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"*Mullus barbatus* L., 1758 and *Mullus surmuletus* L., 1758 as bioindicators of marine environmental contamination: analysis of exposure and effect biomarkers"

"*Mullus barbatus* L., 1758 e *Mullus surmuletus* L., 1758 come bioindicatori di contaminazione in ambiente marino: analisi di biomarcatori di esposizione e di effetto"

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Abstract

Demersal fish stocks are a fundamental component for fishing and the fish market throughout the Mediterranean basin. The mullet, *Mullus barbatus* and *Mullus surmuletus*, are highly appreciated for their organoleptic properties among the coastal demersal fish.

However, these species are too sensitive to pollution in waters and sediments, passing throughout their adult lives. In fact, they search for their prey in the sediments and below them, such as crustaceans, mollusks, and polychaetes. This feature makes them excellent bioindicators of environmental contamination conditions.

This research project was conducted on different areas of the eastern Sicilian coasts, using the two above as key species. The condition of coastal waters was analyzed through multidisciplinary approaches. Thanks to the different biomarkers used, it was possible to have a clear picture of either the contaminants present in each area or the type of pollution present in a specific area under examination.

Keywords: *biomarker; bioindicator; fish; Mullus; Mullidae; Ionian sea; gonadal histology; gene expression; vitellogenin; heavy metals; endocrine disruptors; EDs; pesticide; PAHs.*

Riassunto

Gli stock ittici demersali sono una componente fondamentale per la pesca e per il mercato ittico di tutto il bacino Mediterraneo. Fra i pesci demersali costieri, le triglie, *Mullus barbatus* e *Mullus surmuletus*, sono estremamente apprezzate per le loro proprietà organolettiche.

Tuttavia, tali specie sono estremamente sensibili alla qualità delle acque ed in particolare dei sedimenti, con i quali rimangono in contatto nella fase adulta della loro vita. Nei sedimenti, e al di sotto di essi, ricercano le loro prede, come crostacei, molluschi e policheti. Tale caratteristica li rende ottimi bioindicatori delle condizioni di contaminazione ambientale.

Questo progetto di ricerca è stato condotto sulle coste orientali siciliane, utilizzando come specie chiave le due sopraindicate. Attraverso approcci multidisciplinari si è analizzata la condizione delle acque costiere. Grazie ai diversi biomarcatori utilizzati è stato possibile avere un quadro chiaro o dei contaminanti presenti in ogni area o del tipo d'inquinamento presente in una specifica area in esame.

Parole chiave: *biomarcatore; bioindicatore; pesci; Mullus; Mullidae; Mar Ionio; istologia delle gonadi; espressione genica; vitellogenina; metalli pesanti; interferenti endocrini; EDs; pesticidi; IPA.*

1 Introduction

The actual condition of the marine environment is highly alarming. The pollutants come into rivers, lakes, and finally into the oceans in many ways. Since the second half part of '900, the contamination of seas and oceans was studied accurately and with worry for the future (Hardy, 1973; Jackson & Taylor, 1992; Kindt, 1984; Waldichuk, 1977). What was made to contrast the increase in pollution in the last 40 years? Paradoxically, too little. The contamination of the aquatic environment increased according to the developing industry. The growing demand for new technologies, more fossil fuel, a greater quantity of disposable things caused a necessary production of new chemical compounds, new materials, and new forms of contamination. About 80,000 chemicals have been introduced into the environment in the past 50 years (Curtis and Skaar, 2002). There is growing evidence that some of these chemicals present on a large scale, even globally, may threaten any living organism (Vos et al., 2000).

Marine pollution is characterized by a lot of different types of contaminants. First of all, approximately more than 10 tons of plastic are thrown into the oceans every year (Jambeck et al., 2015). The majority, about 80 percent of marine litter, are plastics (EU Parliament 2019). The problem is the massive presence of plastic floating in the

sea and also its sizes and complex chemical compositions (Carney and Eggert 2019). On every scale of size, plastic affects the food chain of many water ecosystems. From the macro plastic to the nanoparticles, they can be ingested by aquatic organisms (Eriksen et al., 2014).

Moreover, plastic's chemical composition is not unique but is very heterogenic, depending on the utility of the considered plastic material. For this reason, in addition to the mechanical problem of plastic's presence, the worst issue is the input of tons of chemical compounds in the water. In a study by De Frond et al. (2019), it was estimated that moreover than 87000 tons of plastic trash had been thrown into the oceans in 2015 and, with them, 190 tons of 20 different chemical compounds. Naturally, it is impossible to accurately estimate the enormous number of pollutants that plastic releases into the water. Thousands of chemicals can mix and become more or less toxic for the aquatic fauna and flora. The chemicals mainly present in the oceans are oil, toxic metals, and persistent organic pollutants. How is shown in the following infographic (Figure 1), seawater contamination is caused by many different factors: industries, wastewater, ship pollution, etc.

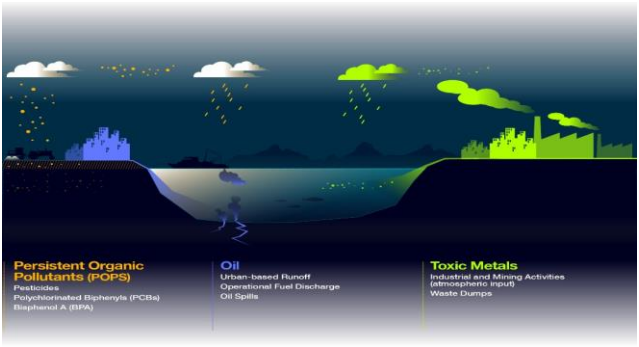


Figure 1. Water Chemical Pollution Infographic by Ocean Health Index

Most of the compounds mentioned above fall into the large category of endocrine disruptors (Endocrine-Disrupting Compounds -EDCs-). They are among the most persistent contaminants in the environment. They have been defined as "endogenous substances capable of causing adverse health effects by modifying the reproductive functions of an organism, affecting the reproductive endocrine system, causing complications also to the offspring". The effect of these contaminants has been found in humans as in many wild species, especially marine organisms, since the aquatic environment

facilitates the distribution of these substances (Colborn et al., 1993; Colborn, 1998; EEA Report, 2002).

Some heavy metals such as Cd, Hg, As, Pb, Mn, and Zn are able to influence the endocrine system, producing alterations in physiological functions (Iavicoli et al., 2009a).

Although various researches already exist, all the mechanisms by which heavy metals act as endocrine disruptors are not yet fully known, in particular because the action varies from species to species and based on the chronicity of exposure to these substances. For example a reduction in plasma testosterone levels was found in a species of teleosts (*Pimephales promelas*) treated with Hg or Pb (Drevnick and Sandheinrich, 2003),

Other examples of endocrine disruptors can be flame retardants (Szabo et al., 2009), phthalates (C. Wang et al., 2016), bisphenols (Molina et al., 2018), dioxins (Mocarelli et al., 2008), pesticides (Sifakis et al., 2011) and so-called emerging contaminants, which include drugs and personal hygiene products.

How is it possible to study the effect of so many free substances in the environment? Bioindicators and biomarkers have long been the fastest and most comprehensive way to study environmental contamination, particularly in the marine environment.

Through bioindicator organisms, especially from the benthos,

pollution's negative effect was highlighted in animal physiology. It is not simple to understand these effects by studying pollutants only from the chemical point of view, given that those effects derive from many compounds' synergistic and simultaneous presence. So, biomarkers and bioindicator organisms can act as a starting point for such a vast problem (Rank, 2009; C. R. Tyler et al., 1998; Warwick et al., 1990). A biomarker is a signal within the bioindicator organism that is particularly sensitive to environmental changes or pollutants. In a chemical safety meeting, the International Program on Chemical Safety, conducted by the World Health Organization (WHO), the biomarker was defined as "any substance or physiology response, which can influence or predict a pathology or a disturb" (United Nations Environment Programme, International Labour Organisation, World Health Organization, and International Program on Chemical Safety., 2001).

In another report on the validity of biomarkers in environmental risk assessment, the WHO proposed a complete definition of them: "any analysis that reflects an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The analyzed response can be functional and physiological, biochemical at the cellular level, or a molecular interaction" (WHO Task Group on Biomarkers and Risk Assessment: Concepts and

Principles. et al., 1993). Studying these molecules/signals in research has become very common as the first endpoint to be reached and verified, both for environmental and clinical studies. On the other hand, an even broader definition considers not only the incidence or outcome of a disease but treatments or exposure, even involuntary, for example, to chemicals. Specific biomarkers must be well characterized, and their functionality must be demonstrated several times to predict certain conditions correctly and justify their appropriate use in a particular research aim (Strimbu and Tavel, 2010). However, these biological indicators have been considered an integral part of environmental monitoring analyses in both national and EU regulations in recent years. Pure chemical analyses were previously carried out, but biodiversity and the biological component have been highlighted (C.I.B.M. "G. Bacci" 2015). There is still a lot to do, both in terms of improving analytical techniques and in terms of management. In Europe, measures to safeguard the marine environment are inefficient, and chemical and molecular analyses are not enough, but greater coordination between the scientific sector and political forces is needed (Katsanevakis et al., 2020).

1.1 Aim of the Doctoral Research Project

The current project aims to evaluate the possible pollution of coastal marine waters in three areas of the Sicilian eastern coast characterized by different anthropogenic stresses. This evaluation will be carried out using bioindicators, the two Mediterranean fish species of *Mullus*, and specific exposure and effect biomarkers.

The peculiarities of the two fish species and the wide range of biomarkers used will allow us to picture the type of pollutants present in eastern Sicilian waters. It avoids using direct analyses of the sediment and the waters, which are often limiting and expensive. Three areas of the coast were considered in this project due to their anthropogenic characteristics.

The tract from San Marco beach (Calatabiano, CT) to AciCastello (Acitrezza, CT) was considered for its high urbanization rates. Augusta basin (SR) is a zone characterized by the presence of the petrochemical area of 'Priolo', a high environmental risk site due to the massive industrialization and high level of pollution. The third studied area is the tract from Avola to Portopalo di Capo Passero (SR), a heavily agricultural area.

1.2 Sicilian seawater pollution

The island of Sicily is separate from mainland Italy from the Straits of Messina, characterized by different water masses that present a complex distribution, also in the neighboring portions of the Ionian and Tyrrhenian Seas.

In 2019 the ARPA Sicilia (Regional Agency for the Protection of the Environment) published the yearbook on the environmental data of the Region. Various parameters have been assessed regarding coastal waters, including their chemical status classification based on water and sediment matrices analyses. According to this document, "The chemical status of coastal marine surface waters is defined based on the concentration of specific pollutants in the water matrix, sediments, and biota. The water body's chemical status is classified as good if the water body meets all the Standards of Environmental Quality (EQS) for the substances in the priority list (Tab. 1/A water and biota matrix, Tab. 2/A sediment matrix Legislative Decree 172/2015). If not, the water body is classified as a water body in which good chemical status is not recognized." According to this report, the classification as "not good" chemicals status is a union between the quality of sediment and water analysis. All the areas (except Portopalo) resulted "not good" only in water analysis despite the quality of the sediment was "good". Only one area showed "not good"

status in both water and sediment: Acicastello (Yearbook ARPA Sicilia, 2019).

As now well known, there are at least five primary sources of water pollution: domestic wastewater, agricultural runoff and industrial wastewater, sewage from septic tanks, and rainwater (Maulin P., 2017). First, urban wastewater is a significant pollution source, especially with intense urbanization, and the treatment plants are obsolete or malfunctioning. These systems often also receive industrial and agricultural wastewater, significantly increasing the range of chemical compounds and contaminants to be removed. Unfortunately, as the production of new chemicals increases, it is difficult to purify the water completely. Therefore, in wastewater, it is possible to find all types of contaminants, from heavy metals, PCBs, pesticides, and herbicides, up to the so-called "emerging contaminants" such as pharmaceuticals and personal care products (PPCPs) (Kasprzyk-Hordern et al. 2008; Mason et al. 2016; Ratola et al. 2012). Considering the fundamental role of wastewater treatment plants, the 2019 "Treatment plants control" ARPA Sicily Report is somewhat alarming: of the approximately 5 million inhabitants residing in Sicily, distributed in 390 municipalities, only about 61% are served a wastewater treatment plant. Furthermore, in the report mentioned above (which refers to 2018), most Sicilian wastewater

treatment plants have been sanctioned or have requested the plant's adjustments (Report ARPA Sicilia 2019).

Furthermore, in many areas of the Mediterranean, like Sicily, the cities characterized by a substantial tourist increase during the summer, appear to have more significant problems of water pollution due to an overload of wastewater and the presence of contaminants present in detergents, pharmaceutical and personal care compounds (Orhon et al. 1999; Paraskevas et al. 2002; Renzi et al. 2012).

In addition to wastewater, marine contamination by heavy metals is remarkably persistent on the Sicilian eastern coast, particularly in the Priolo Gargallo area (Augusta Bay, SR), where there is a petrochemical industry. According to a study conducted by Sprovieri et al. (2011), the Augusta basin can release a large amount of Hg to the Mediterranean due to its hydrological and geological characteristics of the continental slope present outside the basin favored by the Levantine Intermediate waters for the transport of pollutants.

Another form of pollution is to be considered that of PAHs, ubiquitous (Gianguzza and Orecchio, 2006; Orecchio, 2007; Bansal and Kim, 2015), volatile substances deriving from incomplete combustions, direct dispersions of oil products, and biogenic natural sources, which are released into the environment by various human

activities: urban runoff (Hoffman et al., 1984; Boonyatumanond et al., 2007), household heating (Vuković et al., 2015), manufactured gas plant sites et al., 2012). There are many studies about PAHs in Sicily, those substances were found in the air, on the surface of monuments, on leaves plants, and in superficial seawater (Governanti et al. 2004; Orecchio, 2007; Romagnoli et al., 2016; De Guidi et al., 2017; Vecchiato et al., 2018). In particular, some research evidence PAHs presence in the sediment of a lake like Ganzirri (ME) and coastal sea sediment (Giacalone et al., 2004: Frenna et al., 2013). The effects of these contaminants on the benthonic compart are also known, as shown in a study about the Augusta basin, where the micropaleontological analyses, performed on three sedimentary sequences deposited after 1940, underline the modification in the benthic foraminiferal assemblage (Di Leonardo et al., 2007). A study by Perra et al. (2011) highlighted how the MPA of Isole dei Ciclopi (Acitrezza, CT) sea sediments are characterized by a high PAH rate, like other marine protected areas characterized by a high tourist impact.

Another type of contamination comes from the agriculture industry in Sicily, representing a large portion of the economic activity, particularly in the southeast. Pesticides and herbicides are chemical compounds resistant and persistent in the environment.

During a monitoring campaign carried out on the southeast coast of Sicily (Ragusa), Licciardello et al. (2011) observed high pesticide contamination of groundwater in two of the five monitoring wells. About the persistence of these compounds, they said: "At least 25 % of the observed pesticides that have not been for sale for some years (from 1 to 9), were observed, probably due to the residue in groundwater or the use by the farmers of the remaining amount also after the deadline set by the law. Moreover, it has to be noted that most of the observed pesticides (about 71 %) were not applied in the selected farms (at least officially). Most of the observed pesticides are insecticides and fungicides; 26.8 % of them resulted in the highest risk classes set by the Pesticides Working Group of Italian Environmental Agencies".

1.3 Biology, ecology and socio-economic importance of Mullus sp

Mullus barbatus and *Mullus surmuletus*, respectively red mullet and red striped mullet, are sympatric species with a vast and similar geographical distribution that includes the coastal areas and the continental shelf of the Mediterranean Sea and the northwestern Atlantic until 200-300 m depths (Hureau, 1986; Tserpes et al., 2002). The two species show many common characters, including

morphological characters (Gosline, 1984; Aguirre and Lombarte, 1999), feeding habits (Gharbi and Ktari, 1979; Golani and Galil, 1991), biological and genetic similarities (Cammarata et al., 1991; Arculeo et al., 1999).

