UNIVERSITY OF CATANIA

DEPARTMENT OF CHEMICAL SCIENCES

INTERNATIONAL PhD IN CHEMICAL SCIENCES - XXXIII CYCLE

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Monitoring of Persistent Organic Pollutants (POPs) in marine biota from the Catania Gulf (Sicily, Italy) and development of new sensitive analytical methods for their detection

PhD Thesis

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ABSTRACT

This PhD thesis focus on the development of new analytical strategies, using either the common gas-chromatography mass spectrometry (GC-MS) methodologies and the fluorescence spectroscopy, to assess the distribution of different classes of Persistent Organic Pollutants (POPs), including polychlorinated biphenyls (PCBs), organochlorine pesticides (POCs), and polycyclic aromatic hydrocarbons (PAHs), in environmental matrices (biota, volcanic ashes and seawater), as well as on the monitoring of these pollutants in *Mullus Surmuletus* of the Catania Gulf (Sicily, Italy) and on the evaluation of the potential human health risks associated to the consumption of fish.

As concerns the development of more sensitive analytical methods to detect POPs in fish muscle using GC-MS, after preliminary investigations on different extraction and clean-up techniques to remove potential interferences, a modified QuEChERS extraction and GC-MS methodologies were developed and implemented for the quantification of 16 priority PAHs, 29 PCB and 23 OCPs in muscle fish and the developed methods were validated according to UNI ENI ISO 17025. The methods for PAHs and PCBs show good linearity and optimal recoveries for almost all analytes, as well as satisfactory accuracy, yielding percent deviations within the range 100 ± 15%. All experimental CV% were lower than 15% for PAHs and PCBs, proving that the methods have good within-run precisions. On the other hand, since the quantification of OCPs was carried out without IS, only recoveries were tested, and this method was used only with external calibration and mainly for qualitative purposes.

The developed methods were used to assess the distribution of POPs in *Mullus Surmuletus* from Catania Gulf (Sicily), as well as the potential human health risks associated to the consumption of fish. The determined PAHs concentration values ranged from 0.25 to 6.10 ng/g wet weight, whereas for the other classes of pollutants some PCBs (PCB 028, PCB 138 and PCB 153) and only the pesticides 4,4′-DDE were detected in trace. The most abundant PAHs detected were lower molecular weight (LMW) compounds with 2 to 3 rings. Relying on the PAHs concentration values, on the consumption data and on the total Toxic Equivalent (TEQ), the incremental lifetime cancer risk (ILCR) was assessed and its calculated value (2.97×10^{-7}) is far below the "maximum acceptable risk level" (ARL), suggesting a low potential carcinogenic risk on consuming *M. surmuletus* for local population. Even though the study shows a quite low contamination level in *M. surmuletus*, intensive monitoring programmes are still highly needed in order to provide a better picture of the PAHs distribution in Catania Gulf and of the human health risk linked to fish consumption.

Considering the natural pressures to which the area investigated is subjected, an ultrasonic extraction followed by an SPE clean-up, and GC-MS methodology was optimized and developed

to detect PAHs in Etna volcanic ashes in order to identified a potential baseline of natural sources of these pollutants and to evaluate the potential impact of volcanic eruptions to the marine environment. Experiments carried out spiking the samples with 10 μg/Kg provided recovery ranged from 70 to 120% for almost analytes. Preliminary characterization of Etna volcanic ashes, sampled at high altitude (~3000 m), showed the presence of the three- to six-ring PAHs, suggesting a mixture of petrogenic and pyrogenic sources. Considering that the data reported in literature are very few and controversial, the results obtained by this preliminary study clearly indicate the need of additional and in-depth investigations.

Finally, a new method for the detection of pyrene in ultrapure water has been developed by using an aromatic anion highly fluorescent, i. e. fluorescein. Preliminary results suggest an efficient energy transfer from pyrene donor to fluorescein acceptor, which probably occurs via the stacking interactions of the two aromatic systems that result to be an important driving force in solution. In order to evaluate if the presence of a metal core could enhance this effect, novel fluorescent nanoparticles capped with fluorescein (Fluo-AgNps) were synthetized and characterized by TEM and UV-Vis spectroscopy. The efficiency of energy transfer was measured by monitoring both the quenching of the pyrene emission and the enhancement of the fluorophore emission, which is greater with the free ligand fluorescein than that observed with Fluo-AgNPs. The developed method allows to detect pyrene in aqueous solutions at ppt level, using a very simple system, without additional preconcentration of the samples, obtaining a sensitivity much better than that reported with the direct fluorescence methods. The method for the quantitative determination of pyrene in seawater is still ongoing and the preliminary data showed that a pre-treatment of the seawater sample is required to remove potential interferences and to optimize the efficiency of the transfer.

The data here reported are preliminary and further investigations, among which fluorescence lifetime measurements and thermodynamic investigations in order to assess the nature and the entity of the non-covalent binding are highly needed.

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INTRODUCTION AND AIMS OF THE PROJECT

In the last decade marine pollution has obtained great attention due to the macroscopic phenomena of plastic debris as well as to the increasing and continuous dumping of wastes which affect the environment, the daily life of fish and other marine species. Many organic compounds enter the marine environment as a result of man's activities, originating, for example, from discharges into rivers, estuaries and coastal waters. Moreover, compounds that are dispersed into the atmosphere as a result of volcanic eruptions, forest fires and anthropopressure also contribute to the pollution of aquatic environment.[1]

Persistent Organic Pollutants (POPs) are widely distributed throughout marine ecosystems, and they have been the focus of research in environmental chemistry since its inception in the 1960s.[2,3] POPs, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (POCs), and polycyclic aromatic hydrocarbons (PAHs) are well known environmental pollutants, which pose health risks to marine biota at higher trophic levels and to human consumers of fish species.

