Università Politecnica delle Marche

Department of Life and Environmental Sciences

PhD in Life and Environmental Sciences

POLYCYCLIC AROMATIC HYDROCARBON (PAH) POLLUTION IN WILD ADRIATIC FISH

from the main determining factors of PAH accumulation to some biological responses of fish

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Polycyclic Aromatic Hydrocarbon (PAH) pollution in wild Adriatic fish

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PhD Thesis

Written by Emanuela Frapiccini

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To me the sea is a continual miracle,
The fishes that swim, the rocks, the motion
of the waves, the ships, with men in them,
What stranger miracles are there?

(Walt Whitman)

CONTENTS

PREFACE

This research thesis was submitted to the Università Politecnica delle Marche in partial fulfilment of the requirements for the Doctor Philosophy Degree (PhD) in "Life and Environmental Sciences". The research project was taken place both at the Università Politecnica delle Marche and at the Institute of Marine Biological Resources and Biotechnologies (IRBIM) of the National Research Council (CNR). The PhD project received financial support from the FAO-ADRIAMED regional project, the Marine Strategy Framework Directive (MSFD), and the Biological Sampling of Fisheries catch – Data Collection Framework (FEAM 2014-2020).

The principal aim of this PhD thesis, entitled "*Polycyclic Aromatic Hydrocarbon (PAH) pollution in the wild Adriatic fish – from the main determining factors of PAH accumulation to some biological responses of fish*", were to examine a type of priority pollutants, namely polycyclic aromatic hydrocarbons (PAHs), in two most commercially relevant fish species (*Mullus barbatus* and *Solea solea*) of the Adriatic Sea (Mediterranean Sea). According to MSFD's implementation, all environmentally relevant contaminant types and pollution effects in the marine environment need to be considered (Gago et al., 2014). Therefore, the thesis examines which determinant factors are involved and can affect PAH accumulation in wild fish and related PAH levels and some biological responses of fish exposed to PAH pollution, within the context of human consumption.

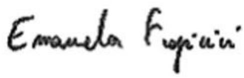
For this research activity, an innovative and "green" method of PAH extraction was applied: the QuEChERS approach coupled with UHPLC-FLD system. For the first time, the physicochemical properties of PAH compounds, some key biological variables of fish (body size, lipid content, age, sex and reproductive stage), the environmental feature and the different seasons were taken into account, in order to increase the knowledge of these contaminants. For first-time some antioxidant defense enzyme activities in the muscle of fish evaluated in the Northern Adriatic Sea.

This PhD project has been possible thanks to the support of many people who have contributed to this activity of research. So, I would like to express my sincere gratitude to my main supervisor Dr. Anna Annibaldi and my co-tutors Dr. Monica Panfili and Dr. Mauro Marini, for their continuous guidance and useful knowledge. I would like to thank my colleagues Dr. Stefano Guicciardi, Dr. Alberto Santojanni, Dr. Silvia Illuminati and Dr. Cristina Truzzi, for having work together and for many comments and support. Thanks to Dr. Francesco Alessandro Palermo and Dr. Paolo Cocci for having dedicated their time and expertise to me. I would like to extend my thanks to Dr. Silvia Franzellitti and Dr. Monia Perugini for providing helpful suggestions.

I would like to express my heartfelt thanks to my husband for his love and because he stands by me like a pillar in my life. I would like to thank my parents, my sister and, my best friends for the patience and endless affection they give me.

Sincerely,

Emanuela Frapiccini



THESIS SUMMARY

This thesis entitled "*Polycyclic Aromatic Hydrocarbon (PAH) pollution in the wild Adriatic fish – from the main determining factors of PAH accumulation to some biological responses of fish*" looks at the Polycyclic Aromatic Hydrocarbons (PAHs). These hydrophobic pollutants are the most widespread organic pollutants, whose sources can be petrogenic, biogenic and pyrogenic. This thesis focuses on the 16 PAHs, classified as priority pollutants by the European Union (EU) and the United States Environmental Protection Agency (US EPA) due to their carcinogenic and mutagenic effects and, included in the Descriptor 8 and 9 of the Marine Strategy Framework Directive (MSFD). The PAH level and distribution were investigated in different tissues of two fish species (*Solea solea* and *Mullus barbatus*) and in marine sediments of an important fishing ground located in the Northern and Central Adriatic Sea, in order to understand what factors (physicochemical, environmental, seasonal, and biological) could significantly be affected by PAH accumulation. Moreover, this thesis analyzed the biological response of fish, examining some molecular biomarkers of the oxidative stress in muscle tissue of fish exposed to PAHs.

In particular, the aim of this study is to increase the knowledge of which factors exert the greatest influence on PAH accumulation and to assess the effects of PAH in fish tissue, within the context of human consumption. Physicochemical properties of PAH compounds, some key biological variables of fish (body size, lipid content, age, sex and reproductive stage), environmental feature and different seasons were taken into account. Additionally, some possible biological responses of fish exposed to PAH pollution were examined.

The present thesis consists of seven chapters and three papers and opens with a general introduction describing PAHs in marine environments (***Chapter 1***). Then, the ***Chapter 2*** discusses the overall aim of the PhD research, and summarizes the papers included in the PhD thesis and the ***Chapter 3*** describes the main results and discussions. ***Chapter 4*** examines in detail the methodology for PAH extraction: the QuEChERS, a non-traditional method. In the ***Chapter 5***, the study area is described and in the ***Chapter 6*** the main biological characteristics of two fish species selected

for this study (*S. solea* and *M. barbatus*) are illustrated. Finally, a concluding remark of the overall results achieved in the present study is elaborated in ***Chapter 7***.

A collection of three manuscripts included in the PhD research is listed. Two of them have recently been published in international journals (with Q1 ranking), while the last paper has just been submitted. These studies have demonstrated how the PAH level and distribution were dependent on both the physicochemical properties of compounds, as well as habitat characteristics and surrounding pollution (*paper 1*). When the PAH compounds are grouped according to their molecular weight (MW) in three groups (low, medium and high MW), the heavier PAHs (medium and high MW), showed higher levels during the pre-spawning period and in the winter season (*paper 2*) and low MW PAHs induced oxidative stress in the muscle tissue of the fish (*paper 3*).

At the end of this, I have listed other products and publications not included in this thesis but performed during my PhD period.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are one of the most important categories of organic compounds consisting of two or more fused benzene rings, widely distributed in the air, water, soil and sediment. They are hydrophobic pollutants and semi-volatile compounds with relatively low water solubility and high lipophilicity. They have high values of log K_{ow} (octanol/water partition coefficient) inversely proportional to their number of aromatic rings and molecular weight (MW). They can be divided into three categories: low MW – PAHs, with two and three rings (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene); medium MW – PAHs, with four rings (fluoranthene, pyrene, benzo[a]anthracene and chrysene) and high MW – PAHs with five or more rings (benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene and benzo[g,h,i] perylene) (Moraleda-Cibrián et al., 2015; Barhoumi et al., 2016). Their scientific concern is because these compounds are persistent, toxic, bioaccumulative and undergoing long range atmospheric transport.

Certain PAHs are listed in the Stockholm Convention for their potential adverse health effects and sixteen of them are considered in the priority pollutant list of the United States Environmental Protection Agency (US EPA) and European Union (EU) (Figure 1). Certain PAHs are classified as probably and potentially carcinogenic to humans according to the International Agency for Research on Cancer (Combi et al., 2020; Anyakora et al., 2005; Neff, 1982; Valavanidis et al., 2008). Some priority pollutant PAHs, such as benzo[a]pyrene and ∑PAH4 (sum of benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene and chrysene) are generally used as an indicator of the presence of PAHs in food by the European Food Safety Agency (EFSA, 2008; Bansal and Kim, 2015) and their presence in foodstuffs has been restricted within the EU (EC, 2006; 2011).

Due to these well-known toxic effects that can induce cell damage, mutagenesis, teratogenesis and carcinogenesis, the 16 PAHs priority pollutants are widely monitored and are the subject of numerous environmental studies (Zhao et al., 2019; Balcioğlu E.B., 2016). In addition, the EU Marine Strategy Framework Directive (MSFD, 2008/56/EC) implementation – that represents the EU's Integrate Maritime

Policy tool – requires that the European Union member states should establish ecological monitoring programmes and, especially in Descriptors 8 (concentrations of contaminants giving rise to pollution effects) and 9 (contaminants in fish and other seafood for human consumption), that concentrations of several contaminants, including PAHs, should be at levels that do not give rise to pollution effects. This is recommended to achieve Good Environmental Status (GES) of marine waters by the Member States, with an initial target for 2020 (Kammann et al., 2017; Giani et al., 2019; Danovaro et al., 2020; Azaroff et al., 2020; Gago et al., 2014).

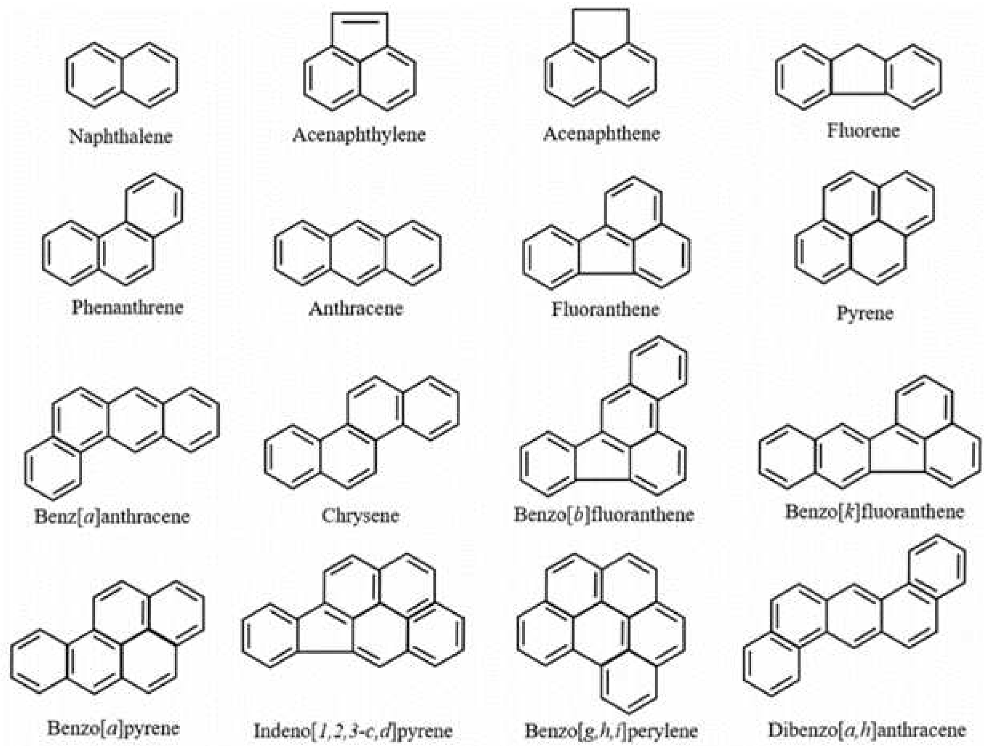


Figure 1. Chemical structure of the 16 priority pollutant PAHs, according to US EPA.

The main PAH contamination sources are due to the incomplete combustion of organic compounds (oil, wood, fossil fuels and organic matter) during industrial processes and other anthropogenic activity, as well as ship traffic and oil spills. Furthermore, PAHs can also be produced in nature because of volcanic activity,

forest fires, natural oil spills, diagenesis processes and biomass burning (Kim et al., 2013; Rovere et al., 2020; Magi et al., 2002; Maisano et al., 2017).
A wide range of PAHs enter in aquatic environment systems through atmospheric deposition, industrial and effluent discharges, vehicle combustion emissions, sewage sludge, storm runoff, riverine inputs and other possible ways. (Arienzo et al., 2017; Abdel-Shafy and Mansour, 2016; Zhang et al., 2015; Combi et al., 2020; Hoffman et al., 1984). Once in the water column, thanks to their lipophilic nature, PAHs tend to be absorbed into suspended particulate matter and transferred from the surface to the deep waters and sediments, which are considered to be the final organic pollutant sink in marine environments (Wetzel and Van Vleet, 2004; Nellier et al., 2015; Sun et al., 2016; Sun et al., 2017; Cardoso et al., 2016). Considering that marine sediments are the ultimate sink for pollutants, benthonic and demersal fish due to their contact with sediment, could be more prone to PAH intake (Duarte et al., 2017).
Generally, aquatic organisms are able to easily absorb organic pollutants into their bodies from sediment, water or through the ingestion of suspended particles and contaminated food (van der Oost et al., 2003; Qin et al., 2020). Physicochemical properties of PAHs (i.e., high lipophilicity and log K_{ow}), as well as the sediment ones (e.g., organic carbon, grain size), control most of the interactions between contaminants and sediment and, consequently, the bioavailability of PAHs and their accumulation in marine organisms (Fernandez & Gschwend, 2015; Fisk et al., 1998; Rose et al., 2012; Yu et al., 2019).
The bioavailability of pollutants is controlled by several interactions between sediment, water and biota, and very complex sorption – desorption mechanisms, associated with them occur continuously. For these reasons, predicting the bioavailability of pollutants and understanding their behaviour often requires laborious research, and it is important to keep in mind these interactions, as well as other biological and environmental factors that could affect PAH bioaccumulation.

Once PAHs enter in marine organisms, all the biotransformation processes that can increase the hydrophilicity of PAHs take place to facilitate the expulsion of PAHs from organisms (Santana et al., 2018; Lawal AT, 2017).
Aquatic vertebrates, such as fish, have more biotransformation systems capable of converting and metabolizing PAHs into more water-soluble derivates in comparison to invertebrates (Cocci et al., 2019; Boelsterli, 2007). The PAHs are subject to these biotransformation mechanisms, in a first step by metabolic enzymes of the cytochrome P450 (CYPs) system, and then their products are coupled to chemical

groups by phase II enzyme catalysis (Oliva et al., 2010; Santana et al., 2018). During these biotransformation reactions, the PAHs could exert a toxic effect leading to oxidative stress response by fish or damage to DNA, lipids and proteins. Oxidative stress can be neutralised by several antioxidant defence mechanisms, both enzymatic and non-enzymatic (Abele et al., 2017; van der Oost et al., 2003). In order to evaluate the impact of PAHs on oxidative stress in marine organisms, including fish, several antioxidant enzymes are used as biomarkers (Gorbi et al., 2005; Regoli&Giuliani, 2014).

In the Mediterranean Sea, although many studies focus on the source, level and risk assessment of PAHs in sediments and marine organisms (Baumard et al., 1998; Perugini et al., 2007; León et al., 2013; Ferrante et al., 2018; Guerranti et al., 2016; Galgani et al., 2011; Mercogliano et al., 2016; Morelada-Cirbián et al., 2015), trophic transfer of PAHs (Carrasco et al., 2013) and the PAH effect on marine organisms (Cocci et al., 2018; Costa et al., 2016; Solè et al., 2013) have received less attention, as well as few field studies. Indeed, in the Adriatic Sea, knowledge of the relationship between levels of PAHs in organisms and environmental factors, the PAH accumulation in different tissues of fish and the factors influencing this accumulation are still limited. In addition, mechanisms of the gene expression of the antioxidant response in wild fish tissue have been poorly investigated.

2. AIM OF THE STUDY

The assessment of PAH levels in environmental matrices and in the edible part of aquatic organisms is an important issue. The interest in PAH pollutants and their possible transfer to edible seafood has increased since the implementation of the MSFD. PAH uptake, accumulation, and availability, as well as the possible effects of PAH exposure on living organisms are complex, species-specific phenomena, and further studies are needed to characterize the factors influencing PAH bioavailability in aquatic organisms.

The overall objective of this study is to increase knowledge of which factors exert the greatest influence on PAH accumulation and to assess PAH levels in fish tissue, in the context of human consumption.

For this end, the following questions have been taken into account:

- How do the environmental characteristics of different sampling areas affect the bioaccumulation of PAHs in fish? (*paper 1*)
- How are PAHs distributed in different fish tissues? (*paper 1*)
- What is the behaviour of PAHs in relation to their physicochemical properties? (*paper 1* + *paper 2*)
- Which biological factors are involved in the accumulation of PAH in fish tissue? (*paper 1* + *paper 2*)
- Is there a seasonal effect on PAH levels in fish tissue? (*paper 2*)
- What is the biological response of fish exposed to PAH pollutants? (*paper 3*)

In addition, the QuEChERS extraction method was applied in this thesis to provide a methodological advancement towards a suitable protocol for quantifying PAHs in fish tissue (***Chapter 4***).

2.1 Summary of the studies

This sub-section summarizes each paper included in my PhD thesis.

Paper 1: Polycyclic aromatic hydrocarbon (PAH) accumulation in different common sole (*Solea solea*) tissues from the North Adriatic Sea peculiar impacted area

The present study focuses on the bioaccumulation of PAHs in marine organisms through the assessment of the PAH levels both in common sole, *Solea solea*, and marine sediments. PAH levels were measured in the different tissues of the wild population of *Solea solea* and in the surrounding sediments where the fish lives. Three impacted areas of the Northern Adriatic Sea, with different anthropogenic inputs of PAH pollutants were selected and the presence of PAH and their anatomical distribution in fish were investigated. Since *S. solea* lives in close association with sediments, feeds on the seafloor and makes little movements, it was selected for this study. Three study areas of the Northern Adriatic Sea characterized by a different anthropogenic input of PAHs were chosen (the Venetian Lagoon, the Po Delta and fishing grounds off Chioggia). This research includes the analysis of PAH concentration in various fish tissues (gill, liver and muscle), in relation to the spatial distribution of PAHs in the marine sediments, where the fish live. In addition, the physicochemical properties of PAHs (rings and K_{ow}) and some key biological variables (i.e. lipid content of tissue and body size) are correlated with PAH bioaccumulation.

Paper 2: Effects of biological factors and seasonality on the level of polycyclic aromatic hydrocarbons in red mullet (*Mullus barbatus*)

Herein, it was decided to investigate some aspects concerning which physicochemical, biological and seasonal factors could influence the accumulation of PAHs in muscle tissue, i.e., the edible part through which the pollutant reaches humans. It has been evaluated how PAH levels in edible fish interact with some biological parameters of fish, in particular the fish reproduction and the different seasons. In addition, the physicochemical properties of PAHs (rings and K_{ow}) were also analysed. For this purpose, *Mullus barbatus* was selected as fish species because it can be caught easily and is available all year round, and because it is used as a bioindicator for pollution monitoring. Furthermore, it spends its life in contact with the bottom, has a short life cycle and a quite simple reproductive cycle that takes place at a specific time of the year. Sampling was carried out in the Northern and Central Adriatic Sea. *M. barbatus* was caught monthly with a bottom trawl net, in a rich deep-sea fishing ground across the Northern and Central Adriatic Sea, over the period of one year. Edible fillets of 380 specimens were analysed for PAH concentrations. Relationships with morphometric (length and weight) and biological characteristics of fish (age, sex, reproductive stage and lipid content), with the main physicochemical properties of PAH and, finally, with the seasonality were took into account for evaluating the accumulation of PAHs in muscle fish.

Paper 3: Polycyclic aromatic hydrocarbons (PAHs)-induced oxidative stress in muscle tissue of non-spawning red mullet (*Mullus barbatus*) females from the Adriatic Sea

Finally, the *paper 3* describes in more detail the PAH effect in *Mullus barbatus* by investigating some molecular biomarkers of the oxidative stress in muscle tissue of wild red mullet, *M. barbatus*, exposed to PAHs. Specimens were collected from a rich deep-sea fishing area in the Northern and Central Adriatic Sea. Only females at the end of the spawning season, sexually mature but inactive, were selected, in order to exclude possible effects on PAH levels due to reproduction and sex, since in the previous study (*paper 2*) some effects on the reproductive stage were observed. For the first time, some preliminary insights into the association between PAH levels and mRNA expression profiles of some antioxidant genes and lipid peroxidation in muscles of non-reproductive red mullet females were provided.

3. MAIN RESULTS AND DISCUSSIONS

In this chapter, the main results, and discussions, of three papers included in the PhD thesis, are described.

Paper 1: Polycyclic aromatic hydrocarbon (PAH) accumulation in different common sole (*Solea solea*) tissues from the North Adriatic Sea peculiar impacted area

In the *paper 1*, PAH levels were measure in fish tissues (n = 50) of common sole *S. solea* (gills, liver and muscle) and, at the same time, in marine sediments where the species lives. Samples were collected from three areas of the Northern Adriatic Sea, characterized by different anthropic input (Venetian Lagoon, Po Delta and off Chioggia, the last one used as control).

Individual PAHs were divided into three groups (low, medium and high) based on their molecular weight (MW). In terms of frequency of detection, low MW-PAHs are the most present in all fish tissues. Instead, in terms of PAH accumulation, medium MW-PAHs are the main group both in fish tissue and marine sediment. On the contrary, high MW-PAHs are extremely low or undetectable, and recorded only in fish tissue. A negative relationship between PAHs and Log K_{ow} was found in the different tissue of *S. solea*, but it was significant ($p < 0.05$) only in gills, suggesting that a part of PAHs, that accumulate in the gills, depends on its physicochemical properties, such as equilibrium partition (K_{ow}). In addition, the direct exchange between water and sediment through the gills, acts as an important mechanism of bioaccumulation mechanism in *S. solea*. Indeed, in this study, a significantly ($p < 0.05$) higher level of PAH accumulation was found in gill tissue, followed by the liver and then in the muscle, in all three examined study areas (Figure 2). As expected, PAH levels in fish and sediments from the Venice Lagoon and the Po Delta were higher than the ones from fishing grounds off Chioggia. Furthermore, a significant relationship between PAH in fish tissues and sediment were found ($r = 0,970$, $p < 0,001$; Figure 3). These impacted areas are characterized by human activities as well as strong riverine inputs and other direct and indirect discharges which could have increase PAH contamination.

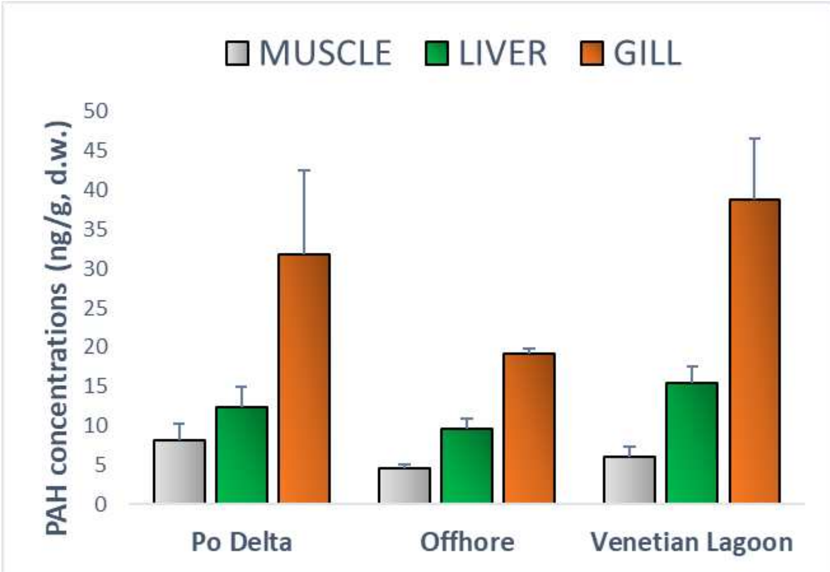


Figure 2. PAH concentrations (ng/g d.w.) in muscle, liver and gills of *S. sole*a caught in three study areas (Po Delta, off Chioggia - control - and Venetian Lagoon).

