The female gonad of the control organisms showed an organization typical of the maturation period (December-January) corresponding to stage III (Rosati et al., 2019; Duinker et al., 2008) for which mature oocytes were found and some pear-shaped ones that filled the lumen of the follicles surrounded by a small amount of VCT and ADG cells, in line with what was reported for the species that demonstrates an inverse relationship between the maturation stage and the distribution of the connective tissue (Duinker et al., 2008; Suárez et al., 2005), since the gonad is ready for the deposition of the ovule. The female follicles, like the male ones in the control group, presented with intact walls and well delineated gametic cells (Figure 5.5: A). It was evident that also in female gonads PS MPs had a certain influence, highlighting an intensification of the effect in relation to the increase in dose (Figure 5.5: B and C). In fact, although the gonadal tissue maintained a good structural integrity in the organisms treated with the lowest dose of microplastics (0.5 µg/mL), it is worth highlighting the presence of some degenerating oocytes, a condition that is highlighted by the irregular shape of the nucleus and the formation of cellular wrinkles, a potential sign of apoptotic events (Figure 5.5: B). As anticipated, this condition showed a worsening at the highest concentration (1 µg/mL) with, in addition, an alteration of the structural organization of the reproductive tissue (Figure 5.5: C).

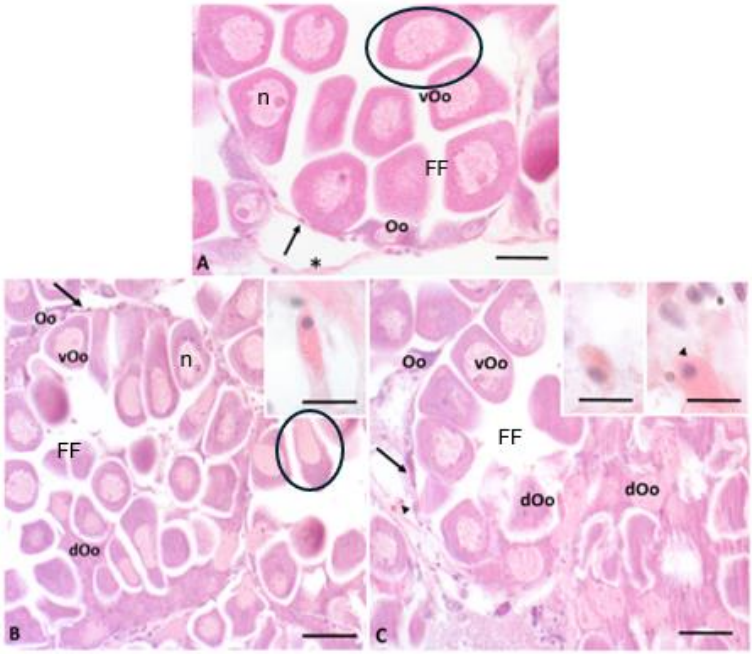

Figure 5.5 Sections of female gonads of *M. galloprovincialis* stained with the colorimetric method hematoxylin-eosin (H/E). (A) Control: canonical organization with intact follicles rich in vitellogenic oocytes (vOo) and follicular cells (arrow), (B) samples exposed to 0.5 µg/mL PS MPs show follicles with intact walls with vitellogenic and also degenerated oocytes (dOo), (C) samples exposed to 1 µg/mL PS MPs in which the presence of degenerated oocytes increases. Female follicle (FF), ovogonia (Oo), pear-shape oocytes (circle), nucleolus (n), connective cells (*), hemolymph cells (arrowhead, in insets). Scale bars: A-B- C: 30 µm; B and C insets: 10 µm.

5.3.2. Histochemical data

A histochemical analysis (PAS reaction) was also performed to verify the potential alterations in the level of glycogen, the major storage form of carbohydrates, in the gonads of both sexes (Meyerholz et al., 2018). When present, the homopolymer was detected in red magenta since the periodic acid selectively oxidizes glycosidic bonds making available closely contiguous aldehyde groups that react with the Shiff reagent determining the detection of the neutral polymer (Alonso et al., 2019).

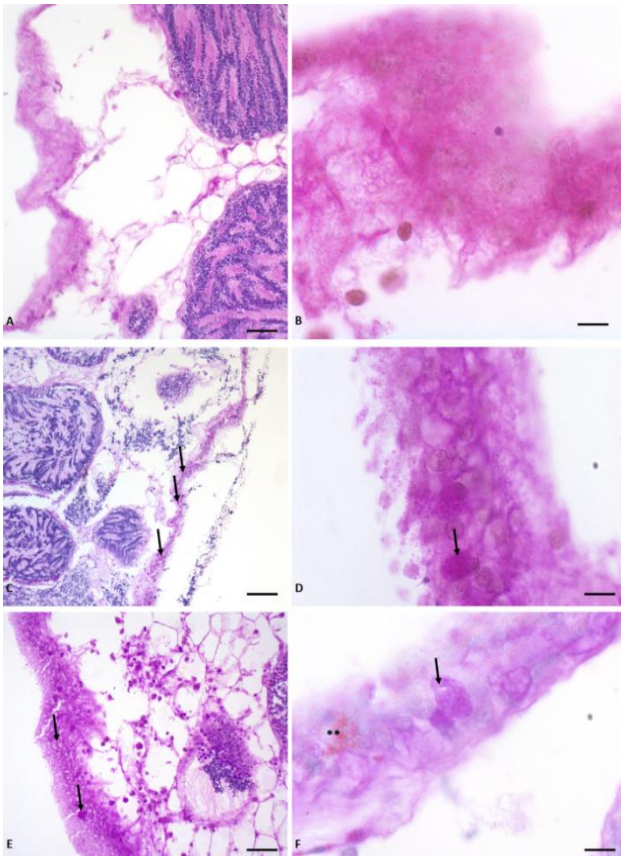

Figure 5.6. Sections of male gonads of *M. galloprovincialis* with PAS reaction. (A and B) Control samples. (C and D) Samples exposed to 0.5 µg/mL PS MPs. (E and F) Samples exposed to 1 µg/mL PS MPs. PAS-positive hypertrophic goblet cells (arrow), lipofuscin granules (double asterisk). Scale bar: A: 50 µm, B-D-F: 10 µm, C-E: 60 µm.

From the histological observation, it was noticed that both in mussel females and males the scarce connective tissue did not show a marked positivity to PAS neither at the level of VCT nor ADG cells. It is likely that the amount of glycogen was so low, in line with the reproductive period in which mussels were collected (Osterheld et al., 2024; Rosati et al., 2019; Prisco et al., 2017; Pipe et al., 1987; Gabbott & Whittle, 1986; Gabbott, 1979) that histochemical analysis was unable to detect it, unlike more sensitive analyses such as metabolomics. Actually, a positivity was found at the level of goblet-shaped cells located along the peripheral area of male follicles (Figure 5.6: C, D, E and F), which tended to increase proportionally to the PS MPs exposure dose. Additionally, colored agglomerates were also noticed that would seem to describe lipofuscin deposits in the peripheral area of the male gonadal tissue (Figure 5.6: F).

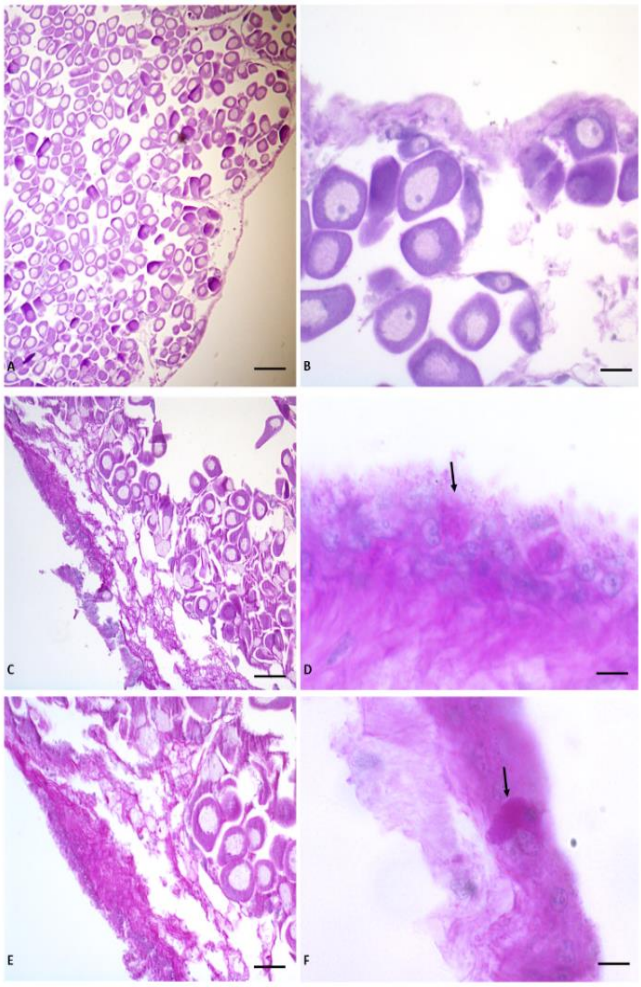

Figure 5.7. Sections of female gonads of *M. galloprovincialis* with PAS reaction. (A and B) Control group of female gonads in which no PAS positivity was detected, (C and D) samples exposed to 0.5 µg/mL PS MPs in which PAS positivity was detected, (E and F) samples exposed to1 µg/mL PS MPs in which PAS positive hypertrophic cells are evident (arrow). Scale bar: A: 100µm, B: 30µm, C: 60µm, D-F: 10µm, E: 50µm.

Figure 5.7 highlights the female gonadal tissue by PAS reaction. In the control group, the presence of glycoprotein accumulations (PAS positive) was not detected (Figure 5.7: A and B), in line with what was found in males, however, in the treated group, a moderate presence of hypertrophic goblet cells was detected that were marked with red-magenta, a sign of positivity to the PAS reaction, distributed towards the peripheral areas of the tissue (Figure 5.7: C, D, E and F).

5.3.3 Metabolomic data

The 1-D ^{1}H NMR spectra obtained by the metabolomics analysis of male (Figure 5.8, blue) and female (Figure 5.9, pink) gonads of mussels *M. galloprovincialis* taken from the control group, show the polar metabolite composition. In detail, within the metabolome of both sexes it was possible to identify amino acids (BCAAs, arginine, etc.), osmolytes (betaine), neurotransmitters (choline), energy metabolism-related metabolites (glucose), and tricarboxylic acid cycle intermediates (malonate). Interestingly, from the overlapping of male and female NMR spectra of mussel gonads it was noticed an almost identical metabolite profiling in terms of molecules present into the metabolome, with differences related to sex only in relation to metabolite concentrations (Figure 5.10)

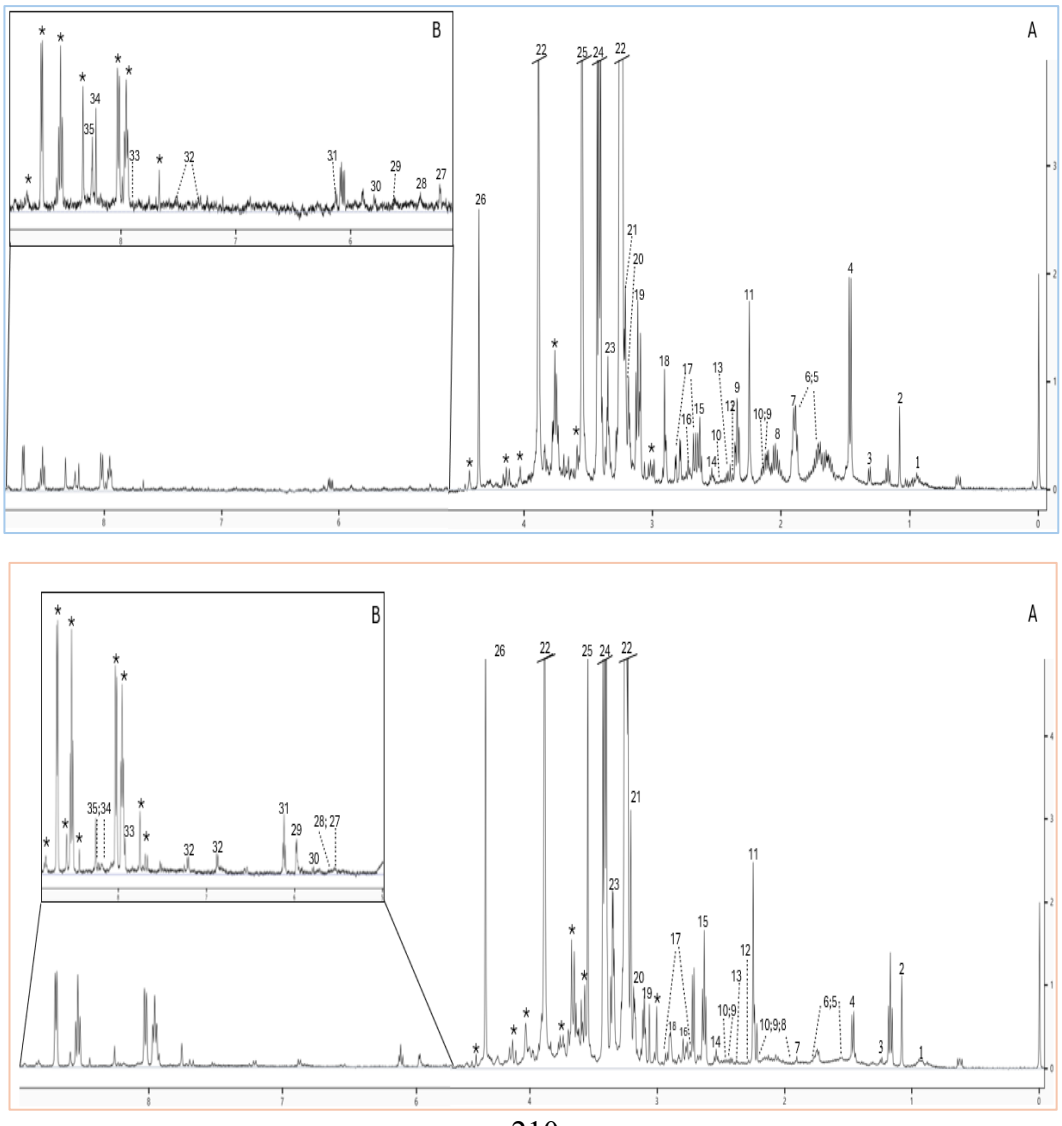

Figure 5.8 and Figure 5.9 Representative 1H 1-D 500 MHz NMR spectrum of the male (blue) and female (pink) gonads of control of *M. galloprovincialis* with (A) showing the aliphatic region and (B) a vertical expansion of the aromatic region. Keys: 1-BCAA (leucine, Isoleucine, Valine), 2-Mytilitol, 3-Lactate, 4-Alanine, 5-Arginine, 6-Lysine, 7- Acetate, 8- Proline, 9-Glutamate, 10-Glutamine, 11-Acetoacetate, 12-Pyruvate, 13-Succinate, 14-βAlanine, 15-Methionine, 16-Sarcosine, 17- Aspartate, 18-N,N-Dimethylglycine, 19-Malonate, 20-Choline, 21-O-Phosphocholine, 22-Betaine, 23- Theophylline, 24-Taurine, 25-Glycine, 26-Homarine, 27-Glucose, 28-Glycogen, 29-UDP Glucose, 30-Uracil, 31-ATP/ADP, 32-Tyrosine, 33-Histidine, 34-IMP, 35-NADH/NADPH, *-Unknown.