Since both species are benthic carnivores, they feed on small invertebrates (crustaceans, mollusks, polychaetes) that live on or within the bottom substrates (Gharbi and Ktari, 1979; Golani and Galil, 1991). For these ecological habits, they pass their entire adult life in contact with the sediments. For this reason, they can be considered the primary target species of Mediterranean demersal fishing and are fished using more than one type of gear, like the bottom-trawl net and gill net (Reñones et al., 1995; Demestre et al., 1997). Demersal fishing represents one of the most productive fisheries of the Mediterranean Sea, including the Sicilian fishery compart (Lleonart, 2005).

These two species are fundamental for the sector of small Sicilian artisanal fishing, which represents 69% of the whole fleet, involving 2530 vessels (Battaglia et al., 2010; Cannizzaro et al., 2011).

1.3.1 Mullus surmuletus

The Striped Red mullet has an elongated body with many shades of color: it has a reddish-brown back, whitish sides with three

or four yellow-golden horizontal stripes, and a pink belly. The body's anterior and ventral position have two appendages, the barbels, used to search for food on the seabed; they can be hidden in a groove on the lower jaw during rest (Louisy and Trainito, 2006). The life cycle of mullets comprises different pelagic phases represented by eggs, post-larvae, and juvenile specimens (Palomera et al., 2007). Growth varies between the two sexes: females are characterized by a faster growth rate and a larger size, while males grow slowly and are smaller (Voliani, 1999). Commonly, the male ranges from 11 to 28 cm in length, while the female ranges from 12 to 32 cm. Both females and males reach sexual maturity about one year after birth (Reñones et al., 1995). The period of reproductive activity in females runs from March to June, with a peak in April and May; in males, on the other hand, the period of reproductive activity is more expansive and extends from December to June, with a peak in March and April (Reñones et al., 1995).

1.3.2 Mullus barbatus

On a morphological level, *M. barbatus* is very similar to *M. surmuletus*. It is distinguished only for a few details; the head has a vertical profile, opposite to that of the red mullet, which, on the other hand, is more pointed. It has two barbels with a sensory function,

which it uses to track down prey in sediment. The body's coloring is generally pink on the back and white on the belly, with a longitudinal reddish stripe that extends from the eye to the caudal end of the body (Louisy and Trainito, 2006). The reproductive period coincides with that of *M. surmuletus.*

1.4 Biomarkers of exposure

Exposure biomarkers are used to quantify specific chemical residues or metabolites of xenobiotic compounds in different biological matrices, taking into account the kinetics of the biomarkers of interest (Frontiersin.org). Sometimes measuring the exposure biomarkers says nothing about the effect. For example, measuring the response activated by metals, hydrocarbons, or halogenates in some cases, does not necessarily indicate that the organism is undergoing toxicity.

1.4.1 Concentrations of heavy metals

Heavy metals are those metals that have a relatively high density, greater than 4 g/cm^3, five times greater than water and are toxic or poisonous even at low concentrations. Metals are considered essential and highly toxic pollutants in the various environments in which they can be found and have demonstrated this due to their high

toxicity and persistence in the habitats in which they are found (Naggar and Ghorab, 2018).

Heavy metals can be released into the environment from both natural and anthropogenic sources. They are natural components of rocks and soils and enter the environment due to atmospheric agents and erosion. Important natural sources are continental atmospheric agents, forest fires, particles released by vegetation, and volcanic activity.

Aquatic systems, in particular, are susceptible to the presence of metals as pollutants, probably to a greater extent than terrestrial habitats. In summary, all living organisms in any ecosystem are variously contaminated, bioaccumulating and biomagnifying these compounds along the food chain.

Chemical characterization of metals directly in a tissue can be a quick first approach for assessing the contamination of that species at a particular sampling site. This biomarker of exposure is widely used. The analysis of the concentrations of these metals can be helpful both in terms of environmental assessment and risk assessment in human consumption of some foods by humans. For example, fish often have very high levels of these compounds, which build up in the muscle (Copat et al., 2013).

The maximum limits of contamination of meat permitted by

Italian law have been established only for some metals such as Lead, Cadmium, and Mercury (Reg. CE1881 / 2006), for metals for which no maximum limits are envisaged, it is only possible to make a comparison with the levels found in similar studies for these elements.

1.4.2 Concentrations of polycyclic aromatic hydrocarbons (PAHs)

The ecological risks of polycyclic aromatic hydrocarbons (PAHs) in aquatic sediments will vary with toxicity and bioavailability to aquatic biota. PAHs are ubiquitous compounds from multiple sources such as petroleum, spilled fuel, street run-off, coal tar from coal gasification, creosote treatment of wood, or coke ovens at steel plants (Ringuette, 1993). Furthermore, these compounds are easily retained and, in particular conditions released by plastic material, ubiquitous garbage of our seas (Heintzman et al., 2015; Lohmann et al., 2009; Tan et al., 2019).

One of the major problems is that the biodegradation of PAHs is highly variable and depends not only on PAH structure, but also on the physicochemical characteristcs of the site and the number of types of microorganisms present. PAHs sorb to the organic matter in soils and sediments, and the rate of their desorption influences the rate at which microorganisms can degrade the pollutants (Shuttleworth and

Cerniglia, 1995).

Another criticality of the presence of these compounds in the environment is that they are often not present alone but in the form of a complex mixture, for which risk assessment remains problematic (Le Bihanic et al., 2014).

The use of fish, and aquatic organisms in general, as bioindicators remains a crucial point for assessing the presence of these contaminants in water. In a study conducted by Escartín and Porte (1999), it was pointed out that fish living in areas subject to a strong impact of industry tend to accumulate more PAHs. However, attention must be paid to the species chosen as a bioindicator as there are substantial differences in the responses to these exhibits. In this case, between the two species examined, *Mullus barbatus* and *Serranus cabrilla*, the species of Mullidae accumulate much more 1-pyrene than the other, with orders of magnitude tens of times greater.

1.4.3 Concentrations of pesticides

The most used pesticides are insecticides, herbicides, and fungicides. Pesticides are of two types, natural and synthetic pesticides. Pesticide's usage in agricultural fields to control pests is highly toxic to non-target organisms like fish and affects fish health through metabolism impairment, sometimes leading to mortality. The

effects of these contaminants are different at many physiological levels, such as effects on acetylcholinesterase, chromosomal aberrations, carcinogenic effects, and changes in protein content (Shankar et al., 2013). Before 1940, most chemicals used as pesticides were based on these same toxic groups, in such compounds as lead arsenate, copper sulfate, sodium arsenite, sodium cyanide, and phenolic mixtures. In addition to these, naturally- occurring organic compounds derived from plants, such as pyrethrum, derris, and nicotine were widely used as insecticides (Holden, 1973).

Regarding the fishes, the bottom feeders play a significant role in the process of resuspension, during which the contaminant adsorbed on the sediment would be recycled into the water column. OCPs contained in farmland run-off can enter the river and persist for an extended period; they can therefore be adsorbed in the sediment and onto suspended particulate matter, transferred into food chains, accumulated in the fatty tissues of fish, and finally reach human beings. According to the diet, it would seem that carnivores accumulate more pesticides than omnivores and herbivores (Zhou et al., 2007).

The use of pesticides as biomarkers must have a multi-approach, as each substance acts and interferes with the various tissues differently. In an in vitro experiment, exposure to different

contaminants separated and combined together causes different responses in tissues such as liver, gills, and brain, resulting in different oxidative damage to the lipid component (Bacchetta et al., 2014).

The most common pesticide always found in the environment is now DDT, although its ban dates back to many years ago. In a study conducted by Wang et al. (2012), DTT accounts for nearly 83% of all pesticides detected in the samples.

The effects of these substances on aquatic fauna are manifold: histological alterations of the branches, interaction with the protein synthesis process, decrease in erythrocyte count, abnormal behaviors, poor oxygen transport capacity in the blood, decrease in glycogen in the liver, physiological and histological disorders in the gonads, necrosis and osmoregulatory dysfunctions (Napit, 2013).

Furthermore, the strong bioaccumulation power of these substances affects the trophic chain, exposing possible predators and even humans to intense exposures of these substances, consequently to the consumption of contaminated fish. Therefore there is a high health risk (Buah-Kwofie et al., 2018).

1.4.4 Vitellogenin (VTG) gene expression and plasma levels

Vitellogenin (VTG) is a phosphoglycoprotein produced by the liver, and it is the yolk's precursor in all oviparous and ovoviviparous vertebrates. Vertebrate VTGs are composed of two lipovitellins and a phosvitin, distributed as follows within the precursor: the domain representing lipovitellin I is located at the N-terminal end, while the domain representing lipovitellin II is located at the C-terminal end. Finally, between these two domains, there is the domain of phosvitin. Therefore, the domains of VTG are arranged linearly as follows: NH2 - heavy chain lipovitellin - phosvitin - light chain lipovitellin - component β '- COOH (Lubzens et al., 2010).

It is one of the most widely used biomarkers of reproduction for the study of EDCs (Endocrine Disruptor Compounds) for many animal species, from fish to birds. (Fossi et al., 2002; Jiménez et al., 2007). VTG responds to estrogenic signals since estradiol, like estrogen-like, binds to specific intracellular receptors in liver cells. It forms a complex that interacts with the promoter of the VTG gene family, inducing its transcription.

Vitellogenesis is a crucial event of reproductive function and occurs during oogenesis, the main regulatory system of which is the hypothalamic-pituitary-gonadal axis. Natural environmental factors,

such as temperature and photoperiod, the availability of food, suitable places for reproduction, and nesting influence the central nervous system and the hypothalamus-pituitary-gonadal axis in regulating gonadal maturation and hormone secretion sexual. Subsequently, therefore, under favorable conditions, the secreting neurons of the hypothalamus release GnRH, which stimulates the pituitary to release LH and FSH. Once released into the bloodstream, these gonadotropins reach the gonad where they produce estrogens used for ovogenesis and stimulate the hepatocytes to synthesize VTG.

After the protein synthesis, it is released in the blood and spreads first in the theca cells, associated with blood vessels, and then in those of the granulosa. Here, it passes through channels and enters the radiated area, taking it for a mechanism of receptor-mediated endocytosis. The internalization of vitellogenin occurs in pits coated in the plasma membrane on oocytes from which the coated vesicles are formed (Wallace, 1985), VTG is taken up by membrane-bound vitellogenin receptors (VtgR). The VtgR, which belongs to the low-density lipoprotein receptor family, interacts with the N-terminal region of VTG via electrostatic attraction. Once in the oocytes, VTG is cleaved into yolk proteins (lipovitellin and phosvitin), stored as nutrients for the developing embryo (Ding, 2005).

In males, these genes are heavily methylated and “silenced”.

Therefore, this protein expression in males can be considered a valid biomarker of effect to these contaminants (Forner-Piquer et al., 2020). It has also been shown that endocrine disruptors such as bisphenol A have transgenerational effects on the larval stage of male F1 born from females exposed to these substances (Santangeli et al., 2016). Many studies have also highlighted using these biomarkers (VTG-like) in marine invertebrates, essential for studying endocrine disruptors as they are part of the trophic chain (Matozzo et al., 2008).

Numerous studies have also been done in the laboratory to associate the effect of specific concentrations of environmental pollutants with estrogenic activity under controlled conditions. One of the main compounds tested is the ubiquitous compound nonylphenol (NP). It mimics estrogens' action by binding to specific receptors and induces the expression and synthesis of VTG and other molecules related to reproduction. This effect has been evidenced in organisms such as *Salmo salar*, *Pimephales promelas,* and *Oncorhynchus mykiss* (Arukwe et al., 2001; Giesy et al., 2009; Yadetie and Male, 2002).

In general, estrogens and 17β-estradiol, in particular, are potent inducers of vitellogenesis. In fact, since the first experiments, the response to the administration of estrogens has been necessary for Chondrichthyes, teleosts, amphibians, reptiles, and birds. Estrogens can also increase the number of their receptors in hepatocytes of

immature males or females and stabilize the VTG messenger against cytoplasmic degradation.

In a study by Scholz et al. (2004), it has been shown that small teleosts such as medaka (*Oryzias latipes*) are an advantageous model for studying the endocrine disorders caused by various chemicals. The medaka has a short reproductive cycle, which allows the study of the effects of EDs during a complete life cycle. Furthermore, it has been shown to be very sensitive to hormone exposure leading to complete and functional sexual inversion. In the above study, increasing concentrations of ethinylestradiol and genistein were administered, and a significant increase in vitellogenin synthesis was observed in young and adult males.

Another study by Zezza et al. (2020) considered specimens of wild trout (*Salmo trutta*) from Lake Liri in Abruzzo. The results showed that 92% of male fish exhibited serum VTG concentrations above 10 ng / mL. The elevated VTG levels found in male trout could be interpreted as a sign of an effect induced by exposure to endocrine disruptors, particularly environmental estrogen.

Among the adverse effects of the increase in the synthesis of vitellogenesis in fish were found kidney damage, increased susceptibility to adult disease of testicular growth, reduction in the rate of spermatogenesis, and changes in sexual maturity. These have clear

negative consequences on the reproduction of the organisms themselves and are therefore highly relevant from an ecological point of view (Solé et al. 2001).

Several studies have also been conducted on various species of invertebrates, especially mollusks (for example, several species of bivalves and gastropods). In particular, mollusks are taken into consideration as they are capable of filtering water: by doing this, however, most of the pollutants present in the water are bioaccumulated and cause physiological alterations to the organisms themselves. As for fish, it has been seen that the synthesis levels of VTG increase when these species are exposed to chemicals with estrogenic activity (Tran et al., 2019).

That confirms that the analysis of the gene expression level and its serum concentration in immature females and males can be used as a biomarker of alteration of the endocrine system in the examinations and, therefore, their eventual exposure to endocrine disruptors present in the environment.

1.5 Biomarkers of effects

The use of effect biomarkers usually involves quantifiable changes in biochemical or physiologic parameters. Measurement of oxidative stress markers, cytogenetic endpoints, such as micronuclei

induction, chromosome aberrations, sister chromatid exchange, has been classically used as effect biomarkers (Links and Groopman, 2010).

1.5.1 Gonadal histology

The analysis of histological changes in different fish tissues has been widely used for decades as an instrument in aquatic toxicology to monitor acute and chronic situations and provide additional information to physicochemical analyses. In addition, techniques have been used to assess environmental pollution for over 20 years, providing details on the effects of many substances in the aquatic environment (Yancheva et al., 2016).

The histology of gonads offers a powerful method in studying the reproductive health of marine organisms, particularly in fish. It is routinely used for sex verification, identifying the stage of development, documenting the presence of intersex, tumors, parasites, and other abnormalities, and quantifying atresia. It can also be used for more subtle changes such as the thickness of the vitelline envelope at various stages, yolk appearance, necrosis of sperm, and Sertoli cell proliferation. Gonadal histology, in conjunction with hormone and vitellogenin measurements, morphological and fecundity studies, can provide insights into the effects of various environmental stressors on

reproductive health (Blazer, 2002).

1.5.1.1 Presence of intersex

Intersex is defined as the simultaneous presence of male and female gonadal tissue in a gonochoristic (fixed-sex) species. The intersex condition has been documented in both wild and laboratory animals, including fish, amphibians, and reptiles (Charles et al., 2008). In a review of Bahamonde et al. (2013), approximately 37 species of fish have been reported to exhibit some degree of intersex in the environment, and evidence is not conclusive that changes in gonad size, plasma VTG, and sex steroid hormones are correlated to intersex. For this reason, it is always recommended to conduct every type of analysis, particularly on new fish species, to understand better which biomarkers could be the best for this fish bioindicator.

1.5.1.2 Melanomacrophage centers (MMCs)

Melanomacrophage centers (MMCs) are distinct groups of pigment-containing cells, generally found in the reticuloendothelial support matrix of hematopoietic tissues. In teleosts, hematopoiesis is typically located in the spleen's stroma and the kidney, and there is also an element of hemopoiesis in the peri-portal areas of the liver, the

intestinal submucosa, and the thymus. There are also reports of their occasional occurrence in gills, brains, and gonads (Agius and Roberts, 2003; Macchi et al., 1992).