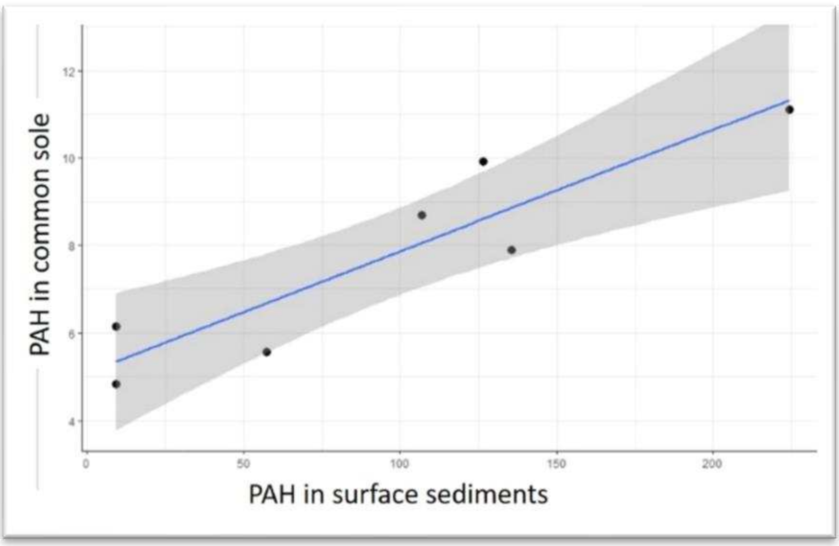


Figure 3. Relationship between PAH levels in *S. solea* and in surface sediments collected in three study areas (Po Delta, off Chioggia and Venetian Lagoon).

Total lipid content was determined in different fish tissues. The higher values of lipid content were found in liver (15%), followed by gills (9%) and muscle (2%). Even though PAHs are lipophilic compounds, a weak positive correlation between PAH bioaccumulation and the lipid content was found in three tissues, but statistically significant only for gills ($p < 0.05$). Furthermore, any relationship between body size and PAH levels in fish tissues have been recorded. Therefore, in this study, in which particularly impacted areas were considered, it is possible that lipid content might not be the only determinants of PAH bioaccumulation in fish tissues. These findings suggest that bioaccumulation and bioavailability of PAHs are closely related to the physicochemical properties of compounds, habitat characteristics and surrounding pollution, rather than biological factors (body size and lipids).

Paper 2: Effects of biological factors and seasonality on the level of polycyclic aromatic hydrocarbons in red mullet (*Mullus barbatus*)

The *paper 2* describes some aspects relating to which biological, chemical, and seasonal factors could influence the PAH accumulation in muscle tissues of the red mullet *M. barbatus*. PAH level were measured in muscle tissues of about 400 individuals of red mullet, collected monthly for a whole year. Total PAH concentrations average about 100 ng/g w.w., and it is considered as minimally polluted. PAHs compounds were grouped into three categories according to their number of rings and Molecular Weight. The low MW PAH predominated in muscle tissue. In particular, naphthalene accounting more than 70% (Figure 4). This result could be because low MW-PAHs have higher values of solubility, and bioavailability and lower capability to be metabolized compared to heavier PAHs. Therefore, a negative relationship ($p < 0.05$) between the individual PAH concentrations and their values of Log K_{ow} has been recorded, suggesting that PAH levels is strongly dependent on the physicochemical properties of compounds.

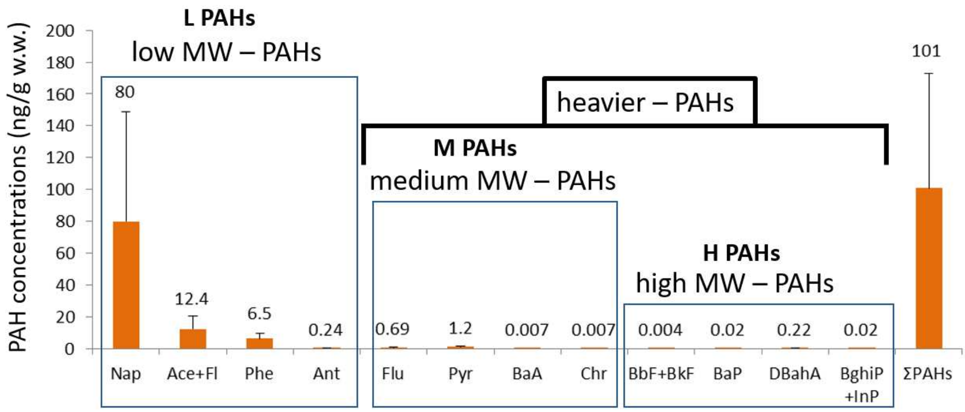


Figure 4. PAH concentrations (ng/g w.w.) of 400 red mullets individuals collected in the Adriatic Sea. PAHs were grouped according to their molecular weight, MW, in three groups: low, medium and high.

In this study, the reproductive stage and the different seasons seem to play an important role in the accumulation of heavier PAHs, whereas low MW-PAHs are not affected. When PAH levels was related to some biological parameters of fish, there were no significant differences in the PAH accumulation neither body size nor age of fish. In this study, the specimens were grouped according to their gonadal development stage (young, pre-spawning, spawning and post-spawning) to study the effects of reproduction on PAHs accumulation in muscle tissue. Low PAHs appeared to be unaffected by this, while heavier PAHs were significantly higher during the pre-spawning period in female ($p<0.05$). This effect was not so evident for males. This could be due to the spawning in red mullet females of the Adriatic Sea, is concentrated in a short period (late spring – early summer), while mature males are present almost all year round. Therefore, during the period that precedes reproduction, females need to accumulate a large amount of lipid for egg production, consequently, females could be subjected to a major lipophilic pollutant intake. For this reason, total lipid content in each reproductive stage in females and males were measured. The results showed a maximum of lipid content during the pre-spawning and a minimum in the post spawning in both sexes, but significantly only in females (Figure 5).

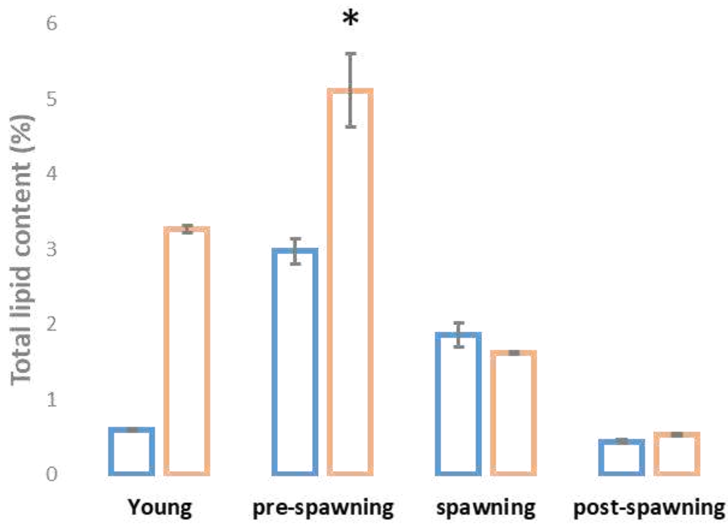


Figure 5. Total lipid content (%) in muscle tissues of red mullet grouped according to their reproductive stages (young, pre-spawning, spawning and post-spawning), in males and females.

When the effect of the different seasons was analysed, there was no evidence of any effects on the Low PAHS, instead, heavier PAHs showed higher values in winter than in other season one for both sexes. This finding could be explained by several reasons: the seasonal variability of PAH emission in atmosphere, which increases during the winter due to increased domestic heating and discharge by urban runoff. Moreover, the intensive bottom sediment resuspension, that occurs during the strong autumn and winter storm events, could lead to the mobilization of pollutant more recalcitrant, increasing their bioavailability portion.

Paper 3: Polycyclic aromatic hydrocarbons (PAHs)induced oxidative stress in muscle tissue of non-spawning red mullet (*Mullus barbatus*) females from the Adriatic Sea

In the *paper 3* the oxidative stress response by fish exposure to PAHs, through molecular biomarker were investigated. PAH concentrations in muscle tissue was related to molecular biomarker of some antioxidant gene (SOD, CAT, GST) and LPO. A negative relationship between some specific PAH congeners and Fulton's condition factor (K index) was found, underlining a general stress in fish, because the K index is a parameter of well-being and suggests sublethal effects due to chemical environmental stressors, such as changes in energy storage and metabolism. Furthermore, when we investigated up or down regulation of gene expression of some antioxidant defence systems, a negative correlation was found between the CAT (catalase) and the GST (glutation S transferase) and some PAHs, statistically significant ($p < 0.05$) for LMW PAHs and phenanthrene, respectively. Conversely, a positive correlation between LPO (lipid peroxidation) and some PAHs was found. These findings suggest that fish exposed to PAH have shown a down regulation of the mRNA levels of antioxidants, causing a lipoperoxidative response and, in general, highlighted the oxidative damage potential of LMW-PAHs, particularly PHE (Figure 6).

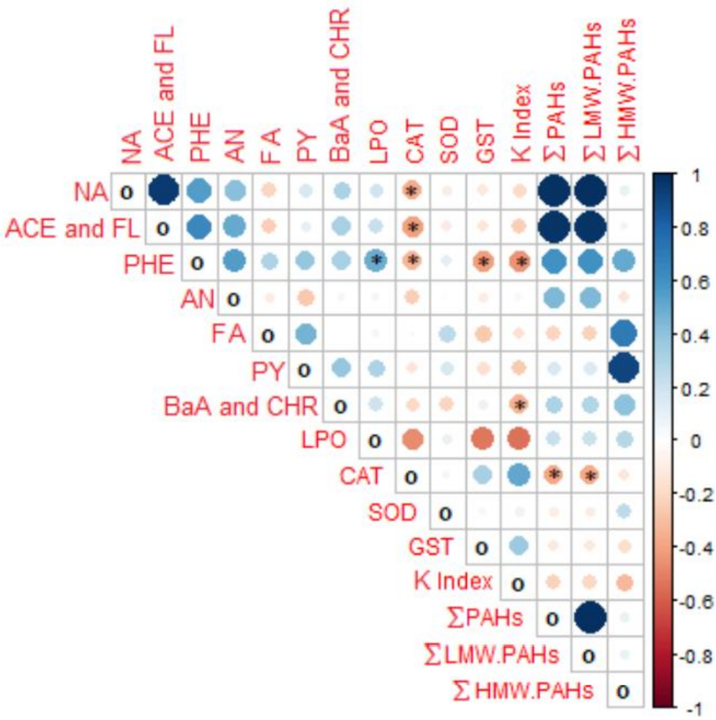


Figure 6. Heatmap of the Spearman correlation coefficients (ρ) between PAH concentrations and oxidative stress biomarker levels in muscle tissues of red mullet, *Mullus barbatus*. Colour indicates whether the correlation is positive (blue) or negative (red) while size and darkness of the circles indicate the strength of the correlations, with stronger correlations being larger and darker than weaker ones. (*, $p < 0.05$).

4. THE QuEChERS METHOD

In the present thesis, the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) technique combined with the established UHPLC-FLD (Ultimate High Performance, Liquid Chromatography – Fluorescence Detector) conditions was used to rapidly determine the EU priority PAHs in fish tissues.

The QuEChERS approach is a novelty technique of preparation of samples and extraction of contaminants. At the beginning, it was used for pesticide extraction in fruit and vegetables (Anastassiades et al., 2003; Lehotay et al., 2005; Xu et al., 2011), but since then this approach has been continuously modified for other matrices (i.e. food and environmental matrices) and for the analysis of a wide range of organic contaminants, including PAHs (Ramalhosa et al., 2009; Smoker et al., 2010; Norli et al., 2011; González-Gurbelo et al., 2015). Therefore, an increasing trend in QuEChERS research regarding PAH extraction is evident, from 2009 (when this approach was introduced for the first time for PAHs extraction) to 2020 (Figure 7).

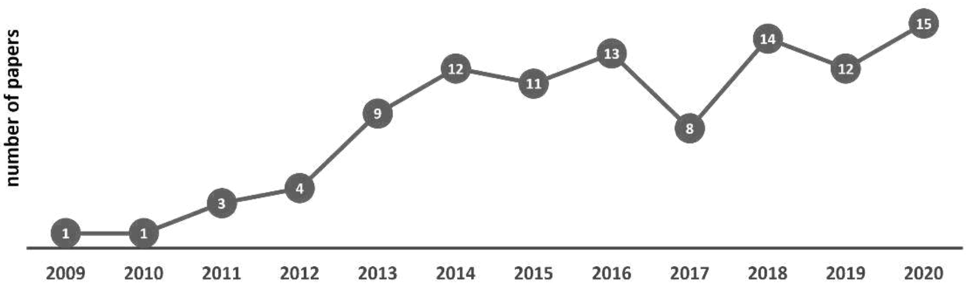


Figure 7. Trends in the QuEChERS research from 2009 to 2020 (December) based on a search on Web of Science of article titles, abstracts and keywords using the combination of the two terms “QuEChERS” and “PAHs”.

QuEChERS presents several characteristics, which make up its name, such as quick, easy, cheap, effective, rugged and safe. It is a rapid technique as it requires only two steps: the first is the extraction and partitioning of samples, while the second step is the clean-up in dispersive solid phase extraction (d-SPE) (Chiang et al., 2021). In the first step, an important advantage of this method is that in the QuEChERS extraction, samples were prepared using acetonitrile as a solvent, which can be used directly for both GC and LC analysis. Acetonitrile is an apolar solvent but can be mixed with water and it can penetrate the pore water matrices for the extraction of the selected analytes. When the salts (magnesium sulphate, $MgSO_4$ and sodium chloride, NaCl) are added, acetonitrile is sufficiently partitioned from the aqueous matrix. For this reason, it is recommended that sample should have about 70% of water (humidity). Moreover, the complete homogenization of the sample is very important for the first step of the QuEChERS technique to effectively execute the extraction of analytes (Lehotay SJ, 2011). In the second step of the QuEChERS method, the extracted and partitioned samples are purified using d-SPE to remove sources of potentially interfering compounds, including organic acids or polar pigments (Kim et al., 2019) (Figure 8).

Extraction and partitioning	5 g of homogenized fish are transferred to a 50 mL centrifuge tube Add 10 mL of acetonitrile Vortex for 1 min Add salt packet (4 g $MgSO_4$ and 1 g NaCl) to the centrifuge tube Vortex immediately for 3 min Centrifuge samples for 3 min at 3400 rpm
Clean-up and dispersive SPE	Take the upper layer of the clear supernatant (3 mL) Add supernatant to d-SPE tube (900 mg $MgSO_4$, 300 mg PSA and 150 mg C_{18}) Shake for 1 min Centrifuge for 1 min at 3400 rpm Take the upper layer of the supernatant Filtered and prepared for instrumental analysis in UHPLC-FLD

Figure 8. Simplified diagram of the QuEChERS process

Indeed, the QuEChERS technique is simple, fast and it is considered a valid alternative in terms of time (less time for preparation of samples) and solvent consuming, for this reason it could be considered as “green” method of extraction. Comparing the QuEChERS method with traditional techniques of extraction (i.e., Accelerated Solvent Extraction, sonication and Soxhlet), it is more efficient and economic than other ones (Lehotay S.J., 2011).

In the present thesis, the QuEChERS method was applied for extraction and purification of PAH compounds from fish tissue samples (*paper 1, 2* and *3*). Whereas, for marine sediment samples, the traditional method of ultrasonication was used (Baldrighi et al, 2019 "*ADDITIONAL PRODUCTS*", P3). The analysed PAHs were extracted with the QuEChERS kit using anhydrous $MgSO_4$ and NaCl. The main important phase of laboratory is the purification of the samples, particularly for biological samples that are more complex and with a high lipid content, therefore, these samples have a complex matrix that could cause a deleterious effect, such as interference in the analytical system (Lucas & Zhao, 2015). In this thesis study QuEChERS clean-up was performed, so the samples were purified by a d-SPE clean up with $MgSO_4$ and primary secondary amine (PSA). After shaking and centrifuging, an aliquot of the supernatant was collected for PAH analysis in UHPLC-FLD.

4.1 Comparing the QuEChERS method with ASE

Appling the QuEChERS extraction method was another aim of this thesis, in order to provide an alternative method in respect to traditional ones and a suitable protocol for quantifying PAHs in fish. Therefore, a comparison between QuEChERS and a traditional method, such as the Accelerated Solvent Extraction (ASE) was performed by analysing standard reference material (SRM NIST 1974c). Therefore, selected PAHs were extracted with both methods and the purified extracts were concentrated and recovered with acetonitrile for chemical analysis in a UHPCL (Ultimate 3000, Thermo Scientific) equipped with a fluorescence (RF2000, Thermo Scientific) detector, as mentioned above.

The comparison between QuEChERS extraction and the traditional ASE method for some selected PAHs (Ace, Fl, Flu and Py) is shown in Table 1 (Caroselli et al., 2020). The accuracy (%) of the QuEChERS method and ASE method are 93 and 91, respectively. These findings suggest that the performance of the QuEChERS technique was compared to ASE using the same SRM NIST 1974c. Therefore, it was suitable for PAH extraction in fish matrices in terms of accuracy and recoveries, as also demonstrated by Shen et al. (2020).

Table 1. Comparison of determined PAH concentration (wet weight basis) between QuEChERS and accelerated solvent extraction (ASE) methods for SRM NIST 1974c[a]. (Caroselli et al., 2020, "*ADDITIONAL PRODUCTS*", P2)

PAH	**Certified reference NIST 1974c**	**QuEChERS method**		**ASE method**	
	value ng g^{-1} ± SD	Measured value ng g^{-1} ± SD, *n* = 4	% Accuracy (RSD, %) *n* = 4	Measured value ng g^{-1} ± SD, *n* = 4	% Accuracy (RSD, %) *n* = 4
Ace	0.343 ± 0.019	0.319 ± 0.068	93 (21)	0.311 ± 0.072	91 (23)
Fl	2.31 ± 0.04	2.27 ± 0.28	98 (12)	2.17 ± 0.33	94 (15)
Flu	45.3 ± 0.8	45.5 ± 2.7	100 (6)	45.9 ± 0.9	101 (2)
Py	23.9 ± 1.6	22.6 ± 2.0	94 (9)	23.0 ± 1.4	96 (6)

[a]https://www-s.nist.gov/m-srmors/certificates/1974C.pdf

5. THE STUDY AREA

The Adriatic Sea is a semi-enclosed basin (138,600 Km^2) located in the northeast part of the Mediterranean Sea, between the Italian peninsula and the Balkan Region. It is generally divided into three sub-basins: theNorthern Adriatic, the Middle Adriatic and the Southern Adriatic (Artegiani et al., 1997a, b). The North Adriatic is very shallow (50 – 70 m) and characterized by freshwater plume of the Po River, the Middle Adriatic Sea is characterized by the Mid Adriatic Pit, also called Pomo Depression or Jabuka Pit, which reaches the maximum of 270 m (Central Pit), 255 m in the Western Pit and 240 m in the Eastern Pit (Marini et al., 2006). The South Adriatic Sea extends from the Gargano Promontory to the Otranto Strait and represents the deepest part of the Adriatic Sea (over 1200 m), called South Adriatic Depression (SAD). Generally, the depth of the Adriatic Sea gradually decreases from south to north, and about 74% is less than 200 m deep (Trincardi et al., 1996).

The hydrodynamics of the Adriatic Sea, as well as the transport of sediments, materials, and pollutants in three sub-basins, are characterized by a general cyclonic circulation, dense water formation and fluvial runoff derived primarily from the Po River. Therefore, the Adriatic Sea is characterized by a general anti-clockwise circulation, influenced by two main currents: the West Adriatic Current, WAC and the East Adriatic Current, EAC, which are linked to the dominant winds of the Adriatic basin (Bora and Scirocco). This typical cyclonic circulation plays a fundamental role in the distribution of nutrients, materials and pollutants transported by rivers, especially along the west coast of the basin.

In the Adriatic Sea, three water masses can be clearly distinguished: the Adriatic Surface Water (AdSW) flowing on the western Italian side, the Levantine Intermediate Water (LIW) coming from the eastern Mediterranean and, finally, the Adriatic Dense Water (AdDW) i.e. cold, high salinity waters that could be trapped for one or more years in the sea bottom (Marini et al., 2008, 2010, 2016).

The Northern Adriatic Deep Water (NAdDW) and the Southern Adriatic Deep Water (SAdDW) form in the northern and southern Adriatic sub basins, respectively. The role of the AdDW is essential to the sediment transport and to the transfer of particles, sedimentary materials, pollutants, nutrients and lipids, to the deeper water columns (Salvadó et al., 2017). On the northern shelf, NAdDW forms during the winter by strong cooling and surface evaporation, associated with episodes of north-east Bora wind, and flows southward close to the coast (Vilibić&Supić, 2005).

Such complex morphology and hydrodynamics of the Adriatic Sea cause the waters and the finer sediments discharged by the Po and Apennine rivers to flow along the western coast. These tend to extend offshore in summer and to be confined near the coast in winter (Spagnoli et al., 2014; Frascari et al., 2006; Cozzi and Giani 2011). Rivers flowing into the Adriatic Sea (Figure 9) contribute about 20% of the all river runoff in the Mediterranean Sea. Particularly, river runoff derived primarily from the Po River, plays a key role in driving the coastal dynamics, the physical and biogeochemical processes throughout the basin. It flows down along the western coast and crosses one of the most industrialized areas in Europe, the Po Valley, which is subjected to intense urban, rural and industrial pressure (Langone et al., 2016; Campanelli et al., 2011; Combi et al., 2020).