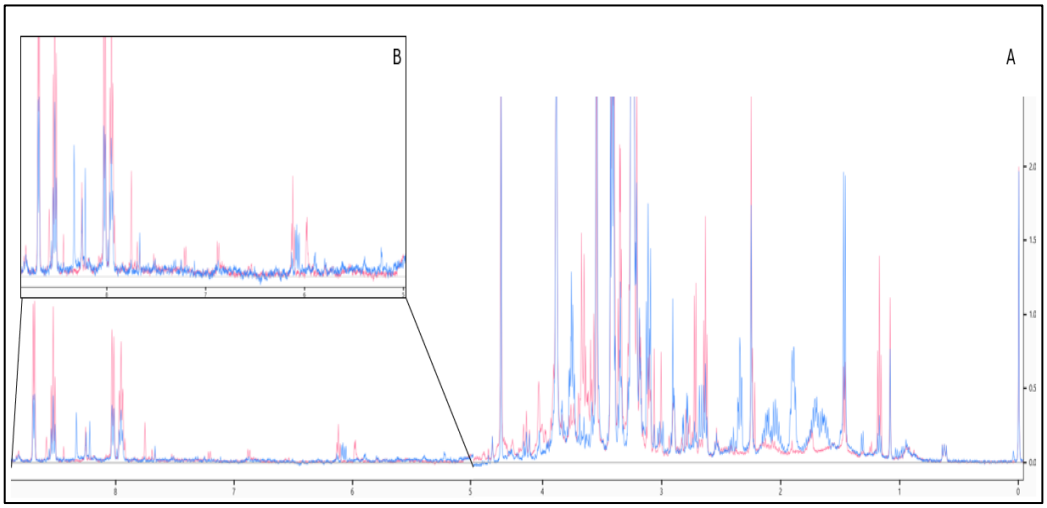

Figure 5.10 Overlapped 1H 1-D NMR spectra at 500 MHz of *M. galloprovincialis* representing male (blue line) and female (pink line) gonads to highlight differences in metabolite concentrations with (A) showing the aliphatic region and (B) a vertical expansion of the aromatic region.

Metabolomic analysis of gonadal sub-samples treated with PS MPs produced ^{1}H NMR-1D spectra for both sexes, showing some divergences compared to those obtained from mussels of the control group, highlighting how the micro-polymer exerted an action on both male and female specimens of *Mytilus galloprovincialis* concerning, above all, the energy pathway (glucose, glycogen, malonate) and protein turnover (BCAA, arginine). Below a summary table (Table 5.1) is reported in which the most significant variations in metabolites from mussel gonads that underwent changes following 48 h exposure to PS MPs (0.5 µg/mL and 1 µg/mL) are listed and expressed as percentage changes, measured with respect to the control group and grouped by metabolic pathway in which they are involved.

METABOLITES INVOLVED TO	CHEMICAL SHIFT AND PEAK SHAPE (ppm)	CHANGE IN %			
		0.5 µg/mL		1 µg/mL	
AMINO ACID METABOLISM		48 h		48 h	
Arginine	3.8 (t), 3.2 (t), 1.9 (m), 1.7 (m)	↑51	↓17	↓24	↓46
Glycine	3.6 (s)	↑3	↓27	↓18	↓57
Glutamate	3.8 (q), 2.4 (m), 2.1 (m)	↑7	↓49	↓24	↓71
Histidine	4.0 (m), 3.2 (q), 3.1 (m)	↑5	↓19	↓69	↓27
Isoleucine	3.7 (d), 1.0 (d), 0.9 (t)	↑19	↓18	↓38	↓43
Leucine	3.7 (m), 1.7 (m), 1.0 (t)	↑13	↓15	↓21	↓52
ENERGY METABOLISM					
Glycogen	5.4 (s), 3.8 (m), 3.6 (m), 3.4 (m)	↑930	↓49	↑71	↑150
Glucose	5.2 (d), 3.8 (m), 3.7 (m), 3.5 (m)	↑77	↓60	↓40	↑168
Malonate	3.1 (s)	↓35	↓44	↓55	↓62
OSMOREGULATION					
Betaine	3.9 (s), 3.3 (s)	↑6	↓11	↓20	↓57

Table 5.1 Summary table of the percentage changes (%) in response to the lowest (1 µm/mL) and highest (0.5 µm/mL) dose of PS MPs of the concentrations of the most significant metabolites in male (blue) and female (pink) gonads of *M. galloprovincialis* after a 48-h exposure in respect to the sex related control. (One ONOVA test, $p < 0.05$) s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet.

More in detail, in the male gonads of *Mytilus galloprovincialis* after 48 h of exposure (Figure 5.11: M), glucose levels, the primary for of cellular energy, underwent an alteration demonstrating a bell shape in its level with an increase at the lowest concentration (0.5 µg/mL) and a depletion at the highest one (1 µg/mL), in both cases significant. Instead, the female counterpart (Figure 5.11: F) showed an opposite response (inverted bell shape), equally significant, presenting a marked decrease in glucose at the lower concentration (0.5 µg/mL) and an important increase at the higher PS MPs concentration (1 µg/mL).

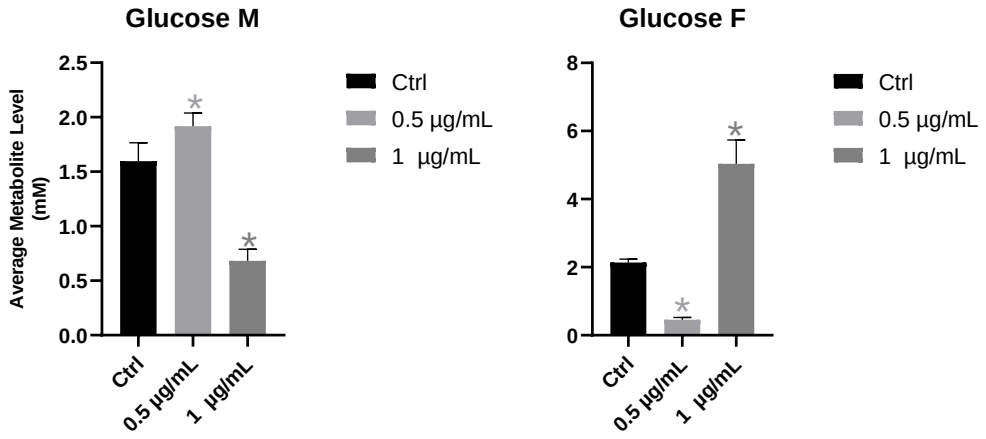

Figure 5.11 Graphs of concentrations of glucose (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Similarly, the energy reserves represented by glycogen, which are relatively low in the control groups of both sexes, have shown the same trend previously observed for glucose. Indeed, in males at the lower PS MPs dose, a significant and marked increase of glycogen was recorded, while the higher dose provoked its decrease (Figure 5.12: M). Contrarily, this trend was found to be inverted in female gonads, reflecting the same profile already observed for glucose in females (Figure 5.12: F).

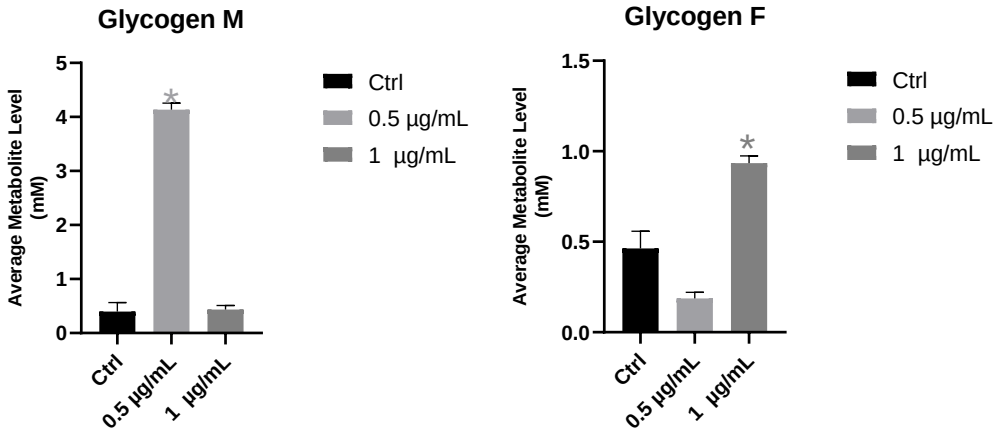

Figure 5.12 Graphs of concentrations of glycogen (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL)

and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

The trend of malonate was univocal between the two sexes, presenting a significant and dose-dependent decreasing trend (Figure 5.13: M and F).

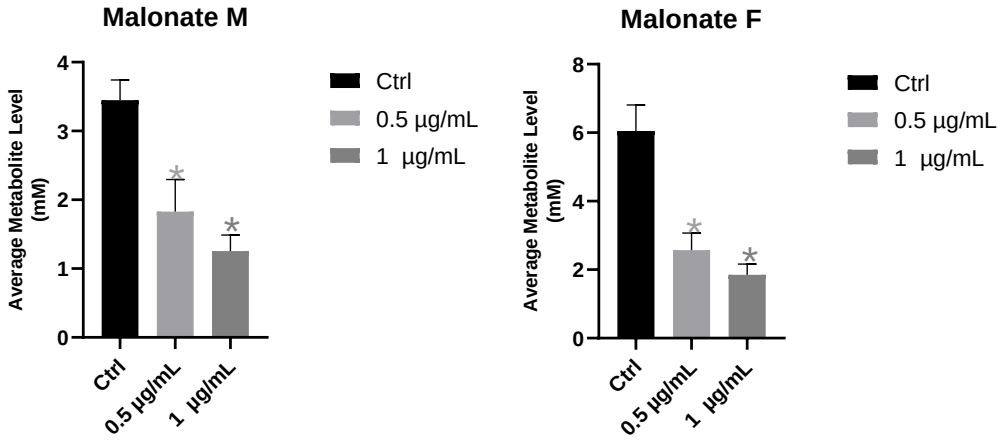

Figure 5.13 Graphs of concentrations of malonate (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Moreover, in male gonads the exposure to PS MPs influenced the metabolism of some amino acids such as BCAAs that, like other free amino acids such as arginine, showed a bell-shaped trend, significant for all the doses tested (Figure 5.14-15-16: M). In the female counterpart, however, it seemed that the gonads underwent a dose-dependent decrease in the same amino acids mentioned above, with $p < 0.05$ especially for the group exposed to the highest PS MPs concentration (Figure 5.14-15-16: F).

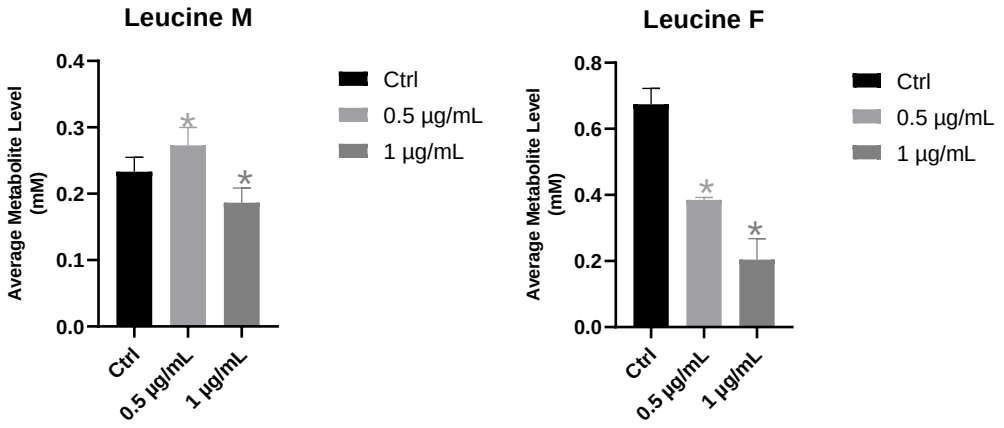

Figure 5.14 Graphs of concentrations of leucine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

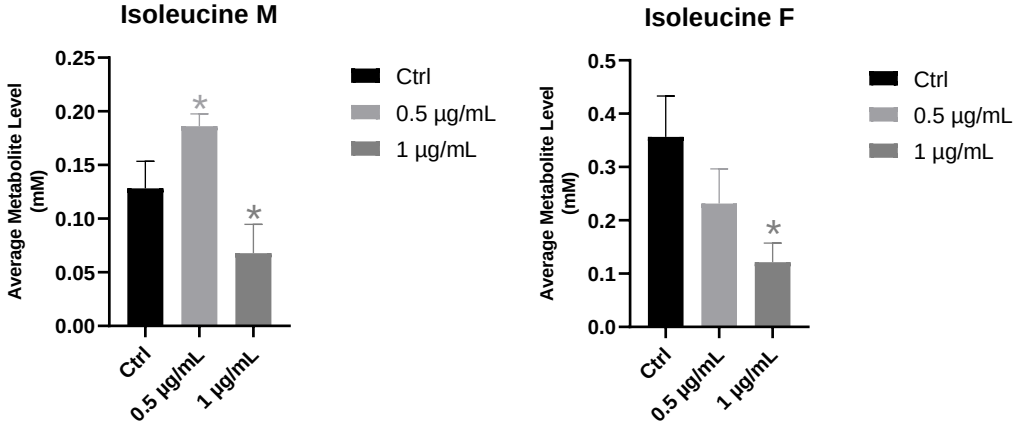

Figure 5.15 Graphs of concentrations of isoleucine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

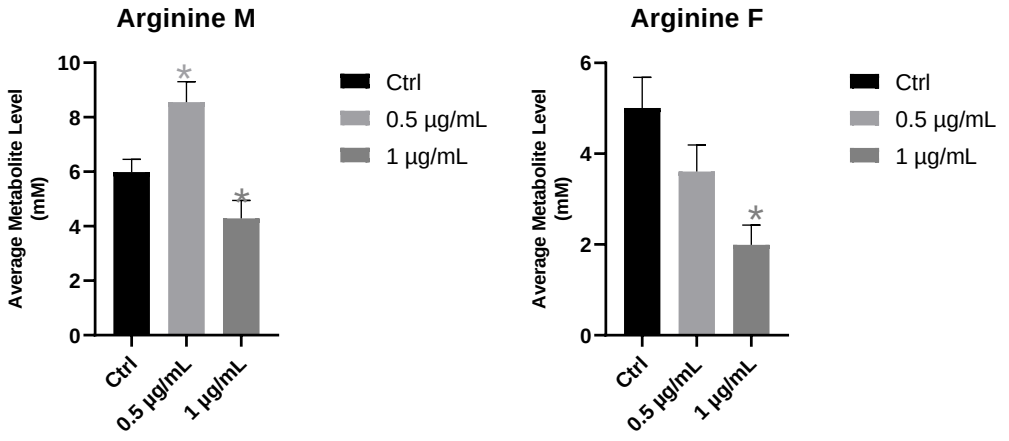

Figure 5.16 Graphs of concentrations of arginine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

As for organic compounds such as betaine, which also plays the role of osmolyte in the gonads, it is evident that males and females tend to respond differently to the two treatments (Figure 5.17: M). In fact, in males a non-monotonic reaction was recorded with an increase of betaine at the lowest dose of 0.5 µg/mL PS MPs and a decrease at the highest dose of 1 µg/mL, both significant, while in females a monotonic and dose-dependent response was recorded represented by a significant decrease in its level (Figure 5.17: F).

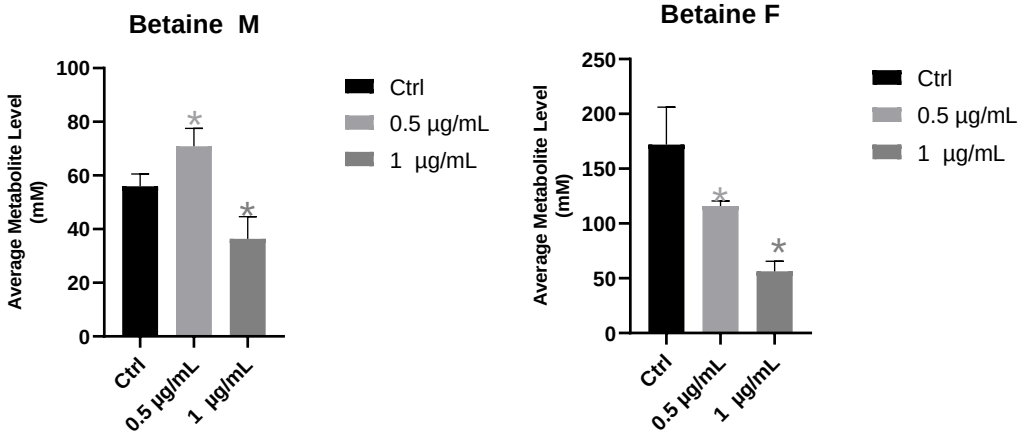

Figure 5.17 Graphs of concentrations of betaine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and

high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

From the analysis of polar metabolites of both sexes it emerged that glycine and glutamate, two amino acids constituting the tripeptide glutathione (GSH), also underwent a consistent alteration in males, resulting in a not significant increase at the lowest dose of PS MPs and significantly decreased at the highest dose compared to the control (Figure 5.18-19: M). Conversely, female gonads responded with a significant dose-dependent decrease (Figure 5.18-19: F).