Although the term "melanomacrophage centers" was applied for the first time for these structures found in teleosts (Ellis and Sousa, 1974; Ribelin, 1975), analogous structures have also been described in the liver of cartilaginous and primitive bony fish (Agius, 1980).

In the most evolved bony fishes, the melanomacrophage centers have larger dimensions and may also contain leukocytes; in salmonids and cartilaginous fish, they are smaller in size, and have a considerable amount of dark pigments, and are distributed somewhat randomly throughout the tissues in which they occur (Agius, 1985).

It has been suggested on both functional and structural grounds that melanomacrophage centers may represent the primitive analogs of the lymph nodes' germinal centers in birds and mammals (Ellis, 1980; Ferguson 1976). Similar pigmented macrophage structures have also been described in amphibians and mammals (Pintucci et al., 1990; Scalia et al., 1988).

The term "melanomacrophage centers" was initially proposed by Roberts (1975), based on the definition of melanin given by Edelstein (1971). He defined the centers as periodic acid Schiff, Schmorls, and Ziehl Neelsen positive staining, generally yellow to

black, aggregates of pigment cells within the hemopoietic tissues, and mature chronic inflammatory tissue.

These aggregates are usually nodular with a delicate argyrophilic capsule and are generally closely applied to vascular channels. They are enlarged after active phagocytoses of heterogeneous materials, such as cell debris, melanin pigments, haemosiderin granules, and lipofuscin residues (Agius, 1985; Agius and Agbede, 1984), as well as lipid droplets, essential protein aggregates, and neutral mucopolysaccharide (Herraez and Zapata, 1986).

The morphological appearance of the melanomacrophage centers may vary in the different species, organs, and also in different physiological conditions within the same species, such as age, starvation, tissue breakdown, iron and hemoglobin metabolism, pathological and inflammatory conditions and immunological processes. There are also published data on changes in macrophages' number and capacity caused by environmental changes (Fournie et al., 2001; Kranz and Gercken, 1987). Furthermore, Peters and Schwarzer (1985) suggested environmental stress itself may induce cellular changes in fish tissues, the main effects including increased macrophage-like cells and enhanced red blood cell degradation.

Many of these studies suggest that the centers' general function

is focused on the destruction, detoxification, or recycling of endogenous and exogenous materials. Therefore, one of the key functions of melanomacrophage centers is their role of metabolic dump for the transfer of debris of dead or damaged cells, including red blood cells (Fulop and McMillan, 1984).

As expected in a structure with such function, these centers have also been observed to increase in size and number as fish age and tissues degenerate (Brown and George, 1985). Melanomacrophage centers also play an essential role in the response of fish to foreign materials, including infectious agents.

Changes in the size of the centers of melanomacrophages can occur physiologically, in association with aging. However, since there are also numerous cases of increasing their size or spleen or kidney size or, in some cases, reducing their frequency in relation to pollutants persuasively supported their use as biomarkers for measuring the effects of environmental exposure to polluting chemicals (Hargis, 1985).

In the ovaries and testes, melanomacrophage centers' presence was correlated with the degradation and reabsorption, respectively, of oocytes and sperm cells not regenerated during gonadal regression (Grier and Taylor 1998; Kumar and Joy, 2015; Ravaglia and Maggese 1995). Like splenic and hepatic melanomacrophage centers, gonadal

ones could be possible tissue biomarkers of environmental stress because they have been found particularly abundant in the testicles of fish collected in polluted areas (Blazer, 2002; Patiño et al., 2003).

A recent study by Micale et al. in 2019 along the north coast of Sicily opened the way for further studies and insights into melanomacrophage centers and their impact on fishes' male and female gonads. These authors suggested that these MMCs presences in the female gonads could be related to spontaneous ovarian regression after spawning. In males, it could be mainly due to environmental factors.

2 Material and methods

2.1 Areas of interest and sampling

Red mullet and Striped Red mullet individuals were caught on the Italian side of Food and Agriculture Organization (FAO) General Fisheries Commission for the Mediterranean (GFCM) Geographical Sub Area 19 (GSA 19) and 16 (GSA 16), along the Sicilian east coast, from October 2018 to October 2019 using bottom trawl nets and gill

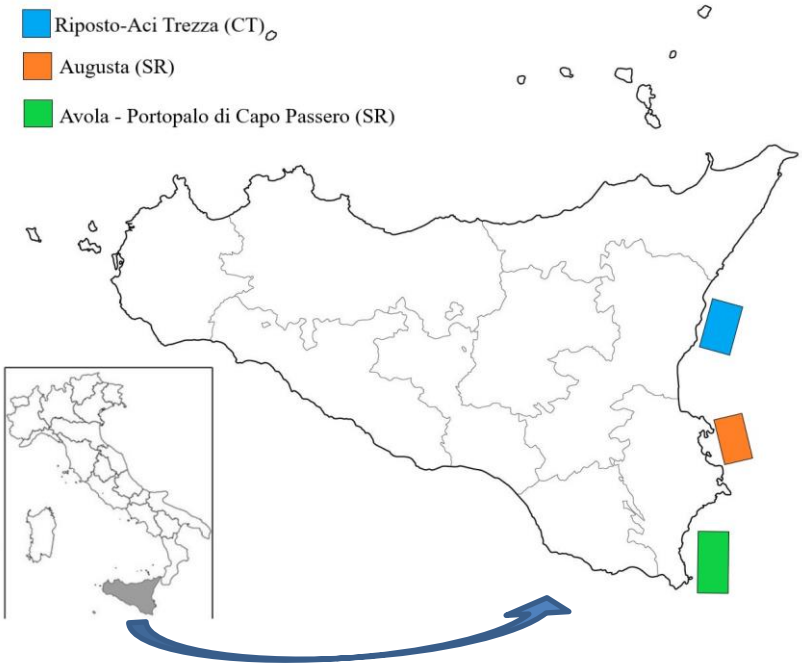


Figure 2. Maps of Sicily with the three studying areas.

nets. The first area of interest is the tract between Riposto and Aci Trezza (NORTH SITE). The second one is Augusta (CENTRE SITE). The third is the tract between Avola and Portopalo di Capo Passero (SOUTH SITE).

219 fishes were sampled. Each sample was collected using sterilized dissection material (knives, scalpels, tweezers, and scissors) made of stainless steel to avoid every type of possible contamination. The fishes were anesthetized, bled by caudal puncture (heparinized 23-G needles), killed by a sharp blow to the head, weighed and measured, the gonads and the livers removed, and weighed, and the sex identified. The livers for qPCR analyses were immediately preserved in RNAlater (Ambion, USA) and stored at -20°C for analysis.

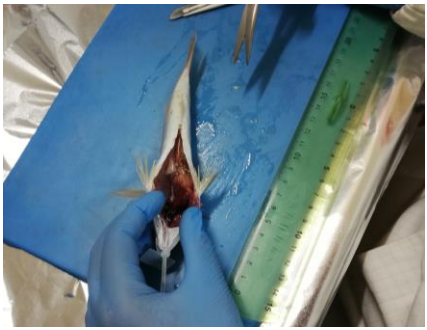


Figure 3. Sampling of a Red mullet.

For each sample, were also collected two aliquots of muscle for chemical analysis.

2.2 Detection of heavy metals

From each specimen, aliquots of 0.5 g of muscle tissue were removed, and metals of interest were extracted and quantified. The samples were mineralized in a Digestion Block System (DigiPrep) using a heated mixture of strong acids. A digestion solution was prepared with 6 ml of 65% nitric acid (HNO3) (Carlo Erba) and 2 ml of 30% peroxide hydrogen (H2O2-Carlo Erba) over a 50 min operation cycle at 80 °C (Figure 4).

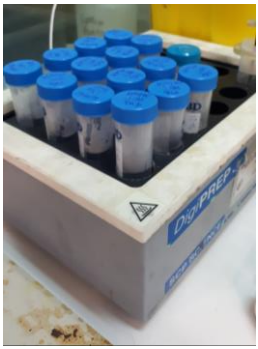


Figure 4. Digiprep at 80° C with the samples

After mineralization, the vessels were opened if a temperature < 25 °C was reached. The content was decanted in falcon tubes, and ultra-pure water (Merck) was added to the samples up to 50 ml. For quantification ICP-MS Elan-DRC-e (Perkin–Elmer, USA) was used. Analytical blanks were processed the same way as the samples, and concentrations were determined using standard solutions prepared in the same acid matrix. Standards for the instrument calibration were prepared with a multi-elements certified reference solution ICP Standard (Merck). The method detection limits (MDL) estimated with 3r of the procedure blanks were (mg/kg w. w.): As 0.273, Cd 0.009, Co 0.045, Cu 0.181, Hg 0.035, Mn 0.262, Pb 0.163, Se 0.026 and V

0.02. For each batch of mineralization, a laboratory-fortified matrix (LFM) was processed for quality control, and we obtained recovery rates between 91.5 and 110%.

2.3 *Detection of IPA and pesticides*

Sample Preparation and Extraction. For each specimen of collected fish, the muscle was homogenized, and aliquots of 2 g were transferred in 10 ml plastic tubes. In each one, 3 ml of phosphate buffer (pH 6) were added to improve the contaminants' extraction in weakly acid pH. Phenanthrene-d10 (Restek) was used as an internal standard at a final concentration of 150 ng/g. After vortexing, all samples were exposed to the ultrasonic bath for 6 minutes at 20 °C. After another vortex step, 3 ml of ethyl acetate were added to each sample. The tubes were vortexed again, and the samples were centrifugated twice for 5 minutes at 4000 rpm (2500 rcf). The supernatants were transferred, through fiberglass filtration, in new 10 ml plastic tubes.

Extraction with ethyl acetate was repeated. Supernatants were added to respective firsts collected.

Extracts were concentrated under a weak nitrogen flow approximatively at a final volume of 50 µl and transferred in glass vials for the analysis (Figure 5).

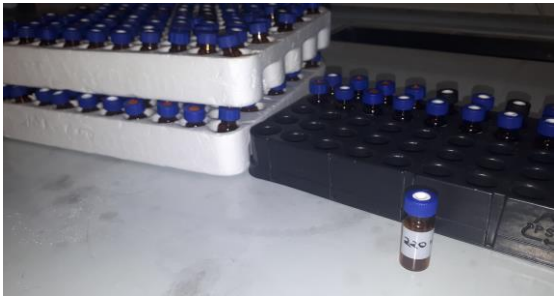


Figure 5. Glass vials with extracted samples.

Pesticides Analysis. The residues were analyzed in gas chromatography coupled with mass spectrometry (GC-MS).

For this purpose, a Shimazu Gas-chromatographer coupled with an ECD detector was used. Each run was conducted at a programmed temperature, from 100 ° C to 180 ° C with increments of 30 ° C per minute, followed by an isotherm of 3 minutes at 180 ° C, and from 180 ° C to 250 ° C with increments of 10 ° C per minute, followed by a 5-minute isotherm at 300 ° C, using an Eclipse-XLB (Perkin Elmer) capillary column, 30 meters long (ID 0.25 mm and DF 1.4 mcm).

Investigated pesticides were *Heptachlor, Pirimiphos Methyl, Fenitrothion, Malathion, Aldrin, Chlorpyrifos, Parathion (Ethyl), Isodrin, Heptachlor Epoxide, Chlorfenviphos, Bromophos Ethyl, Endosulfan I, Tetrachlorvinphos, 4,4'-DDE, Dieldrin, Endrin, Endosulfan II, 4,4'-DDD, 4,4'-DDT, Chlorpyrifos Methyl*
Endrin Aldheyde, Azinphos methyl.

PAH Analysis. The residues were analyzed by liquid chromatography coupled with a UV-Visible detector (HPLC-UV).

For this purpose, an Agilent HPLC-UV was used. Chromatography run was conducted in gradient with mobile phases consisting of water and acetonitrile at the wavelength of 360 nm.

Investigated PAHs were *Fluoranthene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Benzo(ghi)perylene, Indeno(1,2,3-cd)pyrene, Acenaphthene, Acenaphthylene, Anthracene, Benz(a)anthracene, Crysene, Dibenz(ah)anthracene, Fluorene, Naphthalene, Pyrene.*

2.4 Histological analysis of gonads

The fresh gonad's samples were stored in 4% paraformaldehyde overnight; subsequently, they were subjected to two washes in 10x PBS, one in 50% ethanol, and, finally, stored in 70% ethanol overnight. For the paraffin inclusion process, the samples were dehydrated in ethanol with increasing concentration. Subsequently, they were then treated with xylene and, finally, embedded in liquid paraffin at a temperature of 60° C.

Once the paraffin inclusions on plastic support were obtained, sections with a thickness of 3, 5, 20 µm were obtained with the

microtome and then positioned on the slides.

Finally, the slides were subjected to staining with hematoxylin-eosin: first, the tissue sections were rehydrated with passages in ethanol at decreasing dilution percentages, and then carried out the first pass in hematoxylin and then in eosin. Finally, rapid washes were carried out in ethanol at increasing percentages of dilutions and ended with a final wash in xylene. Once this last step was completed, the slide cover was applied.

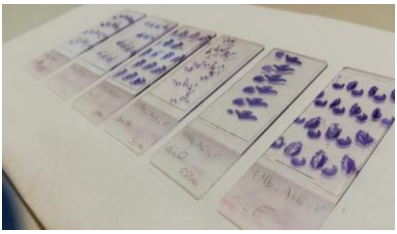


Figure 6. Series of slides after the HE coloration.

All the slides obtained were then subjected to careful analysis with an optical microscope.

To analyze possible intersex phenomena and evaluate the presence of melanomacrophage center, the same method was used, considering all the males of each species under examination. Eight slides per same fish were chosen along the entire gonad, observing all the sections for areas of 1 mm and assigning a degree of abundance between 0 and 3 based on the number detected, according to the following scheme:

- "0" in total absence;

- "1" if 1 to 3 MMCs or oocytes were present in at least three slides;
- "2" in the presence of 4-6 MMCs or oocytes in at least three slides;
- "3" number of centers of melanomacrophages or oocytes greater than 6 units.

2.5 *vtg gene expression*

Liver samples were preserved in RNAlater™ Stabilization Solution (Invitrogen) and stored at -20°C until the moment of the molecular analysis.

Extraction. 25 mg of each sample were taken and transferred into a 1.5 ml RNase-free tube. Total RNA was extracted from the liver using the PureLink™ RNA Mini Kit (Invitrogen).

Purification. The purification from genomic DNA was performed using the On-column PureLink® DNase Treatment during the extraction phase. All eluates of 50 µl were collected into RNase-free tubes and stored at –80°C.

Quantification. To evaluate the quantity of extracted RNA and the

purity of the samples, spectrophotometric analyses were carried out using Nanodrop ND-1000.
Following the loading of 1 µl of the sample, the instrument will evaluate the absorbance level at 230, 260, 280 nm and the respective ratios.

Retrotrascription. Total RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen). Each cDNA was transcripted from 1000 ng of total RNA, with a final concentration of 55 ng/µl.
The obtained first-strand cDNA was stored at -20°C until use.

In this case, PCR was conducted as a control to verify that the previous analyzes were performed correctly and were successful.
PCR was carried out with Platinum Taq DNA Polymerase of Invitrogen.
After PCR amplification, the reaction products were run at 110V on a TAE-0.8% agarose gel.

qRT-PCR was conducted to quantify the expression level of the *vtg* gene, taking into consideration a housekeeping gene (18S) used as an endogenous control. The primers sequences of both genes (target and

housekeeping) are shown in Table 1.

Gene	Sequences 5'-3'	Annealing temperature	Bibliography
vtg	F: GCCATGGTCCATGAAGATGC R: GCTTTGAACTGAGCCCAGAGAG	60°C	(Mushirobira et al., 2021)
18s	F: AGGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACC	60°C	(Sepulcre et al., 2007)

Table 1. Gene primers sequences

Real-time PCR reactions were conducted using Fast SYBR Green Master Mix (Applied BioSystems).

The thermal cycling conditions were as follows:

- initial denaturation at 95 ◦C for 20 sec;
- 40 cycles of amplification at 95 ◦C for 3 s, 60 ◦C for 30 s, and 72 ◦C for 30 s;
- the melting curve at 95 ◦C for 15 s, 60 ◦C for 1 min, 95 ◦C for 5 acquisition;
- cooling at 40 ◦C for 10 s.