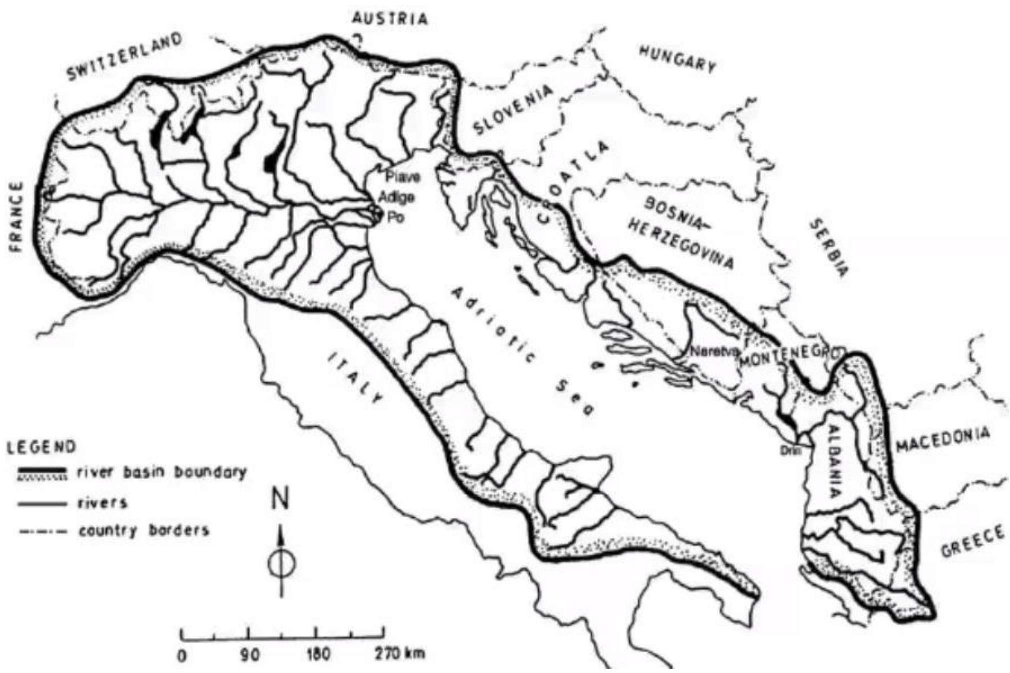


Figure 9. Rivers that flow into the Adriatic Sea (Cushman-Roisin et al., 2001).

According to the General Fisheries Commission for the Mediterranean Sea subdivision (GFCM, 2009 – FAO), the Northern and Central Adriatic Sea is also referred to as Geographical Sub Area, GSA 17. Since rich rivers flow over the shallow shelf of the North Adriatic and introduce large fluxes of nutrients and sedimentary materials (Cozzi and Giani 2011, Campanelli et al., 2011), this part of the Adriatic is one of the richest fishing grounds in the Mediterranean Sea. Furthermore, the mixing of the bottom sediments due to the complex hydrodynamics of the Adriatic Sea, makes this area highly productive. The productivity rate of the coastal area is greater than the open sea one, due to the relatively lower depth, vicinity of the land and fresh-water inflow. The resulting biomass richness of commercial species makes GSA 17 one of the most intensively fished areas in Europa. However, the GSA 17 is also characterized by shallow eutrophic waters, and aquatic organisms (mainly benthic and demersal species) that live there are highly vulnerable to anthropogenic impacts and the presence of pollutants.

In the present thesis, the study areas selected in *paper 1* were located in a particularly impacted zone of the Northern Adriatic Sea sub-basin. They are two well-known and complex transitional environments, such as the Po Valley and the Venetian Lagoon, and a third area offshore, less contaminated, and selected as a control (a fishing area off Chioggia). Instead, the Northern and Central basins of the Adriatic Sea were chosen in *paper 2* and *paper 3*. These areas are characterized by sediments of varying composition and grain size, becoming clayey and then sandy from east towards the centre of the basin (Droghini et al., 2019, *ADDITIONAL PRODUCT, P4*; Frignani et al., 2005). In addition, this area is also characterized by strong inputs from Italian rivers flowing through highly industrialized, densely populated and intensively farmed areas, making these areas extremely susceptible to pollution, particularly organic contaminants such as PAHs (Magi et al., 2002; Marini&Frapiccini, 2013; Illuminati et al., 2019).

6. THE FISH SPECIES

Two fish species, *Solea solea* and *Mullus barbatus*, were chosen in this PhD thesis in order to investigate the PAH contamination in fish tissues. *S. solea* and *M. barbatus* were selected for their high ecological and economical importance inthe Mediterranean Sea and, in particular, in the Adriatic Sea (FAO, 2020). Moreover, these species represent a good source of proteins, vitamins and other nutrients of high quality, and common use in the Italian diet (Bodin et al., 2014; Durmuş et al., 2018). In addition, taking into account the numerous and peculiar characteristics of the species (i.e. habitat, migration, life cycle) and in view of the implementation of the MSFD (Descriptor 8 and 9), *S. solea* was selected for the study carried out in *paper 1* and *M. barbatus* for the studies carried out in *paper 2* and *paper 3*.

6.1 *Solea solea*

Common sole (*Solea solea*; Linnaeus, 1758, Figure 10) was selected for the study carried out in different particularly impacted areas of the North Adriatic Sea, the Venetian Lagoon, the Po Delta and ground fishing off Chioggia (*paper 1*). This demersal flatfish is one of the most commercially important species in Adriatic fisheries, contributing around 23% to the overall sole catch of the FAO-GFCM (Food and Agriculture Organization – General Fisheries Council for the Mediterranean) area, particularly northern parts of the Adriatic basin (Scarcella et al., 2014; Pellini et al., 2018). *S. solea* lives in sandy and muddy bottoms, feeding on the seabed and lives in close contact with sediment. It has been suggested that demersal detritivores such as common sole, could be the link between contaminated sediment and the aquatic organisms. Since it is highly vulnerable to anthropogenic impacts, such as the presence of contaminants, it is widely accepted as a sentinel of chemical contamination in seawater and biomarker studies in relation to PAH exposure (Claireaux and Davoodi, 2002; Sagratini et al., 2008; Sun et al., 2016). Considering these important aspects and since the northern Adriatic Sea is an important spawning and aggregation area for common sole (Scarcella et al. 2014), this species was chosen for the *paper 1* research.

The catch of *S. solea* in the North Adriatic Sea is dominated by age 0 – 1 year old individuals, with little presence of large individuals. This spatial distribution is due to the fact that young specimens are mostly concentrated in the north-western Adriatic Sea, along the Italian coast (up to 30 m depth) and around the mouth of the Po River. Instead, the main aggregation grounds of adults are in the central Adriatic, from the inshore waters in the north to the deeper waters (70 m) in the south (Grati et al., 2013). Findings in *paper 1* confirmed this, showing that the morphometric of individuals exhibited no significant differences in the two environmental transition areas (Venetian Lagoon and Po Delta), whereas the individuals caught in ground fishing areas off Chioggia were heavier and longer ($p < 0.05$).

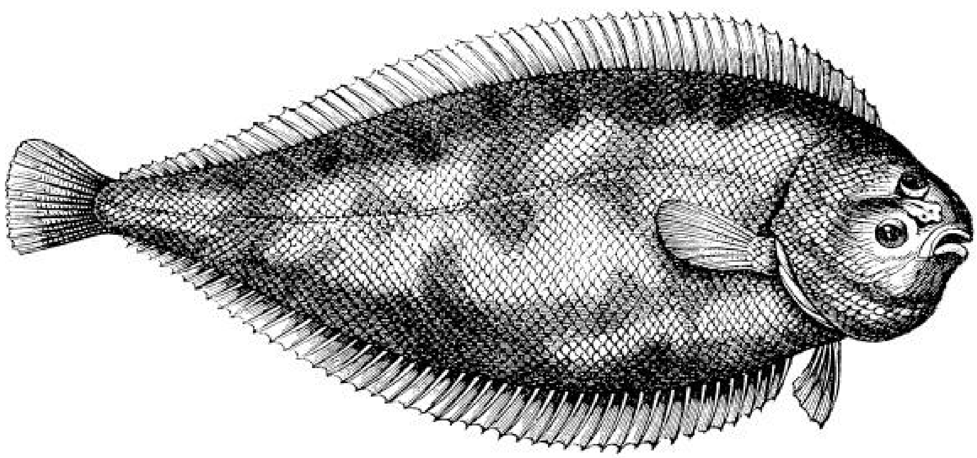


Figure 10. Common sole, *Solea solea* (Linnaeus, 1758)

6.2 *Mullus barbatus*

Red mullet (*Mullus barbatus*; Linnaeus, 1758, Figure 11) was chosen for two studies about the effects of several factors on PAH levels in muscle tissue (*paper 2*) and the oxidative damage potential of PAHs on wild fish (*paper 3*). This demersal species represents one of the most important fishery resources widely distributed in the Mediterranean Sea. Moreover, it is also a target species in the Adriatic, accounting for about 13% of the landings of all demersal species excluding bivalves and gastropods (but including crustaceans and cephalopods) and for 3.5% of the landings of all species (EU Data Collection Framework, 2017). Red mullet is caught all year round especially during the autumn, the period of maximum recruitment when, the catches are characterized by the contemporary presence of young and adult specimens. For these reasons, red mullet was selected for the study carried out in *paper 2*, in which the specimens were caught monthly throughout a whole year, in order to consider the PAH level throughout the reproduction cycle of the fish.

Red mullet lives in close contact with sandy and muddy bottoms, feeding mainly on benthic organisms (Corsi et al., 2002; Della Torre et al., 2010; Giani et al., 2019). *M. barbatus* is widely suggested as bioindicator species in the Mediterranean Sea. Since it tends to accumulate pollutants more largely than other species, red mullet has therefore been used for pollution monitoring of PAH and other contaminants (Della Torre et al., 2010; Guerranti et al., 2016). Its reproductive peak usually comes from May to July (Carbonara et al., 2015; Follesa & Carbonara, 2019).

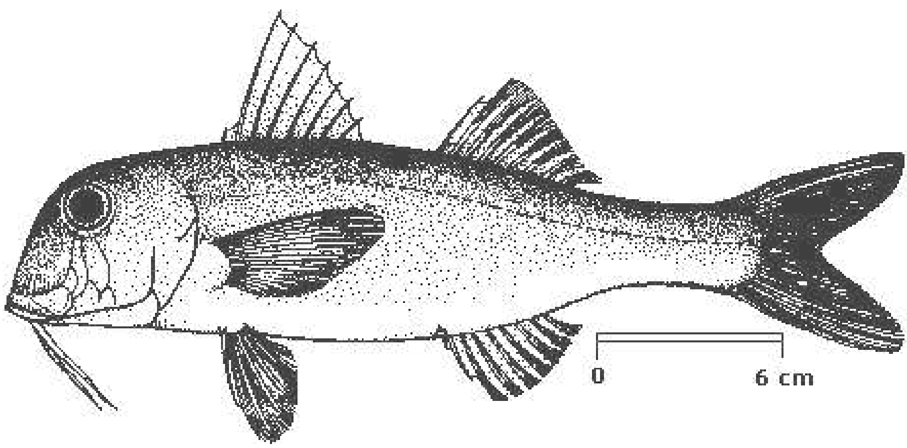


Figure 11. Red mullet, *Mullus barbatus* (Linnaeus, 1758)

PAPER 1

Polycyclic aromatic hydrocarbon (PAH) accumulation in different common sole (*Solea solea*) tissues from the North Adriatic Sea peculiar impacted area

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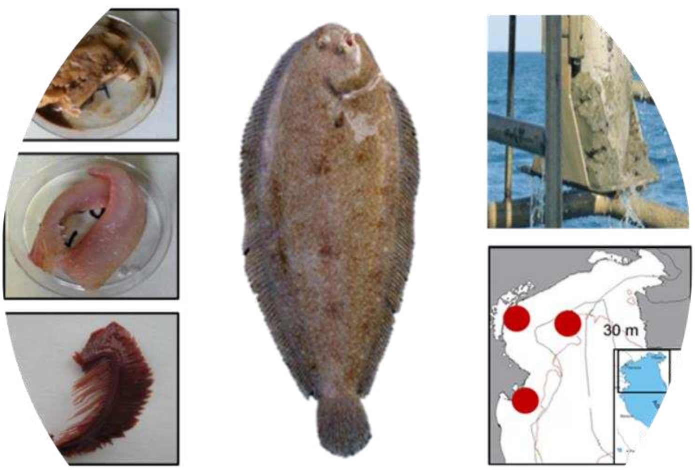

Polycyclic aromatic hydrocarbon (PAH) accumulation in different common sole (*Solea solea*) tissues from the North Adriatic Sea peculiar impacted area.

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Keywords: PAH bioaccumulation; determining factors; fish tissue; Solea solea; northern Adriatic Sea.

The present study of PAH levels and distribution in fish tissue aims to investigate the main factors affecting PAH bioaccumulation. Although several investigations have examined the impact of PAHs on marine organisms of the Adriatic and Mediterranean basins (Amodio Cocchieri et al., 1990; Corsi et al., 2002; Perugini et al., 2007; Galgani et al., 2010; Trisciani et al., 2011; Storelli et al., 2013; Cocci et al., 2018; Ferrante et al., 2018), comparisons between various fish tissues and environmental samples are limited. Moreover, the interest in these pollutants and their possible transfer to food of marine origin has increased after the Marine Strategy Framework Directive (MSFD), the first EU legislative instrument (EC, 2008) regulating the protection of marine biodiversity to achieve Good Environmental Status (GES).

For this reason this study were to analyze and evaluate the residue levels and distribution of some PAH priority pollutants in the tissues (gills, liver and muscle) of common sole (*Solea solea*, Linnaeus, 1758) individuals collected from three areas of the northern Adriatic Sea and to examine the main factors involved in PAH bioaccumulation, namely the lipid content of tissue, the biometric characteristics of fish, environmental features, and the physicochemical properties of PAHs (number of ring and K_{ow}), to establish which factors exert a major influence on PAH bioaccumulation in fish tissue, especially S. solea.

Aquatic ecosystems like coastal marine areas act as a sink for many harmful substances, including persistent organic pollutants (POPs) such as polycyclic aromatic hydrocarbons (PAHs) (Webster et al., 2011; Van Ael et al., 2012; Marini and Frapiccini, 2014). PAHs are among the most widespread organic contaminants and constitute a class of widely studied pollutants; in particular, 16 of them have been classified as priority pollutants by the European Union and the US Environmental Protection Agency (US EPA) due to their carcinogenic and mutagenic effects (Vives et al., 2004; Pandey et al., 2011; Xia et al., 2012). PAHs derive mainly from anthropogenic activities, including the combustion of organic matter, oil, wood, fossil fuels, and the release of hydrocarbons by crude oil (Neff et al., 2005; Stogiannidis and Laane, 2015; Barhoumi et al., 2016). Natural inputs into the environment are ascribable to volcanism, forest fires and petroleum seeps, but represent a small contribution to the overall PAH concentration in the environment (Baumard et al., 1999; Lima et al., 2003; Zhang et al., 2015). Due to their lipophilicity, low solubility in water and high persistence, PAHs naturally tend to be adsorbed in suspended matter, and then result in relatively rapid deposition in marine sediments (Cheung et al., 2007; Koelmans et al., 2010; Cui et al., 2016). Their hydrophobicity, namely octanol-water partition coefficient (Kow), is the dominant physical parameter for the fate of PAHs, as it determines their capacity for transport and distribution between different environmental compartments as well as their uptake and accumulation by living organisms (Chu & Chan, 2000; Frapiccini and Marini, 2015; Hussein et al., 2016). PAH bioaccumulation is also influenced by other physicochemical characteristics, such as number of rings, type and origin, environmental conditions (e.g. sampling area, PAH emission sources nearby), and the characteristics of the species itself (e.g. trophic position, body size, lipid content, tissue) (Varanasi et al., 1985; Dominguez et al., 2011; Van Ael et al., 2012; Bodin et al., 2014). Accordingly, they can accumulate in the fatty tissues of organisms, biomagnify, and be transferred through the food chain, thus also affecting consumer health (Perugini et al., 2007; Bandowe et al., 2014; Zhonghua et al., 2014). The evaluation of PAH levels in environmental matrices and the edible part of aquatic

organisms is therefore an important issue (Bodiguel et al., 2009; Zhao et al., 2014). However, PAH bioaccumulation is a complex phenomenon governed by many factors including their uptake and elimination. Therefore, further studies are necessary to characterize the factors affecting PAH bioavailability to marine organisms and their distribution in fish tissues (Soclo et al., 2000; Bodin et al., 2014; Moraleda-Cibrián et al., 2015; Sun et al., 2016).

The northern Adriatic Sea is a shallow basin (less than 100 m deep) located in the northern part of the Mediterranean Sea. It is characterized by strong inputs from Italian rivers that flow through highly industrialized, densely populated, and intensively farmed areas (Sagratini et al., 2008). The River Po is the largest river, characterized by a mean annual discharge rate of 1500-1700 m3/s, accounting for about a third of the total riverine freshwater input to the Adriatic Sea (Campanelli et al. 2004, Marini et al. 2008; Campanelli et al., 2011). The three areas selected for this investigation were two well-known and complex transitional environments, the Po Valley, a very important agricultural region and the industrial heart of northern Italy (Castellarin et al., 2011), and the Venetian Lagoon, which is characterized by complex interactions between natural factors and human activities (Secco et al., 2005); the third area was a slightly less contaminated area 40 km off Chiogga (Fig. 1).

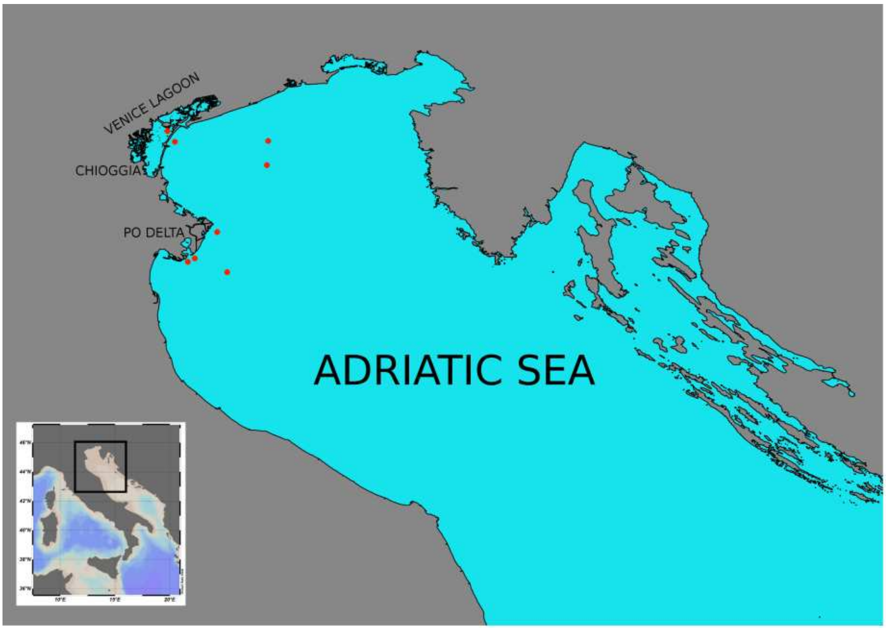

Fig. 1. Map of the study area (northern Adriatic Sea) with the location of *S.solea* and marine sediment sampling sites.

Common sole (S. solea) is one of the most commercially important species in Adriatic fisheries, with high ecological and economic value and high relevance for human consumption (Scarcella et al., 2014; Pellini et al., 2018). Since it feeds on the bottom and lives in close association with sediment, it is a widely accepted sentinel of chemical contamination in seawater and biomarker studies in relation to PAH exposure (Claireaux and Davoodi, 2002; Sagratini et al., 2008; Wessel et al., 2010; Bodin et al., 2014; Gonçalves et al., 2014; Sun et al., 2016). These considerations, and the fact that the northern and central Adriatic Sea are important spawning and aggregation areas for common sole (Scarcella et al. 2014), led this species and area to be chosen for the study.

Sampling activities were carried out in November and December 2014 in the framework of the "rapido" Trawl SoleMon Survey (Grati et al., 2013). Individuals of S. solea (n= 48) were caught with modified rapido trawls in 8 zones, and surface sediments were collected at the same sites using a box corer (Table a). All soles in the catches were measured (total length, mm) and weighed (wet weight, g) and examined for sex. Samples of muscle, liver and gill tissue were dissected out using acid-cleaned scalpels and scissors in the on board laboratory. Tissue samples were stored at -18 °C for PAH determinations.

Table a. Geographical coordinates of fish and sediment collection sites and number (n) of individuals of *S. solea* caught there.

Sites	Latitude	Longitude	*n* S. solea
Po Valley1	44°48,813N	12°27,985E	5
Po Valley2	44°48,714N	12°30,955E	7
Po Valley3	44°53,432N	12°36,644E	5
Po Valley4	44°43,497N	12°44,377E	5
offshore Chioggia1	45°14,773N	12°51,274E	6
offshore Chioggia2	45°20,180N	12°52,448E	5
Venetian Lagoon3	45°25,547N	12°20,613E	7
Venetian Lagoon4	45°21,687N	12°22,225E	8

A standard PAH solution with dichloromethane:methanol (1:1 v/v) containing the 16 priority pollutants (EPA 610 PAHMIX, Supelco, Bellafonte, PA, USA) was used for preparing PAH standard multipoint calibration. The chemicals used were dichloromethane, acetonitrile for HPLC gradient grade, acetone and petroleum ether (all purchased from VWR International, Fontenay- sous-Bois, France). 18.2 MΩ water was prepared by a Milli-Q system (Millipore, Billerica, MA, USA). Sediment samples certified IAEA code 408 and IAEA code 383, and material certified for fish homogenate IAEA code 406 were obtained from the International Atomic Energy Agency (Vienna, Austria). Quick easy cheap effective rugged and safe (Quechers)

(ECMSSC-MP) extraction kits containing 4 g MgSO4 and 1 g NaCl and a centrifuge tube ECMPSC1815CT containing 0.9 g MgSO4, 0.3 g PSA, and 0.15 g endcapped C18 were purchased from CPS Analytical (Milano, Italy). The Quechers method was applied and developed for the extraction and purification steps of PAHs from fish tissue, in agreement with various works (Ramalhosa et al., 2009; Sapozhnikova et al., 2013; Pfannkoch et al., 2015; Morrison et al., 2016). It is a simple and fast method that requires the use of a small volume of organic solvent and fewer steps than traditional extraction methodologies (Albinet, 2013; Sapozhnikova et al., 2013). It is therefore a valid alternative to other methods, because it employs a multiresidue sample preparation procedure adapted for extraction and clean-up (Morrison et al., 2016). Based on this procedure, 5 g of homogenized fish tissue was placed into a screw-capped tube with 10 mL acetonitrile and shaken in a vortex for 1 minute. Then, MgSO4 and NaCl in a ratio 4:1 (g/g) were added to the extract and the tube was shaken again in a vortex for 3 min. The tube was immediately centrifuged at 3400 RPM for 3 minutes at 4°C. Subsequently, 3 mL of the supernatant was recovered for clean-up and transferred to another screw-capped tube containing 0.9 g MgSO4, 0.3 g PSA (primary and secondary amines), and 0.15 g of C18 phase. The tube was shaken in a vortex for 1 minute and centrifuged at 4°C at 3400 RPM. The upper phase was collected in a round bottom flask and left under a laminar flow until complete evaporation: the dry residue was recovered with acetonitrile (0.4 mL), placed in a vial, and stored at -18°C until HPLC-FLD analysis.