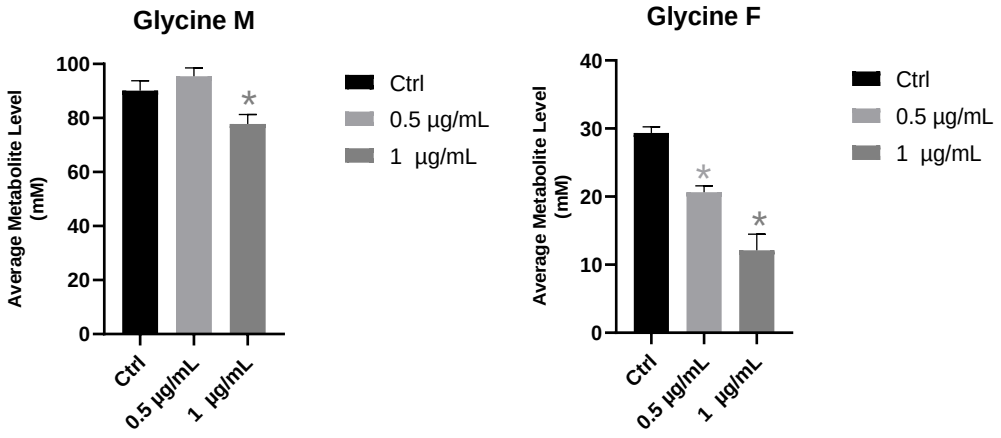

Figure 5.18 Graphs of concentrations of glycine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

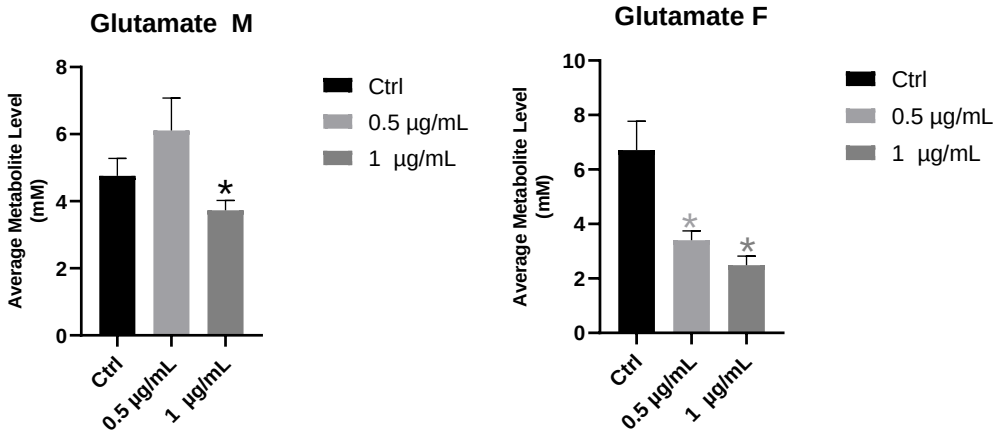

Figure 5.19 Graphs of concentrations of glutamate (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

The amino acid histidine, a constituent of the detoxifying peptide ovothiol, appears to have undergone a depletion following exposure to PS MPs, which is detected more markedly in female gonads (Figure 5.20: F) than in male (Figure 5.20: M) counterpart, since a decrease in the amino acid occurs already at the lowest concentration and it is only significantly relevant for the highest concentration in both sexes.

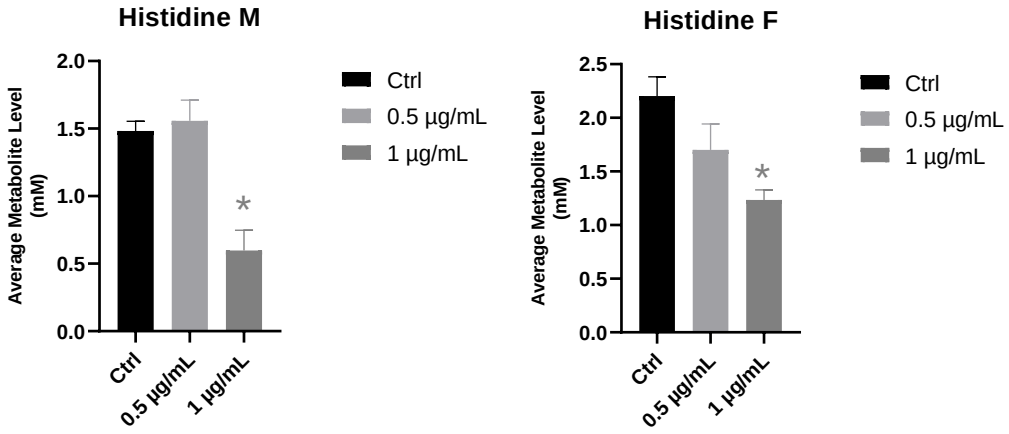

Figure 5.20 Graphs of concentrations of histidine (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

Choline, a constituent of membrane phospholipids and of the neurotransmitter acetylcholine, showed a similar trend in both males and females, depicting a significant dose-dependent depletion in respect to the group of control (Figure 5.21: M and F).

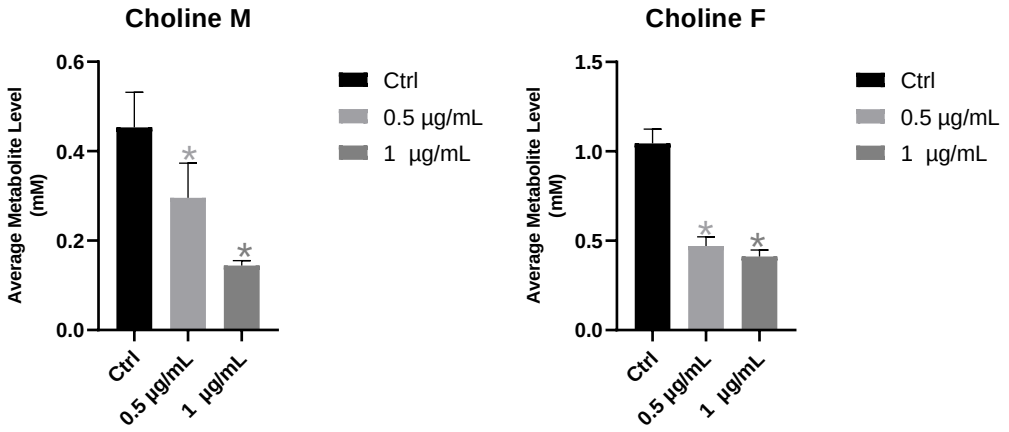

Figure 5.21 Graphs of concentrations of choline (mM, expressed as means) in male (M) and female (F) gonads of the mussel *M. galloprovincialis* after 48 hours of exposure to a low (0.5 µg/mL) and high (1 µg/mL) concentration of PS MPs and control group (Ctrl). Significant differences compared to the control (One ANOVA test; $p < 0.05$) are indicated with asterisks (*).

5.4 Discussions

Today's world is a human-scale world of plastics, so what in the late years of the 19th century had been originated as a semi-synthetic polymer as a prelude to innovation, practicality in the 21st century has become a worrying reality that harms many ecosystems. Due to the not always suitable capacity of WWTPs to retain these plastic materials (Xu et al., 2023), illicit deposits, and the worsening caused by the spasmodic use of disposable personal protective equipment to deal with the SARS CoV-2 pandemic, rivers, seas and oceans have become the collection basins of these materials whose presence is widely distributed from the surface areas to the water column reaching the sediments (Han et al., 2024; Sharma et al., 2024). Thus, plastic in all its forms (macro, meso, micro and nano) and qualities (PS, PVC, etc.) holds 85% of the contribution in the scale of marine pollutants (UNEP, 2021).

Polystyrene is one of the most widely used polymers for packaging, construction, etc. as it has peculiar properties such as rigidity, thermoplasticity that make it performant in many industrial sectors (Turner et al., 2020). Due to its difficult biodegradability (Liu et al., 2023), it is one of the most widespread and more abundant polymers found in the environment (Ullah et al., 2023), mainly the

coastal areas (Han et al., 2024; Chan & Not, 2023). The impact of the polymer in the form of microparticles can be found both on the abiotic component (physical and chemical environment) and on the non-target biotic component, so it is essential to understand its biological impact, especially considering possible consequences on reproductive health of biota, seeking information at both the tissue and cellular level (Dong et al., 2024; Gupta et al., 2023; Qiang & Cheng et al., 2021).

Therefore, the aim of this research project developed in collaboration with the University of Naples Federico II was aimed at evaluating the potential biological effects induced by PS MPs at the level of the reproductive system of male and female organisms of the marine mussel *Mytilus galloprovincialis*, a gonochorous species with important ecological value in the construction of coastal ecosystems and socio-economic value within the global food chain (Lopez et al., 2023). To address this aim, a multi-biomarker approach including histomorphological, histochemical and metabolomic techniques was applied on the selected model organism at the level of gonads after a short-term exposure of 48 h of mussels to two different doses of PS MPs. The approach used successfully demonstrated how males and females of mussels can respond early to an external stress, implementing different physiological strategies based on their phenotype. This further accredited the entire thesis work supporting the original idea that a xenobiotic can elicit biological responses dependent on sex, and that these can also diverge based on the reproductive period of the animal under investigation (Dong et al., 2024; Murano et al., 2023).

The fact that the damage found in the gonads of both sexes is directly related to the presence of microplastics is accompanied by the awareness that a sessile and filter-feeding organism such as *M. galloprovincialis* was able to internalize already after 24 h of exposure PS MPs of similar size (3 μm) both in organs more in direct contact with the aqueous medium, such as the gills, and in those more internal, such as the digestive glands (De Marco et al., 2023; Cappello et al., 2021; Wei et al., 2021).

This is in line with the data herein obtained from the histo-morphological analysis of mussel gonads, which attested the interference of the contaminant by demonstrating an alteration of the male follicle organization after a very short time (48 h) highlighting, already from the lowest PS MPs concentration (0.5 μg/mL), a

disorganization of the spermatic cells inside the lumen associated with the disconnection of the germinal cells from the wall, a condition that worsened in a dose-dependent manner determining a disintegration of the order of the cells located more in the periphery that grouped together in rosette structures. The malformations highlighted by the H/E colorimetric method could be ascribed to a purely mechanical cause, due to the steric hindrance exerted by the size of the PS MPs once internalized and absorbed by the tissue, since multi-organ studies have described how, depending on the size, the PS MPs can be more easily internalized by one tissue than another and compromise it (Zhang et al., 2024). This hypothesis is supported by the finding of 10 μm PS MPs within the ocular apparatus of zebrafish embryos where retinal detachment was evidenced (De Marco et al., 2022). This could explain how the increased concentration of particles at the 1 μg/mL treatment caused greater damage than 0.5 μg/mL, given the greater bulk exerted by the higher quantity of MPs. It is also likely that the detachment of germ cells from the seminiferous epithelium in male mussels was the result of a perturbation of the cellular architecture affecting the tight junctions, as demonstrated at the level of the blood-testis barrier of mouse models exposed to PS MPs in which a down-regulation of genes for connexin 43 and occludin was recorded (Xu et al., 2024; Hassine et al., 2023).

Even in the female gonad, after both PS MPs treatments, some alterations occurred, more moderate however compared to the male counterpart. In fact, while maintaining a good structural integrity unlike the males, the presence of some degenerating oocytes was evident, the number of which increased in relation to the dose, a sign of a probable apoptosis event. This could be consistent with the pro-inflammatory state that the PS MPs induced, in fact a study on rat gonads highlighted that PS-MP could, due to the increase in ROS, promote pyroptosis and/or apoptosis at the level of the ovarian granulosa with NLRP3/Cas1 signaling mechanisms (Hou et al. 2021). In fact, the confirmation at the level of the gonadal tissue of both sexes, of the direct influence of the contaminant on the development of an inflammatory state was the manifestation of an early immune response represented by haemocyte populations along the connective tissue. The implementation of an innate mechanism and the haemocyte recall is typical of invertebrates, and it is particularly rapid in the species *M. galloprovincialis* when

exposed to contaminants and specifically to PS (Gonçalves et al., 2023; De Marco et al., 2023; Impellitteri et al., 2022; Auguste et al., 2020; Sendra et al., 2020). The presence of an inflammatory condition that occurs in a short time frame was confirmed by metabolomic analysis. In fact, the metabolism of glutathione (GSH) in both male and female gonads, albeit in different ways, underwent changes following the two treatments that were reflected in the variation of metabolites such as glycine and glutamate, which are two precursor amino acids for the synthesis of the non-protein antioxidant (Sokolova et al., 2024). The tripeptide together with the GST enzyme play a fundamental role in phase II of the biotransformation mechanisms to counteract the stress induced by an oxidative condition, conjugating harmful molecules with GSH to produce fewer toxic products (Capo et al., 2021; Zhang et al., 2021b). An alteration of the building blocks of GSH may be a sign of a dysregulation of this process that could impact the redox balance of the gonad and cause serious damage to the functional state of the tissue and a transgenerational effect, as demonstrated for other organisms exposed to PS MPs (Sun et al., 2022; Wang et al., 2021; Luo et al., 2019). It is typical in the genus *Mytilus* that exposures to PS MPs (2 μm) lead to destabilization of the redox balance and the production of ROS (Huang et al., 2021). Sussarellu et al. (2016) confirmed that long-term exposure to PS MPs between 2 and 6 μm at 10, 10^4, 10^6 particles/L resulted in a negative effect on the reproductive health of oysters. Overall, high doses of PS MPs induced a significant decrease in both gonads of the two metabolites, which could underlie their mobilization for the formation of GSH, as a greater turnover of the molecule is necessary to cope with the oxidative stress commonly induced by this type of contaminant that, in fact, is able to induce a high production of ROS at the level of the gonads of males and females, even at the expense of gametic cells (Mottola et al., 2024; Sun et al., 2022; Qiang & Cheng, 2021).

It is also possible that at the gonad level of both sexes, the detoxifying mechanisms are also supported by other molecular structures, such as ovothiol. The two amino acids cysteine and histidine are conjugated by the enzyme Ovo A (iron-dependent sulfoxide synthase II) to form a molecule with non-enzymatic activity to detoxify from the H_2O_2 species (Diaz de Cerio et al., 2020). In this study, a depletion of histidine was noted more for females, already from the lower concentration, than

for males, indicating a probable sign of the construction of new ovothiol molecules to cope with the stress induced by ROS.

Further attesting to the perturbation of the redox state in the male gonad is the fact that, thanks to the little percentage of carbohydrates within the "decay pigment" (so called because it is proportional to the cellular age), PAS positive granular deposits were found, which indicated the presence of lipofuscins. It is known that PS MPs can create ROS, which in turn can attack the unsaturated lipids constituting the membranes, modifying them and producing as many reactive substances that interact with denatured proteins constituting molecular scaffolds such as lipofuscins (Saraceni et al., 2023; Wu et al., 2023).