Amplifications were carried out with a systematic negative control (control containing no cDNAs).

Each sample was amplified in triplicate.

No amplification was observed in RT negative controls, and no primer-dimer formation occurred in the NTC. The observed single peak in the dissociation (melt) curve at the end of the amplification

cycle indicated the absence of primer-dimer formation and primer specificity. Furthermore, to validate the correct amplification of the sequence, the PCR product was sequenced, resulting in a 104 bp vitellogenin fragment. Sequencing of *vtg* from the PCR amplification was carried out in the examined samples. A BLAST search was performed to verify the gene's correct amplification, which matched with an isoform of vitellogenin of *Thunnus thynnus*.

Analysis method. Data were analyzed using the comparative CT method (2-ΔΔCT) described by Livak and Schmittgen (2001), so that, in quantitative analysis, Real-time PCR based on threshold counts (Ct) of the tested specimens (treatments fed diets containing different amounts of VE, treated with dietary foods without VE) with control samples (negative control) and using the formula ΔΔCt, the ratio of the target gene to reference gene was calculated using the following formula: R=2-ΔΔCt.

2.6 VTG and steroid hormones in plasma

ELISAs for VTG and four sex steroids were validated for the two species under study (*M. surmuletus* and *M. barbatus*), based on methods developed at IATS-CSIC for *Solea senegalensis* (Guzmán et al., 2008). Regarding VTG, a species-specific protein, we validated

heterologous ELISAs, using antigen (VTG) and antibodies (AbVTG) purified and obtained from S. senegalensis (Guzman et al., 2008) because specific reagents are not available for our species of interest.

2.6.1 Levels of VTG measured by ELISA

The wells of 96-well microplates are first coated with antigen (VTG). Then, the VTG coated wells are incubated with a solution containing the sample/STD (free VTG) and specific VTG antibodies (AbVTG); during this time, free VTG (contained in the sample or STD) will compete with the coated VTG to bind to the AbVTG. A subsequent washing step will eliminate free antigen-antibody complexes, while AbVTG bound to coated VTG will remain attached to the wells. For further sample calculation, these antigen-antibody complexes are further detected with secondary antibodies (labelled with an enzyme, HRP) and color development obtained with TMB.

2.6.2 Levels of steroid hormones measured by ELISA

The wells of 96-well microplates are first coated with monoclonal anti-IgG antibodies. Then, the coated wells are incubated with a solution containing: 1) sample/STD, 2) tracer (AChE-labelled steroid) and, 3) specific steroid antibodies. During this time, the

steroid antibodies will bind to the coated antibodies and also a free steroid. Both the tracer and the one contained in the sample or STD will compete to bind to the steroid antibodies. A subsequent washing step will eliminate free antigen-antibody complexes, while the complexes of steroid antibody with either tracer or steroid in the sample/STD will remain attached to the wells. The tracer-antibody complexes are further detected colorimetrically with Ellman´s reagent for further sample calculation.

2.6.3 *Validation of ELISA methods*

All five ELISAs were validated for our species by testing the parallelism of serially diluted plasma samples of *M. barbatus* and *M. surmuletus* with the STD curves.

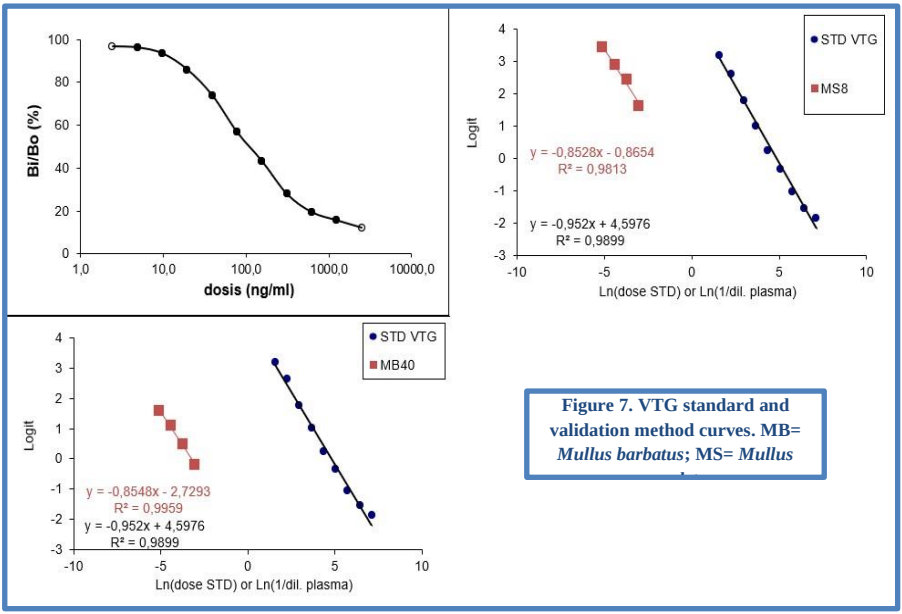


Figure 7. VTG standard and validation method curves. MB= *Mullus barbatus*; MS= *Mullus*

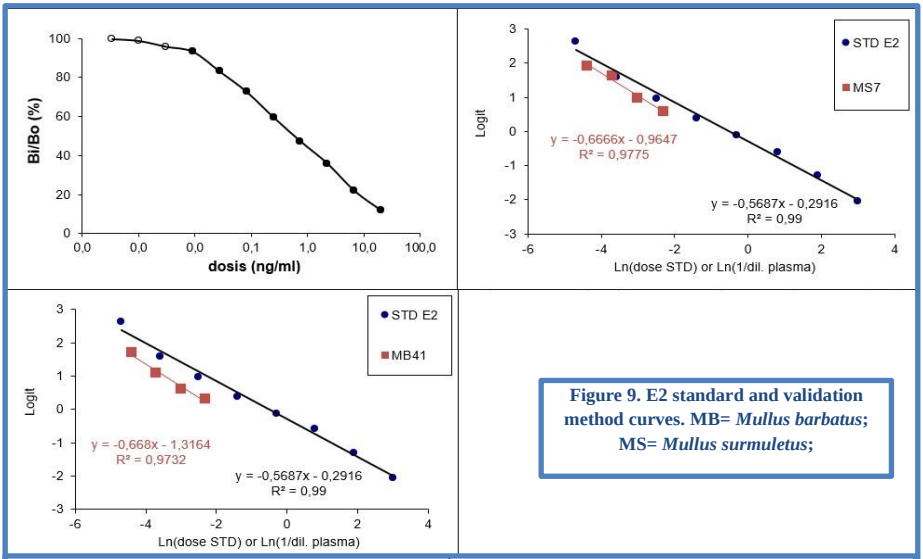


Figure 9. E2 standard and validation method curves. MB= *Mullus barbatus*; MS= *Mullus surmuletus*;

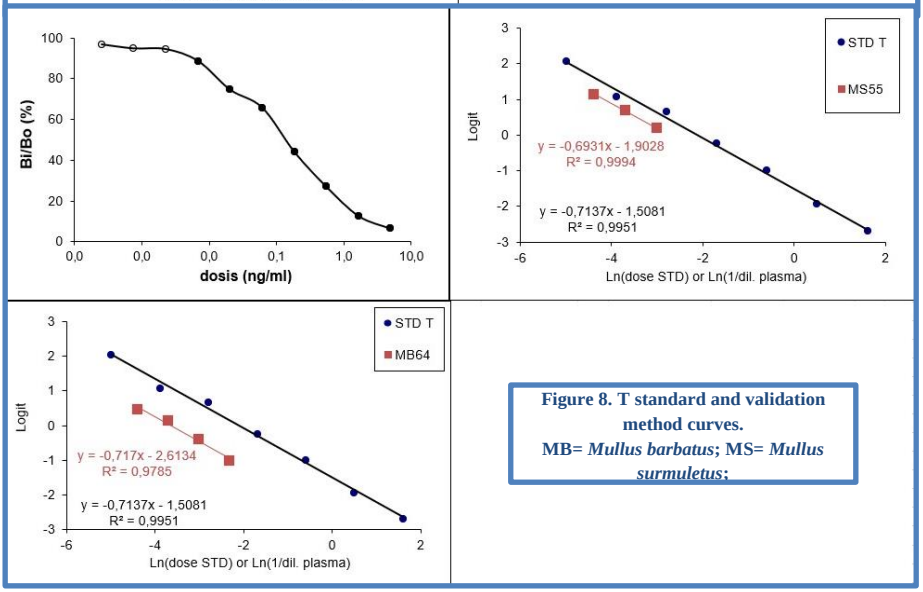


Figure 8. T standard and validation method curves.
MB= *Mullus barbatus*; MS= *Mullus surmuletus*;

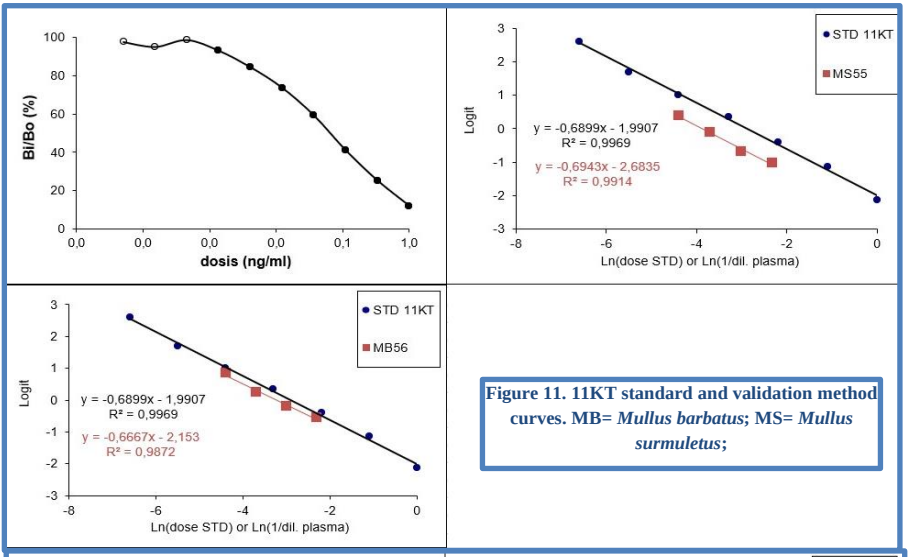


Figure 11. 11KT standard and validation method curves. MB= *Mullus barbatus*; MS= *Mullus surmuletus*;

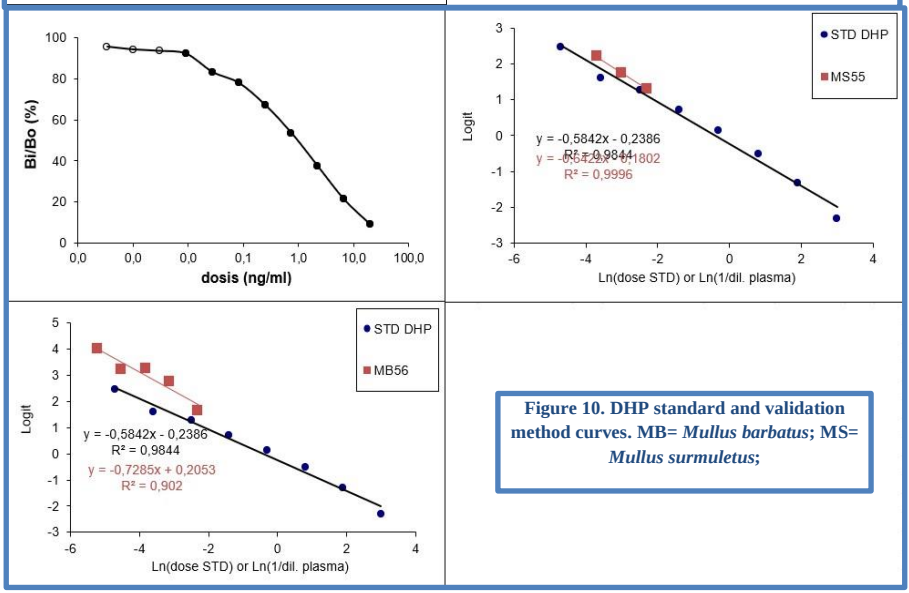


Figure 10. DHP standard and validation method curves. MB= *Mullus barbatus*; MS= *Mullus surmuletus*;

The characteristics of all four ELISAs were checked and confirmed to be optimal. The precision (inter-assay coefficient of variation, IE) of the ELISAs was determined specifically for the analysis performed in this study by adding the same plasma sample in all microplates of the analysis (n=7-13). The sensitivity of the assays was determined according to the limits of detection of the standard curves of the ELISAs (15-85 % of binding) and considering that plasma samples were used at a minimum 10-fold dilution to avoid non-specific matrix effects. The characteristics of the method are shown in Table 2.

ELISA	Nº of analysis (n)	Precision (IE CV%)	STD curve range (15-85%, ng/ml)	Limit of detection in plasma
VTG	13	12.7 %	919.5 – 16.7	167 ng/ml
E2	7	12.4 %	11.9 – 0.039	0.39 ng/ml
T	8	18.1 %	1.39 – 0.0094	0.09 ng/ml
11KT	8	15.5 %	0.77 – 0.0036	0.036 ng/ml
DHP	8	14.0 %	11.92 – 0.035	0.35 ng/ml

Table 2. Characteristics of ELISA methods.

2.7 Statistical analysis

The statistical elaboration of the data was performed by the software SPSS for Windows (version 26.0.0.1). Pearson's correlation was used to verify the relationship between Hg and Se concentration in muscle and validate the trend of *vtg* gene expression with plasma VTG concentration in female individuals. One-way ANOVA analyzes with Post hoc Dunnet T3 were used to relate the various sampling sites for each biomarker used. Wilks' Lambda discriminant analysis was performed to compare biometric variables and metals between sample groups.

3 Results

3.1 Heavy metals

The following tables (Table 3 and Table 4) show the descriptive analysis of the data on heavy metals for each compound, the number of samples in which it was detected, the average concentration expressed in µg/kg, minimum value, maximum value, and standard deviation.

Table 3. Descriptives analysis of heavy metal concentration (µg/kg) in *Mullus surmuletus* samples

	N	Minimum	Maximum	Mean	Std. Deviation
As	119	1,3063	34,0692	13,294464	7,5842677
Cd	119	,0090	,4140	,027950	,0520992
Co	119	,0452	,6077	,054334	,0626174
Cu	117	,1817	1,1817	,231210	,1230920
Hg	119	,0390	,7385	,212619	,1741822
Mn	116	,2627	,6680	,273971	,0574763
Pb	119	,1633	,9994	,170768	,0767559
Se	119	,1565	,8384	,422368	,1257710
V	119	,0293	,3431	,035145	,0340301

Table 4. Descriptives analysis of heavy metal concentration (µg/kg) in *Mullus barbatus* samples

	N	Minimum	Maximum	Mean	Std. Deviation
As	99	4,3326	65,3870	20,763844	10,5197467
Cd	99	,0090	,2354	,028795	,0429891
Co	99	,0452	,0452	,045200	,0000000
Cu	99	,1817	1,0297	,230831	,1235896
Hg	99	,0163	1,5440	,297702	,2231801
Mn	99	,2627	,5981	,267535	,0348161
Pb	99	,1633	,3635	,166791	,0244952
Se	99	,2821	,9164	,510908	,1219871
V	99	,0293	,0536	,029696	,0027210

Regarding *cadmium* (Cd), *cobalt* (Co), *manganese* (Mn), *lead* (Pb), and *vanadium* (V), no statistically significant differences were found for each species between the three sampling sites.

The concentrations of *arsenic* (As) in the muscle of both species do not show significant differences between the sampling sites. However, they are very high, with the mean concentration for *M. surmuletus* around 35 µg/kg, while for *M. barbatus* the mean doubles exceeding 65 µg/kg. Furthermore, in both species, the highest average of this metal is found at the South site.