PAH extraction from marine sediments was carried out according to a previous work by our group (Marini and Frapiccini, 2013), with some modifications and improvements. In brief, PAH extraction from 10.0 g sediment samples was obtained by three 20 min cycles in an ultrasonic bath using methanol:dichlorometano (1:1 v/v) as the solvent to achieve liquid-liquid separation. The PAH enriched solvent was removed initially by rotary evaporation (T = 30±2°C) and later by gentle nitrogen flow. The final volume of the analytical samples was adjusted to 0.4 mL with acetonitrile and stored at -18°C until HPLC-FLD analysis.

To determine the water percentage in tissue, they were lyophilized by a freeze-drying process that enables complete loss of water at low temperature (-20 °C) and pressure. Tissues were accurately weighed and freeze-dried (Edwards EF4 modulyo, Crawley, Sussex, England) until constant weight (±0.2mg).

PAH identification and quantification in fish tissue and surface sediments samples were performed by the same methods using an HPLC system (Ultimate 3000, Thermo Scientific, Waltham, MA, USA) equipped with a fluorescence (RF-2000) detector (Thermo Scientific). A Hypersil Green PAH (µm2.1 x 150 mm, 1.8 µm, 120 Å) column in a reversed-phase liquid chromatography with a water:acetonitrile (v/v) gradient elution was used. The mobile phase consisted of an initial composition of 60% acetonitrile (held for 6 min) that, after 15 min, reached 90% (held for 10 min) and then returned to initial conditions. The duration of the analysis was 31 min with the equilibrium time condition of 9 min. The flow rate was 0.3 mL min-1 at 40 °C. The wet weight (w.w.) of fish tissues and sediment was corrected to d.w. after determination of percent humidity in the samples.

The lipid content of muscle, liver and gills was estimated in a group of sole (n = 6 per tissue) by microwave-assisted extraction MARS 5 (CEM Corporation, Matthews, NC, USA) using 15 mL of a 2:1 petroleum ether/acetone (v/v) solvent mixture and 0.5 g of Na2SO2 (Truzzi et al., 2017; 2018). The amount of extracted fat was determined by the gravimetric method. The laboratory analytical balance was a Model AT261 apparatus from Mettler Toledo (Greifensee, Switzerland), which has a readability of 0.01mg and repeatability as SD of 0.015 mg. Its accuracy was tested using two certified reference ''weights'' (OIML class E1) of 100 and 10 mg (both with a tolerance as 2SD of 0.0020 mg).

For the quality control, the procedural blanks were analyzed and the external standard multipoint calibration technique (from 0.05-1.00 to 1.00-20.00 µg/mL) was used to determine the linear response interval of the detector; the average of the correlation coefficients was 0.997 for all analytes. PAHs were identified by comparison of their retention time with those of the authentic standards. The detection limit (LOD) and the quantification limit (LOQ) was calculated for each PAH using the following equations, according to ICH Q2B (ICH, 2005):

LOD = 3.3 Sa/b and LOQ = 10 Sa/b

where Sa is the standard deviation of the intercept of the regression line and b is the slope of the calibration curve (Truzzi et al., 2014a, b). The LODs and LOQs ranged from 0.02 (BkF and BaP) to 1.20 (Nap) ng mL-1 and from 0.05 (BkF) to 4.00 (Nap) ng g-1 d.w., respectively.

The whole analytical procedure was validated by analyzing the reference materials (IAEA code

106) and the recovery fell with the confidence interval of 95%. In addition, recovery rates were obtained for each congener PAH from fortified fish tissue samples (n = 5); these samples were extracted and analyzed by the procedure described above. The range of percentage of recoveries in fortified fish tissue was 50-100%, 53-98%, and 48-96% for muscle, liver and gills, respectively.

For sediment samples, the reference materials used were IAEA code 408 and IAEA code 383 and the recovery percentage ranged from 53 to 88%and from 61 to 82%, respectively, for IAEA -383 and IAEA -408. Statistical analysis of method performance data, particularly the evaluation of the linearity range and LOD and LOQ quantification, was performed using Statgraphics Plus software, version 5.1 (Statgraphics, 2000). A p value lower than 0.05 was considered to indicate significance. PAHs were grouped in three different categories according to Mashroofeh et al. (2015): low molecular weight PAHs (LMW-PAHs) including 2-3 ring PAHs (naphthalene, (Nap), acenaphthene (Ace), Fluorene (Fl), phenanthrene (Phe), Anthracene (Ant); moderate molecular weight PAHs (MMW-PAHs) including 4 ring PAHs (fluoranthene (Flu), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr),); and high molecular weight PAHs (HMW-PAHs) including 5-6 ring PAHs (benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo(a,h)antracene(DahA), indeno[1,2,3,-cd]perylene + benzo[ghi]perylene (InP+BghiP).

The basic data obtained from the sole individuals caught in three areas of the northern Adriatic Sea are presented in Table b. The morphometric data exhibited no significant differences in the two environmental transition areas, whereas the individuals from the third site, off Chioggia, were significantly heavier and longer ($r = 0.93$, $p< 0.05$). These data agree with the spatial distribution of sole reported by Grati et al. (2013) in the Adriatic Sea, who found that S. solea juveniles are mostly concentrated in the north-western Adriatic Sea along the Italian coast down to 30 m depth and around the mouth of River Po, whereas the main aggregation grounds of adults are in the central Adriatic, from the inshore waters in the north to the deeper waters (70 m maximum) in the south. No adults were recorded around the Po estuary in the present study. As expected, length and weight always positively correlated in all areas ($r > 0.88$, $p < 0.05$).

Table b. Average (±SD) of standard length (Ls, mm) and total wet weight (t.w.w., g), and number of *S. solea* individuals and sediment samples analyzed in three areas of the northern Adriatic Sea.

area	Ls, mm	tww, g	n. *S. solea*	n. sediment
Po Delta	239.7 (26.2)	126.9 (38.7)	22	4
off Chioggia	276.8 (24.8)	207.0 (72.0)	11	2
Venetian Lagoon	244.5 (26.9)	118.3 (39.2)	15	2

The content in individual and total PAHs was measured in muscle, liver and gill tissue of 48 common sole individuals caught in the Venetian Lagoon, the Po Delta, and off Chioggia (Table c). Of the Σ16PAHs, acenaphthylene could not be analyzed with FLD due to lack of fluorescence, whereas acenaphthene, fluorene, and anthracene were not available. Dibenzo(a,h)anthracene, benzo(g,h,i)perylene, and indeno(1,2,3-cd)pyrene were below the LOQ (0.03-0.05ng mL^{-1}).

The average total PAH concentration was 6.7 ng g-1 d.w. (range: 1.3-16.3 ng g^{-1} d.w.), 13.1 ng g^{-1} d.w. (range: 2.1-31.8 ng g^{-1} d.w.), and 32.0 ng g^{-1} d.w. (range: 2.0-112.6 ng g^{-1} d.w.), in muscle, liver and gill tissue, respectively. The highest and lowest concentration in muscle were found in sole caught respectively in the Po Delta (8.06 ± 3.2 ng g^{-1} d.w.) and off Chioggia (4.5 ± 2.9 ng g^{-1} d.w.).

Table c. Concentration (ng g-1, d.w.) of individual and total PAHs in *S. solea* tissues from three areas of the northern Adriatic Sea: the Po Delta, the Venetian Lagoon and off Chioggia.

Area	Site	tissue	Nap	Phe	Flu	Pyr	BaA	Chr	BbF	BkF	BaP	DahA	InP+ BghiP	Σ PAHs
Po Delta	1 P	liver	0.91 (1.91)	1.00 (0.70)	1.10 (0.81)	6.95 (4.22)	0.76 (0.27)	< loq	< loq	< loq	< loq	< loq	< loq	10.42 (2.55)
		gills	1.80 (3.73)	1.93 (1.94)	1.90 (3.26)	12.04 (12.70)	< loq	< loq	< loq	< loq	< loq	< loq	< loq	17.67 (19.54)
		muscle	0.64 (1.34)	0.71 (0.25)	0.66 (0.50)	3.37 (1.78)	1.36 (1.78)	< loq	< loq	< loq	< loq	< loq	< loq	6.19 (2.55)
	2 P	liver	1.53 (0.97)	1.32 (0.81)	2.16 (1.34)	10.66 (6.21)	0.26 (0.20)	0.09 (0.25)	0.23 (0.64)	0.10 (0.10)	0.05	< loq	< loq	16.21 (9.31)
		gills	3.72 (2.82)	3.63 (2.97)	4.74 (5.70)	30.33 (21.70)	0.57 (0.29)	0.09	0.38	0.07	0.06	< loq	< loq	43.17 (32.70)
		muscle	0.62 (0.20)	0.71 (0.30)	0.99 (0.50)	4.77 (1.70)	0.11 (0.02)	0.10 (0.07)	0.19 (0.15)	0.10 (0.04)	< loq	< loq	< loq	7.57 (2.50)
	3 P	liver	1.03 (0.36)	1.05 (0.28)	2.09 (1.05)	7.71 (2.62)	0.07	0.02	0.04	0.05 (0.03)	< loq	< loq	< loq	12.02 (4.10)
		gills	2.19 (0.37)	3.69 (3.17)	6.72 (6.00)	22.62 (8.79)	0.66 (0.52)	0.05	0.14	0.12 (0.08)	< loq	< loq	< loq	35.79 (17.40)
		muscle	0.71 (0.36)	0.78 (0.28)	0.98 (1.05)	4.61 (2.62)	0.09	0.04	0.09	0.04 (0.03)	< loq	< loq	< loq	7.30 (4.10)
	4 P	liver	0.60 (0.67)	0.92 (0.67)	0.91 (0.71)	8.29 (1.02)	< loq	< loq	< loq	< loq	< loq	< loq	< loq	10.72 (7.28)
		gills	2.41 (1.50)	2.58 (1.08)	2.85 (1.39)	22.36 (8.56)	0.70	< loq	0.05	< loq	< loq	< loq	< loq	30.39 (11.10)
		muscle	1.02 (0.66)	0.91 (0.38)	0.94 (0.31)	8.34 (3.04)	0.16	< loq	< loq	< loq	< loq	< loq	< loq	11.24 (3.89)
off Chioggia	1 C	liver	0.63 (0.77)	0.73 (0.44)	1.24 (0.82)	6.16 (3.41)	< loq	< loq	< loq	< loq	< loq	< loq	< loq	8.75 (4.98)
		gills	1.89 (2.19)	1.59 (1.14)	2.66 (1.63)	12.52 (8.83)	0.03	< loq	< loq	< loq	< loq	< loq	< loq	18.67 (13.29)
		muscle	0.45 (0.40)	0.43 (0.21)	0.39 (0.43)	2.91 (1.83)	< loq	< loq	< loq	< loq	< loq	< loq	< loq	4.19 (2.83)
	2 C	liver	0.35 (0.29)	0.54 (0.23)	5.50 (7.26)	4.10 (1.67)	< loq	< loq	0.02	< loq	< loq	< loq	< loq	10.51 (8.25)
		gills	1.33 (0.21)	1.47 (0.55)	4.71 (10.25)	11.9 (14.26)	< loq	< loq	0.15	< loq	< loq	< loq	< loq	19.57 (10.04)
		muscle	0.47 (0.38)	0.36 (0.17)	0.37 (0.43)	3.75 (2.26)	< loq	< loq	< loq	< loq	< loq	< loq	< loq	4.95 (3.19)
Venetian	1 V	liver	1.37 (0.41)	1.09(0.41)	1.74 (1.32)	9.57 (4.38)	0.41	0.06	0.11	0.03	< loq	< loq	< loq	14.05 (6.60)
		gills	2.65	2.45 (1.38)	5.68 (3.46)	22.15	0.39	0.08	< loq	0.04	< loq	< loq	< loq	33.18

			(1.38)			(10.22)	(0.25)							(16.14)
		muscle	0.53 (0.11)	0.46 (0.11)	0.47 (0.24)	3.70 (1.76)	< loq	< loq	< loq	< loq	< loq	< loq	< loq	5.16 (2.38)
	2 V	liver	0.78 (0.27)	1.42 (0.27)	1.69 (0.66)	13.02 (2.31)	0.13	< loq	< loq	< loq	< loq	< loq	< loq	16.92 (2.36)
		gills	2.36 (0.78)	3.20 (0.78)	4.20 (2.85)	34.56 (13.70)	< loq	< loq	< loq	< loq	< loq	< loq	< loq	44.32 (18.27)
		muscle	0.30 (0.13)	0.60 (0.15)	0.57 (0.38)	5.39 (1.80)	0.04	< loq	< loq	0.02 (0.03)	< loq	< loq	< loq	6.89 (1.98)

The highest and lowest concentrations in liver were measured respectively in the Venetian Lagoon (16.06 ± 4.6ng g-1 d.w.) and off Chioggia (9.5 ± 6.4ng g-1 d.w.). The highest and lowest concentrations in gill tissue were recorded respectively in the Venetian Lagoon (39.5 ± 17.7ng g-1 d.w.) and off Chioggia (19.1 ± 11.1 ng g-1d.w.). Although several studies have investigated fish and seafood pollution, they have tended to focus on edible tissues and the risk for human health, whereas limited data are available on contaminant bioaccumulation and on the comparison of their content in fish tissue and local sediments (Sagratini et al., 2008; Sun et al., 2016; Moraleda-Cibriàn et al., 2015). A comparison of the PAH tissue concentrations found in the present study and those reported in previous investigations in the Mediterranean Sea was performed, taking into account that data comparability could be hampered by methodological differences, the compounds studied, and concentration reporting (Vives et al., 2004). Overall, our PAH levels were comparable to those found in S. solea muscle tissue from the north-western Adriatic (Trisciani et al., 2011; 3.5-3.9 ng g-1d.w.). Similar data have also been reported by Perugini et al. (2007) for other marine organisms (red mullet, 16.52 ng g-1 w.w.) from the Adriatic Sea, and by Ferrante et al. (2018) for common sole from the Gulf of Catania (Mediterranean Sea;20-38 ng g-1 w.w.). Slightly higher PAH concentrations (from 125.4 to 231.5 ng g-1 w.w.) were found by Storelli et al. (2013) for brown ray caught in the Adriatic Sea.

Among the LMW-PAHs group, only naphthalene and phenanthrene were investigated. However, considering that the concentration of naphthalene in fish tissues represents a high percentage of total LMW-PAH (Darilmaz et al., 2012; Mashroofeh et al., 2015; Moraleda-Cibriàn et al., 2015), in the present work, it can be affirmed that a preferential accumulation of MMW-PAHs in the analyzed fish tissues was recorded (about 80% of the total PAHs). PY was the dominant MMW-PAH congener, with 72.1%, 64.7%, and 68.6% of total PAHs for muscle, liver and gills, respectively. Previous studies of marine organisms from the Adriatic Sea have found the same predominance of 3 or 4 ring PAHs, with a dominance of PHE and PY congeners (Perugini et al., 2007; Storelli et al., 2013; Capolupo et al., 2017). However, the three groups of PAHs in terms of frequency of detection in all fish tissues sampled were LMW (100%), MMW (52%), and HMW (9%). Considering that PAH solubility decreases in inverse proportion to molecular weight (Porte and Albaigés, 1993), LMW-PAHs, due to their low Log Kow (< 5), higher bioavailability, and lower depuration rate, tend to have a higher uptake compared with HMW-PAHs (Sun et al., 2016; León et al., 2014). In addition, LMW-PAHs (e.g. Naphthalene) are not metabolized in the liver of some species of fish as efficiently as HMW-PAHs (Varanasi and Gmur, 1981; Broman et al., 1990; Meador et al., 1995; Mashroofeh et al., 2015; Morelada-Cibrián et al., 2015). For this reason, the presence of LMW-PAHs has been widely documented in various studies of polluted fish where NA and PHE were the

dominant PAH congeners, followed by MMW-PAHs, where Flu and PY were predominant, while HMW-PAHs were usually very low or undetectable (Mashroofeh et al., 2015; Morelada-Cibrián et al., 2015; Ke et al., 2017).

Marine surface sediments were investigated because they may constitute a major sink for many POPs introduced into the aquatic environment (Guerra, 2012; Solé et al., 2013; Marini and Frapiccini, 2014) as well as a key matrix in the contamination of fish living in contact with sediment (Cheung et al., 2007; Solé et al., 2013). The sediment concentrations of individual and total PAHs were also measured, to understand how the sampling areas affected their bioaccumulation in fish tissues (Table d). All compounds were detectable in sediment samples except for acenaphthylene, acenaphthene, fluorene and anthracene, which were not available.

Table d. Mean concentration (± SD) of individual and total PAHs at the three sampling sites in northern Adriatic Sea: the Po Delta, the Venetian Lagoon, and off Chioggia.

Area	Nap	Phe	Flu	Pyr	BaA	Chr	BbF	BkF	BaP	DahA	InP+ BghiP	Σ PAHs
Po Delta	146.47 (10.77)	16.79 (2.52)	234.49 (39.25)	126.14 (11.17)	16.40 (0.60)	23.84 (0.68)	20.25 (5.52)	8.66 (1.83)	10.63 (0.32)	0.19 (0.05)	21.23 (4.93)	625.08 (62.34)
Off Chioggia	25.25 (1.69)	2.20 (0.33)	15.79 (0.30)	9.51 (0.03)	0.85 (0.20)	1.74 (0.41)	1.00 (0.01)	0.68 (0.05)	n.d.	n.d.	1.50 (0.04)	58.51 (2.20)
Venetian Lagoon	171.43 (10.70)	18.65 (0.01)	378.10 (7.78)	275.86 (17.35)	23.88 (0.33)	31.76 (0.32)	22.38 (0.37)	10.18 (0.05)	17.91 (0.03)	0.55 (0.37)	28.07 (3.37)	978.79 (2.19)

PAH concentrations were highest in the Venetian Lagoon and the Po Delta, where mean total PAH concentrations were 979.8±2.2 and 625.1±62.3 ng g-1 d.w., respectively, whereas off Chioggia they were 58.5±2 ng g-1 d.w. The Venetian Lagoon is a highly urbanized and industrialized environmental transition area, where the anthropogenic sources (e.g. urban activities, boat traffic, agriculture, and tourism) can induce PAH accumulation. Similar PAH concentrations have been measured close to the Venetian Lagoonby Frignani et al.(2003), Acquavita et al. (2014), Parolini et al. (2010), and Secco et al. (2005), who reported concentrations ranging respectively from 71 to 730, from 66 to 734, from 15 to 389, and from 20 to 502 ng g-1 d.w. High PAH concentrations have also been described in the Po Delta sediments, maybe due to large amounts of PAH-containing suspended particles brought to this area by the River Po and its effluents (Webster et al., 2011; Sun et al., 2016). The PAH levels measured in our study were comparable to those found by Notar et al. (2001; 30-600 ng g-1d.w.) and Magi et al. (2002; 313-455 ng g-1d.w.). The lowest concentrations were found off Chioggia (57.0 ng g-1, site 56), possibly due to the distance of the sampling sites from the Italian coast (40 km) and, therefore,

from the main PAH inputs from the coast. In this area, Magi et al. (2002) have found values similar to ours (24-183 ng g-1d.w.).
Analysis of PAH sediment concentrations based on molecular weight showed that they were dominated by 4 ring PAHs (MMW-PAHs), which accounted for 72.5%, 47.7%, and 64.7% in the Venetian Lagoon, off Chioggia and the Po Delta, respectively. Similar data have been described in this region (Notar et al., 2001; Alebic-Juretic, 2011; Acquavita et al., 2014). Fluoranthene was the dominant congener in sediments from the Venetian Lagoon and the Po Delta, accounting for 38.6% and 37.5% of total PAHs, respectively. Naphthalene was the first-ranking congener (43.1%) off Chioggia. Therefore, the contribution of LMW-PAHs off Chioggia (46.9% of total PAHs) was higher than the one recorded in the two transition areas (19.4% in the Venetian Lagoon and 26.1% in the Po Delta). As regards the combustion-related PAHs (Pyr, BaA, Chr, BbF, BkF, BaP, InP, and BgP), they accounted for 80.6%, 53.1%, and 73.9% of total PAHs in the Venetian Lagoon, off Chioggia, and in the Po Delta, respectively.

To explore the mechanism of PAH bioaccumulation in fish tissue, a correlation between the log- transformed concentration of individual PAHs and the respective log Kow values was performed for each type of tissue. A negative relationship was found between Log Kow and log PAH residual in the different tissues of S. solea. PAH concentrations in fish gills correlated significantly with their Kow values ($r = 0.69$-0.98, $p < 0.05$) in the common sole individuals collected from the Venetian Lagoon, the Po Delta and off Chioggia. Although the same trend was seen in muscle and liver, it was not significant ($r = 0.49$-0.64 for muscle and $r = 0.43$-0.65 for liver, $p > 0.05$). This finding suggests that a quota of PAHs accumulating in fish gill depends on PAH physicochemical features, such as equilibrium partition. Moreover, direct exchange with the water and sediment through the gills act as a major bioaccumulation mechanism in common sole (Bandowe et al.,2014; Mashroofeh et al., 2015) The matrix with higher PAH bioaccumulation was gill tissue, confirming earlier reports that the gills are the primary tissue for pollutant accumulation through gill-water and sediment transfer (Yang et al., 2007; Bandowe et al., 2014; Zhao et al., 2014).

As mentioned above, the S. solea individuals collected off Chioggia were longer and heavier than those caught in the other two sampling areas. As expected, length and weight always correlated positively and significantly in all areas ($r = 0.93$, $p < 0.05$). A moderately strong, negative correlation between body size and PAH concentrations was seen in liver (the correlation coefficient ranged from -0.60 to -0.72) and gill (range, from -0.54 to -0.80) in the individuals collected from the Po Delta and off Chioggia ($p < 0.05$). As regards muscle, there was a relatively weak relationship between body size and PAH concentrations, whereas there was a negative but not significant relationship between body size and PAH concentrations in the three tissue types from sole caught in the Venetian Lagoon. PAH tissue concentrations decreased with the increase in body size, in line with the data reported by León et al. (2014) on red mullet from the Mediterranean coast of Spain and with Pellini et al. (2018), who highlighted that the abundance of microplastic particles, which may adsorb persistent hydrophobic compounds such as PAHs, decreased with increasing sole body size in the Adriatic Sea. In fact, a reduced induction of xenobiotic biotransformation processes with age has been documented in fish (Whyte et al., 2000; Coulillard et al., 2004), as has a relatively low resistance of PAHs to biotransformation and a high depuration rates by adult organisms (Bodiguel et al., 2009).