Literature reports that for marine bivalves, exposure to MP can cause an alteration of the feeding method (Wang et al., 2020), inducing a sense of satiety that is not true as they discriminate food intake based on size and not quality. The reduction of energy intake could be approached by a revision of the bioenergetic plan that would be redirected towards protective effort, with the use of energy to extrude the xenobiotic from the organism (Opitz et al., 2021; Green et al., 2019). In fact, it has been demonstrated that in *Mytilus coruscus* both exposure to single PS MPs and that combined with *Prorocentrum lima* lead to the alteration of the metabolism of ATP-binding cassette (ABC) transporters with the increase of gene expression aimed at improving the detoxification mechanism (Tang et al., 2022). In this perspective, the idea that the short-term response involves a greater energy requirement to satisfy defence strategies arises, and this is in line with the evident modulation of glucose, the first energy source to be made available in response to stress. As confirmed by Cappello et al. (2021), glucose in the metabolic extracts of the digestive glands of *M. galloprovincialis* tended to undergo an increase already after 24 h of exposure to polystyrene MPs, acquiring the highest value after two days of treatment (48 h), in line with what was highlighted in this study at the lower concentration of PS MPs for males. As stated in the reported study (Cappello et al., 2021), the increase in glucose could have been caused by an early gluconeogenic mechanism, implemented to cope with the high energy demands to implement defences and to support the last reproductive effort aimed at releasing gametes into the external environment (Hassan et al., 2018). Furthermore, it is also likely that the high glucose accumulation is a strategy to ensure the success of external

fertilization, mostly for a species like *M. galloprovincialis* in which a high rate of maintenance of sperm mobility is required. This depends on the content of ATP, and therefore on the substrates that provide it such as glucose and glycogen, produced both by the glycolytic pathway and by that of mitochondrial oxidative phosphorylation at the level of the head and flagellum of spermatozoa, as supported for *M. edulis* (Kong et al., 2024).

Instead, in female mussels exposed to the same concentration of PS MPs (0.5 μg/mL) an opposite event occurred with a decrease in glucose, as shown in other species (Ye et al., 2021). In this regard, it is reasonable that the deposition of gametes, especially for the female sex that supports the growth of larger cells with a considerable glyco-lipo-protein content, constitutes a stress event for the genus *Mytilus* with consequent expenditure of energy reserves (glucose and glycogen) and proteins (Mredul et al., 2024). Otherwise, as supported by Sussarellu et al. (2016), it is also plausible that, depending on the needs of the entire organism, energy may be redirected to activate and maintain high the defence mechanisms. The high variability in the level of glucose recorded between the two sexes as well as the concentrations of PS MPs tested, defines a divergence that may be the result of a hormetic event. This is typical in bivalves, such as it was previously documented in the Australian oyster *Saccostrea glomerata* following exposure to single polyethylene MPs and in combination with estrogenic mixtures, or in *M. galloprovincialis* exposed to heavy metals (Kumar et al., 2024; Lettieri et al., 2023).

Subjected to a xenobiotic stress, the species *M. galloprovincialis* can increase the cellular content of glucose, as well as of the intermediate products of the Krebs cycle (Paul et al., 2024), and also of glycogen as a strategy to counteract the possible persistence of unfavourable conditions. A study in mice has shown that polystyrene plastics can regulate glucose and lipid metabolism, inducing during a short-term exposure an increase in serum glucose and glycogen levels (He et al., 2024). Furthermore, 5 μm PS MPs can, in the liver of zebrafish, disturb glucose metabolism by altering the expression of some genes such as *pyruvate kinase* and *glucokinase* (Zhao et al., 2020). Glycogen plays a fundamental role in the gametogenic process (Gabbott et al., 1979), that may be found at low level in mussels during the reproductive period (Duinker et al., 2008; Zandee et al., 1980; De Zwaan & Zandee, 1972). Thus, it could be possible that the male gonads respond

to the lower dose of PS MPs by increasing the reserve that will be used to support the mobilization of spermatozoa in the aqueous medium (Kong et al., 2024). Conversely, the behaviour recorded in female gonads took a divergent trend with a decrease of the metabolite, similarly to what reported by Shang et al. (2021) on *M. coruscus* challenged with PS MPs. Indeed, the exposure caused a decrease in energy reserves due to the suppression of filtration rate and energy assimilation, and so potentially altered the carbohydrate reserves at the level of the connective tissue cells (VCT and ADG), a sign of the greater sensitivity to xenobiotics of females compared to males. The biphasic trend in the level of glucose and glycogen observed in mussel males and females would seem to depend not only on MPs. In fact, the two metabolites behave in unison within the phenotype but diverging between sexes. This supports the fact that like other pollutants such as drugs, MPs can induce hormetic events, in this case also influenced by sex (Agathokleous et al., 2022; Sun et al., 2021).

The finding of a unique dose-dependent decrease in malonate, known inhibitor of the enzyme succinate dehydrogenase (Kim, 2002), in the gonads of both sexes supports the idea that the aerobic pathway has been hindered and that a subtraction of the metabolite to be used as a substrate by the enzyme succinate dehydrogenase (Lisi et al., 2013) leads to an impediment of the normal tricarboxylic acids cycle, as also reported at the level of the digestive glands of *M. galloprovincialis* exposed to PS MPs (Georgoulis et al., 2023; Cappello et al., 2021). The divergence between male and female biological responses at the metabolomic level could be due to a greater or lesser reactivity of one sex compared to the other as well as to the different physiological functions between the two types of tissues (Kumar et al., 2024; Zhang et al., 2023) and the reproductive phase as found for the species *M. galloprovincialis* (Blanco-Rayón et al., 2020; Zhong et al., 2020).

In males it was evident that the insult exerted by PS MPs caused a higher energy demand at the lowest concentration, which would lead to explain how there was a high concentration of arginine. Arginine can be interpreted as the product of the dephosphorylation of the high-energy compound phosphoarginine which, by reacting with ADP, leads to the formation of arginine and ATP (Yang et al., 2020), a strategy that coincides with what was previously described as occurring in the male gonads, where ATP is of fundamental importance in the mobility of

spermatozoa preparing to be emitted (Kong et al., 2024). Instead, the decrease in the metabolite, which in females occurred already at the lowest concentration, could be the sign of a strong bioenergetic imbalance or the implementation of compensatory strategies typical of the gender to cope with the greater stress (Barrett et al., 2022; Seuront et al., 2021), to which females seem to be more susceptible or an attempt to reserve energy for future offsprings by forming molecules with a higher energy level, playing a role in the metamorphic process in the genus *Mytilus* (Zhu et al., 2023).

The increased presence of branched-chain amino acids (BCAAs) is a sign in bivalves exposed to high stress of an intense protein turnover (Chen et al., 2021) or of a suppressed protein synthesis due to the presence of MPs (Shang et al., 2021), an event that occurred in *M. galloprovincialis* after short-term exposures to the same quality of microplastics used in this study, but in another storage organ such as the hepatopancreas (Cappello et al., 2021). Instead, in a study on the same species, exposure to MPs induced a depletion of BCAAs, which have important roles in the regulation of nutrient metabolism and energy homeostasis, being leucine and isoleucine ketogenic and gluconeogenic amino acids, respectively (Park et al., 2024). This was in line with what occurred in female gonads at the same concentration (0.5 μg/mL) and for higher (1 μg/mL) doses in both sexes. Overall, the results of the metabolomic analysis highlighted how PS MPs could interfere with carbohydrate and protein metabolism in a divergent way between the two sexes of the Mediterranean bivalve. As for other classes of pollutants, this could have depended on the stage of differentiation and cellular differences between male and female gametes that, in the reproductive period during which the experimental trial occurred, occupy most of the gonadal tissue to the detriment of the connective tissue (Peng et al., 2023; Ramirez et al., 2022; Duinker et al., 2008). Given the coordinated action only at the highest dose between male and female cellular responses, it is possible to hypothesize that females are more easily affected by the action of microplastics and that already from the lowest concentration tested they feel the blow by more ferociously destabilizing the metabolic balances within the gonads, while males seem more resilient by responding to stress in a compensatory way but only at lower doses.

This attitude is proven by the trend of the organic osmolyte betaine. Alterations of the normal physiological condition in the gonads due to a stressed environment such as plastic can lead, together with genetic anomalies, to the alteration of the gametes. Therefore, having an impact on the fertilization process, even if the mechanisms are specific to the species, comparative studies show that in general there are some points in common (Gallo et al., 2020). As for other pollutants, such as dexamethasone (Chapter 4), in mussel males an accumulation of betaine was highlighted, at the lowest concentration, as a probable sign of hyperosmotic stress that must be compensated in a tissue in which osmolarity plays an important role in the motor activity of sperm cells (Boulais et al., 2019). Furthermore, this increase, considering also that of glutamate at the same concentration, can be interpreted as a further confirmation of a compensation attempt that is more marked in males than in females since betaine, in addition to being involved in osmotic regulation, plays other roles within the cell as a donor of methyl groups to synthesize homocysteine. Thus, these parameters underline an overregulated glutathione synthesis to maintain the antioxidant state, as reported in *Crassostrea gigas* (Paul et al., 2024). Conversely, at the higher dose of PS MPs and in a dose-dependent manner for females, a marked decrease in osmolyte was observed in line with what reported for the same mussel species after 48 h of exposure to PS MPs (De Marco et al., 2023), suggesting a sign of a further mode of adaptation to high concentrations of the pollutant to maintain the integrity of the cellular volume.

Choline is one of the products of the reaction of the enzyme acetylcholinesterase (AChE) on the cholinergic neurotransmitter acetylcholine which, in fact, splits into choline and acetate (Wang et al., 2023). According to the works of several authors, AChE inhibition is considered a biomarker of neurotoxicity in ecotoxicological studies on both invertebrates (Cappello et al., 2018; Ciacci et al., 2012; Tsangaris et al., 2010) and marine vertebrates (De Domenico et al., 2013) because it can compromise the normal function of nervous signal transmission. The study by Ma et al. (2023) on the gonads of *Mytilus unguiculatus,* confirmed at the genetic level the presence of *nicotinic acetylcholine receptors* (AChRs) in invertebrates. AChRs belong to a family of acetylcholine-dependent cation channels and are similarly expressed in molluscs in various areas of the body. This supports the hypothesis of the involvement of acetylcholinesterase

(AChE) in many crucial functions, from frontal ciliary movement in the gill epithelium to embryonic development. The results of the study would suggest that AChRs are involved in the regulation of the neuroactive ligand-receptor interaction pathway in male *M. unguiculatus*, highlighting their contribution to gonadal development in this sex. This would provide further insight into the understanding of the neuroendocrine pathway at the gonad level of *Mytilus* species, supporting the possibility that some metabolites may act as a key to understanding the transduction of environmental information at the level of gonadal development (Ma et al., 2023). An idea that finds support in studies on vertebrates, where castration of male mandarin finches *Taeniopygia guttata* leads to a reduction in AChE activity and a 40% decrease in the number of acetylcholine receptors (AChR) (Bleisch & Luine, 1981; Luine et al., 1980). The lower presence of choline in both gonads compared to control organisms could highlight a malfunction of the AChE enzyme that would not be able to metabolize acetylcholine. Eom et al. (2020) suggested that PS MPs in a range between 1 and 10 μm can cause the inhibition of the AChE enzyme in the early life stages of the brine shrimp. Another study on gills of *M. galloprovincialis* during a short-term exposure to 3 μm PS MPs reported an acetylcholine deficit at branchial level, which would demonstrate the alteration of the neuronal signalling pathway by this polymer of microplastics (De Marco et al., 2023).

5.5 Conclusions

In conclusion, the results obtained from this study showed how in the intertidal species *Mytilus galloprovincialis* the male and female gonadal tissues are uniquely affected by the influence of both concentrations of polystyrene microplastics tested during acute and short-term exposure (48 h). The picture of a pro-inflammatory state was confirmed by the early immune response found in the connective tissue of both phenotypes.

Furthermore, it was possible to observe how the male organisms tried, at least at the lower concentration of PS MPs tested, to implement compensatory strategies, showing a divergence in the interpretation of the biological responses compared to the female counterpart, to which mussel males tended to conform at the higher dose tested.

The alteration of the energy pathway (glucose and glycogen) accompanied by that of the amino acid metabolism (BCAA and arginine) highlighted how PS MPs can interfere with the normal bioenergetic process, soliciting a greater demand for energy, in a particular reproductive period such as the one preceding the deposition of the gametes. This could interfere not so much with the maturation of germ cells, but with the vitality of gametes that must face the challenges of external fertilization (Kong et al., 2024). Added to this picture is the disturbance of osmotic balance, another piece of fundamental importance for the success of fertilization (Boulais et al., 2019). Overall, it can be stated that in the non-target species *M. galloprovincialis*, even PS MPs can exert an endocrine disrupting action in both sexes, causing degeneration events that could threaten the functionality of the reproductive system. It is true that the doses used are off the natural scale, but they are realistic enough to describe a potential anthropic scenario that with the continuation of economic development could become an everyday reality.

The aim of this study was also to draw attention to the impact that the anthropogenic activities, such as those occurring close to the coastal areas of large urban centres where WWTPs wastewater, tributaries of urban, sanitary and industrial wastewater, also flow into, could have on the environment and human health.

5.6 References

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CHAPTER 6

Biological responses of Mytilus galloprovincialis gonads exposed to polylactic acid microplastics (PLA MPs)

CHAPTER 6

Biological responses of *Mytilus galloprovincialis* gonads exposed to polylactic acid microplastics (PLA MPs)

6.1 Introduction

As discussed in the previous chapter, today the environment, due to the SARS CoV-2 pandemic, the state of pollution, and the reduced capacity of wastewater treatment plants (WWTPs) to support the pressure of various contaminants, is facing a serious problem represented by primary microplastics (MPs; directly produced as such, present in cosmetic and personal care products) and secondary MPs (obtained from subsequent degradation of the original product) (Akdemir & Gedik, 2023). It should be emphasized that plastic in all its declinations (macro, meso, micro and nano) on the globe has the tendency to concentrate in specific areas, such as along coastal areas, at the intersection of ocean vortices and in semi-closed basins such as the Mediterranean Sea (Anderson & Shenkar, 2021), representing a threat reluctant to disappear for these ecosystems.

Although it is not a new topic, the idea of finding a sustainable alternative to plastic in recent years has been making waves in public awareness, so much so that the development of green and bio-based materials has found wide consensus (Mangal et al., 2023) (Figure 6.1). Indeed, the so-called bioplastics (BPs) are finding a wide use in everyday life proving, like conventional plastics, to be a material of ductile use in packaging, manufacture of toys, pharmaceutical industry, biomedical applications, cosmetics, agricultural and electronic sectors, and in basic and applied research (Ahmad et al., 2024; Kong et al., 2023; Venkatachalam & Palaniswamy, 2020).

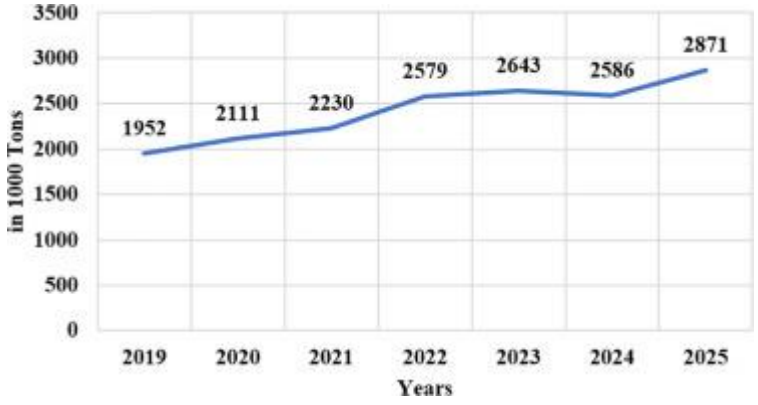

Figure 6.1 Global bioplastic production between the years 2019-2025 (Mehmood et al., 2023).

Furthermore, preferring a biodegradable material is also advantageous for the environment, given that the disposal of polymers of fossil origin involves high economic resources and the emission of products such as carbon dioxide and methane, the so-called greenhouse gases. Additionally, it has been proven that after the incineration of plastic products, the resulting microplastics are still dangerous, because they can settle in the form of particulate matter in the air and be inhaled (Swetha et al., 2023a, 2023b; Shen et al., 2021), while the use of renewable material such as that of vegetal origin (starch, gluten, etc.) would support the reduction of the carbon dioxide intake since the one emitted to process the product and for recycling would be compensated by the one absorbed by the growth cycle of the plants used (Ibrahim et al., 2021; Shah et al., 2021). It follows from this that adapting to a new lifestyle, implementing the use of bioplastics or, even making a transition to biobased plastics, would be beneficial in socio-economic terms, as well as for the health of the Earth ecosystem (European Bioplastics e.V, 2022).