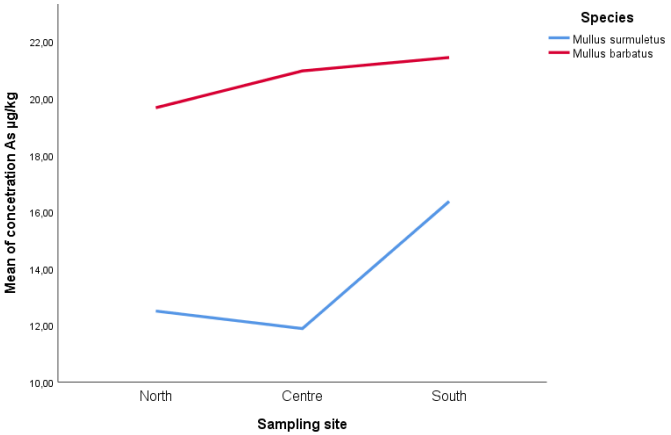


Figure 12. Mean concentrations by sampling points in both species.

Regarding the *copper* (Cu), significant differences in concentrations were found in *M. surmuletus* between the North and Centre sites, no difference between those two sites and the South site (Figure 13). Statistically significant differences were found for *M. barbatus* between the North and South sites, but none with the Centre site.

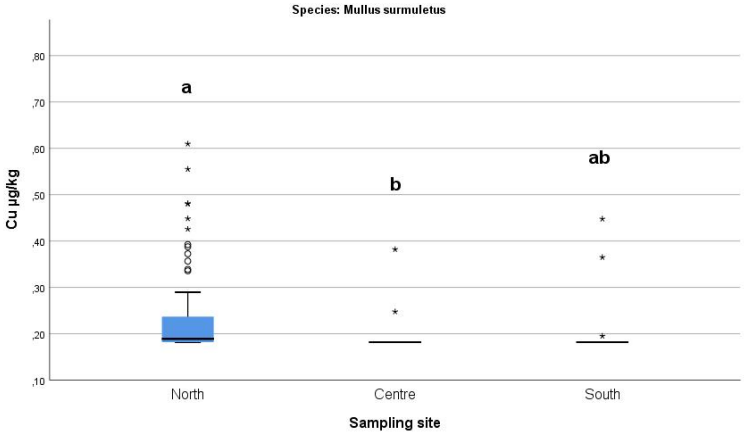


Figure 13. Cu concentrations in *M. surmuletus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisks indicate outlier values

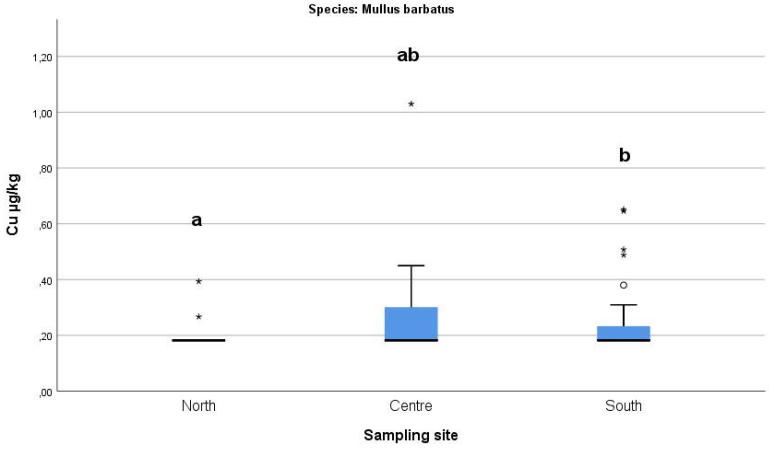


Figure 14. Cu concentrations in *M. barbatus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisks indicate outlier values

About *mercury* (Hg) in both species, significant differences were found between the Centre site and the other two sites (Figure 15 and Figure 16).

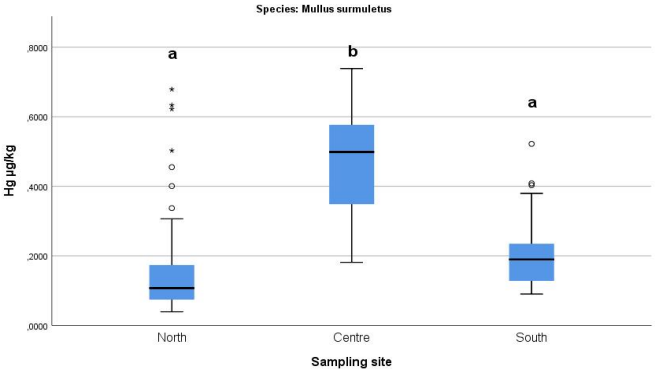


Figure 15. Hg concentrations in *M. surmuletus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisks indicate outlier values

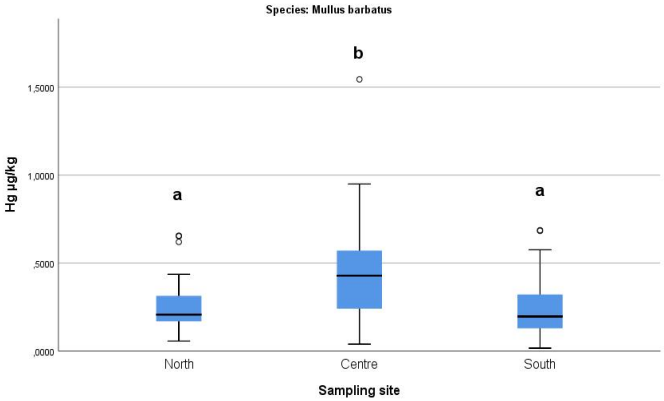


Figure 16. Hg concentrations in *M. barbatus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisks indicate outlier values

Selenium (Se) analyses revealed significant differences in *M. surmuletus* between the South and the North and Centre sites (Figure 17). In contrast, in *M. barbatus*, a significant difference was detected between the North (lower concentrations) compared to both the central and southern sites (Figure 18).

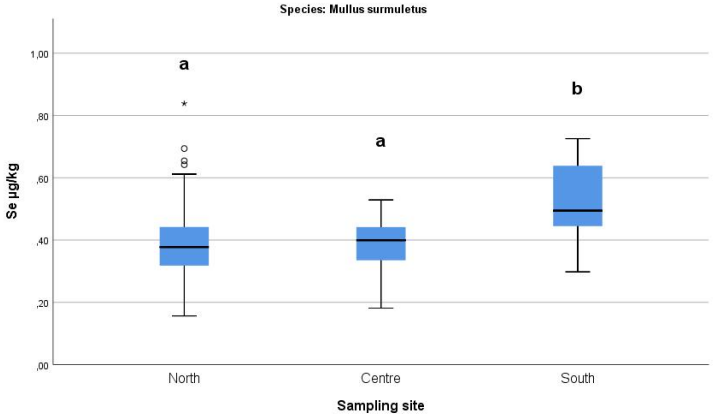


Figure 17. Se concentrations in *M. surmuletus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisks indicate outlier values

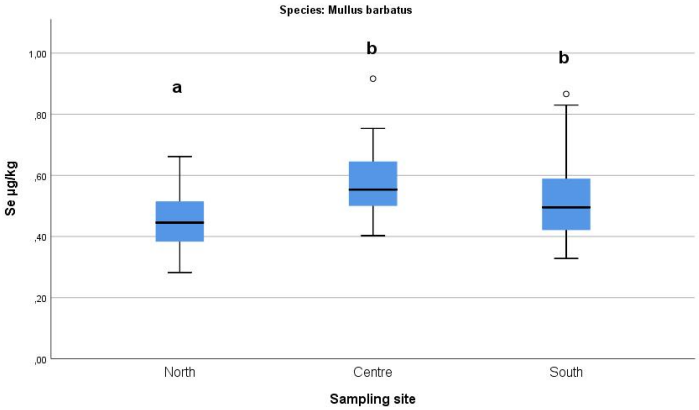


Figure 18. Se concentrations in *M. barbatus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisks indicate outlier values

A good significant positive correlation was found between Hg levels and Se in both species. The Pearson's correlation is around 0,485 with

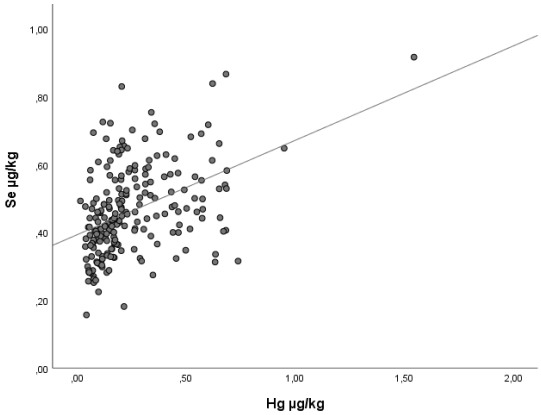


Figure 19. Correlation between level concentration of Hg and Se in both species

a significance > 0,01 (2-tailed).

A Wilks Lambda discriminant analysis was conducted by relating some biometric data and metals. The discriminated groups were four: all the samples divided by sex and by species.

Table 5. Discriminant functions for the biometric variables and heavy metals concentrations.

	Lambda di Wilks	F	df1	df2	Sig.
Lenght sample (cm)	,692	31,235	3	211	,000
Arsenic (As µg/kg)	,842	13,154	3	211	,000
Cadmium (Cd µg/kg)	,984	1,118	3	211	,343
Cobalt (Co µg/kg)	,998	,144	3	211	,933
Copper (Cu µg/kg)	,989	,814	3	211	,487
Mercury (Hg µg/kg)	,957	3,182	3	211	,025
Manganese (Mn µg/kg)	,989	,772	3	211	,511
Lead (Pb µg/kg)	,989	,780	3	211	,506
Selenium (Se µg/kg)	,874	10,150	3	211	,000
Vanadium (V µg/kg)	,993	,514	3	211	,673
GS[1]	,807	16,827	3	211	,000
ES[2]	,802	17,406	3	211	,000

1. Gonadosomatic index
2. Hepatosomatic index

Table 6. Discriminants and functions

	Root		
	1	2	3
Cm	,607*	,308	,023
Hg	-,183*	,131	,087
ES	-,076	,662*	-,093
Se	-,256	,369*	-,024
Mn	,012	-,138*	-,096
GS	-,370	,395	,486*
As	-,308	,382	-,431*
Cu	-,070	-,058	,374*
Pb	,070	,059	,356*
Cd	-,076	-,126	-,171*
V	,076	-,040	,098*

* Largest absolute correlation between each variable and any discriminant function

Discriminant functions 1 and 2 have the highest percentage of cumulated variance (approximately 98%), weighing entirely concerning the third function. Function 1 has as preponderant variables the length of the sample (cm) and the mercury

concentration. Function 2 has a hepatosomatic index, selenium, and manganese as variables. Figure 20 depicts the discriminant analysis according to these two functions, and it results in that function one tends to divide groups according to species, while function two divides groups according to sex.

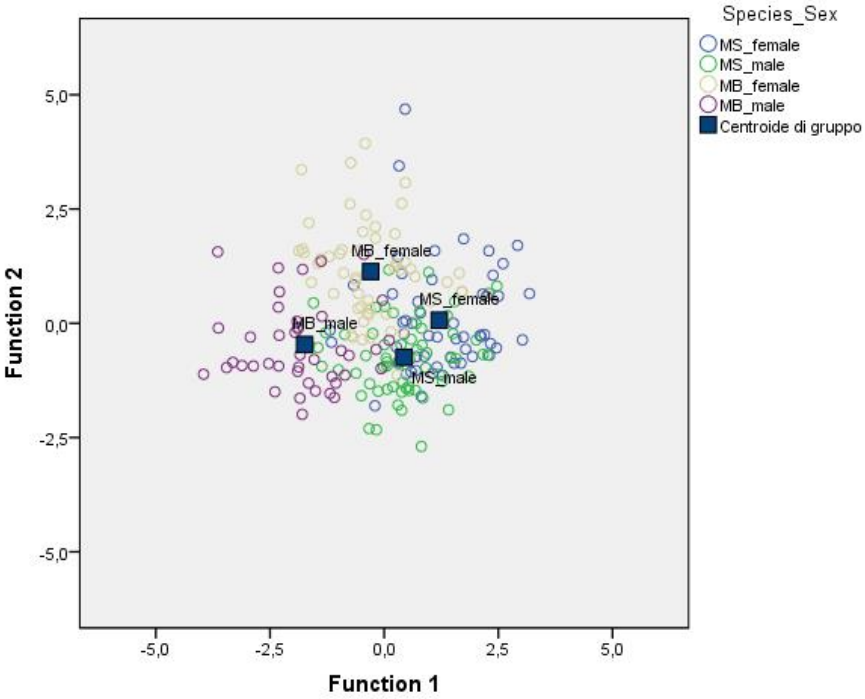


Figure 20. Plot of the individual observation discriminant scores obtained with the canonical discriminant functions for four groups of samples.

3.2 IPA and pesticides

Pesticides. All 21 pesticides considered were searched for in the muscle of all the samples. However, no individual presented relevant levels of these contaminants.

PAHs. All 14 PAHs examined were analyzed in the muscle of each sample. Their presence is not well distributed, and most of the samples presented values below the detection limit of the instruments. Therefore, the following descriptive tables show the number of samples in which it was detected, the average concentration expressed in µg/kg, minimum value, maximum value, and standard deviation for each compound.

Table 7. Descriptives analysis of PAHs concentration (µg/kg) in *Mullus surmuletus* samples.

	N of samples	Minimum	Maximum	Mean	Std. Deviation
Naphthalene	7	,5799	4,1196	1,966814	1,1484922
Acenaphthylene	32	,2512	27,2166	3,829059	5,6358966
Acenaphthene + Fluorene	17	,4676	154,2895	24,550441	40,8406442
Anthracene	25	,2614	9,1803	3,215260	2,3501963
Fluoranthene	51	,9008	23,3853	5,126094	4,2332629
Pyrene	28	,7537	56,2103	3,867689	10,3277352
Benz(a)anthracene	29	,8819	19,9386	4,039245	4,1932169
Crysene	29	2,5960	32,4841	11,543579	8,9515212
Benzo(b)fluoranthene	35	,9543	58,1714	14,885523	13,3033658

Benzo(k)fluoranthene	51	1,1615	77,6818	10,499157	15,5439195
Benzo(a)pyrene	66	,4233	27,0212	5,889691	6,1984542
Dibenz(ah)anthracene	26	1,1639	109,8127	25,117654	37,0113951
Benzo(ghi)perylene + Indeno(1,2,3-cd)pyrene	27	2,0866	75,0921	11,247322	15,0243862

Table 8. Descriptives analysis of PAHs concentration (µg/kg) in *Mullus barbatus* samples.

	N	Minimum	Maximum	Mean	Std. Deviation
Naphthalene	39	,3514	14,5671	4,727728	3,4828924
Acenaphthylene	25	,3100	33,8099	3,158600	6,6351267
Acenaphthene + Fluorene	19	,8346	16,0756	5,240205	4,2812874
Anthracene	29	,2848	38,3761	6,207103	7,4751782
Fluoranthene	46	,7333	12,6699	3,939483	2,4726643
Pyrene	21	,7510	6,8451	1,782676	1,4756935
Benz(a)anthracene	31	1,1668	59,3278	8,481332	13,7426473
Crysene	42	2,4000	133,0888	24,325040	32,0242845
Benzo(b)fluoranthene	17	2,1944	171,8085	26,701682	39,7168453
Benzo(k)fluoranthene	41	3,0536	599,6988	65,194780	134,2727728
Benzo(a)pyrene	69	,3782	266,8896	31,643264	51,2525077
Dibenz(ah)anthracene	8	54,9552	581,5010	329,704263	180,7120813
Benzo(ghi)perylene + Indeno(1,2,3-cd)pyrene	9	1,1000	137,1501	60,733311	48,3909691

It was impossible to perform statistical analyses on all the PAHs analyzed due to the lack of a significant sample for each sampling area. However, some PAHs of particular relevance to health risk has been examined.

Benzo(a)pyrene. This contaminant was detected in most muscle samples of both species (163 out of 220). In particular, significant differences were found in *Mullus surmuletus* between the North sampling site and the Centre site, as shown in Figure 21.