Total lipid content was determined in muscle, liver and gills of some S. solea individuals ($n = 6$ per tissue). The lowest values of the lipid percentage range were found in muscle (1.9-2.1), followed by gill (8.7-10.6) and liver (14.6-15.8), similar to Xu et al. (2011) and to Sagrantini et al. (2008) and Ameur et al. (2013) in common sole from the Adriatic Sea and the Mediterranean Sea. Since PAHs are lipophilic, they preferentially accumulate in tissues with higher lipid content, such as liver (Zhao et al., 2014; Bodiguel et al., 2009; Xu et al., 2011). However, box and whisker plots demonstrated a different PAH bioaccumulation in the three tissues and a significantly higher PAH accumulation in gills, followed by liver and muscle (Fig. 2); this trend is consistent with earlier reports (Vives et al., 2004; Liang et al., 2007). Further, statistical analysis of total PAH concentrations in muscle, liver and gills from the same site (3 tissues, one site) showed that differences between muscle and liver concentrations were not significant ($p > 0.05$), whereas both values were significantly different from those found in gills ($p < 0.05$). These findings do not support the hypothesis that higher fat content in the fish tissue should result in higher PAH content and they indicate that lipid content might not be the only determinant of PAH bioaccumulation in fish tissue (Sagratini et al., 2008; León et al., 2014; Zhao et al., 2014; Sun et al., 2016). Liver, which contains a higher lipid content, has not reported a greater PAH content. In fact, the liver is the main detoxifying tissue and contains relatively high levels of detoxifying enzymes, e.g. cytochromes P450, (León et al., 2013; Nagaraju et al., 2014; Sun et al., 2016). So, in the liver, PAHs can be biotransformed by detoxification enzymes to more hydrophilic products which is more easily excreted by organisms (Porte et al., 2002; van der Oost et al., 2003; Sette et al., 2013). In our study, the relatively lower PAH concentrations found in muscle at all sites demonstrate that muscle is not a target organ for PAH accumulation (Zhao et al., 2014).

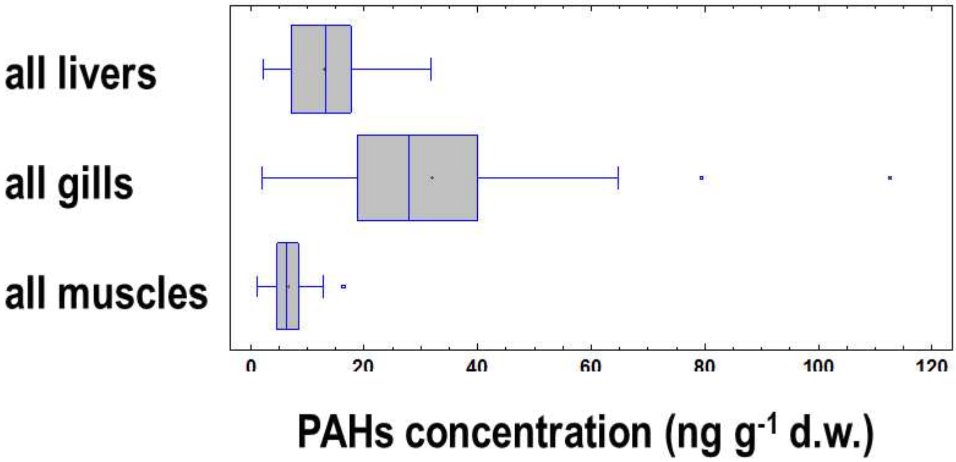

Fig. 2. Box and whisker plots of the total PAH concentrations (ng g-1 d.w.) in tissue from fish caught in the Po Delta, off Chioggia and in the Venetian Lagoon.

With regard to the mean PAH concentration measured in each tissue type at each site (one tissue, 3 sites), the box and whisker plots highlighted a statistically significant difference between the Venetian Lagoon and the site off Chioggia for liver and gills ($p < 0.05$), whereas analysis of the data from muscle showed statistically significant differences between the Po Delta and the site off Chioggia ($p < 0.05$). Notably, the PAH values found in all three tissue types from the latter site were the lowest. In terms of PAH sediment concentrations, Po Delta and, especially, Venetian Lagoon areas were highly contaminated compared to that Chioggia, on the contrary, PAHs in fish tissues are only slightly elevated in these areas. This finding can be explained by the distance from the more impacted coast, since it's closely related to the PAH concentrations in the sediment (Vives et al., 2004; León et al., 2014). On the contrary, more factors exert a major influence on PAH bioaccumulation in fish tissues. Therefore, PAHs are easily biodegradable compounds and their tissue levels do not reflect levels in the surrounding environment.

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PAPER 2

Effects of biological factors and seasonality on the level of polycyclic aromatic hydrocarbons in red mullet (*Mullus barbatus*)

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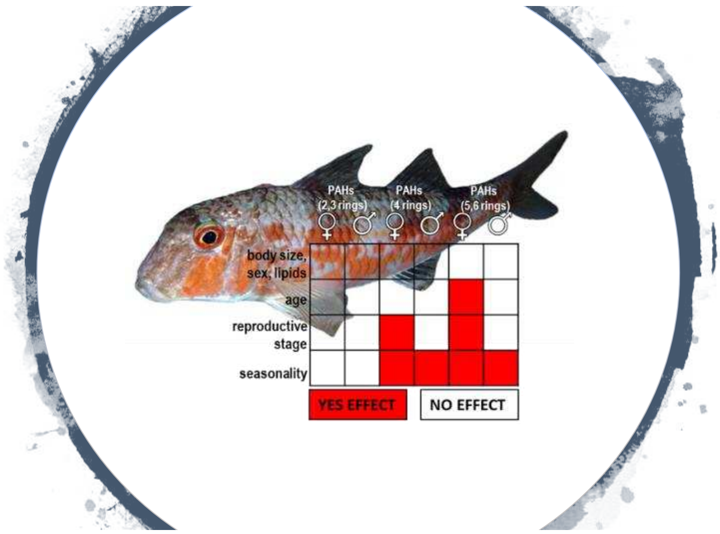

Effects of biological factors and seasonality on the level of polycyclic aromatic hydrocarbons in red mullet (*Mullus barbatus*)

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Abstract

This study evaluates the effects of biological factors of fish and seasonality on Polycyclic Aromatic Hydrocarbon (PAH) accumulation in red mullet (*Mullus barbatus*) tissue. Specimens were collected monthly with a bottom trawl net in an offshore fishing ground in the Northern and Central Adriatic Sea (Geographical Sub Area 17) throughout 2016. The edible fillets of 380 individuals were analyzed for the concentrations of individual PAH, total PAH, and low, medium and high molecular weight (MW) PAHs. PAH bioaccumulation was related to their physicochemical characteristics (MW, and logarithm of the octanol-water partition coefficient, log K_{ow}), some biological parameters of fish (body size, age, sex, reproductive stage and total lipid content), and catch season.

The PAH bioaccumulation pattern and the effects of the different factors varied according to PAH MW. The heavier (medium and high MW) PAHs showed higher levels in winter-autumn and in pre-spawners compared with spawners and post-spawners. Our findings suggest that an important detoxification mechanism, albeit limited to the heavier PAHs, acts in the spawning and post-spawning stage. Low MW PAHs appeared to be unaffected by reproductive stage, lipid content and seasonality. Reproductive stage and seasonality seem to play an important role in the accumulation of heavier PAH, whereas total lipid content and age seem to exert a limited influence, and body size no effect at all.

Keywords

PAH bioaccumulation; red mullet (*M. barbatus*); molecular weight; reproductive stage; seasonal factor.

1. Introduction

In the Mediterranean Sea, chemical contamination with Polycyclic Aromatic Hydrocarbons (PAHs) – a class of organic compounds consisting of two or more fused benzene rings – has become a cause for grave concern. The main PAH sources are the products of incomplete combustion or pyrolysis of organic materials, although some natural events such as diagenesis and forest fires are additional important sources (Neff et al., 2005; Dudhagara et al., 2016; Thompson et al., 2017). The largest PAH emissions are into the atmosphere, however PAHs reach the aquatic environments through direct atmospheric deposition, via contaminated soil and by rain washout (Stogiannidis and Laane, 2015).

PAHs are persistent contaminants. This is due to their physicochemical properties: low water solubility, high octanol-water partition coefficients (K_{ow}), and strong lipophilicity (Louvado et al., 2015; Ferrante et al., 2018). Their toxic effects are related to their biotransformation into toxic and potentially reactive metabolites that can bind covalently to cellular macromolecules such as proteins, RNA and DNA and which can induce cell damage, mutagenesis, teratogenesis, and carcinogenesis (Reynaud and Deschaux, 2006; Cocci et al., 2019). Currently, 16 PAHs are regulated in EU legislation and are included in the U.S. Environmental Protection Agency (EPA)'s priority pollutant list (Vives et al., 2004; Neff et al., 2005; Esposito et al., 2017; Ferrante et al., 2018).

Their lipophilic nature facilitates PAH accumulation in aquatic organisms and fish tissues, which pass them up the food chain (Van der Oost et al., 2003; Wetzel and Van Vleet, 2004; Balcıoğlu, 2016; Frapiccini et al 2018). In particular, fish exposed to polluted ecosystems can absorb contaminants directly from the water through the skin and gills or through ingested food. Although most PAHs are metabolized a short time after uptake (Moraleda-Cibrián et al., 2015; Barhoumi et al., 2016; Kammann et al., 2017), a fraction accumulates in lipid-containing tissues such as liver, eggs or muscle (Murawski et al., 2014; Romero et al., 2018). The increase in PAH bioaccumulation in edible organisms and the attendant risk of long-term effects on human health are a cause of special concern (Bandowe et al., 2014; Miniero et al., 2014; Zhao et al., 2014).

Several studies have assessed PAH contamination sources and concentrations in aquatic environments (water, sediments) and marine organisms (Sette et al., 2013; Barhoumi et al., 2016; Cocci et al., 2018, 2019; Frapiccini et al., 2018; Ferrante et al., 2018); however, there is a notable lack of studies investigating the factors that determine PAH accumulation and availability and the possible effects of PAH exposure on aquatic organisms, particularly edible fish. Notably, alteration of the chemical form of contaminants by biological (biotransformation) or physical (photo-oxidation) processes or by environmental factors has the potential to modify their bioavailability through changes in solubility or reactivity (Oris and Giesy, 1985). Moreover, the mechanisms of absorption, accumulation, transformation and elimination of PAHs, and of other persistent organic pollutants, are complex and often species-specific and are affected by chemical, biological and environmental factors (Vives et al., 2004; Romero et al., 2018). The interactions among these numerous variables greatly hamper data interpretation (Rojo-Nieto and Perales, 2015; Run-Xia et al., 2016; Loughery et al., 2018).

The Adriatic Sea is a relatively enclosed basin located in the central Mediterranean, between the Italian peninsula and the Balkans. The northern and central Adriatic Sea is characterized by shallow, highly productive coastal waters that support rich fishing grounds for several species (Russo and Artegiani, 1996; Artegiani et al., 1997 a,b); however, its relatively enclosed nature and the several rivers, particularly the Po, that discharge natural and anthropogenic debris (Marini et al., 2002, 2008; Cozzi and Giani, 2011) make it extremely susceptible to pollution (Magi et al., 2002; Marini and Frapiccini, 2013, 2014; Fossi et al., 2018). In recent years, the increasing density of the population living in coastal areas, tourism, and shipping traffic have significantly increased PAH concentrations and are threatening the ecosystem (Mandić et al., 2018).

Moving from these considerations, this study was designed to evaluate how PAH physicochemical properties and concentrations in fish tissue interact with seasonality and some biological parameters. The study involved the analysis of the edible tissue of red mullet, *Mullus barbatus*, caught in a rich fishing area in the Adriatic Sea. Red mullet (*M. barbatus* L., 1758) is a demersal fish and a major target species in the Adriatic, accounting for about 13% of the landings of all demersal species excluding bivalves and gastropods (but including crustaceans and cephalopods) and for 3.5% of the landings of all species (EU Data Collection Framework, 2017). It is widely distributed in areas characterized by sandy and muddy bottoms along the coasts of the Mediterranean Sea, the Black Sea, Northwest Africa and Northern Europe (Gündoğdu and Baylan, 2016; Durmuş et al., 2018; FAO 2018). It is an abundant species much appreciated by consumers that in 2016 was worth €10.6 million (FAO 2018). Red mullet is caught throughout the year but autumn is the period of maximum recruitment; its reproductive season extends from May to July-August (Carbonara et al., 2015). *M. barbatus* is considered as a sentinel species in the Mediterranean. As a demersal species living close to the bottom and feeding mainly on benthic organisms (crustaceans, worms, mollusks) (FAO 2018), it accurately reflects contaminant bioavailability and concentration in the marine environment and has extensively been used as a bioindicator for pollution monitoring (Corsi et al., 2002; Porte et al., 2002; Della Torre et al., 2010; Giani et al., 2019).

The present study was performed: 1) to explore PAH accumulation in *M. barbatus* fillets in relation to PAH physicochemical characteristics such as molecular weight (MW) and the logarithm of the octanol-water partition coefficient (log K_{ow}); 2) to determine the relationships between PAH concentrations in fillets and some biological characteristics of fish, i.e. body size, age, sex, reproductive stage and lipid content in muscle; and 3) to evaluate the seasonal variation of PAH bioaccumulation in fillets.

2. Material and methods

2.1 Fish sample collection

Red mullet individuals were caught monthly on the Italian side of Food and Agriculture Organization (FAO) General Fisheries Commission for the Mediterranean (GFCM) Geographical Sub Area 17 (GSA 17), a rich offshore fishing ground spanning the Northern and Central Adriatic (Fig. 1), from January 2016 to December 2016 using a bottom trawl net.

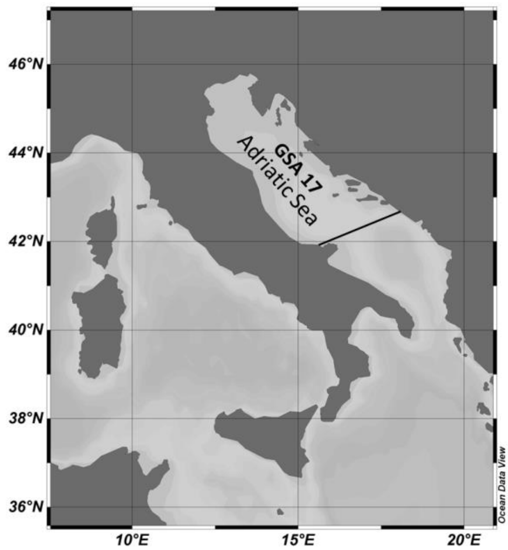

Fig. 1. The fish sampling area spanned the Northern and Central Adriatic Sea, FAO GFCM Geographical Sub Area 17 (GSA 17).

In the laboratory, the body length of each individual was measured to the nearest 0.5 cm, its total weight was recorded to 0.1 g and its sex was determined. The gonadal development stage (maturity) was established macroscopically to assign individuals to one of four categories: Juveniles (J), sexually immature; Pre-spawning (A), adults whose gametes were just beginning to develop; Spawning (S), individuals whose gametes were ready for the spawning season; and Post-spawning (PS), individuals at the end of their reproductive cycle, sexually mature but inactive (Follesa and Carbonara, 2019).

Age was estimated by growth increment counts in the sagittae otoliths according to the method and criteria reported in ICES (2017) and other studies (Mahé et al., 2016; Carbonara and Follesa, 2019). To evaluate PAH concentrations, fillets weighing about 5 g were collected using solvent-cleaned scalpels and scissors, wrapped in aluminum foil, and stored in a freezer at -18 °C.

Fillets from 380 individuals were analyzed for the physicochemical characteristics of PAH (MW and log K_{ow}), lipid content, biological parameters (body size, age, sex, and reproductive stage) and catch season. The distribution of individuals according to these parameters is shown in Table S1.

2.2 PAH extraction and analysis

The concentrations of the 16 environmentally relevant PAHs – naphthalene, Nap; acenaphthene, Ace; fluorene, Fl; phenanthrene, Phe; anthracene, Ant; fluoranthene, Flu; pyrene Pyr; benzo(a)anthracene, BaA; chrysene, Chr; benzo(b)fluoranthene, BbF; benzo(k)fluoranthene, BkF; benzo(a)pyrene, BaP; dibenzo(a,h)anthracene, DBahA; indeno(1,2,3-cd)pyrene, and InP; benzo(g,h,i)perylene, BghiP – in fillets were determined by QuEChERS and HPLC-FD analysis (Ramalhosa et al., 2012; Nácher-Mestre et al., 2014; Frapiccini et al., 2018).

Briefly, 5 g of fillet was homogenized and extracted with the QuEChERS kit using acetonitrile as the reagent. The purified extracts were concentrated under a gentle nitrogen flow and recovered with 0.4 µL acetonitrile for chemical analysis. Samples were individually injected into a UHPLC system (Utimate 3000, Thermo Scientific, Waltham, MA, USA) coupled to a fluorescence detector (RF-2000, Thermo Scientific). Ace was not measured because it is not fluorescent. PAH extraction and quantification have been described in detail in a previous study (Frapiccini et al., 2018). A calibration solution for the standard PAH solution (EPA 610 Mix) was prepared using serial dilutions (1:100-1:800 v/v) with correlation coefficients > 0.997

for all analytes. Analyte peaks were identified in samples by comparing retention times with a standard chromatogram obtained in the same conditions.

The limit of detection (LOD) and the limit of quantification (LOQ) of each PAH were calculated according to ICH Q2B (ICH, 2005; Truzzi et al., 2014). The LOD of the 15 PAHs analyzed was in the range of 0.002-0.070 $ng \cdot g^{-1}$ wet weight (w.w.) whereas their LOQ was in the range of 0.01-0.30 $ng \cdot g^{-1}$ w.w. (Table S2). Method efficiency and accuracy were tested using reference products (NIST SRM 1974c [organics in fillet tissue]). Recoveries in the reference material ranged from 70% (Pyr) to 92% (Ace+Fl), as reported in Table S2, where the plus sign indicates co-eluting PAH peaks. The results were not corrected for recovery.

PAHs were grouped into three MW categories (Moraleda-Cibrián et al., 2015; Barhoumi et al., 2016): low MW (LMW) PAHs included 2-3 fused benzene rings (Nap, Ace+Fl, Phe, Ant); medium MW (MMW) PAHs included 4 rings (Flu, Pyr, BaA, Chr); and high MW (HMW) PAHs included 5-6 rings (BbF+BkF, BaP, DBahA, BghiP+InP).

2.3 Determination of lipid content

Total lipid content was estimated by pooling fillets from individuals of the same reproductive stage according to a protocol adopted in previous works (Truzzi et al., 2017, 2018; Frapiccini et al., 2018). After microwave-assisted lipid extraction using a MARS 5 apparatus (CEM Corporation, Matthews, NC, USA), total lipids were quantified by the gravimetric method.

2.4 Statistical analysis

All statistical analyses and diagrams were performed with the free software R for Windows v. 3.5.1 (R Core Team, 2018). Statistical analysis was mainly used to evaluate the effect of the different factors on PAH concentrations. When the level of a factor was 2, as in the case of sex (male/female), data were analyzed by the t-test, whereas, due to heteroscedasticy, the concentration data were log-transformed to obtain equal variances. When the level of a factor was > 2, as in the case of seasonality, reproductive stage and age, we applied analysis of variance (ANOVA) to the log-transformed data after running another test of the constant variance assumption according to Levene's test (Lomex et al., 2012). In case of data homoscedasticy we ran the usual parametric ANOVA; if the p value was significant, a post-hoc Tukey comparison was performed. In case of data heteroscedasticy, we applied non-parametric ANOVA and, if the result was significant, Games-Howell post-hoc comparisons (Maxwell et al., 2018) as offered by the R package "userfriendlyscience" (v. 0.7.2). The correlation between PAH concentrations and lipid content was tested with Pearson's correlation coefficient (McPherson G., 1990). A reference p value of 0.05 was considered for significance.

3. Results and Discussion

3.1 *PAH concentration and distribution pattern in red mullet fillets*

The concentrations of each PAH, of total PAHs (ΣPAHs), and of total LMW, MMW and HMW PAHs (ΣLMW-PAHs, ΣMMW-PAHs, and ΣHMW-PAHs respectively) are listed in Table 1.

Table 1. Concentrations (ng g^{-1}, w.w.) of individual PAHs, ΣPAHs and ΣLMW-PAHs, ΣMMW-PAHs and ΣHMW-PAHs determined in *M. barbatus* fillets from the Adriatic Sea. Mean value ± standard deviation, range (min, max) and frequency of detection (%).

PAHs	Mean ± standard deviation	Range (min, max)	Frequency of detection (%)
Nap	80 ± 69	[< LOQ, 294]	99.21
Ace+Fl	12.4 ± 7.9	[< LOQ, 43.5]	98.42
Phe	6.5 ± 3.4	[< LOQ, 15.72]	98.95
Ant	0.24 ± 0.14	[< LOQ, 0.80]	70.26
Flu	0.69 ± 0.32	[< LOQ, 1.89]	78.68
Pyr	1.2 ± 0.3	[< LOQ, 2.44]	99.21
BaA	0.07 ± 0.04	[< LOQ, 0.17]	26.84
Chr	0.07 ± 0.04	[< LOQ, 0.17]	23.42
BbF+BkF	0.04 ± 0.02	[< LOQ, 0.09]	8.42

BaP	0.02 ± 0.01	[< LOQ, 0.04]	7.11
DBahA	0.22 ± 0.08	[< LOQ, 0.37]	9.47
BghiP +InP	0.02 ± 0.02	[< LOQ, 0.08]	3.68
ΣPAHs	101 ± 72	[13, 331]*	n.e.
ΣLMW-PAHs	99 ± 72	[12, 329]*	n.e.
ΣMMW-PAHs	1.8 ± 0.6	[0.3, 4.0]*	n.e.
ΣHMW-PAHs	0.13 ± 0.12	[0.01, 0.37]*	n.e.

n.e., not evaluated

* only values > LOQ

ΣPAH concentrations ranged from 13 to 331 ng·g^{-1} w.w., which according to National Oceanic and Atmospheric Administration categories indicates that the fillets were minimally (10 to 99 ng·g^{-1}) to moderately polluted (100 to 999 ng·g^{-1}) (Soares-Gomes et al., 2010; Ranjbar Jafarabadi et al., 2019). Of the 15 PAHs analyzed, Nap was the predominant congener, accounting alone for 74% of ΣPAHs followed by Phe and Ace+Fl. Nap is considered by the International Agency for Research on Cancer as possibly carcinogenic to humans (Group 2B; IARC, 2010). Four PAHs, BaP, BaA, BbF, and Chr, have recently been selected as indicators of the presence of carcinogenic and genotoxic PAHs in foodstuff (European Commission, 2011). Notably, in our red mullet fillets ΣPAH concentrations were always under the maximum permissible level.