However, what is called bioplastic is not always a biodegradable and/or compostable product since this label refers to various types of compounds, including those of biological origin and non-biodegradable, of biological origin and biodegradable, or of fossil origin and biodegradable such as poly-caprolactone (PCL) (Swetha et al., 2023b) (Figure 6.2).

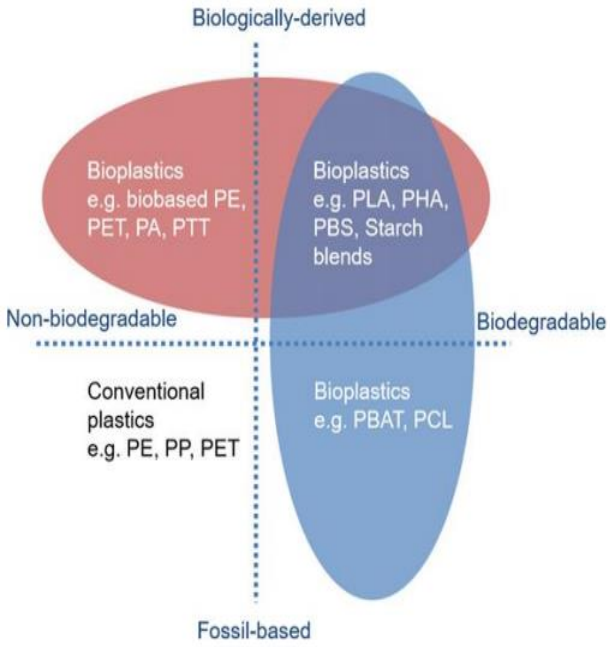

Figure 6.2 Classification of the biodegradable polymers (Shah et al., 2021).

The ability of these promising polymers and/or copolymers to be biodegraded depends both on their physico-chemical composition and rheological properties, and on the conditions of the surrounding environment in which the decomposition process should take place (Post et al., 2024). Bioplastic processing can occur in both aerobic and anaerobic conditions (Folino et al., 2020) and is divided into steps including bio-deterioration (Urbanek et al., 2021) operated by the enzymatic action of microorganisms on the surface of the product, bio-fragmentation always by the microbial component but that determines breakage and decomposition into smaller structures (smaller polymers and copolymers, and/or monomers) (Cao et al., 2024), and then assimilation by which the polymers and/or monomers obtained from the previous process are assimilated by microorganisms and metabolized into intermediates of carbon metabolism to obtain their mineralization into carbon dioxide, water and biomass (Barbe et al., 2024). These are processes exploited by humans at industrial level but that can also occur in nature, and which are influenced by biotic (microorganisms) and abiotic factors such as pH, temperature, humidity and oxygenation (Zoungranan et al., 2020).

Over the years, four main types of polymers have been created, among which there are those of natural origin derived from proteins (casein, gelatin, etc.), from polysaccharides (starch and cellulose), from the synthesis of resources of biological origin (polylactic acid, PLA; polyhydroxybutyrate, PHB; etc.), and from petroleum but biodegradable (aliphatic and aromatic polyesters, PCL, etc.) (Ahsan et al., 2023; Butnaru et al., 2021). Among these, PLA derived from renewable sources (such as starch, glucose, lactose and/or maltose) has made its way into the overview of bioplastics, demonstrating adaptable capabilities to various sectors (biomedical, electronic, packaging, agricultural, etc.) (Figure 6.3).

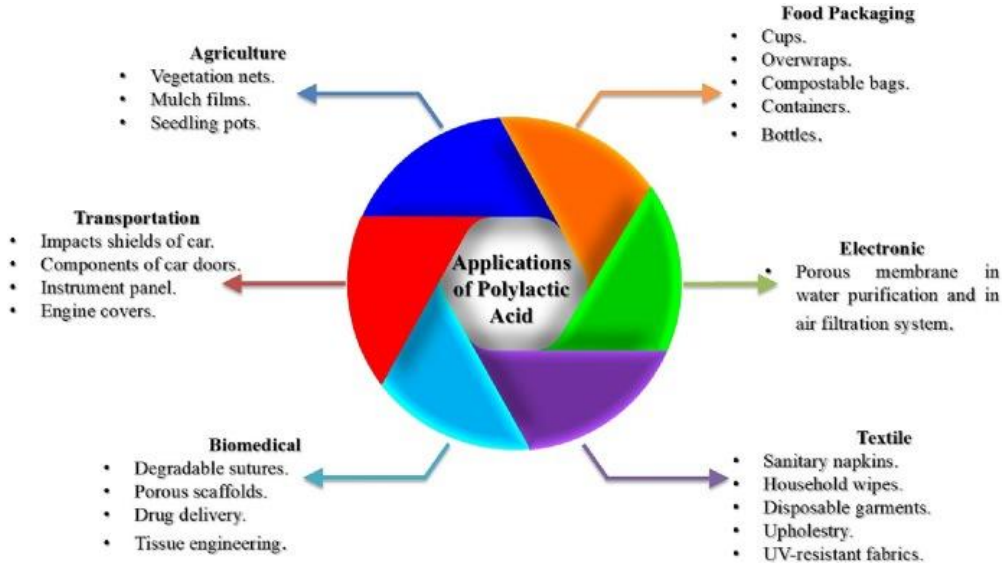

Figure 6.3 Areas of use of polylactic acid (Swetha et al., 2023).

Polylactic acid or polylactate is a thermoplastic polymer that occurs in two enantiomeric forms, such as L-polylactate and D-polylactate, although the first configuration is the most produced by fermentative synthesis processes (Li et al., 2020). It derives from lactic acid (2-hydroxypropionic acid) obtained from the microbial fermentation of raw materials (corn, sugar cane, milk, cotton) with a high percentage of metabolizable sugars by strains of the genus *Lactobacillus* (*L. casei* and *L. acidophilus*), *Lactococcus* (*L. lactis, L. acidophilus*, etc.), *Streptococcus* (*S. salivarius* subsp.) and *Thermophilus*. Industrially, lactic acid is obtained after fermentation, producing calcium lactate which, after filtration, treatment with carbon and acidification, is hydrolysed, esterified, distilled and hydrolysed again to obtain the monomer constituting PLA. To produce the polymer, a cyclic synthesis

process of the lactide is required, which can occur either “in two phases” whereby the lactic acid molecules undergo a polycondensation process at high temperature (130 °C) that leads to the formation of intermediates to be depolymerized at higher temperatures and low pressure to obtain the dimeric ring of the lactide or “in the gas phase” that involves the use of catalysts in a flow of inert carrier gas or even “in the liquid phase with single passage” whereby the water is eliminated during the closure of the ring, therefore the lactide is synthesized by condensation rather than transesterification from an aqueous solution of lactic acid (Balla et al., 2021).

Taking advantage of the good mechanical resistance, the amorphous structure and the flexibility, an estimate made on the production of PLA on the world market sees a growth rate between 2021 and 2028 of 18.1% with turnovers of millions of dollars per year so much so that it has been attributed the title of "polymer of the 21st century" (Ahmad et al., 2024; Mehmood et al., 2023; Swetha et al., 2023; Folino et al., 2020). Also, it has proved to be a worthy substitute in terms of "green economy" of the fossil-based counterpart, due to the high degradation rates in industrial composting processes that involve conditions of high humidity, temperatures above 60 °C, acid pH (which favour autocatalysis) and the presence of oxygen (Chamas et al., 2020). In general, industrial degradation processes are like those that can occur in natural environments, such as hydrolytic, thermo-oxidative, microbial, enzymatic, chemical and photo-induced events that induce chain scission reactions which, however, in nature are temporally longer due to the variability of the chemical-physical-biological conditions (Zaaba & Jaafar; 2020) (Figure 6.4).

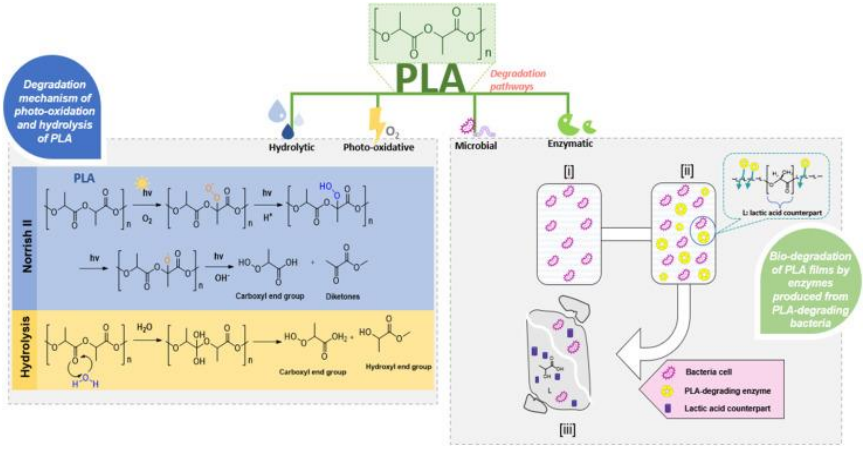

Figure 6.4 Degradation processes of polylactic acid (Ainali et al., 2022).

In anticipation of its greater presence in everyday life, the hypothesis of a consequent tendency to increase the production of waste of this material is possible. A study reported that PLA films after 42 days of burial in sand showed limited degradation events that affect the most superficial part of the artifact without influencing the chemical structure even after 267 days, demonstrating that the polymer is not very predisposed to biodegradation in sandy sediments due to its excessive stability. This highlights the slow degradation process of PLA, mainly when not mixed with other substances, as the hydrophobic degree remains high (De Falco et al., 2021; Emadian et al., 2017; Karamanlioglu et al., 2017) (Figure 6.5).

Source of Bioplastic	Name of Bioplastic	Type of Environment	Conditions	Scale	Biodegradation Indicator	Biodegradability (%)	Period of Biodegradability (Days)
PLA-based	PLA	Freshwater	25 °C—16 h light and 8 h dark	Lab-scale	Weight loss	<2	365
	PLA	Sea water	25 °C—16 h light and 8 h dark	Lab-scale	Weight loss	<2	365
	PLA	Marine	30 °C	Lab-scale	CO_2 production	3.1–5.7	180–365
	PLA	Marine	30 °C	Lab-scale	CO_2 production	4.5–8.4	180–365

Figure 6.5 Degradation time in environmental conditions of PLA (Emadian et al., 2017).

Even in marine environments, PLA is reluctant to degrade like its fossil counterpart, as the availability of dissolved oxygen is relatively low and high temperatures that help the processes of breaking the ester bonds are not reached. This slows down the hydrolysis of the amorphous parts of the polymer, resulting in the release of smaller oligomers and monomers (Chamas et al., 2020; Ribba et al., 2022). This occurs not like other polymers such as polyhydroxyalkanoate (PHA) and Poly-Caprolactone (PCL), which have shown good biodegradability in marine environments where, although the above-mentioned conditions exist and there is more variability of biodegrading microorganisms, there are biological carbon cycles for the mineralization of these (Suzuki et al., 2021).

Okoffo et al. (2022), through a validated analytical method based on pyrolysis-gas chromatography-mass spectrometry, demonstrated how PLA bio-microplastics (PLA BMPs) are present in matrices of wastewater, biosolids and marine sediments at concentrations between 0.09 and 0.18 mg/g (solid matrices) and mg/L (aqueous matrices), showing that, as for conventional plastics (polyester, etc.), the treatments carried out in treatment plants (WWTPs) are not sufficiently suitable for their total biodegradation.

A growing number of studies are questioning whether PLA BMPs could prove to be a threat to biota like their synthetic counterparts and whether the ecosystem, mainly the marine one, faces a new challenge against bioplastic pollution (Ainali et al., 2022). Some studies confirm that PLA BMPs are similarly toxic to conventional MPs (Zimmermann et al., 2020). Like the most common MPs smaller than 5 mm, PLA BMPs can also be taken up by the aqueous medium and mistaken for food. In a study by Duan et al. (2024), the exposure of adult male zebrafish *Danio rerio* to a diet based on bioplastic highlighted that after ninety days of exposure, alterations in the intestinal microbiota, metabolic processes and structural damage occurred at the digestive tract level. These effects are consistent with those recorded by a previous study that showed how the biodegradable nature of PLA was a double-edged sword for the health of the microbiota, as PLA MPs can be degraded by intestinal enzymes and cause the production of smaller particles and a decrease in pH inducing dysbiosis in zebrafish (Wang et al., 2023; Duan et al., 2022). Like their petrochemical counterpart, one of the main characteristics of the cytotoxic effect of PLA BMPs is to induce alterations in the defence mechanisms against stress provoked by oxygen radical species, managing to produce mitochondrial dysfunction and consequent cell death, aggravated by the tendency to bioaccumulation (Chagas et al., 2021; Zhang et al., 2021).

This perspective is even more worrying for filter-feeding organisms, such as most marine invertebrates, including bivalves that can discriminate suspended particulate matter in the aqueous medium based on size. Therefore, it is possible that the frequency of interfacing, due to a mainly sessile lifestyle, affects the accumulation of these biological micro-polymers more seriously, being able to exert greater toxicity.

If there are so many similarities with the toxic effects of the conventional counterpart, it is possible that, like PS MPs, even PLA BMPs could also interfere with the reproductive system of the biota. However, there are not certainties in this regard since few studies are now moving to consider the effect of this new emerging contaminant on the reproductive health. A study on *Caenorhabditis elegans* exposed to environmental concentrations (µg/L) of PLA BMPs showed a reduction in the reproductive capacity of the nematode, alterations in the development of the gonads and apoptosis (Shao et al., 2023). However, a study conducted on the vertebrate zebrafish demonstrated how, in conditions favourable to its degradation, PLA causes an increase in oxidative stress, changes during the differentiation of germ cells in females and in hormone production, as well as transgenerational effects on the offsprings, resulting in high mortality and inhibition of hatching (Zhang et al., 2024). Furthermore, it has been reported that after exposure to PLA, *D. rerio* larvae assumed an anxiety-like behaviour with a reduction in motor activity and neurotoxic alteration (de Oliveira et al., 2021). A study on the sea squirt *Microcosmus exasperatus* showed that disposable items in PLA, from which secondary MPs can derive, can induce toxic effects by reducing the fertility rates of marine invertebrates (Anderson & Shenkar, 2021), or even that PLA can cause reduced reproductive capacity in invertebrates such as *Daphnia magna* (Zimmermann et al., 2020).

All this has led to considering this new type of bio-microplastic of PLA as a potential threat to the reproductive health of non-target organisms. For this reason, by choosing the model organism *Mytilus galloprovincialis*, it was decided to design a seven-day experimental plan to verify whether short-term exposure could impact the organization, redox balance and functionality of the reproductive tissue in both sexes. To simulate environmental conditions, PLA MPs of variable dimensions were used (in the range 0.1 µm - 5 mm), while a single dose of 0.5 mg/L was selected, considering the constant increase in PLA production and its consumption at a global level.

6.2 Materials and methods

6.2.1. Mussel acclimatization and experimental design

At the end of November 2023, organisms belonging to the species *Mytilus galloprovincialis* were acquired from the Messina aquaculture plant S.A.Co.M. (Sicily, Italy). Specimens with an average valve size of 4-5 ± 0.4 cm were transported to the scientific facility "Mesocosm Facility" of the CNR-IRBIM in Messina (Sicily, Italy) and housed randomly, without the possibility of distinguishing sex given the tightly packed valves, in glass aquaria to avoid any contamination by other types of plastic, filled with 15 L of filtered sea water (FSW) at 300 µm (Figure 6.6).