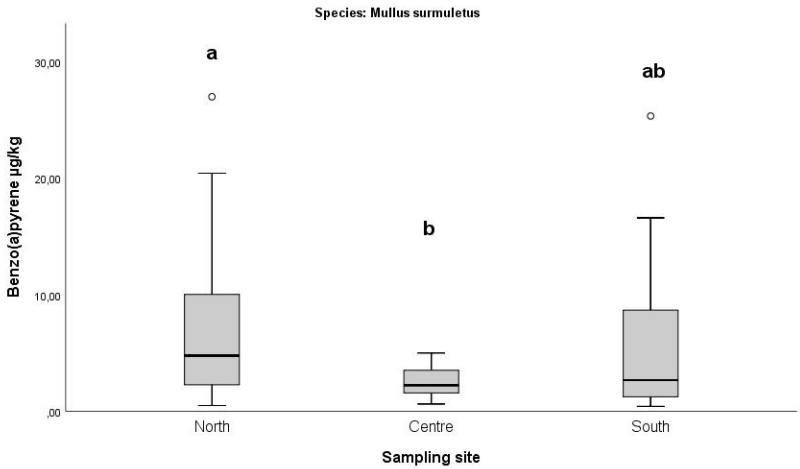


Figure 21. Benzo(a)pyrene concentrations in *M. surmuletus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and *post hoc* Dunnet T3. The circles indicate outliner values

The same statistical analysis was done on *Mullus barbatus*, and no significant differences were found between the three sampling sites

(Figure 22). Although there are no differences between the sampling sites, the concentration values in this species are tens of times higher than those of *Mullus surmuletus.*

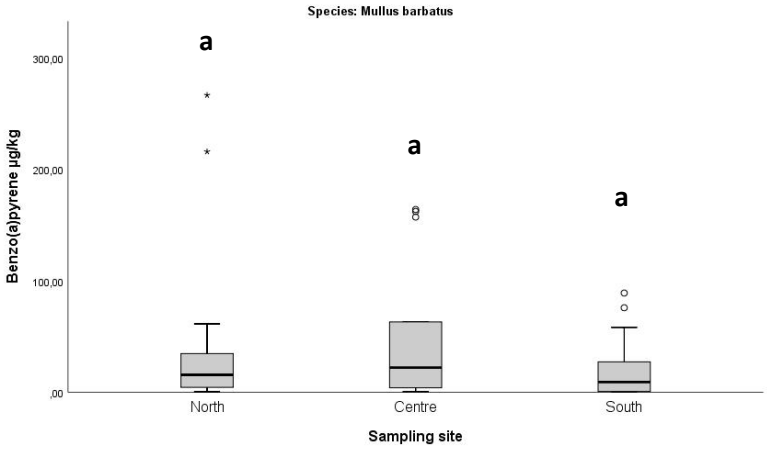


Figure 22. Benzo(a)pyrene concentrations in *M. barbatus* muscle at the three sampling sites. No significant differences (p <0.05) were indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisk indicate outlier values.

Benzo(k)fluoranthene. This contaminant was detected in 92 of 220 samples, 41 for *M. surmuletus* and 51 for *M. barbatus*. Significant differences were found in the first species between the Centre site and the other two sampling sites (Figure 23). No significant differences were found in *M. barbatus* according to the post hoc Dunnet T3. However, a high concentration of this contaminant was detected, particularly in the Centre site, where the average concentration is 201,9 µg/kg (Figure 24), with a sample that reaches a concentration of 599,69 µg/kg.

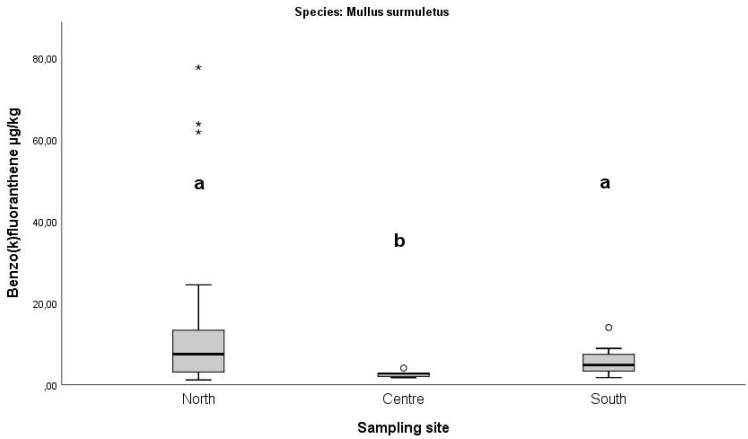


Figure 23. Benzo(k)fluoranthene concentrations in *M. surmuletus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles and asterisks indicate outliner values

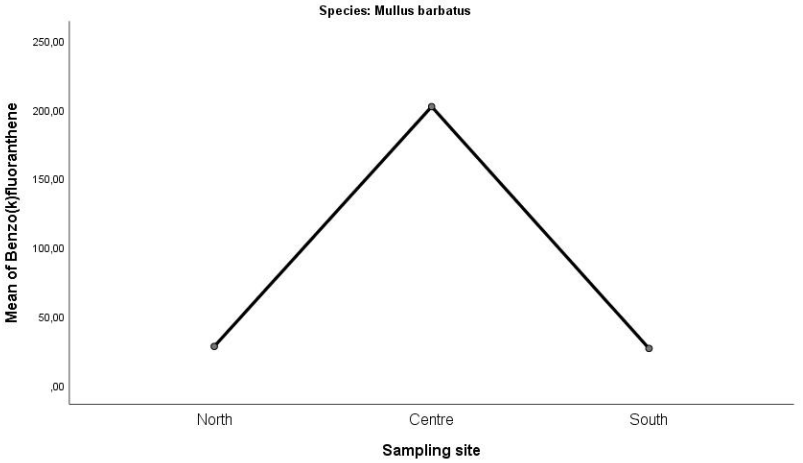


Figure 24. Mean of Benzo(k)fluoranthene concentrations in *M. barbatus* muscle at the three sampling sites.

Crysene. Statistically significant differences were found in the *M. barbatus* samples between the South site and the two North and Centre sites

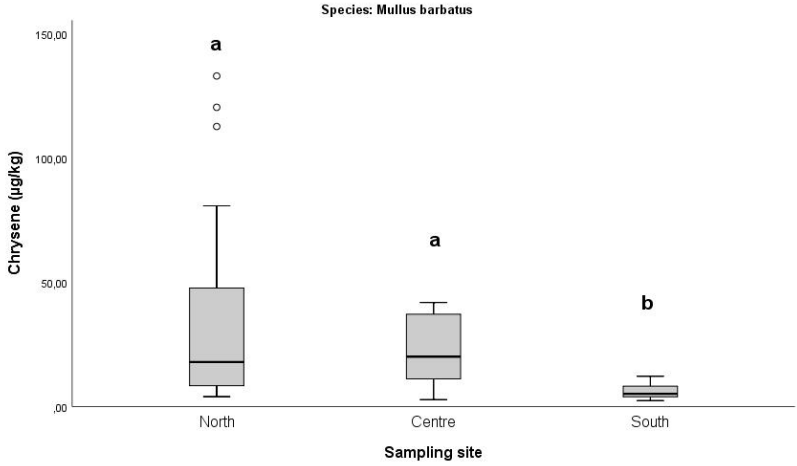


Figure 25. Figure 11. Crysene concentrations in *M. barbatus* muscle at the three sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3. The circles indicate outliner values

3.3 Histology

The histological analysis of the gonads was carried out on both sex, and each individual was assigned a reproductive stage using the

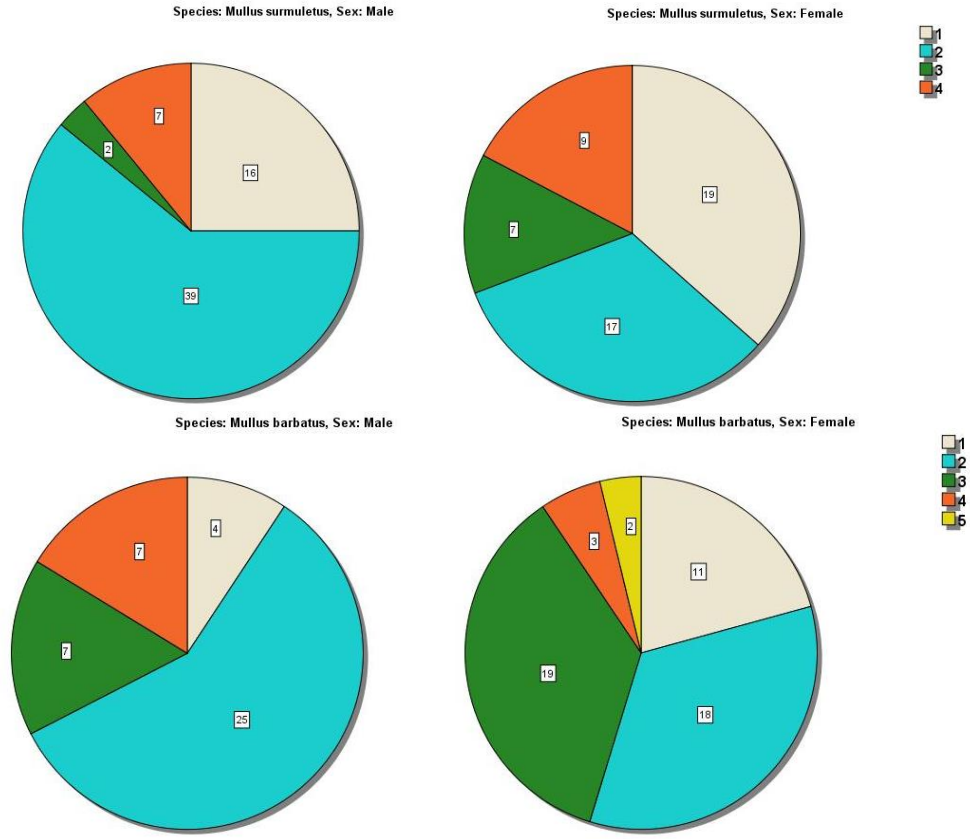
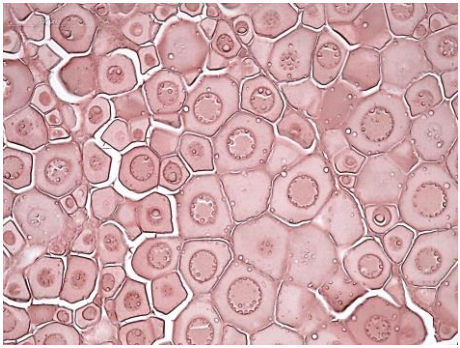
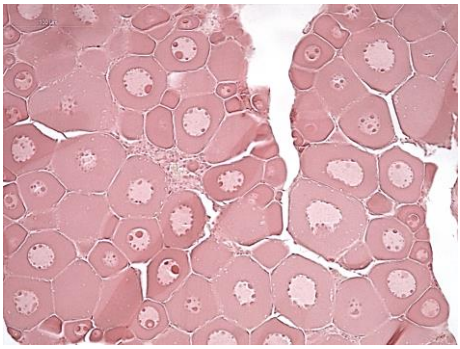


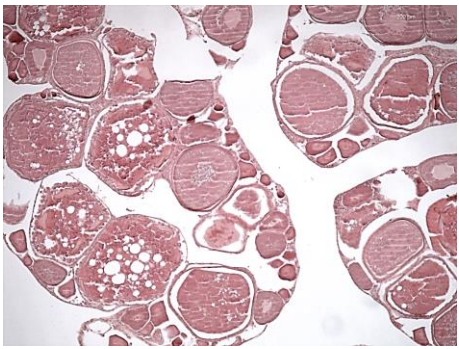
Figure 26. Assigned reproductive stage divided into species and sex groups of samples. The stage "1" corresponds to a IMMATURE, "2" corresponds to a DEVELOPING stage, "3" corresponds to SPAWING CAPABLE, "4" corresponds to REGRESSING and "5" corresponds to REGENERATING phase.

methodology proposed by Brown-peterson et al. (2011). As shown by the graphs in Figure 26, the reproductive stage most present in the various groups of samples is stage 2. No stage 5 samples were found in males of both species and *M. surmuletus* females.

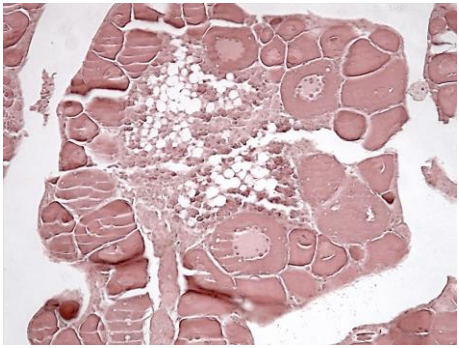
The table shows all the 20x magnification stages in *M. barbatus*, divided into males and females.

Immature female gonad of *M. barbatus* – stage 1 Oocytes exhibiting asynchronous but discontinuous secondary growth (PG = primary growth oocyte)	
Developing female gonad of *M. barbatus* – stage 2 PG, CA, Vtg1, and Vtg2 oocytes present	

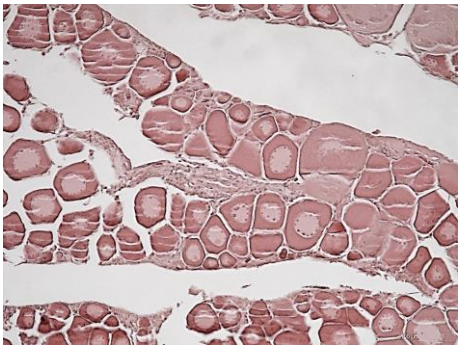
Spawning capable female gonad of *M. barbatus*– stage 3
Individual oocytes visible macroscopically. Vtg3 oocytes and POFs present. Atresia of vitellogenic and hydrated oocytes present.



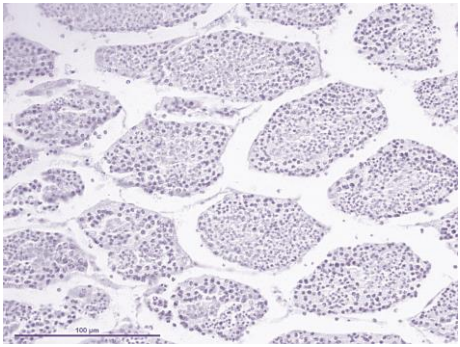
Regressing female gonad of *M. barbatus*– stage 4
Atresia (any stage) and POFs present. Some CA and/or vitellogenic (Vtg1, Vtg2) oocytes present



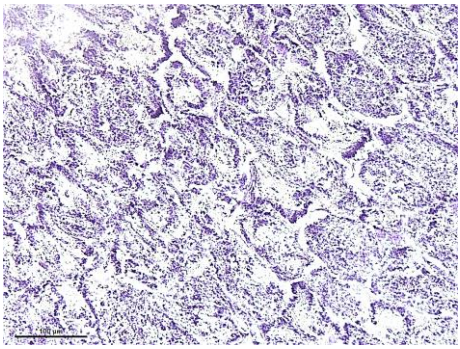
Regenerating female gonad of *M. barbatus* – stage 5
Oocytes exhibiting synchronous, discontinuous secondary growth (A = atresia; POF = postovulatory follicle complex)



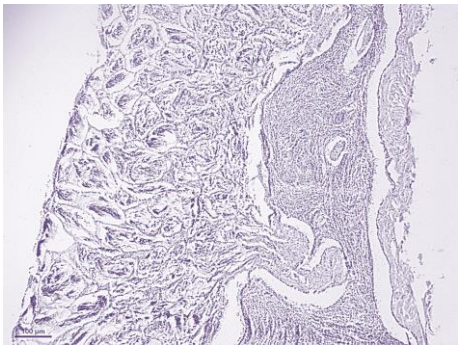
Immature male gonad of *M. barbatus* – stage 1
Only Sg1 present; no lumen in lobules.