LMW-PAHs were the predominant PAHs, followed by MMW-PAHs and HMW-PAHs. Their distribution pattern and concentrations are in line with previous studies of *M. barbatus* in the Mediterranean Sea (Perugini et al. 2007; Della Torre et al., 2010; León et al., 2014; Guerranti et al., 2016).

The PAH distribution pattern correlated with their physicochemical features. Log-transformed PAH content showed negative relationships with MW and log K_{ow} (Fig. 2). For the MW regression line, the slope estimate was -0.046 (standard error = 0.008, p value < 0.001; Fig. 2a) and for the log K_{ow} regression line the slope estimate was -2.117 (standard error = 0.383, p value < 0.001; Fig. 2b).

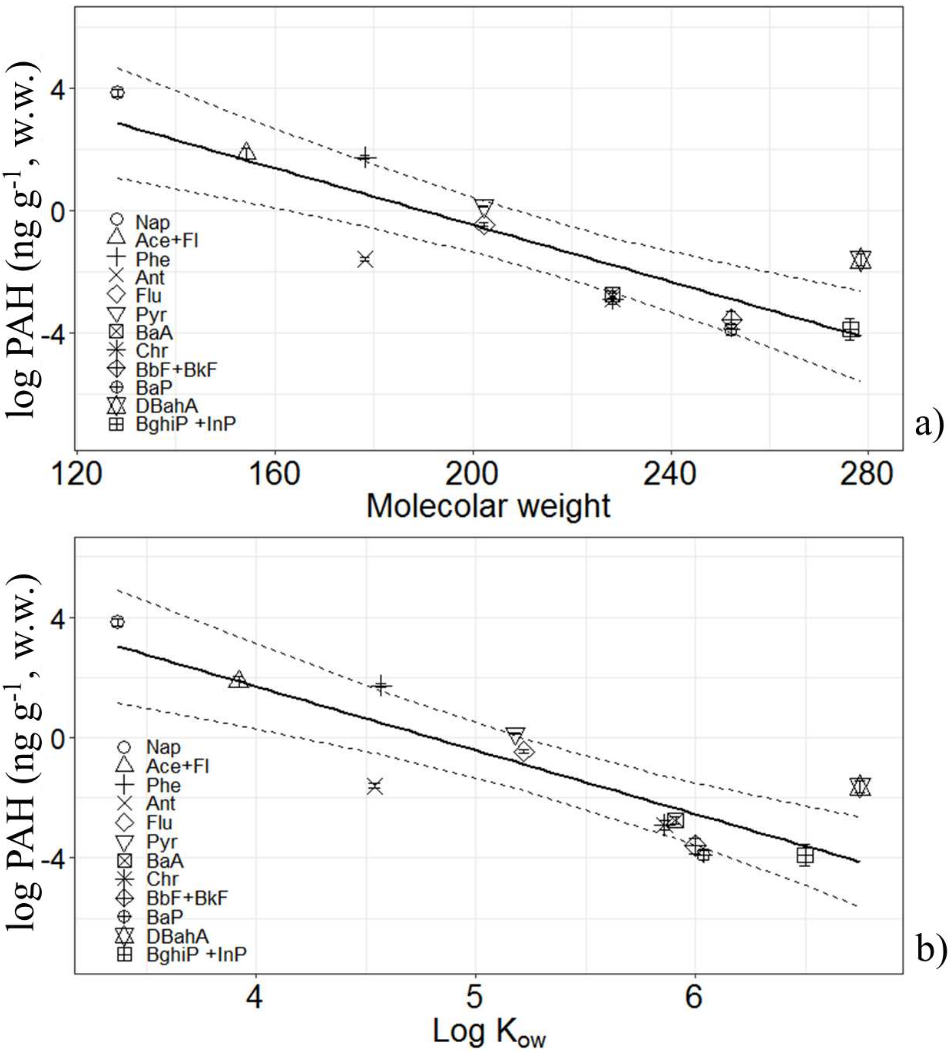

Fig. 2. Log-concentrations (ng g^{-1}, w.w.) as a function of PAH physicochemical features: molecular weight (a) and log K_{ow} (b). The small error bars at symbols are ±1 standard deviation. Solid lines represent regression lines, dotted lines are 95% confidence bands.

The log K_{ow} value reflects the balance between the lipophilicity and hydrophilicity of any compound and is used to predict their ability to migrate, undergo transformation, and diffuse in the environment. PAHs with a low log K_{ow} value (< 5.0), such as LMW-

PAHs, are characterized by a greater water solubility compared with the heavier PAHs, which promotes bioavailability and assimilation; as a consequence, these pollutants are more abundant in muscle tissue than the heavier PAHs (Zhao et al., 2014; Santana et al. 2018; Sun et al., 2018). In contrast, PAHs with a higher log K_{ow} value (> 5.0), such as MMW-PAHs and HMW-PAHs, are closely associated with suspended particulate matter and tend to be stored in marine sediment. However, processes that are quite common in aquatic environments, like transport, sediment resuspension and biogeochemical dynamics can increase their bioavailability and absorption by marine organisms (Perugini et al., 2007; Mashroofeh et al., 2015; Stogiannidis and Laane, 2015; Dong et al., 2016; Parinos and Gogou, 2016).

Our findings highlighted that PAH concentrations in red mullet fillets are strongly dependent on PAH physicochemical characteristics, as reported in other species (León et al., 2014; Moraleda-Cibrián et al., 2015; Run-Xia et al., 2016). The lower HMW-PAHs than LMW-PAHs concentrations found in our fillets can be ascribed to different metabolic transformation pathways (Goksoyr and Förlin, 1992) besides the more limited bioavailability of HMW-PAHs. Notably, fish are capable of metabolizing and eliminating these compounds by a variety of pathways including the cytochrome P450 (CYP) enzyme system. Accordingly, MMW-PAHs and HMW-PAHs are more prone to undergo biotransformation and metabolization than LMW-PAHs (Liang et al., 2007; Wang et al., 2018; Cocci et al., 2018, 2019).

3.2 Biological factors

PAH concentrations in *M. barbatus* muscle were also investigated in relation to some biological factors of the fish like body size (length and weight), age, sex, reproductive stage and total lipid content.

The mean total body length and total mass of our red mullet specimens ranged from 9.0 to 20.0 cm (13.8 ± 2.5 cm) and from 6.0 to 101.6 g (30.7 ± 16.8 g), respectively. There were no significant differences in the accumulation of the three MW groups in relation to body size; in addition, HMWs were never detected in fillets from the larger (i.e. longer and heavier) individuals (Fig. S1). This can probably be attributed to the detoxifying action of the CYP enzyme system. Indeed, the higher levels of ethoxyresorufin-O-deethylase (EROD) – a biomarker of PAH biotransformation – seen in adult fish compared with juveniles as a result of maturity-dependent EROD activity suggest that the biotransformation capacity via the CYP system is more efficient in larger individuals (Barhoumi et al., 2016; Cocci et al., 2018, 2019; Ranjbar Jafaraqbadi et al., 2019).

Age ranged from 0 (11%) to 1 (41%), 2 (42%) and 3 years (5%). Specimens whose age was unclear (n.c.) were not included in this analysis (Table S1). Age did not correlate with MW PAH concentrations except for HMW-PAHs in females after the post-hoc comparison of 2-0 groups. In females PAH concentrations appeared to increase with age (Fig. 3).

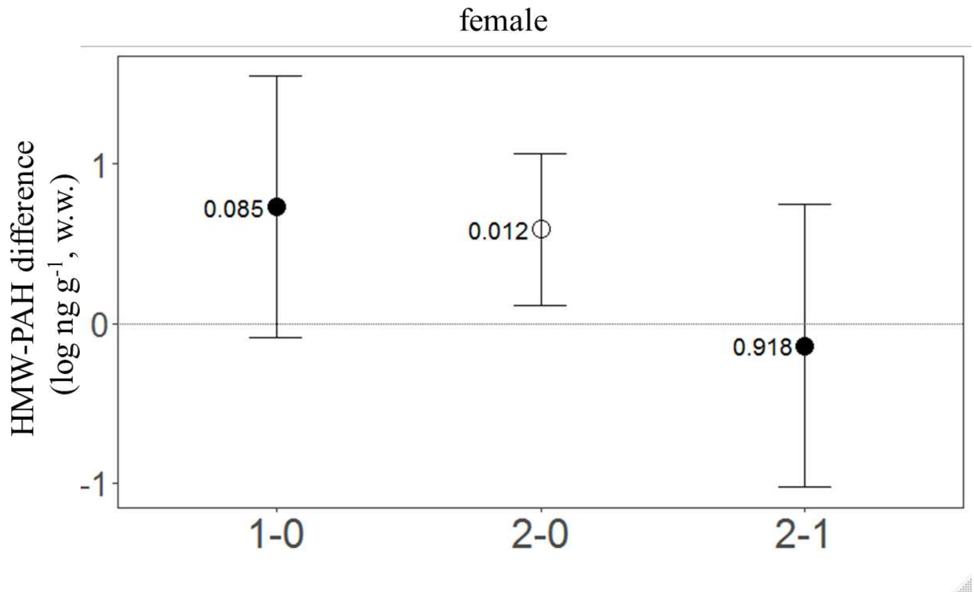

Fig. 3. Post-hoc comparisons of HMW-PAH concentrations (log PAH ng g^{-1}, w.w.) as a function of age in females. Symbols represent the difference between the respective group means reported on the x-axis and the error bars at the 95% confidence intervals. The horizontal line at y=0 was added for reference. The number near the symbol is the p value. Significant differences ($p < 0.05$) are indicated by empty symbols.

There was no sex effect either on ΣPAH concentrations or on any MW category, probably because males and females share the same diet, feeding behavior and living habitats (León et al., 2014).

Data analysis was also performed in relation to the reproduction stage. Red mullet is a batch spawner characterized by continuous gamete maturation and spawning in successive batches (Murua and Saborido-Rey 2003; Kokokiris et al. 2014, Ferrer-Maza et al., 2015). The spawning period in the Adriatic Sea consists of a single event that in females takes place from April to July with a peak in May and June, whereas mature males are present almost throughout the year (Carbonara et al., 2015). In both sexes the reproductive stages are associated with changes in metabolism and genomic and hormonal profiles (Burger et al., 2007) that can determine a different PAH accumulation in muscle. Accordingly, there was no evidence of an effect of reproductive stage on LMW-PAHs, either in the combined sex dataset or in males and females considered separately (data not shown). MMW-PAH concentrations were significantly higher during the pre-spawning period (A) than in the other reproductive stages (J, S, and PS) both in the combined sex dataset and in females (Fig. 4). A similar pattern was detected for HMW-PAHs, except that the differences were not consistently significant; in particular, HMW-PAHs were not detected in J female or PS male individuals (Fig. 4).

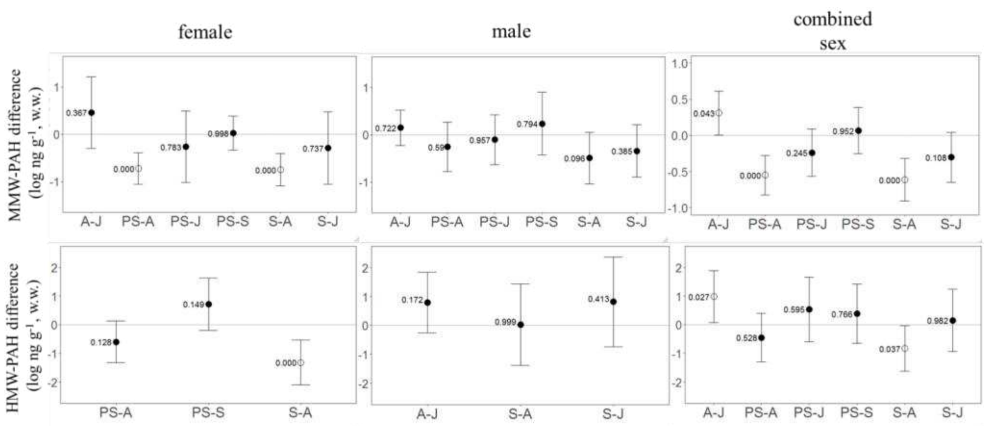

Fig. 4. Post-hoc comparisons of MMW-PAH and HMW-PAH concentration (log PAH ng g^{-1}, w.w.) as a function of reproductive stage (J = juvenile, A = pre-spawning, S = spawning, PS = post-spawning), in females, males, and the combined sex dataset. Symbols represent the difference between the respective group means reported on the x-axis and the error bars at the 95% confidence intervals. The horizontal line at y=0 was added for reference. The number near the symbol reports the corresponding p value. Significant differences ($p < 0.05$) are indicated by empty symbols. The post-hoc comparisons for LMW-PAHs are not reported because they were never significant.

The significantly higher MMW-PAH and HMW-PAH concentrations seen in A females and the lack of a reproduction effect on LMW-PAHs is probably due to the increased metabolic demand during the period that precedes reproduction, when females presumably allocate a greater amount of their energy to egg production and are thus more likely to absorb pollutants. The heavier PAHs declined in the spawning and post-spawning stages, which are characterized by gamete emission and loss of lipids, where PAHs are stored (Ruiz et al., 2011). These findings support the view that a critical detoxification process takes place in females in these two stages (Bodiguel et al., 2009; Xiu et al., 2015). The lack of an effect of reproductive stage on PAH concentrations in males, observed in our study, may be due to mature males are present almost throughout the year, in addition to the need for greater lipid stores for egg production in females (Lloret et al., 2007; Carbonara et al., 2015).

Lipids are critical energy stores, especially during reproductive and non-feeding periods and are the first components to be mobilized (Adams, 1999). Seasonal fluctuations seem to be mainly related to the reproduction cycle (Sargent, 1995), but other factors such as water temperature, food availability, geographical area, habitat quality and stress may also affect muscle lipid content (Lloret et al., 2007). For these reasons total lipid content was calculated in our red mullet fillets in the four reproductive stages. In the J, A, S and PS stages average total lipids were 3.3%, 5.1%, 1.6% and 0.5 % w.w. in females and 0.6%, 3.0%, 1.8%, and 0.4 % w.w. in males respectively (Table S3). Our findings are comparable to those reported in other studies of fillets of *M. barbatus* from the Eastern Mediterranean and the Black Sea

(Roncarati et al., 2012). Di Lena et al. (2016) have found a much higher lipid content in autumn (8.72%) than spring (1.48%) in *M. barbatus* from the central Adriatic Sea, whereas modest fluctuations through the year have been reported in red mullet from the North Aegean and the Eastern Mediterranean (Polat et al., 2009; Tulgar and Berik, 2012). At variance with the lower lipid content found in our fillets during the reproductive period (Table S3), Lloret et al. (2007) and Ferrer-Maza et al. (2015) have reported that *M. barbatus* from the Western Mediterranean feed actively during this period, they store lipids in the gonads and do not experience lipid depletion.

Lipid storage and content depend on reproductive stage and sex. The significant changes found in our fillets likely reflect lipid mobilization from muscle to the gonads during spring and through the reproductive period and their subsequent accumulation in autumn-winter. In both sexes lipids were highest before spawning, they declined during gamete release, when the available energy had to be balanced between the needs of body fitness and reproduction, and were lowest after the spawning period. The presence of sexually mature males throughout the year goes a long way towards explaining the differences in body size and lipid storage found between the sexes. The similar lipid content and concentration of the heavier PAHs (MMW-PAHs and HMW-PAHs) seen in our fillets in the different stages of the reproductive cycle suggest a close relationship between them. Nevertheless, a clear, positive correlation was found only for MMW-PAHs in females (Pearson = 0.908, $p = 0.0007$) and for HMW-PAHs in males (Pearson = 0.859, $p = 0.0285$). Thus, according to our data the lipid content in muscle exerts a limited effect on PAH concentration.

3.3 Seasonality

The effects of seasonality on PAH accumulation in fish muscle were investigated by dividing the year into four seasons according to Artegiani et al. (1997 a, b) as follows: winter (January – March), spring (April – June), summer (July – September), and autumn (October – December). Previous studies suggesting a possible relationship between PAH levels in fish tissue and the wet or dry season have rarely covered a whole year (Liang et al., 2007; Perugini et al., 2007; Ramalhosa et al., 2012;

Wunderlich et al., 2015). Our PAH concentration data show a seasonal trend in relation to their MW (Fig. 5).

LMW-PAH levels were highest in autumn and lowest in winter in both sexes, even though the difference was significant only for the combined sex dataset ($p = 0.038$; Fig. 5). As regards MMW-PAHs, the highest values were found in winter and the lowest in summer for both sexes, both in the combined and the separate dataset. These comparisons showed a clear effect of seasonality on PAH accumulation, especially in the combined dataset and in females, where the differences were significant except for autumn and spring. In contrast, males showed significantly higher concentrations only in winter (Fig. 5). Similarly, seasonal fluctuations with the highest concentrations in winter for both sexes were observed for HMW-PAHs although there was a single value for the combination “autumn/female” (NA; Fig. 5).

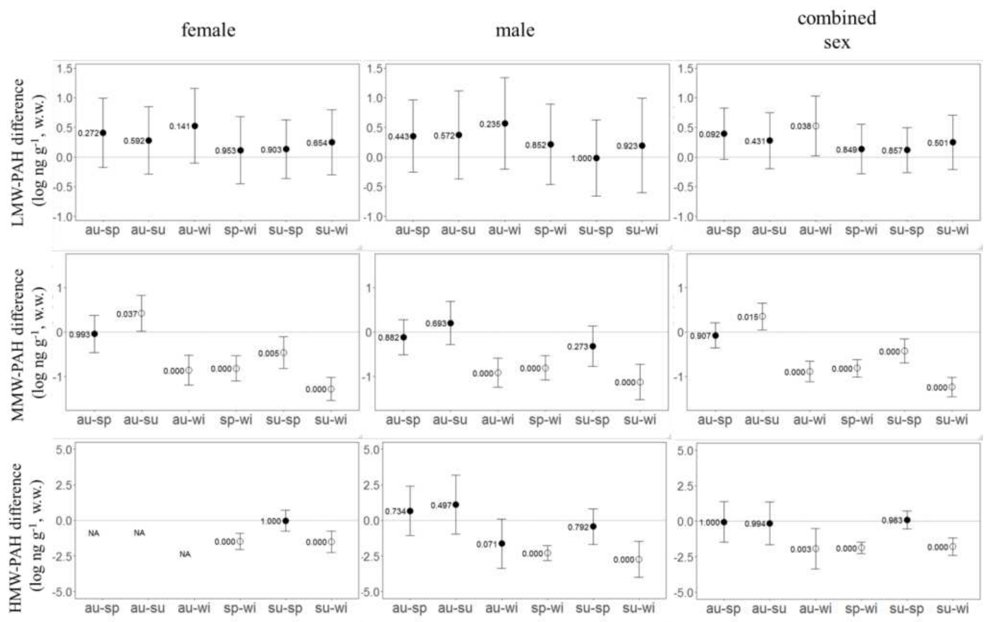

Fig. 5. Post-hoc comparisons of LMW-PAH, MMW-PAH and HMW-PAH concentration (log PAH ng g^{-1}, w.w.) as a function of seasonality (wi = winter, sp = spring, su = summer, au = autumn), in the combined sex dataset, females, and males. Symbols represent the difference between the respective group means reported on the x-axis and the error bars at the 95% confidence intervals. The horizontal

line at y=0 was added for reference. The number near the symbol is the corresponding p value. Significant differences ($p < 0.05$) are indicated by empty symbols.

LMW-PAH concentrations seemed to be unaffected by seasonality; in contrast, MMW-PAHs and HMW-PAHs showed significant changes, with the highest values in winter and the lowest in summer (Fig. S2). LMW-PAHs exhibited a gradual increase from winter to autumn, peaking in November and December. In contrast, MMW-PAHs and HMW-PAHs peaked in winter, dipped in spring – coinciding with reproduction – and plateaued in summer-autumn, after spawning. Previous studies have shown that in red mullet some enzymes involved in the PAH detoxification pathway, particularly the CYP system, were significantly underexpressed in spring compared with autumn (Mathieu et al., 1991; Gorbi et al., 2005). Other studies have described a marked reduction in the antioxidant defense system (glutathione S-transferase) in winter (Goksoyr and Förlin, 1992; Pavlović et al., 2010). Seasonal variability in PAH emission into the environment may also be implicated, since the high summer concentrations of LMW-PAHs may be related to increased ship (liner) traffic, while the reduction in HMW-PAHs could be due to lower pollution pressure (e.g. from domestic heating) (Bajt, O., 2014). In contrast, the increased presence of HMW-PAHs in winter is probably due to greater water hydrodynamics, where sediment resuspension promotes the mobilization of the heavier PAHs (Dong et al., 2016; Sun et al., 2018).

4. Conclusion

To our knowledge this is the first study evaluating the potential effect of some biological and seasonal factors on the accumulation of PAH compounds in edible fillets of *M. barbatus* sampled monthly throughout the year.

Differences in PAH accumulation depended mainly on their degree of lipophilicity and bioavailability. The main finding of the study is the lack of evidence of an effect of the reproductive stage, lipid content and seasonality on LMW-PAH concentrations. In contrast, reproductive stage and seasonality provided an important contribution to the accumulation of the heavier PAHs. MMW-PAH and HMW-PAH values peaked in the pre-spawning stage, when the lipid content was also highest; this was especially true of females. According to our data, the spawning and post-spawning period could provide important fish detoxification mechanisms albeit limited to the heavier PAHs. However, the lipid content was of limited importance. Body size and age did not exert a significant effect on the accumulation of any of the PAH categories, except in females which seemed to show an increase in all PAHs with age. Seasonality affected MMW-PAH and HMW-PAH but not LMW-PAH accumulation. The heavier PAHs peaked in winter and then declined until summer, a finding that could be related more to seasonal factors (such as PAH emission into the atmosphere and resuspension due to hydrodynamic processes) and to the expression of the CYP detoxification system rather than to reproductive stage.

We believe that these findings provide fresh insight into how PAHs interact with the red mullet life cycle and into the health implications of fish consumption.

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Appendix A. Supplementary data

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Supplementary Material

Effects of biological factors and seasonality on the level of polycyclic aromatic hydrocarbons in red mullet (*Mullus barbatus*)

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Table Captions

Table S1. The distribution of individuals (female and male) according to catch season, age and reproductive stage (J = juvenile, A = pre-spawning, S = spawning, PS = post-spawning).

Table S2. The limit of detection (LOD), the limit of quantification (LOQ) and recovery in reference material (NIST 1974c) of each PAH.

Table S3. Summary of water content (%) and total lipid content (% w. w.) of pooled fillet samples of the *M. barbatus* collected from Adriatic Sea (Geographical Sub Area 17, GSA 17), according to reproductive stage of female and male individuals (J = juvenile, A = pre-spawning, S = spawning, PS = post-spawning).