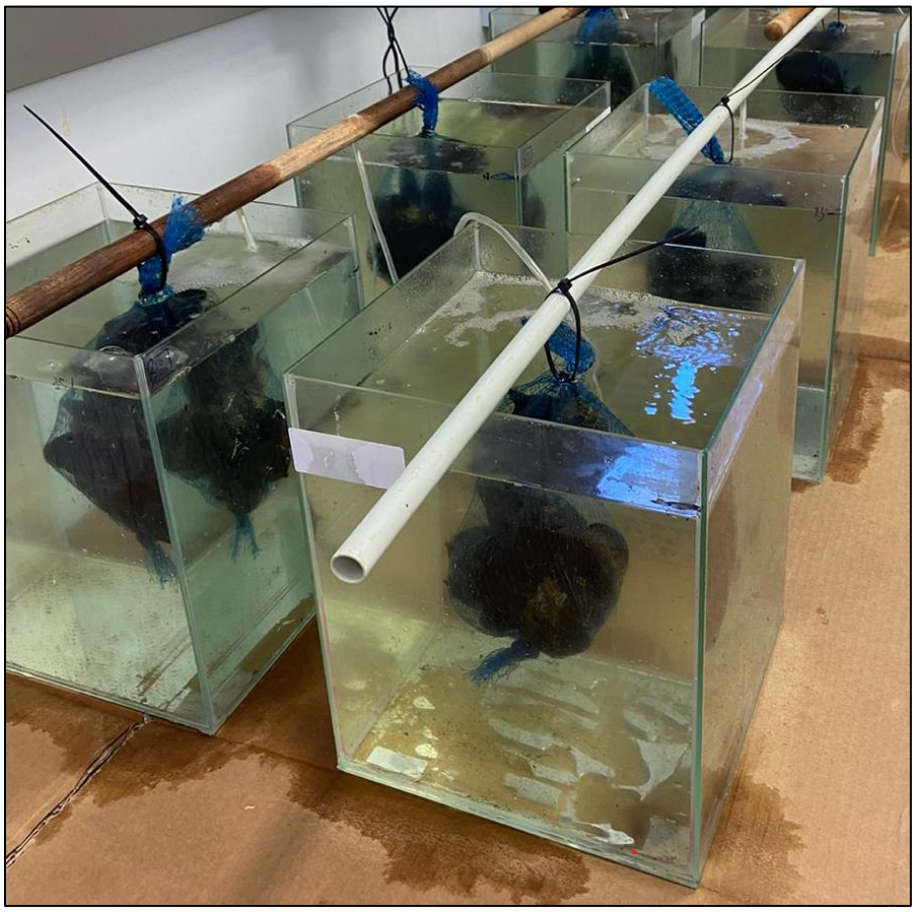

Figure 6.6 Experimental plan set up in triplicate. Second row tanks control (Ctrl: 0 mg/L), first row tanks treated (PLA MPs: 0.5 mg/L).

An acclimatization period of 15 days was performed, during which the following controlled laboratory conditions were maintained, namely temperature at 18 ± 1 °C, salinity 35‰, pH 8 ± 0.02, photoperiod L/D 12 h:12 h, and continuous aeration. Then, the experimental trial was started, which included two conditions: a negative control (Ctrl: 0 mg/L) with mussels exposed only to FSW, and a group of mussels treated with polylactic acid microplastics (PLA MPs: 0.5 mg/L) of variable size (range 0.1 µm - 5 mm). The administration of food, based on commercial algae mix (Liquizell, Hobby), was performed at each water change for a total of seven days.

The contaminant was in powder form, therefore the correct quantities to be used were calculated in relation to the total volume of the medium present in the aquarium (0.5 mg x 15 L), and then aliquots were prepared for each water change. Since the PLA powder tends to be slightly electrostatic, the doses of the contaminant were first suspended in small quantities of FSW taken from each aquarium and then re-introduced with the suspended contaminant at the final concentration to be tested. Everything was done in triplicate, and after seven days of exposure six organisms were taken from each experimental condition (control and treatment with PLA MPs), to have a statistically significant number of mussel males and females.

After fresh sex assessment (see Chapter 3), a small portion of the gonad samples to be used for histological analysis was introduced into dedicated tubes containing PFA fixative solution (Immunofix) at 4% (37% formaldehyde diluted with 1 M PBS at pH 7.4), while the remaining tissue intended for biochemical analysis was weighed and placed in sterile tubes in liquid nitrogen to be transported at the laboratory at the University of Messina (Sicily, Italy), where they were then stored at -80°C.

6.2.2 Histological analysis

The posterior adductor muscle of each mussel was cut and in order to collect the gonads, as previously described by Afsa et al. (2023). Small portion of mussel gonads of both sexes, after 4 hours at 4 °C in fixative, were subjected to two rapid washes in PBS 1 M and pH 7.4 for 10 minutes each, and subsequently to a

dehydrating series in alcohol (ethanol, Mixetan; Cuneo, Italy) (1 h at 50°, 1 h at 70°, 30 min at 80°, 30 min at 95° and 15 min at 100° I and 15 min at 100° II). Afterwards, the diaphanization phase was carried out with the clarifying agent xylene (Sigma-Aldrich, Darmstadt, Germany) mixed at 50% with 100° ethanol for 30 min, followed by the step in xylene for 30 min. Each tissue was treated for 1 h with an ultra-pure xylene-paraffin mixture Paraplast (Bio-Optica, Milan, Italy) and then underwent two further passages of 1 h each in paraffin.

Thin sections of 4 µm were cut manually with a rotary microtome (Leica Microsystems, Wetzlar, Germany) and mounted on a slide holder and left overnight in an oven at 26 °C. The following day, each slide was deparaffinized for 5 min in xylene, twice. After rehydration with a decreasing series of alcohols (100°, 95°, 80°, 70°, 50°, 35°) until reaching DW (5 min for each passage), staining was carried out with hematoxylin/eosin (H/E). Each slide was immersed in a haematoxylin bath (basic dye) for 15 sec and then cleaned with a quick passage in DW, passed in tap water at room temperature for 10 min and rinsed in DW, and then stained with eosin (acid dye) for 8 sec (Cappello et al., 2021). A brief dehydration in alcohol followed these steps (95°, 100° I, 100° II), and then slides were moved to the 100° alcohol and xylene mixture and finally only in xylene, which is the solvent of the mounting resin, Eukitt® Quick-hardening mounting medium (Sigma-Aldrich, Darmstadt, Germany) with which the coverslip was mounted to protect the histological preparation. In this way, it was possible to determine more precisely the sex, the morphological organization and the potential alterations of the gonadal tissue.

All images were acquired with a Zeiss Axio Imager Z1 motorized microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany) (Maisano et al., 2017), by immersion objectives.

6.2.3 Histochemical analysis

To identify the presence of the glycogen, a histochemical reaction with periodic acid-Schiff (PAS; down section) (Abcam, Cambridge, United Kingdom) combined with a d-PAS (diastasis-PAS; up section) was performed on the histological slides of gonads from each experimental condition on the two distinct but serial sections

belonging to the same cutting series, following the modified protocol of Meyerholz et al. (2018).

This allowed to evaluate the presence and quantity of the polysaccharide, comparing the positive PAS (glycogen present) with the negative d-PAS (glycogen absent) using a solution of α-amylase of 15 U/mg in PBS 1 M at pH 7.4 in volumes sufficient to completely cover each section. For the enzyme to be activated, each slide was incubated at 37 °C for 20 min in an oven. After rinsing in DW, periodic acid was left to act on both sections for 5 min. This was followed by two quick washes in DW and then Shiff reagent was used for 10 min. Subsequently, a 2 min bath in hot water (30 °C) was performed with a passage in hematoxylin for 30 sec. The excess of colour was eliminated with two washes in DW.

A rapid dehydration in alcohol (95°, 100° I, 100° II), a step in a mixture of 100° alcohol and xylene, and the two steps in xylene were performed before covering the sample with a coverslip and sealing it with Eukitt (Sigma-Aldrich, Darmstadt, Germany).

The images were obtained with a Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Werk Göttingen, Germany) equipped with an AxioCam digital camera (Zeiss, Jena, Germany) (Maisano et al., 2017) and were exported in tiff format. Using the Image J (Image Processing and Analysis in Java) software version 1.54i, the amount of glycogen highlighted by the PAS reaction was measured. The measurement acquisition process strictly follows what is reported in Chapter 3 in the section "Materials and methods", within the sub-paragraph "Histochemical analysis". The results, for univocal understanding of the data, have been reported in pixels (Mai et al., 2023; Chen et al., 2022; Jones et al., 2021). All images are reported in the table with scale bar 20 μm.

6.2.4 Biochemical analysis

In order to evaluate the response to potential oxidative stress in both sexes of the Mediterranean bivalve *M. galloprovincialis*, male and female gonad fragments of approximately 60 mg were mechanically homogenized in a 0.1 M Tris-HCl buffer adjusted to pH 7.5, in a volume equal to 10 times the weight in grams of the

sample (600 µL total volume overall), using 3.2 mm diameter stainless steel beads and TissueLyser LT (Qiagen, Hilden, Germany) with a cold rotor, set at 50 oscillations/sec for 6 min (De Marco et al., 2023). All homogenized samples were subsequently centrifuged in a centrifuge (Centrifuge 5417R, Eppendorf, Milan, Italy) refrigerated at 4 °C for 20 min at 9000 g. For each sample, taking care not to touch the pellet obtained from the previous step, as much of the supernatant as possible was taken and aliquoted into tubes based on the quantity needed for the subsequent analyses.

A UV-mini 1240 spectrophotometer (Shimadzu, Milan, Italy) (Figure 6.7) was used and for each enzymatic analysis a temperature of 25 °C was maintained, as in mussels the proteins perform their enzymatic activity at this temperature (Maisano et al., 2016). The Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, United States) was used to measure the total protein content and bovine serum albumin (BSA, Sigma-Aldrich, Darmstadt, Germany) as a reference (Bradford, 1976).

Figure 6.7 UV-mini 1240 spectrophotometer (Shimadzu, Milan, Italy).

To evaluate perturbations of the redox balance at the cellular level, the enzyme catalase (CAT) was considered, whose activity was measured by monitoring the variation of the optical density at a wavelength of 240 nm for 90 sec (Sureda et al., 2011). Specifically, 440 µL of 0.1 M phosphate buffer at pH 7.5 were used for 45 µL of sample. After briefly mixing manually the samples, 215 µL of H_2O_2 (diluted 1:100 with the phosphate buffer) were added just before reading and, after mixing well, everything was inserted into a quartz cuvette and placed in the spectrometer to perform the measurement. The results were expressed in µmol/min/mg of protein.

Another enzyme used to evaluate the direct impact of the pollutant on the gonadal tissue was glutathione *S*-transferase (GST). For this analysis, 840 µL of phosphate buffer were introduced into quartz cuvettes, this time obtained by preparing two distinct solutions: one of KH_2PO_4 (Carlo Erba, Milan, Italy) (solution A: 13.6 g in 1 L of DW) and one of K_2HPO_4 (Carlo Erba, Milan, Italy) (solution B: 17.4 g in 1 L of DW) used to equilibrate the final pH to 6.5, 50 µL of 20 mM 1-chloro 2,4 dinitrobenvene (CDNB) solution, and 10 µL of sample. After brief mixing, 100 µL of GSH (reduced glutathione) were introduced. The blank was obtained with equal quantities and by replacing the supernatant volume with phosphate buffer. The reading was performed by acquiring at a wavelength of 340 nm. The data detected after 3 min and indicating the enzymatic activity were expressed in nmol/min/mg of protein according to the method described by Habig et al. (1974).

Additionally, to verify the lipid peroxidative state (LPO) the concentration of malondialdehyde (MDA) was estimated using the thio-barbituric acid reactive method (TBARS) (Testamenti, 1987). The analysis proceeded in sealed glass cuvettes, in which 500 µL of thio-barbituric acid (TBA; Sigma-Aldrich, Darmstadt, Germany) (0.0375% w/v), 500 µL of trichloroacetic acid (TCA; TW Reagents, Monza, Italy) (15% w/v), and 460 µL of DW were added to 40 µL of sample. The samples were placed in a heat bath at 90 °C for 15 min (Figure 6.8), left to cool and then read on the spectrometer at 532 nm. To obtain the MDA calibration curve, 1,1,3,3-tetraethoxypropane (TEP) was used (Botsoglou et al., 1994). The concentration of MDA was expressed as nmol per mg of protein (nmol/mg protein).

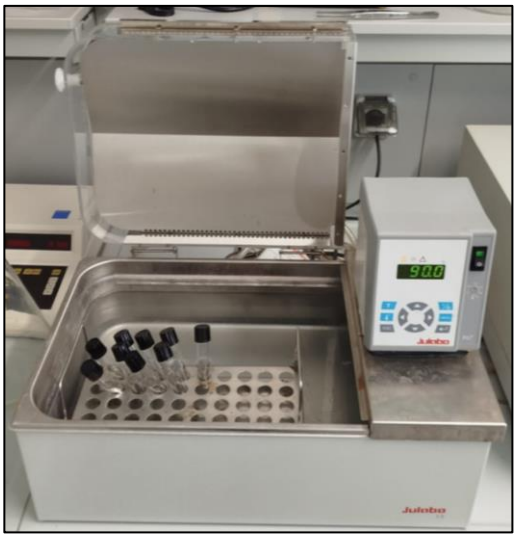

Figure 6.8 Thermal bath (Julabo, Milan, Italy).

6.2.5 Statistical analysis

All the data obtained were processed as an average using the Excel spreadsheet (Microsoft 365) and calculating the standard deviation (± SD). All the results were loaded on the GraphPad software to perform the statistics using the 2-way ANOVA operating module and considering a *p value* less than 0.05 as significant (p <0.05).

6.3 Results

6.3.1 Histological data

Histological analysis, using the H/E colorimetric method, highlighted how the control organisms at the initial time point and after seven days did not show variations in gonadal maturation, demonstrating a tissue organization typical of stage IIIA and III, both in males (Figure 6.9: A) and females (Figure 6.10: A), respectively (Prisco et al., 2017; Rosati et al., 2019). This was consistent with the reproductive season (late November-early December) during which the research project was developed.

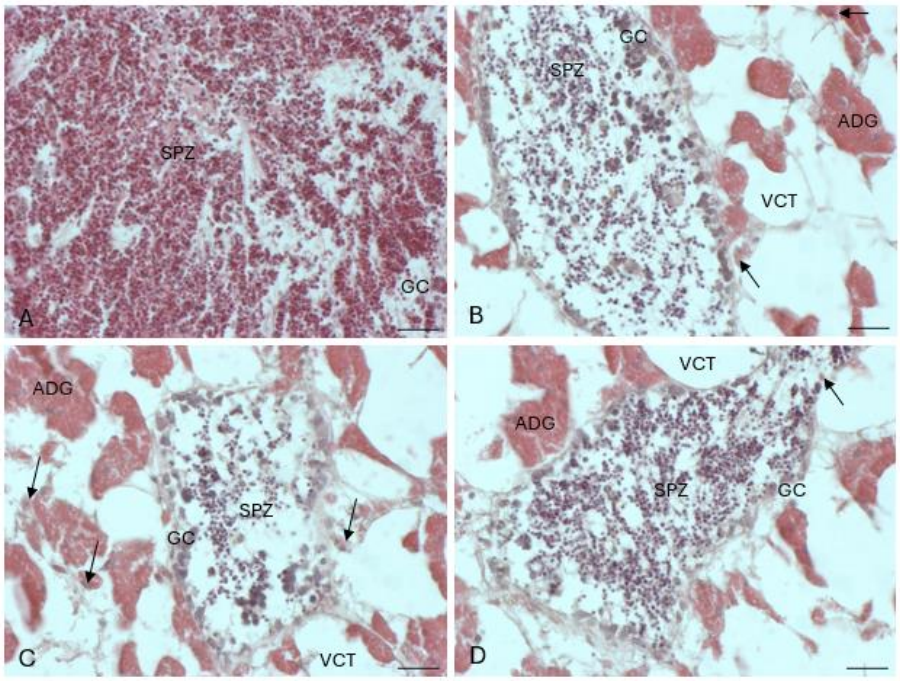

Figura 6.9 Representative histological sections of male gonads of *Mytilus galloprovincialis* of the control group (A) and of the group exposed to PLA MPs after seven days of treatment (B, C, D), showing infiltration of hemocytes (arrows) on the connective tissue. Presence of vesicular connective tissue (VCT) cells and hypertrophic adipogranular (ADG). Cells. Spermatozoa (SPZ), germ cells (GC). Scale bar: 20 µm.