Developing male gonad of *M. barbatus* – stage 2
Spermatocysts evident along lobules. Sg2, Sc1, Sc2, St, and Sz present in spermatocysts. Sz not present in lumen of lobules or in sperm ducts. GE continuous throughout.

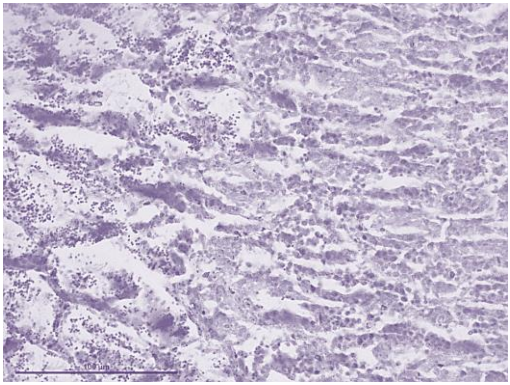


Spawning capable male gonad of *M. barbatus* – stage 3
Sz in lumen of lobules and/or sperm ducts. All stages of spermatogenesis (Sg2, Sc, St, Sz) can be present. Spermatocysts throughout testis, active spermatogenesis.



Regressing male gonad of *M. barbatus* – stage 4

Residual Sz present in lumen of lobules and in sperm ducts. Widely scattered spermatocysts near periphery containing Sc2, St, Sz. Little to no active spermatogenesis



3.3.1 Intersex

By analyzing the gonads of all the male samples, no intersex phenomena were found.

3.3.2 Melanomacrophages centres

In this histological analysis, all the gonads of male individuals of both species were evaluated. The degree of abundance was then assigned to each sample, based on the vision of 8 slides. In the following figures 4 examples are shown, one for each stage of abundance of MMCs. All the slides are colored in H&E stain, and the thickness is 10µm.

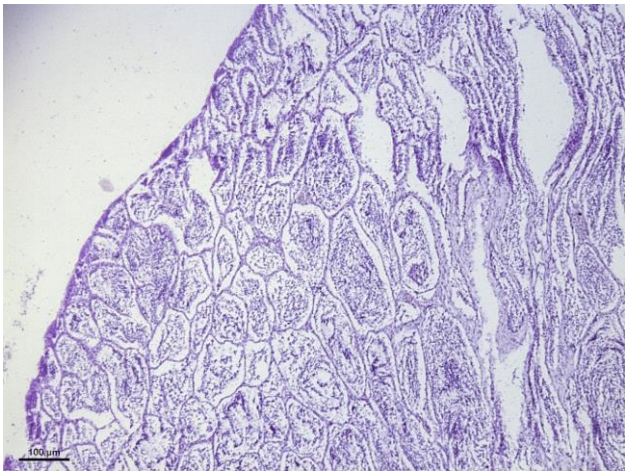


Figure 27. 10x photograph of a 1 mm area of the male gonad without centers of melanomacrophages (degree of abundance "0").

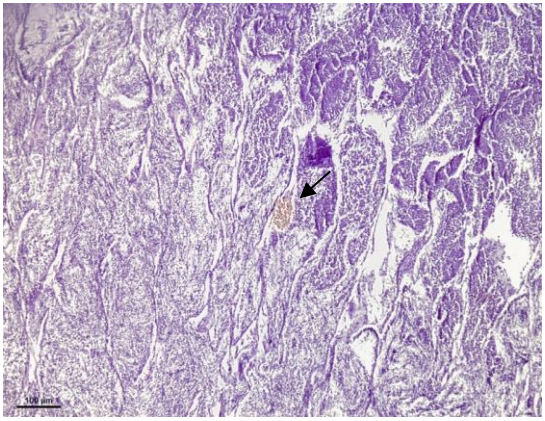


Figure 28. 10x photograph 10x photograph of a 1 mm area of the testis that has only one MMC indicated by arrow (degree of abundance "1").

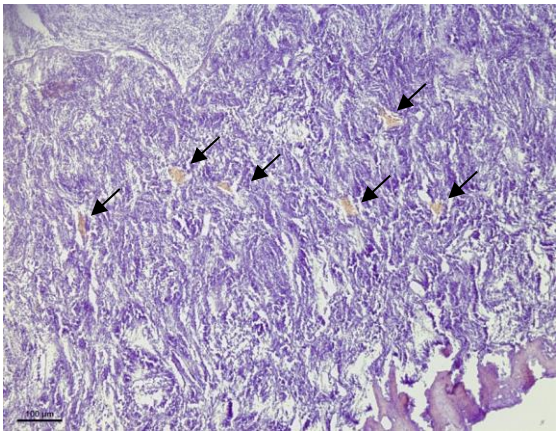


Figure 29. Degree of abundance "2" due to the presence of 6 MMCs (arrows) in an area of 1 mm.

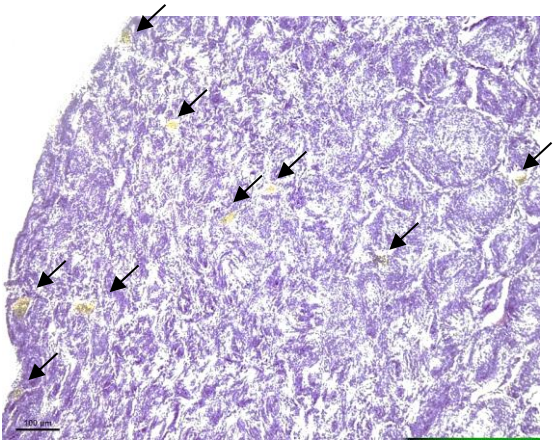


Figure 30. 10x magnification of an area of 1 mm that has more than 6 MMCs (arrows), therefore with degree of abundance "3".

No statistical analyzes were carried out on the results of MMCs. However, from what emerged from a descriptive evaluation that compares the degree of abundance of MMCs and reproductive stage, *M. surmuletus* has the highest degree of abundance in reproductive stage 4 (regressing), while in *M. barbatus* samples, a large majority of specimens have a strong presence of MMCs in reproductive stage 2 and 3 (respectively developing and spawning capable)(Figure 31 and Figure 32).

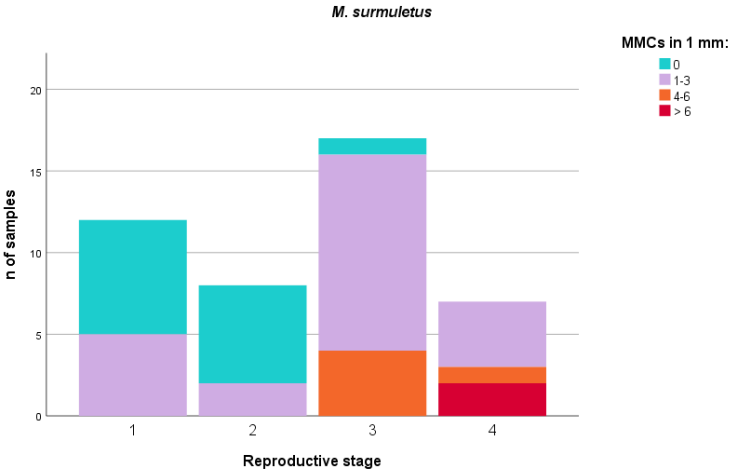


Figure 31. Males of *M. surmuletus* classified into reproductive stage, number of samples and degree of MMCs presence.

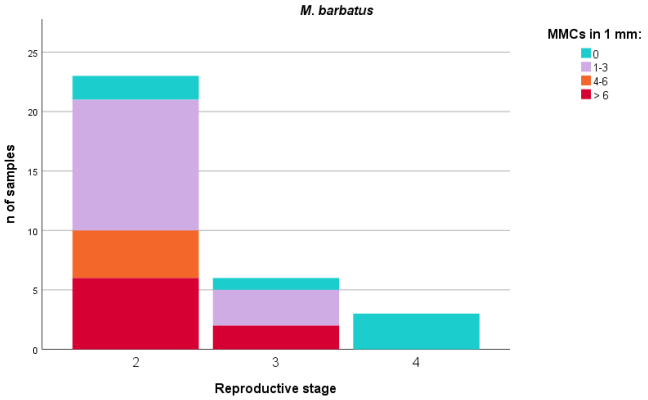


Figure 32. Males of *M. barbatus* classified into reproductive stage, number of samples and degree of MMCs presence.

In *M. surmuletus*, all specimens with the highest degree of MMCs were found to be from the North site, therefore the sites were not compared for this species.

Instead, as regards *M. barbatus*, we compared the sampling sites, and the presence of individuals with a high presence of MMCs is almost constant in each site (Figure 33).

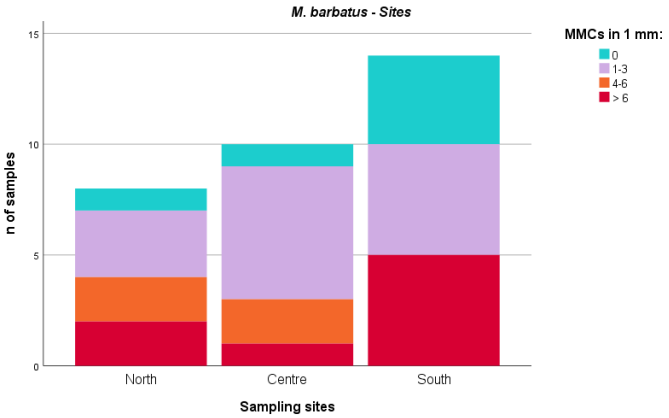


Figure 33. Males of *M. barbatus* classified into sampling sites, number of samples and degree of MMCs presence.

3.4 Steroid hormones and VTG plasma levels

In Table 9, there are the descriptive analyses of the four steroid hormones (E2, T, 11kT, DHP) taken into consideration and vitellogenin protein (VTG).

Table 9. Descriptive analysis of steroid hormones concentration (ng/ml) and VTG concentration (µg/ml) in plasma of all samples.

Females samples of *Mullus surmuletus*					
	N	Minimum	Maximum	Mean	Std. Deviation
VTG	48	,17	2,28	,6496	,59840
E2	48	,39	13,18	4,6590	3,54351
T	48	,11	4,78	,8285	,84268
11KT	45	,03	12,91	,6087	2,23010
DHP	46	,35	2,56	,5011	,38858
Male samples of *Mullus surmuletus*					
	N	Minimum	Maximum	Mean	Std. Deviation
VTG	61	,17	,96	,3002	,24037
E2	60	,39	12,59	5,4055	3,11868
T	61	,09	4,66	,8757	,74175
11KT	59	,04	2,04	,6146	,58411
DHP	57	,34	1,74	,4960	,30525
Female samples of *Mullus barbatus*					
	N	Minimum	Maximum	Mean	Std. Deviation
VTG	51	,17	1,84	,7031	,62034

E2	51	,38	17,00	5,8651	4,69829
T	51	,20	6,46	1,3778	1,16055
11KT	51	,04	,50	,1563	,11719
DHP	51	,32	2,11	,5790	,38215

Male samples of *Mullus barbatus*

	N	Minimum	Maximum	Mean	Std. Deviation
VTG	42	,17	1,72	,3050	,33260
E2	42	1,45	18,77	5,4650	3,40062
T	42	,29	12,39	2,3531	2,40335
11KT	42	,04	16,70	3,4439	4,61711
DHP	42	,35	1,14	,4321	,18149

Two female samples of *Mullus surmuletus* detected abnormal testosterone values and 11-ketotestosterone, with values more like male ones and a low level of estradiol. However, histological analysis of the gonads confirmed their sex as female. These two samples were removed from the analysis of the relationships between reproductive stages and hormone levels.

Figure 34 summarizes the trends in the concentrations of VTG and steroid hormones in the plasma of individuals of *M. surmuletus*, divided into males and females, in relation to the

reproductive stages that were determined by histological analyses.

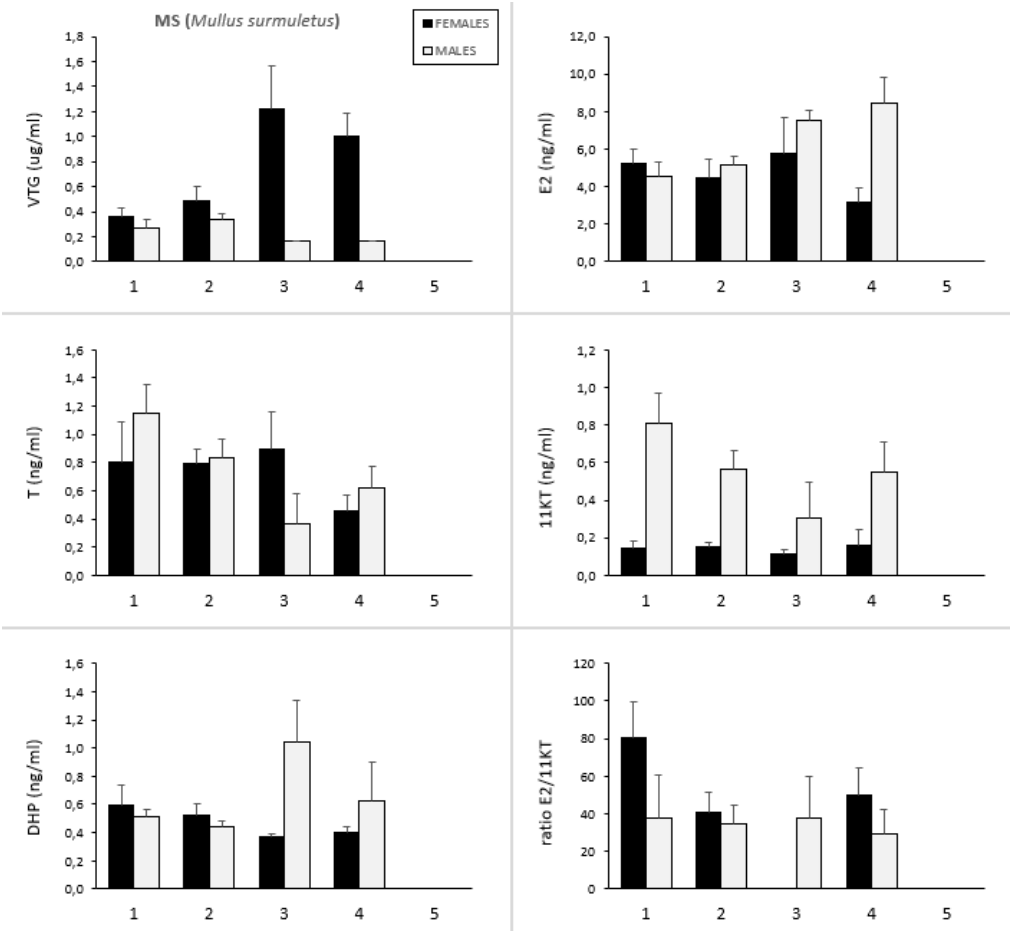


Figure 34. Concentrations of VTG and steroid hormones in plasma of *M. surmuletus* females and males, in relation with their reproductive stage determinated histologically

Figure 35 shows the trends of the concentrations of VTG and steroid hormones in the plasma of individuals of *M. surmuletus*, divided into males and females, in relation to their reproductive stages.