Figure Captions

Fig. S1. Concentrations of the three Molecular Weight PAH groups in relation to fish length, cm, (a) and fish weight, g, (b). The solid lines are regression lines.

Fig. S2. Temporal variation of concentrations in the three PAHs Molecular Weights groups (LMW-PAHs, MMW-PAHs and HMW-PAHs). The symbols represent mean values and the error bars are 95% confidence intervals.

Supplementary Tables

Table S1. The distribution of individuals (female and male) according to catch season, age and reproductive stage (J = juvenile, A = pre-spawning, S = spawning, PS = post-spawning).

female					male			
	catch season				catch season			
	spring	summer	autumn	winter	spring	summer	autumn	winter
	65	71	37	54	66	26	31	30
age	reproductive stage				reproductive stage			
	J	A	S	PS	J	A	S	PS
nc	0	10	6	8	0	2	1	1
0	5	1	3	0	28	3	0	0
1	8	18	8	21	24	45	8	14
2	0	49	35	39	0	18	6	2
3	0	5	7	4	0	1	0	0

n.c., age not classifiable

Table S2. The limit of detection (LOD), the limit of quantification (LOQ) and recovery in reference material (NIST SRM 1974c) of each PAH.

	Unit	Individual PAHs										
		Nap	Ace+Fl*	Phe	Ant	Flu	Pyr	BaA	Chr	BbF+BkF*	BaP	DBahA	InP+BghiP*
LOD	μg·g^{-1}	0.010	0.060	0.070	0.010	0.010	0.004	0.002	0.002	0.010	0.004	0.004	0.003
LOQ	μg·g^{-1}	0.300	0.180	0.220	0.270	0.040	0.010	0.010	0.010	0.020	0.010	0.010	0.010
recovery	%	81.70	91.55	73.88	80.82	92.51	69.69	75.97	73.84	75.20	89.48	90.00	70.15

* the plus sign indicates co-eluting PAH peaks.

Table S3. Summary of water content (%) and total lipid content (% w. w.) of pooled fillet samples of the *M. barbatus* collected from Adriatic Sea (Geographical Sub Area 17, GSA 17), according to reproductive stage of female and male individuals (J = juvenile, A = pre-spawning, S = spawning, PS = post-spawning).

water content (%)								
	J		A		S		PS	
	mean	sd	mean	sd	mean	sd	mean	sd
female	76.88	0.38	72.66	0.47	77.57	0.13	79.11	0.25
male	78.00	0.38	75.54	0.08	75.56	0.33	77.54	0.35

total lipid content (% w. w.)								
	J		A		S		PS	
	mean	sd	mean	sd	mean	sd	mean	sd
female	3.27	0.05	5.11	0.49	1.62	0.02	0.53	0.01
male	0.59	0.02	2.97	0.17	1.85	0.16	0.44	0.03

sd, standard deviation

Supplementary Figures

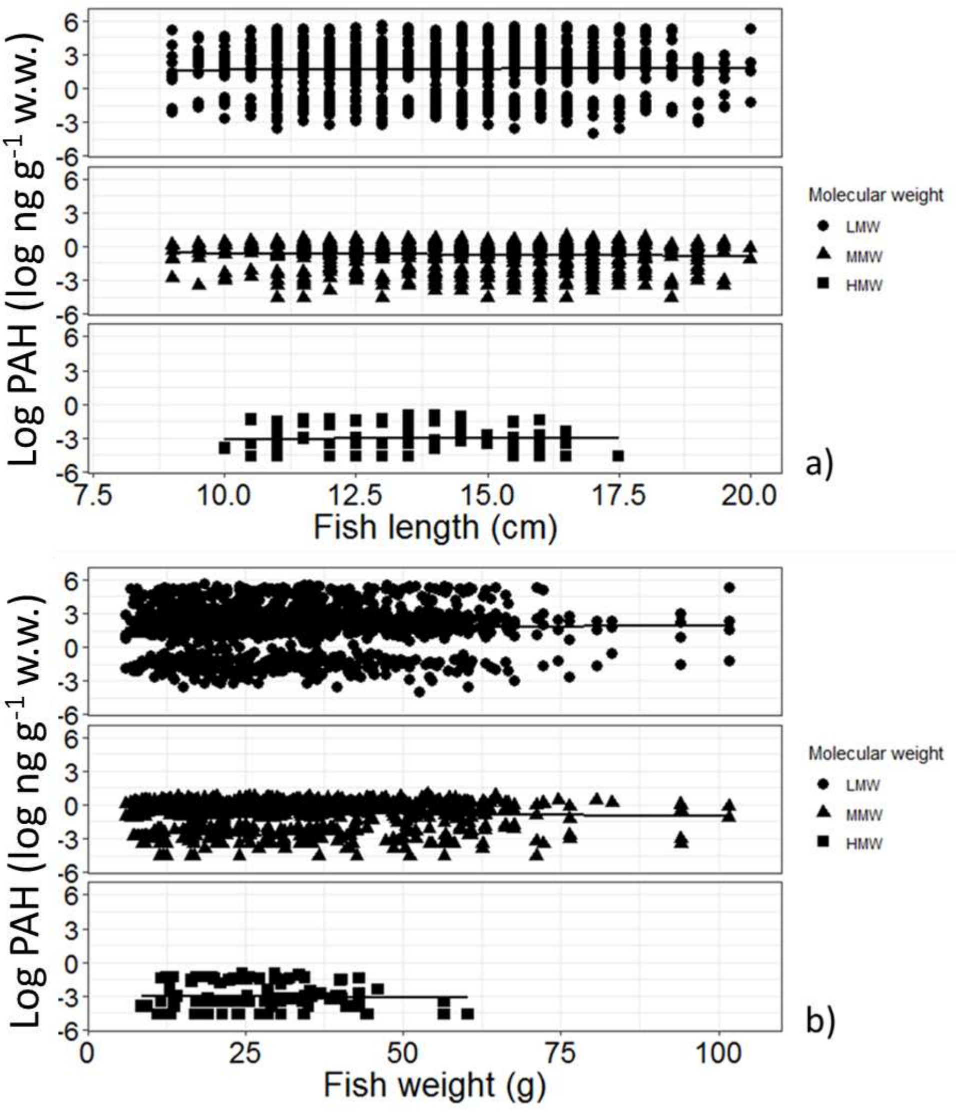

Fig. S1. Concentrations of the three Molecular Weight PAH groups in relation to fish length, cm, (a) and fish weight, g, (b). The solid lines are regression lines.

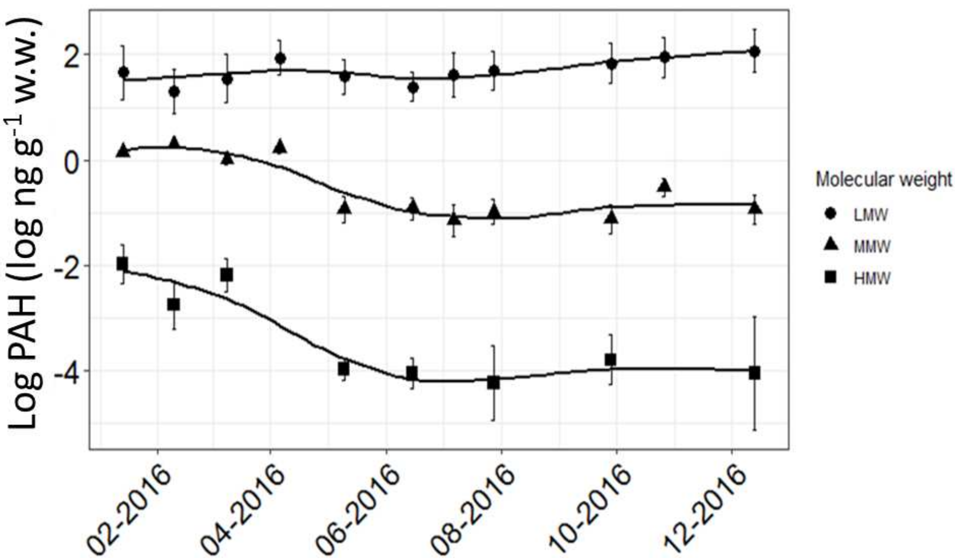

Fig. S2. Temporal variation of concentrations in the three PAHs Molecular Weights groups (LMW-PAHs, MMW-PAHs and HMW-PAHs). The symbols represent mean values and the error bars are 95% confidence intervals.

PAPER 3

Polycyclic aromatic hydrocarbons (PAHs) – induced oxidative stress in muscle tissue of non – spawning red mullet (*Mullus barbatus*) females from the Adriatic Sea

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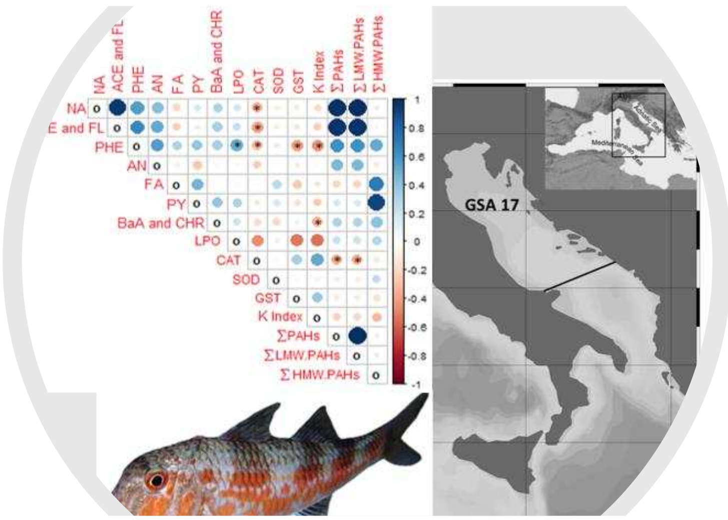

Polycyclic aromatic hydrocarbons (PAHs) - induced oxidative stress in muscle tissue of non-spawning red mullet (*Mullus barbatus*) females from the Adriatic Sea

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Abstract

In this study, we examined the association between PAH concentrations and mRNA expression profiles of some antioxidant genes, as well as lipid peroxidation (LPO), in muscle of wild red mullet (*Mullus barbatus*) collected from a rich offshore fishing area of the Northern Adriatic Sea. Muscle tissues of 36 non-spawning female fish were analyzed for PAH concentrations, applying the QuEChERS extraction coupled with UHPLC-FLD system. Detectable levels of PAHs were found in all samples examined. The average concentrations of total PAH (∑PAHs) were 203 ± 159 ng/g, w.w., with a predominance of low molecular weight (LMW) PAHs. The results showed that catalase (CAT) expression level was negatively associated with ∑PAHs, particularly ∑LMW-PAHs, and some PAH congeners. A negative correlation was also found between glutathione S-transferase (GST) mRNA expression level and phenanthrene (PHE) tissue concentration. Conversely, differences in LPO levels in

muscle were significantly positively related with PHE accumulation highlighting the oxidative damage potential of LMW-PAHs.

Keywords: Adriatic Sea, *Mullus barbatus*, QuEChERS, PAHs, Environmental impact, Endocrine Disruptors, Gene biomarker, Oxidative stress response

Introduction

Aquatic environment is contaminated by several class of pollutants like polycyclic aromatic hydrocarbons (PAHs), organic compounds with two or more fused benzene rings. PAHs are recognized as priority pollutant substances for environmental monitoring programs (Combi et al., 2020; Kammann et al., 2017) and sixteen of them have been identified as most priority by the United States Environmental Protection Agency (EPA). In addition, certain PAHs are classified as probably and possibly carcinogenic to humans by International Agency for Research on Cancer (IARC, 2018). They can cause harmful effects, in both vertebrates and invertebrates, due to well-known of their toxicity, carcinogenicity and mutagenicity characteristics (Balk et al., 2011; Combi et al., 2020; Pampanin et al., 2016).

Seafood and fish can be exposed to PAHs present in water and sediments due to atmospheric pollution or oil spills (Lawal, 2017). In general, sources contribute to environmental input of PAHs are pyrogenic, petrogenic and biological (diagenetic process), but the major are anthropogenic, mainly from the incomplete combustion of fossil fuels, wood, oil spills and discharge from ships and sewage sludge (Lawal, 2017). According to these production sources, PAHs could have different physicochemical properties and characteristic patterns that could vary the toxicity of these compounds, as well as, the processes of uptake, the distribution patterns, the process of metabolism and elimination (Arienzo et al., 2017; Sverdrup et al., 2002; Zhang et al., 2016). Heavy PAHs, those that contain more than four rings with high molecular weight (HMW), are more stable and more toxic than the light PAHs which have low molecular weight (LMW) with 2-3 rings (Lawal, 2017). In addition, as size, number of rings and angularity of PAH compounds increase, the hydrophobicity of molecules increases also. For these reasons, it is crucial to identify the pollution emission sources of PAHs, their levels and distribution in aquatic ecosystems, in order to estimate the potential risks for organisms, to monitor the fate of these contaminants in the food chain and, therefore, for preventing long-term consequences to marine organisms and humans.

Once PAHs enter into marine organisms, xenobiotic biotransformation processes capable of converting PAHs into water soluble compound occur, increasing hydrophilicity of compounds, in order to facilitate their excretion (Santana et al., 2018). PAHs are subject to these biotransformation mechanisms, in a first step by metabolic enzymes of the cytochrome P450 (CYPs) system, and then their products are coupled to chemical groups by phase II enzyme catalysis (Oliva et al., 2010; Santana et al., 2018). During these biotransformation processes, PAHs have the potential to produce reactive oxygen species (ROS), that are responsible for oxidative stress and loss of macromolecules membrane integrity, such as proteins, lipids or DNA damage (Martins et al., 2013; Oliva et al., 2010; Pampanin et al., 2016; Santana et al., 2018). Potential damage of ROS is usually neutralised and/or degraded by enzymatic and non-enzymatic antioxidant defence mechanisms, such as superoxide dismutase (SOD) and catalase (CAT) activities (Abele et al., 2017; Santana et al., 2018; van der Oost et al., 2003).

Antioxidant enzymes have been routinely used as biomarkers for assessing the impact of PAH-induced stress in marine organisms (Bhagat et al., 2016; Cocci et al., 2019), including fish (Pampanin et al., 2016; Sanni et al., 2017). Beside assessing the enzymatic activities, evaluation of gene expression profiles of antioxidant enzymes may be useful as early-warning signal of pollutant-related oxidative stress. In this regard, it has been suggested that molecular biomarkers might be more sensitive than enzymatic biomarkers, especially for monitoring exposure to environmental chemicals (Giuliani et al., 2013; Regoli and Giuliani, 2014). However, information regarding the impacts of PAHs on fish from the Northern Adriatic Sea is scarce;

therefore, we believe that it is important to carry out studies in this sense, in order to support conservation measures for species of great ecological and commercial value within the perspective of the implementation of the Marine Strategy Framework Directive (MSFD, 2008) that represents the EU's important tool to achieve the Good Environmental Status (GES) of marine ecosystems.

The northern Adriatic Sea is a shallow basin (< 100 m water depth, Artegiani et al. (1997) located in the northern part of the Mediterranean Sea. It is characterized by strong inputs from Italian rivers that flow through highly industrialized, densely populated, and intensively farmed areas (Sagratini et al., 2008). The River Po is the largest river, characterized by a mean annual discharge rate of 1500–1700 m^3/s, accounting for about a third of the total riverine freshwater input to the Adriatic Sea (Marini et al., 2008). In addition, this study area is particularly characterized by sediments, which represents the ultimate sink of these pollutants, of varying composition and grain size (mostly sand and mud) that could be transported to this area through a large number of rivers occurring along the Italian north-western coast, especially Po River (Campanelli et al., 2004; Spagnoli et al., 2014).

Taking this into account, red mullet (*Mullus barbatus*, L. 1758) has been considered in the present study. It is an important resource widely distributed in the Mediterranean area, where it is considered one of the most important fishery species (FAO, 2020). It is a demersal fish with a close association with sandy and muddy bottoms where it inhabits. In addition, *M. barbatus* is a target species of bottom trawl fishery and it is often used as the bioindicator species since it is a territorial fish with a high commercial, economic and nutritional values (Di Lena et al., 2016; Durmuş et al., 2018; Kokokiris et al., 2014).

The aims of the present study were to evaluate the PAH accumulation in muscle of wild red mullet (*M. barbatus*), and to investigate the relationship between mRNA expression profiles of some oxidative stress biomarkers, lipid peroxidation and PAH concentrations in muscle tissues.

Materials and Methods

2.1 Samples collection

A total of 36 specimens of wild red mullet *M. barbatus* were caught by bottom trawl net in a rich offshore fishing area of the Northern Adriatic Sea, in the first half 2019 year. Fish sampling were obtained from the Italian side of Food and Agriculture Organization (FAO) General Fisheries Commission for the Mediterranean (GFCM) Geographical Sub Area 17 (GSA 17). Fish were measured for body length, total weight and were sexed. Only female individuals at the end of their spawning season, sexually mature but inactive (Follesa and Carbonara, 2019), were selected for this study. This because our previous study, carried out on this species, showed that reproduction exerts a significant effect on the accumulation of PAHs in females only (Frapiccini et al., 2020). Muscle tissues of each fish were rapidly dissected and stored at -18 °C until use.

2.2 Determination of PAHs

Sixteen EPA priority PAHs (naphthalene, NA; acenaphthylene, AC; acenaphthene, ACE; fluorene, FL; phenanthrene, PHE; anthracene, AN; fluoranthene, FA; pyrene, PY; benzo[a]anthracene, BaA; chrysene, CHR; benzo[b]fluoranthene, BbF; benzo[k]fluoranthene, BkF; benzo[a]pyrene, BaP; dibenz[a,h]anthracene, DhA; indeno[1,2,3-c,d]pyrene, IcP and benzo[g,h,i]perylene, BgP) were extracted from muscle tissues with the QuEChERS (Quick Easy Cheap Effective Rugged and Safe) technique, flowing the general procedure of our previously studies (Caroselli et al., 2020; Frapiccini et al., 2018; Frapiccini et al., 2020). In the first step of extraction, the homogenized muscle tissue (5 g) were put into 50 mL extraction tubes with 10 mL acetonitrile and a salt partitioning packets containing 4 g $MgSO_4$ and 1 g NaCl. The tube was shaken in a vortex during 3 min and it was immediately centrifuged at 3400 rpm for 3 min at the temperature of 4 °C. The supernatant was recovered for the second step of clean-up by a QuEChERS 15 mL dispersive SPE tube containing 900 mg $MgSO_4$, 300 mg PSA sorbent and 150 mg C_{18} sorbent. The d-SPE tube was shaken and centrifuged at 3400 rpm for 1 min at the temperature of 4 °C. After this clean-up step, the extract was transferred to a new vial, concentrated and recovered with acetonitrile at a final volume of 0.4 mL, finally, stored at -18 °C until UHPLC-FLD (Ultimate 3000 - RF2000, Thermo Scientific, Waltham, MA, USA) analysis. A Hypersil Green PAH column (2.1 x 150 mm, 1.8 µm, 120 Å) in a reversed-phase LC with a mobile phase (water:acetonitrile, v/v) gradient elution was used. The flow rate was 0.3 mL min^{-1} at the temperature of 40 °C. The peaks of analytes were identified in the samples by comparing the retention times with a standard chromatogram under the same conditions. Acenaphthylene is not observed in the FLD chromatogram because it has no appreciable fluorescence under the conditions used and, therefore, it hasn't been considered in this study. The method of extraction was evaluated for its linearity, repeatability, recovery, limit of detection (LOD) and quantification (LOQ). The external standard multipoint calibration technique (from 1:400 to 1:3200, v/v) was used to determine the linear response interval of the detector. Three aliquots without fish tissue samples were extracted and analyzed as laboratory blanks. This procedural blank was carried out to ensure that no PAH contamination could happen in the laboratory and no amount of the target PAHs was found in blank samples. LODs and LOQs were calculated for each analyte according to ICH Q2B (ICH, 2005). The recovery and standard deviation of the extraction method were estimated based on the average of 5 replicates of one fortification level in fish tissue matrix samples at a 1:400 v/v of the standard solution EPA 610 PAHMIX (Supelco, Bellafonte, PA, USA). LOQ and LOD, the recovery rate obtained from spiked fish tissue samples and the coefficients of determination of all analytes are given in Table 1.

Table 1. Recoveries (% RDS), LOD, LOQ and coefficient of determination (r^2) of the proposed QuEChERS method.

PAHs	LOD	LOQ	Recovery (% RDS)	Coefficient of determination (r^2)
NA	0.013	0.040	73 (11.3)	0.9992
ACE and FL*	0.004	0.012	79 (9.5)	0.9935
PHE	0.011	0.032	96 (6.3)	0.9994
AN	0.005	0.015	95 (8.8)	0.9998
FL	0.008	0.026	81 (5.9)	0.9991
PY	0.002	0.006	85 (8.0)	0.9996
BaA and CHR*	0.002	0.007	98 (4.5)	0.9997
BbF and BkF*	0.001	0.004	95 (6.3)	0.9999
BaP	0.001	0.003	97 (5.6)	0.9998
DhA	0.002	0.007	86 (5.8)	0.9999
BgP and IcP*	0.003	0.008	87 (5.6)	0.9999
Total PAHs	0.052	0.160	86 (7.0)	0.9991

* (co-eluted PAHs)

2.3 Quantitative realtime PCR (q-PCR) analysis

Isolation and purification of total RNA from muscle were carried out using Trizol™ LS reagent (Invitrogen) procedures. Total RNA (2 µg) was reverse transcribed with the 5X All-In-One RT MasterMix with AccuRT (abm®) according to manufacturer's instruction (Applied Biological Materials Inc.). For molecular analyses, a SYBR Green ABI 7300 Real-Time PCR assay was performed with specific primers for SOD, CAT, GST target genes and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene (Table 2). Given that target gene sequences for *Mullus barbatus* were not available in GeneBank, PCR amplification was performed using primer pairs designed against the known *Sparus aurata* respective sequences. The optimized reaction mixture contained: 12.5 µL BrightGreen 2X qPCR

MasterMix (abm®), 1 μL each of forward and reverse primers (both 10 mmol L^{-1}), 2 μL cDNA template, and sterile distilled water. Thermo-cycling for CAT, SOD, GST, GAPDH reactions was for 10 min at 95 °C, followed by 40 cycle of 20 s at 95° C and 60 s at 60 °C. Specificity of primers pairs were verified using the melting curve analysis produced by the ABI 7300 software. The efficiency of qPCR primer sets was reported in Table 2. Individual cDNA amplifications were run in triplicate. Results were calculated using the relative method implemented with the Pfaffl equation by accounting for different primer reaction efficiencies (Pfaffl, 2001).