Specifically, in the control male gonads, the follicles were full of spermatic cells (SPZ), whose heads were made evident by the blue-violet of the hematoxylin that marked the nucleic acids in the nucleus; while going towards the periphery, germ cells (GC) were distinguished not yet completely differentiated (Figure 6.9: A). In the group exposed to PLA MPs after seven days the tissue maintained a structural organization like the control, with the presence of follicles with intact walls and well delineated by the basal epithelium that underlies the entire germinal region. However, the connective tissue is pervaded by hemocytic cells and by abundant hypertrophic ADG cells that acquired a granular appearance (Figure 6.9: B, C and D). Unlike the control gonads, in the organisms exposed to bioplastics it seems that some follicles have undergone emptying. In fact, few spermatozoa with a blue-violet colored head and eosinophilic tails and abundant germinal cells along

the entire peripheral wall can be distinguished towards the lumen (Figure 6.9: B, C and D).

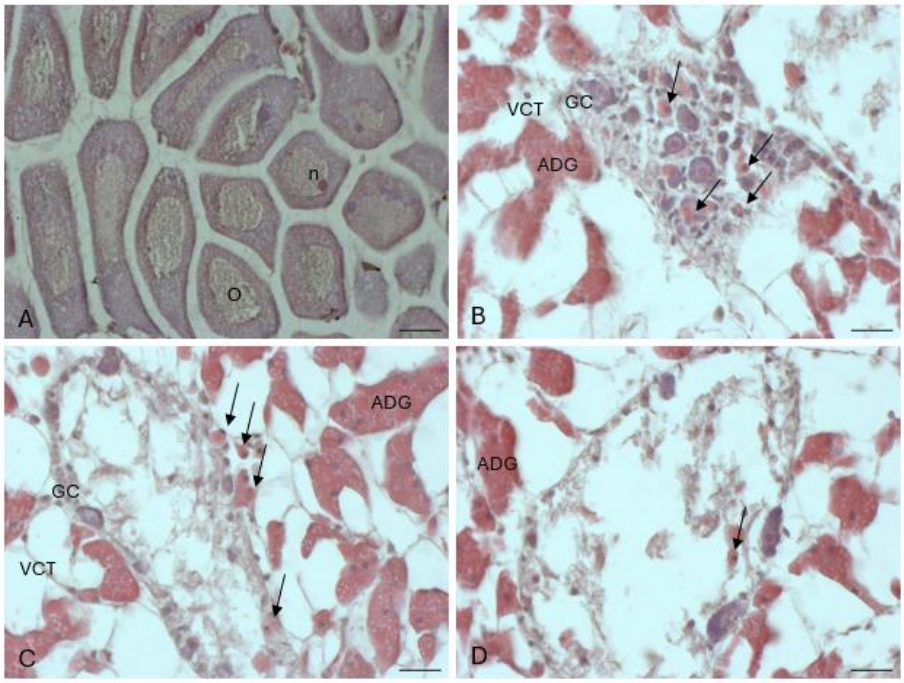

Figure 6.10 Representative histological sections of female gonads of *Mytilus galloprovincialis* of the control group (A) and of the group exposed to PLA MPs after seven days of treatment (B, C, D), showing intense infiltration of hemocytes (arrows) on the connective tissue and also inside the follicles that are emptied. Presence of vesicular connective tissue (VCT) cells and hypertrophic adipogranular (ADG) cells. Ovocites (O), nucleolus (n), germ cells (GC). Scale bar: 20 µm.

In the control female gonads, H/E staining highlighted large follicles filled with abundant oocytes (O), in some of which the nucleolus (n) was evident, more intensely colored in blue-violet than the nucleus. The connective tissue is present, but as in the male counterpart it was more reduced than in the previous stage due to the more advanced maturation phase (Figure 6.10: A). In the group treated with PLA MPs after seven days the tissue seems to maintain the structural organization of the control. However, at the level of the connective tissue some alterations similar to those found in the male counterpart were evident, such as hypertrophic ADG cells, less abundant than in males and a greater presence of hemocytes than in the other sex which, in this case, tended to settle also inside the follicle (Figure 6.10: B). Also in this case, as in the previous one, the gonads seemed to have undergone some spawning event since most of the follicles are emptied (Figure 6.10: C and D).

6.3.2 Histochemical data

PAS reaction was performed on gonadal tissue sections of male and female mussels of *M. galloprovincialis* to verify the presence of glycogen and possible alterations due to the treatment with 0.5 µg/L PLA MPs.

The results obtained showed that compared to the control group (Figure 6.11: A), the male gonads showed, albeit slightly more marked, a positivity to the PAS reaction (Figure 6.11: B and C), highlighted by red-magenta veins inside the VCT cells.

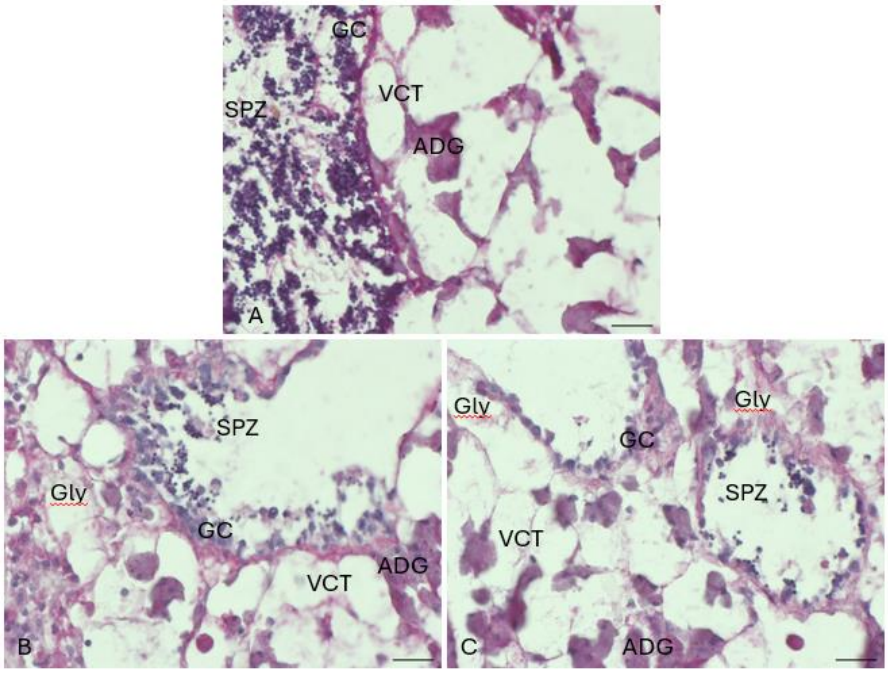

Figure 6.11 PAS reaction on male gonads of *Mytilus galloprovincialis* of the control group (A) and of the group exposed to PLA MPs after seven days of treatment (B e C), showing a slight positivity for glycogen (Gly) inside the vesicular tissue (VCT) cells (B and C) compared to the control (A). Adipogranular (ADG) cells, spermatozoa (SPZ), germ cells (GC). Scale bar: 20 µm.

In the control group of female gonads (Figure 6.12: A) it seemed that in some VCT cells there was a greater responsiveness to the PAS reaction compared to the male counterpart, instead a significant presence of the polysaccharide was detected in the group exposed to PLA MPs at the level of the VCT cells (Figure 6.12: B and C).

The analysis highlighted how the concentration of the polysaccharide was slightly different within the control group between males and females, and how the

divergence increased after seven days of treatment with PLA MPs, as highlighted in the graph in Figure 6.13.

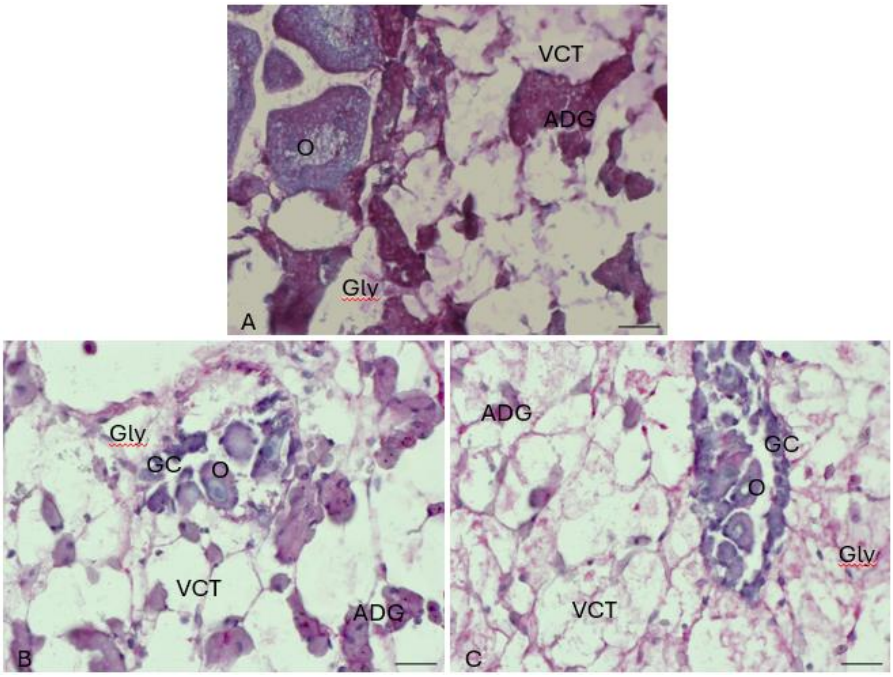

Figure 6.12 PAS reaction on female gonads of *Mytilus galloprovincialis* of the control group (A) and of the group exposed to PLA MPs after seven days of treatment (B e C), showing a slight positivity for glycogen (Gly) inside the vesicular tissue (VCT) cells (B and C) compared to the control (A). Adipogranular (ADG) cells, ovocites (O), germ cells (GC). Scale bar: 20 µm.

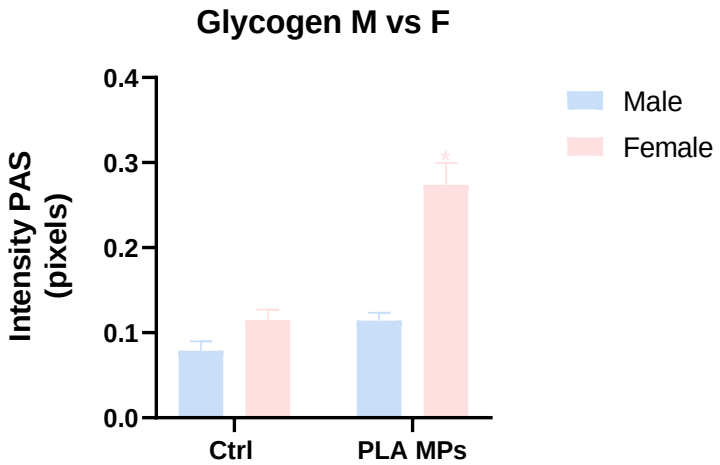

Figure 6.13 Representative histogram of the mean concentration (pixels) of glycogen in male (blue bar) and female (pink bar) gonads of the control and treated groups, after seven days of exposure to PLA MPs. Significant differences compared to the control ($p < 0.05$) are indicated with asterisks (*).

6.3.3 Biochemical data

The biochemical analysis performed on the gonads of both sexes and related to the activity of the catalase (CAT) enzyme did not seem to highlight any relevant alteration following seven days of exposure to 0.5 mg/L of PLA MPs in males and females compared to the control condition. Only a slight increase in the male gonads compared to control was observed, also in respect to the female gonads (Figure 6.14).

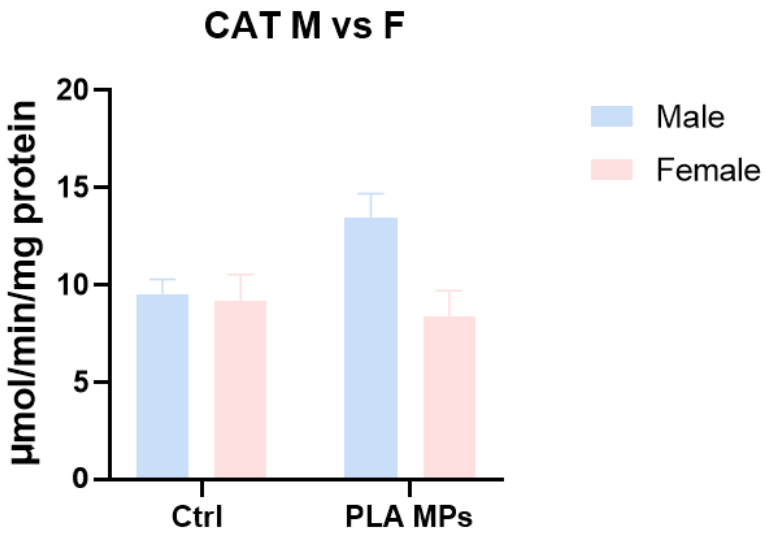

Figure 6.14 Histogram representing the enzymatic activity of catalase (CAT; µmol/min/mg protein).

In the mussels exposed to PLA MPs, no significant modification in the enzymatic activity of GST was highlighted, as reported in Figure 6.15. However, also in this case, the different responsive trend between males and females was noted since a slight increase in GST activity was recorded in male gonads compared to the control (Figure 6.15).

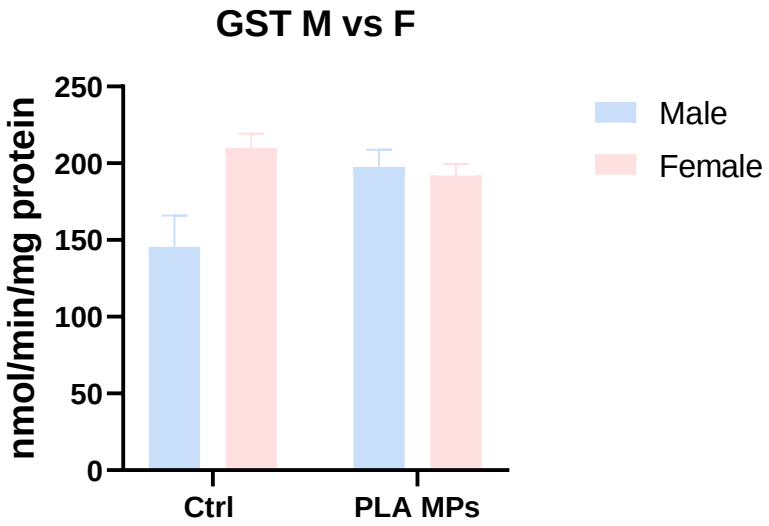

Figure 6.15 Histogram representing the enzymatic activity of glutathione *S*-transferase (GST; nmol/min/mg protein).

The analysis to quantify the concentration of MDA showed that after seven days of treatment, the PLA MPs did not cause any significant perturbation at the level of lipid peroxidation (Figure 6.16). In this case, the trend of MDA appeared to describe the same physiological response for both male and female gonads.

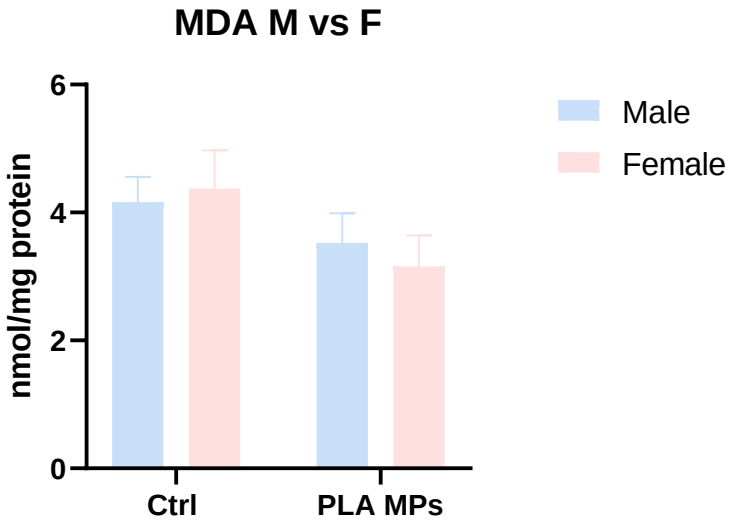

Figure 6.16 Histogram representing the concentration of malondialdehyde (MDA; nmol/mg protein).