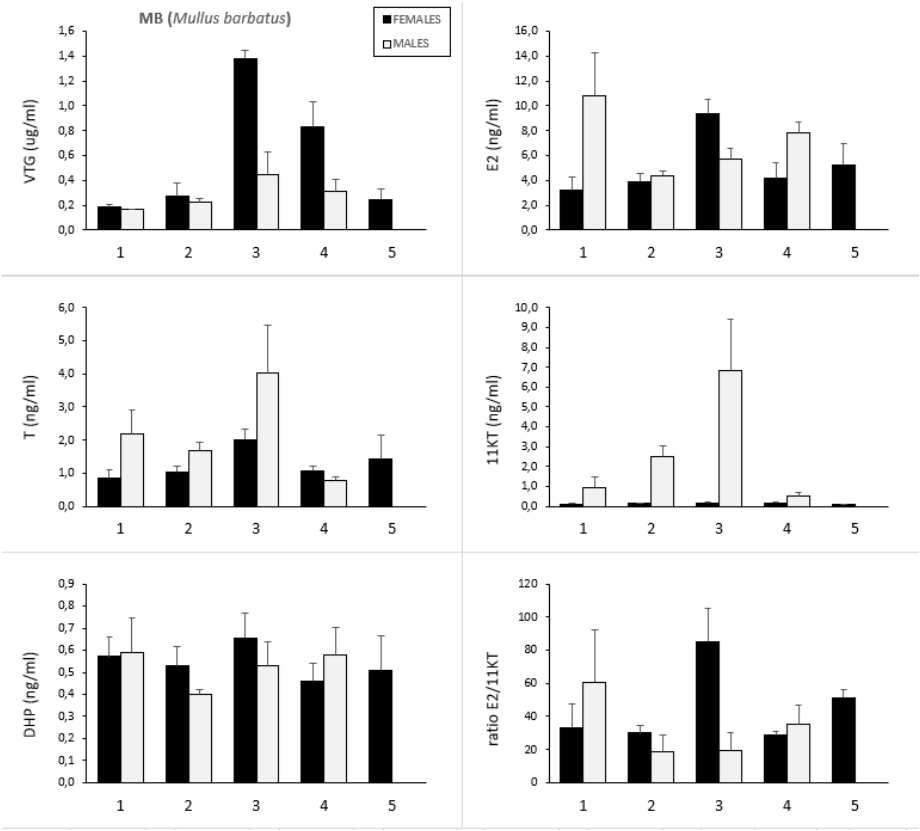


Figure 35. Concentrations of VTG and steroid hormones in plasma of *M. barbatus* females and males, in relation with their reproductive stage determinated histologically

Regarding the concentration of VTG in the plasma of males, significant differences were found between the *M. surmuletus* of the South area and all the other sampling sites. A mean plasma VTG concentration of about 0.3 μg / ml of both *M. surmuletus* and *M. barbatus* is present at all sampling sites, as shown in Figure 36.

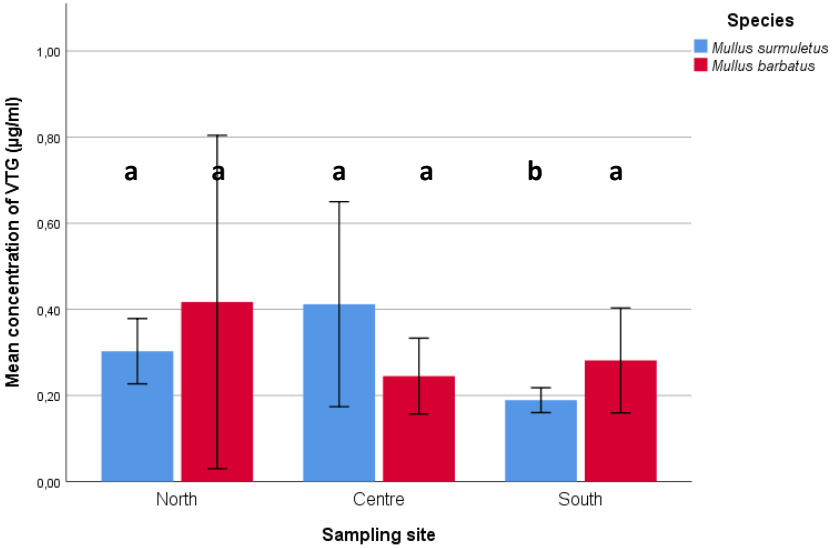


Figure 36. Means of concentration of VTG plasma level in both examined species divided into sampling sites. Letters indicate significant differences (p <0.05), as indicated by one-way Anova and post hoc Dunnet T3.

3.5 *vtg gene expression*

A semi-quantitative analysis was conducted through the ΔΔCt method. The results were transformed according to that formula and relativized to a group of females in phase 2 of reproductive development.

Pearson correlation analysis revealed a strong positive correlation between plasma VTG and relative gene expression levels in both species for female individuals. On the other hand, the same correlation is not significant for males, neither with plasma levels nor with reproductive stages.

The following graphs (Figure 38 and Figure 37) show the relative levels of VTG expression on a logarithmic basis, divided by sampling site. All sites have specimens that express this gene. In general, *M. barbatus* has higher relative values than males of *M. surmuletus*.

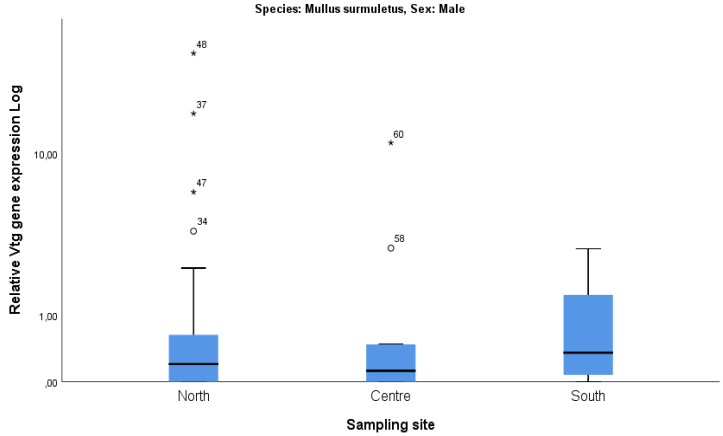


Figure 38. Relative representation of the presence of *vtg* gene expression in *M. surmuletus* in the three sampling points.

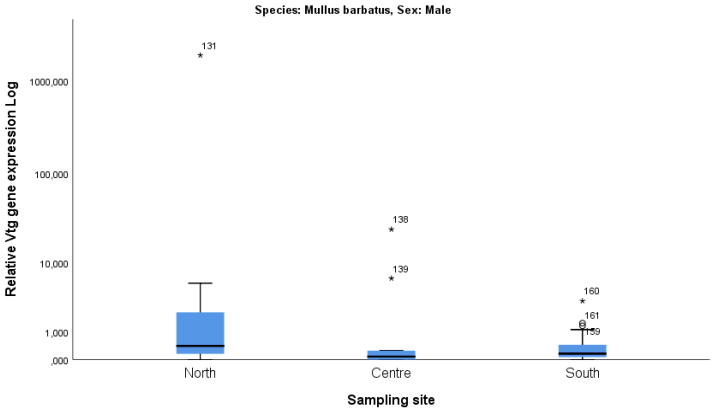


Figure 37. Relative representation of the presence of *vtg* gene expression in *M. barbatus* in the three sampling points.

4 Discussion

Based on the data collected and the analyses made through the series of biomarkers used, no sampling site is free from at least one type of contamination.

Regarding heavy metals, it is not surprising that the Centre site, corresponding to Augusta (SR), is characterized by greater bioaccumulation of mercury by both species. This sampling site has had a powerful anthropogenic impact in recent decades due to the petrochemical industry. Despite the arrest of some activities releasing heavy metals, their presence continued to bioaccumulate and biomagnify through the sediments and the food chain (Sprovieri et al., 2011; Di Leonardo et al., 2014; Ferrante et al., 2017).

The comparative analysis of the bioaccumulation of metals in pelagic, benthic, and dermal species indicates a higher trend in metal contamination, especially regarding the presence of higher values of Hg in demersal species in the Mediterranean (Naccari et al., 2015) such as *M. barbatus* and *M. surmuletus*. Stergiou and Karpouzi (2002) calculated the trophic level of numerous species of the Mediterranean Sea by inserting them into different functional groups, and the two species of *Mullus* were included in the functional group of omnivores with preferences for animal prey. The primary diet of *M. barbatus* and *M. surmuletus* consists of polychaetes, bivalve mollusks, and small

crustaceans (amphipods, decapods, and copepods). Mollusks appear to be the primary source of metal contamination in *M. barbatus*, where high mercury concentration levels found in muscle have been linked with mercury's affinity for thiol groups and muscle proteins in fish (Kontas et al., 2021).

As emerged from the data of this doctoral work, arsenic concentration levels in fish muscle are generally high. These levels of arsenic are in line with many bibliographical works, in which the quantitative analyses of this heavy metal in fish always show very high concentrations, even in the order of mg/kg (Jankong et al. 2007; Shah et al., 2009; Sloth et al., 2005).

The concentration of Selenium is variable, and the results show that it has a positive correlation with mercury. In this regard, the ratio between Se and Hg is essential for understanding the condition of aquatic organisms and, in particular, the safety of their consumption by humans. Selenium tends to have an antioxidant and "sequestration" action of the part of the mercury (Copat et al., 2014). However, in species with higher concentrations of Hg, the concentrations of Se vary in the same direction as the Hg. In fact, Se and Hg usually have a positive correlation, but this can vary depending on the habitat and species (Azad et al., 2019). Although the presence of Selenium appears to help reduction of mercury bioaccumulation (Peterson et al.,

2009), little is known about its effects at high concentrations and prolonged exposure to this metal. For example, in a laboratory experiment with the model fish *Danio rerio*, it was seen how a diet with a high concentration of Selenium decreases reproductive fitness, both infertility and in the survival of the embryo (Penglase et al., 2014). In the context of this research, the positive correlation decreases at the Augusta site for both species under examination, presumably because the Hg values are significantly higher and the positive correlation between the two elements is missing.

Copper was significantly higher in *M. surmuletus* samples at the North and *M. barbatus* at the Centre sites. Although the concentrations are not very high, some samples exceed 1 µg/kg. Copper appears to have adverse effects on both gonadal tissue reproduction and larval survival. In particular, it can damage the functioning of the lateral line (Benoit, 1975; Johnson et al., 2007; Woody and Neal, 2012).

Regarding PAHs contamination, many of the chemicals evaluated were not detected in even half of the samples. Instead, some of them, like Crysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Dibenz(ah)anthracene, seem to have a greater bioaccumulation capacity in the species under consideration. Of those examined through the one-way Anova, the North site is often found

with higher concentrations. Nevertheless, fundamental is to underline that, despite both species bioaccumulating these substances, in *M. barbatus,* the five chemical compounds mentioned above are accumulating with orders of tens of times greater than that of *M. surmuletus*. These substances, when highly concentrated, have adverse effects on essential enzymatic functions blocking cholinergic transmission by inhibition of AChE (Vieira et al., 2008). For example, Benz(a)pyrene can already induce ultrastructural changes in the liver at low concentrations (Au et al., 1999).

The presence of strong bioaccumulation of PAHs and heavy metals, combined with the probable exposure to multiple emerging contaminants that are difficult to assess, resulted in gene expression and subsequent synthesis of vitellogenin in males of both species. Clearly, the VTG does not give information on the type of contamination, except that it is contamination from endocrine disruptors (EDs). Also, among the compounds analyzed in this doctoral work, there are chemical compounds with estrogenic activity, such as benz(a)pyrene itself, but also Hg and As (Iavicoli et al., 2009b; Zhang et al., 2016).

Vitellogenin in plasma has been widely used as a biomarker of EDs contamination in many fish species regarding immature males

and females (Franco et al., 2020; Zezza et al., 2020). The use of this biomarker in the present work did not allow to distinguish an area more or less impacted by EDs, however in both species considered, the presence of vitellogenin in male individuals at all sampling sites underlines a certain exposure to endocrine disruptors. Both *M. barbatus* and *M. surmuletus* males showed a mean plasma VTG concentration of around 0.40 µg / ml.

The plasma hormonal ELISA of the four steroid hormones has allowed, together with the histological reproductive study, to investigate a still unknown aspect of the biology of these two species. The levels found in the ELISA assays are in line with the reproductive stages in both species. However, *M. surmuletus* generally appears to have lower concentrations than *M. barbatus*. Furthermore, the hormone levels in *M. surmuletus* are not always clear, and, for example, estradiol does not follow the precise reproductive trend. Therefore, more analyzes will need to be carried out in the future.

Regarding the possible presence of androgen-like contamination, could the two female samples of *M. surmuletus* who presented a hormonal profile similar to the male one be a case of androgen-like EDs? Even if the two females belong to the same species and come from the same sampling site (Centre), they cannot be the same species and the same sampling site (Centre), two samples

cannot be bioindicators of any contamination. However, androgen-like activities are not rare and affect both the immune (Milla et al., 2011) and the reproductive level. Therefore it would be necessary to investigate the question in the future, perhaps with the analysis of the gene expression of Cyp19a, which could undergo a down-regulation and no longer act as an aromatase catalyst for androgen into estrogen (Leet et al., 2011).

Vitellogenin was also investigated at the gene level in terms of relative expression. The *vtg* gene is notoriously known as a biomarker of contamination by endocrine disruptors. It can be helpful, especially in species with a short life cycle, as it is the first of the many biochemical responses that the organism gives at the moment of exposure to EDs (Arukwe et al., 2000; Chen et al., 2020). Relative *vtg* levels in mature females show a positive correlation with both reproductive stages and plasma VTG levels. However, this correlation is not present in males, suggesting that gene expression does not necessarily involve protein synthesis, probably due to non-chronic exposure to endocrine disruptors. However, the relative levels of *vtg* in *M. barbatus* males are much higher than in *M. surmuletus*.

Although the gene expression of vitellogenin and its protein synthesis in males give information on substantial contamination by EDs that affects the entire reproductive process, no male gonadal

intersex phenomena with the appearance of female tissue have been found. This phenomenon is much more common than the reverse (testis in the ovary), also in the examined *Mullus sp*: most EDs act as estrogen-like and not as androgen-like (Martin-Skilton et al., 2006). Recent studies suggest that the pathways involved in intersex and *vtg* induction in males are not entirely correlated. The manifestation of intersex also involves non-estrogen mediated pathways. These processes may also have different physiological mechanisms; *vtg* induction is related to the liver, while intersex is developed in the gonads as a target of EDCs. That is dependent upon the length and timing of exposure (Bahamonde et al., 2013). The absence of intersex phenomena in the gonads of both species of *Mullus*, examined in the present study, can be linked to the short life of these organisms compared to other fish, especially large pelagic such as tuna and swordfish (Caprioli et al., 2007; De Metrio et al., 2003)

However, the gonad histological analyses revealed another phenomenon linked with the presence of pollutants in the sampling areas. The MMCs are structures generally present only in the final and regressive stages of reproduction in fish. Indeed, their functions are the degradation and reabsorption, respectively, of oocytes and sperm cells not regenerated during gonadal regression

(Grier & Taylor, 1998). Therefore, in *M. surmuletus*, the

presence of MMCs at stage 4 of sexual maturity (regressing phase) can be considered normal, although the number of these structures was very high. Instead, in *M. barbatus*, a reverse trend was found, as the greatest degree of abundance of MMCs is detected in phases 2 and 3, reproductive stages where normally the individual does not need to eliminate cell bodies or waste materials.

5 Conclusion

Thanks to the use of a multivariate approach, using the same bioindicator, aquatic organism, and analyzes it through various biomarkers, it is possible to have a broader picture of what is the situation of contamination of our seas and, above all, the effects that it has on animal, plants, and inevitably also on man.

Through these results, we can again confirm the persistent contamination by heavy metals, mainly Hg, which characterizes the environment and the sea around Augusta (Centre site). Furthermore, the stretch in the North sampling site, between Riposto and Acitrezza, is confirmed to be characterized by a strong urban type of contamination. Both North and South sites show evidence of pollution from types of contaminants linked to the discharge of wastewater and the presence of strong agricultural pressure.

Even if the South sampling site is less critical in statistical significance, it also has some PAHs contamination. Although less densely inhabited, that area has a substantial tourist increase in the summer, which, added to agriculture pressure, both increase the discharge of many substances into the sea.

Considering the two species can be clearly stated that *M. barbatus* is a much more suitable bioindicator for different

biomarkers. In all the analyses that have been carried out, individuals of this species have shown the highest concentrations of both contaminants, gene expression, plasma levels, and the massive presence of MMCs even in the reproductive period. Furthermore, the research of intersex is the most striking bioindicator. However, vitellogenin (expressed or synthesized) may be more beneficial for the most immediate evaluation of a site not chronically contaminated by endocrine disruptors for less long-lived species such as mullet.

In conclusion, all three sampling sites were contaminated by different types of contamination sources and substances, and *Mullus barbatus* is a fundamental species for assessing the state of well-being of the seas as it is ubiquitous demersal and susceptible to contaminations.

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