Table 2. List of primers used in this study

Gene	Primer sequence sense	Primer sequence antisense	Efficiency	GeneBank
CAT	TGCGTCCTCCAAG ATGTGAT	TTAGTGCGTTTGCTCT TACACA	95.2	JQ308823.1
SOD	TGTGTGTGCTGAA AGGAGCC	GTCACAGGTGCTGACT CACT	94.8	JQ308832.1
GST	GTCTGAAGGACA GACCCAGC	AGCATGTCTTGACCCT GTGG	95.8	AY362762.1
GAPDH	TGTTTTCCACGAG AGGGACC	GGCCTTCTCAATGGTG GTGA	97.5	DQ641630.1

2.4 Lipid peroxidation (LPO)

Muscle tissues were removed, weighed, and homogenized in an Ultra Turrax T-50 Homogenizer using 0.1 M chilled sodium phosphate buffer containing 1.17% KCl (pH 7.4) (Beg et al., 2015). The homogenates were centrifuged at 13,000 g at 4 °C for 30 min. The supernatant was separated and total protein concentration was determined by the Bradford reagent containing Brilliant Blue G in phosphoric acid and methanol by comparison to a standard curve (Bradford, 1976). The determination of TBARs was performed through the reaction of an aliquot sample with a solution containing trichloroacetic acid (TCA), thiobarbituric acid (TBA), and butylated hydroxytoluene in a heat bath (60 min at 60 °C) (Beg et al., 2015). Subsequently, samples were centrifuged at 10,000 g for 15 min. The supernatant has been collected and absorbance was read in a spectrophotometer (535 nm; JASCO V- 550). A standard curve was arranged with 1,1,3,3-Tetraethoxypropane (Sigma-Aldrich). Results are expressed as pmol of malondialdehyde (MDA) per mg of total protein content using extinction coefficient 1.56×10^5 M^{-1} cm^{-1} (pmol min^{-1} mg^{-1} protein).

2.5 Fulton's condition index

The Fulton's condition index (K index) was estimated using the equation:

$K = 100(W/L^3)$

where W = the weight of the fish, and L is the length.

2.6 Statistical analyses

Data analysis was performed using the R packages Hmisc (Harrell, 2017) and corrplot (Wei and Simko, 2017). The Spearman's correlation coefficients were calculated between biomarkers of oxidative stress / K index and PAH accumulation levels in muscle replacing PAH concentration values below the LOD with LOD divided by the square root of 2. All PAH congeners that were detected in 90% of the samples were included in the data analysis. Results with $p \leq 0.05$ were considered significant.

Results and Discussion

3.1 PAH accumulation and source identification in muscle of wild red mullet

A total of 36 wild-caught female individuals of *M. barbatus*, sexually mature but inactive (at the end of their spawning season) were analyzed within the study. The individual total length ranged from 15.5 cm to 20 cm with a mean and a standard deviation of 17 cm and 1 cm, respectively. The individual weight ranged from 30.5 g to 73.6 g with a mean and a standard deviation of 50.1 g and 11.4 g, respectively. The presence of total and individual PAHs in muscle tissues are shown in Table 3. All investigated fishes contained some congeners of PAHs and the range of total PAHs (sum of 16 EPA priority pollutants, except AC) was from 22 to 535 ng/g (wet weight, w.w.), with a general average of 203 ± 159 ng/g (w.w.). Total PAHs showed high variability in wild red mullets. However, most of individuals (about 44%) recorded a total PAH concentration up to 100 ng/g (w.w.) and could be classifiable as minimally (10 – 100 ng/g w.w.) and moderately (100 – 1000 ng/g w.w.) polluted, according to

Ranjbar Jafarabadi et al. (2019) and Soares-Gomes et al. (2010). Since edible tissue are investigated, our results recorded none of the regulated PAHs (ΣPAH4, sum of BaA, CHR, BbF and BaP) was present at concentrations exceeding the maximum allowed limit in any investigated samples (EU, 2011). Taking into account that data comparability could be hampered by several factors (*i.e.*, laboratory condition, extraction technique, environmental factors, wild population), the observed PAH accumulation levels were in agreement with earlier studies carried out in Adriatic Sea on fillet of *M. barbatus* specimens by Della Torre et al. (2010) (< LOQ – 124 ng/g w.w.) and by Perugini et al. (2007) (1 – 7 ng/g w.w., 1 – 15 ng/g w.w., 0.05 – 0.7 ng/g w.w. and 2.4 – 5 ng/g w.w for FL, PHE, AN and FL, respectively) and they were in line with other demersal fish caught in the same study area of the Adriatic Sea (100 – 210 ng/g w.w.; Storelli et al. (2013). Within the investigated samples, the most common PAH congeners found in all the muscle samples, were NA, ACE and FL, which accounting for 32% and 56% (ACE and FL were co-eluted) of the total PAHs, respectively. Frequently, BbF, BkF, BaP, DhA, BgP and IcP values were found to be below LOQ.

Table 3. Total and individual PAH concentrations in the 36 wild females of *M. barbatus*

PAHs	**Min**	**Max**	**Mean**	**SD**
NA	0.04*	295.67	93.28	96.03
ACE and FL[a]	19.94	223.63	97.04	63.06
PHE	0.58	9.59	5.65	1.47
AN	0.02*	1.54	0.75	0.41
FL	0.51	3.54	1.95	0.54
PY	1.14	8.03	3.93	1.15
BaA and CHR[a]	0.007*	0.718	0.478	0.194
BbF and BkF[a]	0.004*	0.239	0.050	0.061
BaP	0.003*	0.003*	0.003*	n.a.
DhA	0.007*	0.007*	0.007*	n.a.
BgP and IcP[a]	0.008	0.053	0.009	0.008

Tot PAHs	22.4	535.3	203.1	158.7

[a]co-eluted PAHs

*LOQ values

n.a.: not available

The accumulation pattern of PAHs showed a predominance of LMW-PAHs, representing 94% of the total content of PAHs. Previous investigations highlighted LMW-PAH concentrations were generally higher than HMW-PAH ones in aquatic organisms (Barhoumi et al., 2016; Mashroofeh et al., 2015; Moraleda-Cibrian et al., 2015; Soltani et al., 2019). This is due to LMW-PAHs are more soluble and less capable to be metabolized compared to heavier PAHs (Caroselli et al., 2020; Cocci et al., 2019; Frapiccini et al., 2020; Romero et al., 2018).

3.2 PAH effects on the mRNA levels of related antioxidant enzymes and K index

According to Spearman analysis, most of the assessed parameters were found to be differentially correlated to PAH tissue accumulation (Fig. 2). K index was negatively correlated with the levels of both PHE and BaA and CHR (co-eluted PAHs) suggesting changes in energy storage and metabolism due to chemical environmental stressors. In fish, the Fulton's K condition index is considered as a long term parameter indicative of well-being (Suthers, 2000). Despite the broad fluctuations in response to chemical pollution, reduced K index values have been observed in fish exposed to heavy metals or living in polluted sites (Henry et al., 2012; Kerambrun et al., 2013; Kerambrun et al., 2012a; Kerambrun et al., 2012b). Among oxidative stress-related genes, only CAT showed a significant negative correlation with total PAH concentrations, especially total LMW-PAH, in fish muscle. In this regard, we observed a weak negative correlation between gene expression of CAT and tissue accumulation of some specific PAH congeners (*i.e.* NA, PHE, ACE and FL). Similarly, GST was also negatively associated with PHE, whereas SOD did not show any significant correlation with PAH levels. PHE levels were also found strongly positively correlated with MDA content. Our results substantially confirm what has been previously reported about the capacity of PAHs to induce oxidative stress in aquatic organisms (Douben, 2003). In the present study, the lowest expressions of CAT and GST were detected in fish showing high accumulation of PAHs, particularly the LMW molecules. This demonstrates a PAH-induced ROS generation rate that seems faster respect to the rate and the magnitude of oxidant elimination thus causing oxidative damage to the organism. Indeed, a significant correlation between PHE and muscle TBAR levels was observed in our study. In agreement with these findings, Recabarren-Villalón et al. (2019) reported positive associations between PAHs and muscle lipid peroxidation in *C. guatucupa* juveniles. These results were overall similar to those previously observed for other fish species (Santana et al., 2018). It is highly probable that under the stress of PAH accumulation, the antioxidant defense system might not be sufficient to effectively eliminate oxidative damage as assessed by both the high level of toxic aldehydes produced from lipid peroxide and the down-regulation of CAT and GST gene expression. Reductions in mRNA levels of metabolization and detoxification genes have also been reported in fish fed PAHs (Curtis et al., 2017) and mussels exposed to nitro-PAHs (Chatel et al., 2014). In addition, pyrene significantly downregulated the gene expression of antioxidant enzymes in human liver HepG2 cells (Ma et al., 2019). The present study also highlights the oxidative damage potential of LMW-PAHs, particularly PHE that is widely abundant in aquatic environment (Zhang et al., 2004a; Zhang et al., 2004b) being a major constituent of crude oil and oil products (Mos et al., 2008). Previous studies have showed that PHE can be rapidly accumulated in fish tissues due to a fast metabolic clearance (Sun et al., 2006). The same authors reported that after 1-2 days exposure to PHE, tissue chemical concentrations increased and oxidative–reductive reaction occurred resulting in a modulation of the antioxidant enzyme activities. Interestingly, they found no changes in SOD activity but a significant decrease in that of both CAT and GST. This may be explained by an overproduction of hydroxyl radicals, the most reactive oxygen radical, that can downregulate the cellular antioxidant defense causing cellular damage (Cheng et al., 2002). In addition, acute exposure to PHE was also found to cause histopatological alteration in juvenile African catfish (Karami et al., 2016). From the overall achieved results in the present study, it is clear that downregulation of CAT and GST mRNA expression levels might explain the LMW PAH-induced oxidative stress in the muscle tissues of wild red mullets. We believe that these mechanisms may have contributed to the PHE-related lipoperoxidative response affecting the general well-being of fish. However, further research works are still needed for the evaluation of the extent and severity of chronic PAH exposure on wild populations of red mullet from the Northern Adriatic Sea.

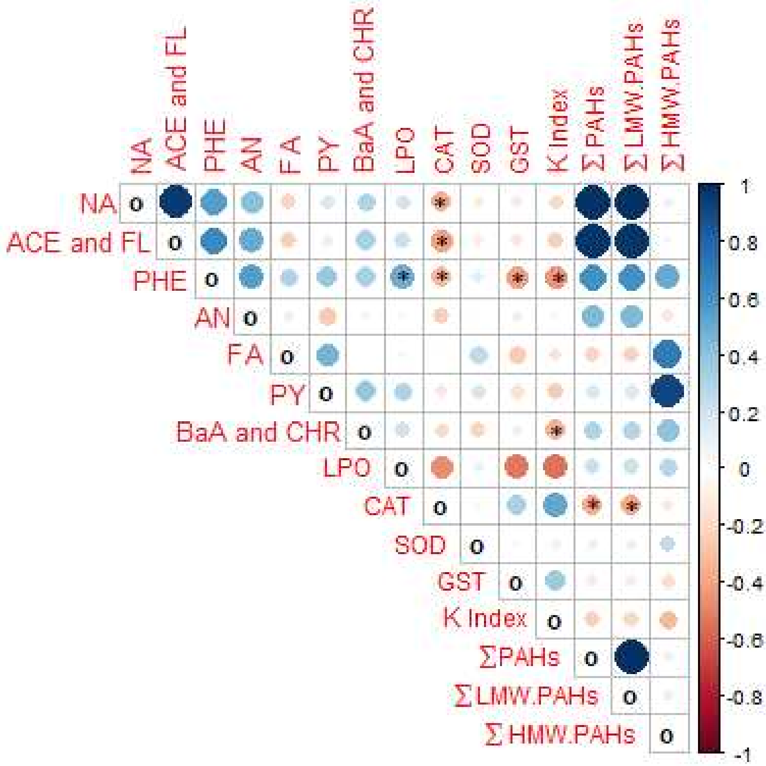

Fig. 2. Heatmap of the Spearman correlation coefficients (ρ) between PAH concentrations and oxidative stress biomarker levels in muscle tissues of red mullet, *Mullus barbatus*. Color indicates whether the correlation is positive (blue) or negative (red) while size and darkness of the circles indicate the strength of the correlations, with stronger correlations being larger and darker than weaker ones. (NA) naphthalene; (ACE and FL) acenaphthene and fluorene; (PHE) phenanthrene; (AN) anthracene; (FA)

fluoranthene; (PY) pyrene; (BaA and CHR) benzo[a]anthracene and chrysene; (LPO) lipid peroxidation; (CAT) catalase; (SOD) superoxide dismutase; (GST) glutathione S-transferase. (*P < 0.05).

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7. CONCLUDING REMARKS

The present PhD thesis provides new insight into the main biological, chemical and environmental effects on the PAH level in fish tissues, including edible tissue. Furthermore, it examines the relationship between the PAH levels and mRNA expression level of some antioxidant enzymes, as well as lipid peroxidation, proving innovative and useful information on the biological responses of wild fish of the Adriatic Sea. For the first time, a rapid multi-residue QuEChERS method was used in the Adriatic Sea to screen for PAHs in *Solea solea* and *Mullus barbatus* fish species.

This is the first research that analyses the muscle tissue of red mullet from the Adriatic Sea for PAH pollution, by studying the entire reproduction cycle of the fish, through monthly sampling over a year. In addition, for the first time, PAH concentrations and mRNA expression profiles of some antioxidant genes, as well as the lipid peroxidation, were investigated in the muscle of wild red mullet from the Adriatic Sea.

In answer to the questions posed above (***Chapter 2***), my PhD research observed that PAH levels in fish (common sole) and sediments from coastal areas (Venice Lagoon and the Po Delta) were higher than those from fishing areas off Chioggia, selected as control. In addition, PAHs with 4 rings medium MW (fluoranthene and pyrene) were accumulated both in fish and sediments. Therefore, PAH bioaccumulation in common sole was closely related to habitat characteristic and surrounding pollution.

PAH levels in different tissue types of common sole, showed highest values in gills, followed by liver and muscle, although the tissue with the highest total lipid content was the liver. This finding could be due to the direct exchange between water and sediment though the gills, which acts as an important bioaccumulation mechanism in common sole. Although PAH are lipophilic compounds, a weak positive correlation between PAH and lipid content was found, suggesting that lipid content might not be the only determining factor.

The physicochemical properties of PAHs had a significant effect on the PAH level in fish tissues. Only in the gill tissue of common sole, the PAH bioaccumulation was significantly affected by their physicochemical characteristics (ring and K_{ow}). These results were also observed in red mullet, where differences in PAH accumulation depended mainly on their degree of lipophilicity and number of rings. In general, consistent differences were found between low and high MW PAHs, demonstrating the strong effect of the physicochemical properties of PAHs.

This PhD study observed that the reproduction stage of fish is an important factor influencing the accumulation of heavier PAHs in muscle tissue of *M. barbatus*. The heavier PAHs showed higher levels in the pre-spawning period and during the winter season in females. In addition, a weak but significant correlation between PAH levels and the total lipid content was recorded in both species. However, total lipid content and age seem to exert a limited influence, on the contrary, the body size of fish showed no effect on PAH accumulation. In general, in red mullet, reproduction stages and seasonality are important factors influencing the accumulation of the heavier PAHs. Specifically, seasonality affects PAH levels in both females and males, whereas reproduction only in females. Low MW PAHs seem to be unaffected by any factors, but chronic exposure to low MW PAH on wild fish could induce oxidative stress by downregulating mRNA gene expression of antioxidant and increasing lipid peroxidation.

The results of this PhD improve the knowledge of the most important factors determining PAH accumulation in the wild Adriatic fish: it confirms that fish not only act as sinking into seawater, accumulating them in some tissues, but also represent the source of exposure to organisms involved in human consumption and provides new information on the biological responses of fish chronically exposed to PAHs.

In conclusion, to know the biological characteristics of fish, as well as the degree of PAH contamination and the mechanisms of action of these contaminants are important issues. In particular, this could be essential to classify the environmental status of marine ecosystems and to produce measures suitable for its support. Thus, the results of my PhD contribute to the dissemination of new knowledge in the marine and toxicological fields.

The understanding of relationships and processes between the presence of contaminants in the marine environment and biota, as well as their ecological and biological effects, are important topics. Therefore, pointed studies in this direction are essential to determine the environmental status of the marine ecosystems and the achievement of the GES, as suggested by MSFD. Furthermore, the results of the present thesis are relevant for the MSFD application, too. Particularly, results and products of this PhD thesis could be used to increase and define the dataset for the development of new environmental quality indexes, as well as to protect the Adriatic fish stocks.

ADDITIONAL PRODUCTS

Below there are other publications not included in the PhD thesis, and the research products that have been shown as contribution to national and international conferences. These additional works have been performed by me in collaboration with some research groups of the CNR, NORCE, UNICAM, UNIBO and UNIURB, during my PhD period.

Other publications

P1 M Rovere, A Mercorella, **E Frapiccini**, V Funari, F Spagnoli, C Pellegrini, AS Bonetti, T Veneruso, AN Tassetti, M Dell'Orso, M Mastroianni, G Giuliani, R De Marco, G Fabi, F Ciccone, I Antoncecchi (**2020**). Geochemical and Geophysical Monitoring of Hydrocarbon Seepage in the Adriatic Sea. Sensors, 20, 5, 1504.
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P2 E Caroselli, **E Frapiccini**, S Franzellitti, S Palazzo, F Prada, M Betti, S Goffredo, M Marini, (**2020**). Accumulation of PAHs in the tissues and algal symbionts of a common Mediterranean coral: Skeletal storage relates to population age structure. Science of the Total Environment 743, 140781 https://doi.org/10.1016/j.scitotenv.2020.140781

P3 E Baldrighi, F Semprucci, A Franzo, I Cvitkovic, D Bogner, M Despalatovic, D Berto, MM Formalewicz, A Scarpato**, E Frapiccini**, M Marini, M Grego (**2019**). Meiofaunal communities in four Adriatic ports: Baseline data for risk assessment in ballast water management. Marine Pollution Bulletin, 147, 171-184.
https://doi.org/10.1016/j.marpolbul.2018.06.056

P4 E Droghini, A Annibaldi, E Prezioso, M Tramontana, **E Frapiccini**, R De Marco, S Illuminati, C Truzzi, F Spagnoli (**2019**). Mercury content in Central and Southern Adriatic Sea sediments in relation to seafloor geochemistry and sedimentology. Molecules, 24, 4467

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P5 P Cocci, G Mosconi, L Bracchetti, JM Nalocca, **E Frapiccini**, M Marini, G Caprioli, G Sagratini, FA Palermo (**2018**). Investigating the potential impact of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) on gene biomarker expression and global DNA methylation in loggerhead sea turtles (Caretta caretta) from the Adriatic Sea. Science of the total Environment, 6149, 49-57 https://doi.org/10.1016/j.scitotenv.2017.11.118

Other Contributions at national and international conferences

C1 F Spagnoli, **E Frapiccini** (**2020**). L'esplorazione degli oceani e dei fondali marini. Villaggio globale (Online) Ed. Mario Adda Editore – Bari. ISSN: 2039-7208, Anno XXIII – N.90 – Giugno 2020 (ID. 426674). https://www.vglobale.it/rivista/natura-e-contronatura/attacco-al-mare/

C2 M Rovere, A Mercorella, F Spagnoli, V Funari, **E Frapiccini**, C Pellegrini, F Ciccone, I Antoncecchi, AS Bonetti, M Dell'Orso, N Tassetti, G Giuliano, R De Marco, G Fabi (**2020**). Cost-effective and relocatable monitoring of natural hydrocarbon seepages in the Italian offshore. Conference Proceeding 2019 IMEKO TC19 International Workshop on Metrology for the Sea: Learning to Measure Sea Health Parameters, MetroSea 2019, ISBN: 978-929900842-3, pp.64-69

C3 **E Frapiccini**, G Pellini, A Gomiero, G Scarcella, S Guicciardi, A Annibaldi, M Betti, M Marini (**2020**). Microplastics and polycyclic aromatic hydrocarbons occurrence in a demersal fish (Solea solea) in the Adriatic Sea. In: Cocca M. et al. (eds) Proceedings of the 2nd International Conference on Microplastic Pollution in the Mediterranean Sea. ICMPMS 2019. Springer Water, Springer, Cham. https://doi.org/10.1007/978-3-030-45909-3_35

C4 M Martinelli, A Gomiero, S Guicciardi, **E Frapiccini**, P Strafella, S Angelini, F Domenichetti, A Belardinelli, S Colella (**2020**). Microplastics in seafood: preliminary results on the occurrence and anatomical distribution in wild

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C7 **E Frapiccini**, E Droghini, A Annibaldi, E Prezioso, M Tramontana, R De Marco, S Illuminati, C Truzzi, F Spagnoli (**2019**). Polycyclic aromatic hydrocarbons in surface sediments of Central and Southern Adriatic Sea. XVIII ABC Congresso di Chimica dell'Ambiente e dei Beni Culturali, Urbino 24-27 Giugno 2019

C8 M Rovere, A Mercorella, F Spagnoli, V Funari, **E Frapiccini**, C Pellegrini, F Ciccone, I Antoncecchi, et al., (**2019**). Cost-effective and relocatable monitoring of natural hydrocarbon seepages in the Italian offshore. In: La geologia marina in Italia. Doi:10.3301/ABSGI.2019.02

C9 A Annibaldi, E Prezioso, E Droghini, M Tramontana, **E Frapiccini**, R De Marco, S Illuminati, C Truzzi, F Spagnoli (**2018**). Determination of mercury in the sediments of the Adriatic Sea in relation to their geochemical and sedimentological characterization. Bioanalitica_Chimica bioanalitica per la saluta, l'ambiente e la sicurezza naturale_Bologna, 21 settembre 2018. ISBN 9788894952056

C10 **E Frapiccini**, A Annibaldi, M Betti, S Illuminati, C Truzzi, G Scarponi, M Marini (**2018**). Polycyclic aromatic hydrocarbon (PAH) accumulation in different common sole tissues by QuEChERs method. Bioanalitica_Chimica bioanalitica per la saluta, l'ambiente e la sicurezza naturale_Bologna, 21 settembre 2018. ISBN 9788894952056

C11 A Mercorella, M Rovere, F Spagnoli, **E Frapiccini**, M Marini, G Giuliani, P Penna, R De Marco, A Soledad Bonetti, M Dell'Orso (**2018**). Cost-effective method for detecting and monitoring natural fluid seepage at sea. CLYPEA-NETWORK PER LA SICUREZZA OFFSHORE – Torino7-8 Giugno 2018

C12 **E Frapiccini**, A Annibaldi, M Betti, A Campanelli, F Grilli, C Truzzi, S Illuminati, M Marini, G Scarponi (**2018**). PAH bioaccumulation in different common sole tissues from the Adriatic Sea. CHIMALI, XII Italian Food Chemistry Congress, Camerino 24-27 Settembre 2018

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