6.4 Discussions

This work arises from the awareness that the great versatility and wide adaptability in different sectors (medical, cosmetic, agricultural, industrial, electro-informatics, 3D printing, etc.) of polylactic acid (PLA), which is due to its mechanical properties, good flexibility and production origin, may determine its marked presence in the global socio-economic market in the near future. Indeed, it is not a detail that today it is recognized as the "polymer of the 21st century" (Ahmad et al., 2024; Mehmood et al., 2023; Swetha et al., 2023; Folino et al., 2020). The US Food and Drug Administration itself accredits the characteristics of PLA, which is why it is considered one of the most promising substitutes for drug delivery, bone implants, tissue engineering, etc. (Abu Hajleh et al, 2020; Li et al., 2020). The polymer's degradation and compostability capacity is a possibility that would seem to be able to solve the problem of plastic pollution, simply by implementing a conversion between the conventional one (such as polystyrene, high-density polyethylene and polyethylene terephthalate) and the one made with polylactic acid. The idea that permeates the perspective of eco-sustainable development is that by failing to reduce the quantity of anthropogenic waste, a burden mainly charged to the most developed countries, the use of a biodegradable material should lead to facilitated industrial disposal activities and a faster elimination of polylactic-based waste in the environment (Zaaba & Jaafar, 2020).

However, alongside its advantageous properties, PLA also hides disadvantages. The very accentuated fragility combines with the slowness of the degradation process in natural environments, determining the persistence of the product and the contextual production of smaller fragments. The possibility that a product made of the polymer or co-polymer degrades exists. However, the speed at which it occurs is low and depends both on the temperature at which the hydrolytic process occurs, which is higher than 55 °C, and on the presence of microorganisms capable of producing enzymes that completely degrade the polymer (such as proteinase K) (Rosli et al., 2021). These are two conditions that are very difficult to achieve in aquatic environments, especially marine ones such as coastal ones, as suggested by a study that showed how low temperatures and the scarcity of bacteria suitable for the degradation of polylactic material can significantly reduce the elimination

process in environments such as the marine one, since water implies a dilution of the microbial communities and a tempering of the thermal conditions (Kliem et al 2020). In this regard, a study by Weinstein et al. (2020) suggested that after 32 weeks in intertidal salt marshes, bioplastic samples represented by PLA, had slower degradation rates than those made of conventional plastics (polystyrene, high-density polyethylene and polyethylene terephthalate). From this it is possible to conclude that persistent PLA bioplastics can be subjected to physico-chemical-mechanical-biological fragmentation like conventional ones and produce smaller polymeric units that can ubiquitously disperse at multiple levels in the aqueous medium, interfacing with the resident biota. Furthermore, like conventional ones, PLA MPs are very predisposed to colonization by organic compounds (microorganisms) and inorganic compounds (such as drugs and metals), demonstrating a Trojan horse mechanism whose effect is accentuated by the release rate of the bioplastic once ingested (Ainali et al., 2022; González-Pleiter et al., 2021; Liao & Yang, 2020).

Considering this, the aim of this work was to shed light on the potential danger of an emerging pollutant such as PLA MPs, considering the effect of realistic concentrations of bioplastic in a variable size range, such as that found in the environment, on both sexes of the Mediterranean mussel *Mytilus galloprovincialis*, to simulate the possible scenario to which the aquatic biota may be exposed.

The possibility that in a relatively short time PLA MPs have managed to reach both the male and female reproductive compartment is proven by the intense immunological presence found after seven days of exposure. The presence of hemocytes in response to a steric insult, such as that represented by MPs with dimensions between 0.1 μm and 5 mm, is typical of the species that can promptly activate immune cells even in more internal organs, such as the digestive glands, against the bulky intruder (Cappello et al., 2021). A pioneer in the evaluation of the toxic effect of bioplastics such as Green et al. (2019) has shown that, after about seven weeks of exposure, PLA MPs can even cause proteomic changes in the hemolymph at the level of C1qDC components that in *M. edulis* are involved in pathogen recognition. The authors agree with the idea that the steric hindrance of MPs may have caused mechanical abrasion, and therefore induced immunological changes. The data obtained from the present study are therefore in line,

demonstrating that the immune response is the bioindicator of a stressed phenotype, as noticed at histological level, that was more accentuated for female mussels. From a similar perspective, the presence of PLA MPs could have been an early stimulating factor for the release of gametes in both male and female gonads, which could have caused, like conventional MPs, a sort of degeneration accredited by the emptying of the follicles (Choi et al., 2022), a possible situation given that in other species of marine invertebrates such as *Paracentrotus lividus*, alterations have been found at the level of the follicles of both sexes exposed to different types of bio-microplastics including PLA (Viel et al., 2024).

When subjected to stress, the Mediterranean mussel *M. galloprovincialis* can implement adaptive strategies to protect its own conservation to ensure physiological balance (De Marco et al., 2023; Yang et al., 2023). The reproductive system, especially that of organisms whose reproductive fitness depends on an external fertilization mechanism, is flexible but it is also capable of adapting to the needs of the entire organism (Danton et al., 1996). It is likely that just as traditional MPs, such as polystyrene, it can cause an alteration of bioenergy in specimens of the genus *Mytilus*. Therefore, also bio-based compounds, such as PLA, can exert a similar action (Shang et al., 2021). This hypothesis is supported by the fact that it was found a modification, compared to the control, of glycogen reserves at the level of VCT cells in conjunction with a hypertrophy of ADG cells. It is known that in bivalves the cells of the vesicular connective tissue are predisposed to the accumulation of neutral polysaccharide that will be used as a fundamental source of energy for the processes of male and female gametogenesis, while the adipogranular cells, in addition to presenting small accumulations of glycogen, are more destined to conserve the protein and lipid component (Pipe, 1987; Gabbott & Whittle, 1986). It is possible that such as conventional MPs, also PLA MPs promoted the redirection and management of the three classes of energy reserves (glycogen, proteins and lipids) for defence strategy in anticipation of the persistence of stress, so that the nutritional support for the gametic cells is preserved, as occurred in other species exposed to conventional MPs (Dong et al., 2024), despite the early emission to which some follicles were subjected.

To date, the limited data in the literature on the effect of PLA MPs do not allow to have a clear vision of the condition to which the biota is potentially being

exposed. Aware of a certain affinity with microplastics and their tendency to induce the formation of reactive oxygen species (Huang et al., 2021), part of the work was aimed at evaluating the activity of key enzymes in the antioxidant process such as catalase (CAT), whose activity allows the reduction (dismutation) of hydrogen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2), and detoxifying such as glutathione *S*-transferase (GST), a phase II enzyme that mediates the conjugation of the xenobiotics/primary metabolite of phase I with an endogenous molecule (reduced glutathione) to make the exogenous one more hydrophilic, and therefore easier to eliminate, both validated to verify early pro-oxidant effects due to various pollutants including microplastics (Cappello et al., 2021). A nine-day study on *Dreissena bugensis*, in which different types of MPs, including PLA, were compared, highlighted specific effects of the type of polymer, reporting the catalase enzyme as a biomarker that demonstrated a significant increase after treatment with PLA (Brehm et al., 2022). A study on *Tenebrio molitor* larvae, insects capable of eating various types of conventional plastic, managing to degrade it at intestinal level, reported that this insect seems to prefer PLA as food. However, after six weeks this caused an increase in ROS levels in the animals compared to the control group fed only bran, confirming almost as much as conventional ones that PLA bioplastics can also induce oxidative stress, even if with a less severe toxic effect (Peng et al., 2023). The study by Shang et al. (2023) on earthworms, in which the effect induced by PLA MPs was compared with that of polystyrene and cadmium and the combination of the two plastics with the metal, confirmed that the bioplastic is capable of inducing ROS production and that the maximum levels, even above the other experimental conditions, were detected at seven days of treatment and led to an increase in the activity of CAT and GSH after seven days, and even twenty-one in the case of glutathione. This would seem to reflect the slight perturbation found herein in the mussel male gonads, in which the levels of CAT and GST activity were slightly higher than those of the control, while in females it seems that the activity of the enzymes had not undergone changes. The low biochemical perturbation exerted on male gonads underlines how males, at this level, may be more susceptible to this type of interference than their female counterparts in a short exposure period, responding with a balanced increase in key defence enzymes. This is also a further demonstration of how the phenotype can influence the induction of

biological responses that can be directed to specific metabolic pathways (Blanco-Rayón et al., 2020). A comparison with marine invertebrates is very difficult due to the scarcity of data in the literature. However, a study by Khalid et al. (2021) on *M. edulis* reports, similarly to the present work, how slight but not significant increases in CAT and GST were found after eight days of treatment with PLA MPs, and that this is the result of an adaptation to counteract the physiological challenge introduced by exposure to the contaminant.

Reactive oxygen species, if not counteracted by the antioxidant system, can affect different structures within the cell compromising its functionality, such as cell membranes. For this reason, another investigation carried out in this study was to verify the concentration of MDA in the gonads of both sexes. The biomarker demonstrated the absence of lipid peroxidation for both phenotypes, a parameter that is not in line with similar studies in which the lipidome is altered in the glycerophospholipid component (Khalid et al., 2021). However, this study focused on another organ than the one examined in this thesis that could respond differently being structurally and functionally different (Cappello et al., 2018; Détrée et al., 2017). In this framework, the finding of the absence of alterations for MDA supports the data obtained on the activity of CAT and GST, accrediting the female specimens as more resilient, which unlike the male ones, do not seem to have undergone particularly marked influences by the PLA MPs tested.

6.5 Conclusions

The work has highlighted how the possibility of using diversified analyses that describe multiple biological levels can provide a more complete understanding of how a pollutant, in this case emerging as PLA, can act in non-target organisms.

The results obtained showed that a short-term exposure (seven days) to concentrations of PLA MPs 0.5 mg/L, although not causing significant changes at the biochemical level (CAT and GST activity, and MDA concentration), promoted an initial alteration of the normal bioenergetic balance in both sexes, with the accumulation of reserves compared to the control state and an early emission of both male and female gametes. The possibility that a strong state of oxidative stress may be established, still early at seven days of exposure, could determine an alteration of the normal state of development of the gonads with a reduction in the reproductive capacity. This could be dangerous for the maturation process of the germ cells in both sexes and consequently for the survival of the species, since the release of gametes that are not yet fully mature can threaten the reproductive fitness rate.

The few data accumulated so far, limited in time, do not allow to judge on PLA, evaluating it as a good or bad substitute for conventional plastics. However, it has been shown that in some way even PLA is similar to conventional plastics since it is able to exert some interfering action on the reproductive health of *Mytilus galloprovincialis,* as it has been seen. Since the production of PLA will increase exponentially in the next few years, it is very likely that the quantity of waste of this material will also undergo an increase. For these reasons, even if PLA is a compostable bioplastic, because of its slow degradation process and the possibility of producing secondary microplastics dangerous for the biodiversity of ecosystems (Green et al., 2016), greater attention should be paid today to not allowing polylactic acid debris to reach natural environments but retained and efficiently disposed by suitable waste management strategies in treatment plants.

6.6 References

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CHAPTER 7

General conclusions

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The results obtained from each individual experimental study, carried out using the Mediterranean mussel *Mytilus galloprovincialis* as model species, have tried to satisfy the ambitious initial purpose of this thesis aimed at understanding more clearly and in its complexity the potential effect of endocrine interference by emerging micropollutants, namely the anti-inflammatory drug dexamethasone (Dex), polystyrene microplastics (PS MPs), and polylactic acid bioplastics (PLA MPs), on the reproductive health of mussels. Specifically, the biological dynamics for which two different phenotypes (male and female) can implement independent morphological, biochemical, molecular and metabolic responses were reported, which in certain contexts were divergent, depending on intrinsic characteristics related to sex.

In order to address the aim of the thesis, conventional techniques (i.e. chemical, histological, histochemical, immunohistochemical) were integrated with more innovative ones (molecular and metabolomics), and this allowed to provide accurate insights of the impact of the micropollutants tested at biological level, returning a network of key information that overall enabled to reconstruct a more comprehensive picture of the reproductive health status of the model species under investigation in different environmental contexts, clarifying the effects of the three key stressors selected to be explored.

Firstly, it is worthy to note that the use of multi-biomarker approach has given the opportunity to acquire new insights about the gonadal system in males and females of the gonochorous species *M. galloprovincialis* grown in a pristine ecosystem such as that of the S.A.Co.M. mussel farm (Messina, Italy), allowing to highlight the inter-sex divergences of physiological biological responses during the maturation period in autumn.

In this thesis it has been demonstrated that, regardless of the phenotype, Mediterranean mussels responded by developing an early immune response (already after three days of treatment) after a sub-chronic exposure of 12 days to environmental doses of the drug dexamethasone, due to the strong inflammatory pressure induced by this synthetic glucocorticoid. Although in sex-related different

ways, the changes in the concentrations of key metabolites of the energy pathway (such as glucose and glycogen) and of secondary pathways (BCAAs, lactate and acetoacetate) suggested that male and female specimens try to deal with the effect of the drug by involving alternative pathways to compensate for the energy demand and satisfy the need during a crucial reproductive period, finalized to obtain healthy gametes suitable for future external fertilization. The negative effect of dexamethasone on the gonads of both sexes was confirmed by a general state of osmotic imbalance that the aquatic organism tried to remedy, implementing metabolic strategies on multiple fronts, in order to restore the canonical physiological condition and thus protect good reproductive fitness. Despite the clear endocrine interference exerted by Dex, also demonstrated by immunopositivity to the anti-FasL antibody, the preservation of a proliferative activity credited to the germ cells (positivity to the anti-PCNA antibody) gives hope that the benthic organism can reorganize itself and counteract stress, maintaining a normal reproductive activity, at least if challenged for a limited time as herein demonstrated.

Moreover, the results obtained from the acute exposure of mussels to polystyrene microplastics (PS MPs) have shown that after 48 h of exposure a similar inflammatory response was established in the two phenotypes of the intertidal mussel *M. galloprovincialis*. It has been noted that both sexes tried to compensate for the strong insult exerted by high doses of conventional microplastics, but that this occurred with two distinct modalities between males and females that have compensated for the alteration of the cellular bioenergetic state by modifying the level of metabolites of the aerobic energy pathway (glucose and glycogen) and secondary pathways such as those of amino acid metabolism (BCAAs and arginine). Such a physiological imbalance, aggravated by the altered osmotic condition and dose-dependent degenerative effects on gametic cells, can cause, in a period close to spawning, a serious threat to the successful fertilization, and therefore to the formation of viable offsprings sufficiently numerous to safeguard the species.

Additionally, the results obtained from the latest experimental study have demonstrated how polylactic acid microplastics (PLA MPs) at concentrations realistically found in the environment can, in a short-time frame (7 days), induce an

imbalance of the normal physiological condition in the male and female gonads of the Mediterranean mussels, as confirmed by a strong infiltration of haemocyte cells throughout the gonadal connective tissue. This evidence indicates that, although in an attenuated way, the effect of bio-microplastics also affected the gonads at cellular level. In fact, an accumulation of glycogen reserves was noted for both sexes within the VCT cells of the gonadal connective tissue. A further confirmation is represented by the early spawning event induced for both males and females. However, even in this case sex-dependent biological responses were observed, reporting at the biochemical level that males implemented an early compensatory strategy with slight increases in CAT and GST. It is worthy to note that imbalances, even slight, in crucial maturation periods such as the gametogenic one, can suddenly threaten the formation of healthy gametes suitable for the subsequent reproductive steps.

Overall, the research provided additional data that allowed to more clearly describe the negative influence of traditional and new emerging pollutants with EDC peculiarities on the reproductive health of non-target organisms, such as the marine bivalve *Mytilus galloprovincialis*. Furthermore, findings of this thesis reveal that the use of a multi-biomarker approach including histological, histochemical, biochemical, molecular and metabolomics investigation, is able to provide insights at multiple levels of biological organization, necessary to understand more clearly the dense network of links on which each organism is built. It is in light of this that the work within this thesis suggests that in designing an eco-cytotoxicological investigation is first necessary to define the basic variability of a species, which may depend on the sex and reproductive stage of the individuals under examination since sex-related peculiarities may be reflected into the biological responses triggered by the exposure to various xenobiotics, leading to an incomplete interpretation of the information that may lead to not correct estimation of the risk to which the biota may be exposed.