POCs and PCBs, resulting from agriculture use and industrial emissions, [4,5] still persist in soil and river sediments as a result of their uncontrolled use in past years, while PAHs still occur either from anthropogenic (coal and wood burning, petrol and diesel oil combustion, and industrial processes) and natural sources (volcanic eruptions and forest fires) and they are widely distributed in colloidal dispersion of aqueous environment as well as in sediments.[6] These organic pollutants exert adverse effect on the aquatic environment and, since they are compounds that are persistent and could bioaccumulate, potentially also on human consumers of fish. Due to their environmental and health risks, the Stockholm Convention on Persistent Organic Pollutants was adopted in 2001 to phase out production, use, and release of POPs. Although banned in many developed countries, some of these POPs are still present in polluted sites or illegally placed into commerce and used in certain developing countries. Understanding the distribution of POPs in marine environment, and identifying pollutant sources, is crucial for assessing and maintaining marine ecosystem health, as well as for evaluating the effectiveness of regulation. In fact, wide-scale chemical monitoring programmes have been developed by international conventions and European Union policies, such as the Water Framework Directive (2000/60/EC) and the new EU Marine Strategy Framework Directive (MSFD). In particular, the directive 2013/39/EU establishes biota Environmental Quality Standard (EQS) for some existing and newly identified priority substances. EQS states that the concentration of a particular pollutant or group of pollutants in water, sediment or biota should not exceed certain given thresholds in order to protect human health and the environment. Since monitoring programmes need to follow up the status of the marine environment, a wide collaboration among the academic research and the environmental agencies is required.

In this context, the development of more sensitive analytical methods that allow the detection of organic pollutants at trace levels is still a golden goal in analytical chemistry, representing a big challenge for the scientific community. In fact, improvements in the sensitivity of analytical techniques are still in progress, due to the low concentration values of these pollutants, to the complexity of the environmental matrix (e.g. biota) which requires laborious pre-treatment of samples, and to the high number of pollutants and congeners which generally require mutiresidual analysis.

This doctoral thesis focuses on the development of new analytical strategies, using either the common GC-MS methodologies and the fluorescence detection, to assess the distribution of different classes of POPs, including PCBs, OCPs and PAHs, in environmental matrices (biota, volcanic ashes and water) and on the monitoring of these pollutants in *Mullus Surmuletus* of the Catania Gulf (Sicily, Italy). The investigated area, the Catania Gulf, is of particular interest, being characterized either by anthropogenic pressures, including industrial and municipal impacts and high maritime traffic, and by natural pressures such as the Etna volcanic activity. The present thesis reports the development of more sensitive analytical methods to detect POPs in environmental matrices, using also alternative methods based on the fluorescence detection, thus contributing to obtain a better picture of the distribution of POPs in the Catania Gulf and providing an overview of the potential health risks due to the human consumption of *M. surmuletus* from the investigated area.

The present PhD thesis is divided in five chapters, the first one reports general information on POPs, including PAHs, PCBs and OCPs, pointing out their proprieties, their source and fate in the marine environment and their adverse effects on human health, while the other chapters focus on the main research activities:

1) the development and validation of analytical methods using GC-MS to detect POPs in biota
2) the biomonitoring of POPs in *Mullus surmuletus* from Catania Gulf;
3) the development of analytical methods to detect PAHs in Etna volcanic ashes;
4) the development of new sensitivity fluorescence-based techniques to assess the presence of PAHs in seawater.

As concerns the development of more sensitive analytical methods to detect POPs in fish muscle using GC-MS, different extraction and clean-up techniques were investigated to remove potential interferences, to clarify the final extracts prior to GC analysis and to enhance the recoveries of analytes. In this respect, a modified QuEChERS extraction and gas-chromatography mass spectrometry (GC-MS) methodologies were developed and implemented for the quantification of

16 priority PAHs, 29 PCB and 23 OCPs in muscle fish and the developed methods were validated according to UNI ENI ISO 17025.

The developed methods were used to assess the status of chemical pollution in marine ecosystems of the Catania Gulf (Sicily) as well as the potential toxic effects due to POPs exposure. Therefore, the biota monitoring was carried out using red mullet, *M. surmuletus,* as bioindicator. This species was selected according to the guidance n. 25[7] by the European monitoring program due to its slow tendency to migrate and its abundance in the investigated area, in addition to being highly consumed from the local population. The potential human health risks arising from the consumption of contaminated fishes were also evaluated using the incremental lifetime cancer risk (ILCR) concept.

Moreover, considering the natural pressures to which the area investigated is subjected, the development and optimization of analytical method to detect PAHs in Etna volcanic ashes were carried out to identified a potential baseline of natural sources of these pollutants and to evaluate the potential impact of volcanic eruptions to the marine environment. Based on EPA methods for analysis of organic pollutants in soil and sediment, an ultrasonic extraction followed by an SPE clean-up, and GC-MS methodologies have been developed and implemented for the quantification of 16 PAHs in volcanic ashes.

Finally, by referring to the development of new sensitivity fluorescence-based techniques, which could be a smart alternative to the widely utilized trace-analytical methods to assess the presence of PAHs in seawater, several fluorescence experiments were carried out using pyrene as model compound to study a possible interaction between PAHs and an aromatic anion highly fluorescent, i. e fluorescein, either as it is and as silver nanoparticles capping agent (Fluo-AgNps). In this respect, different fluorescence titrations were carried out in aqueous solution and in seawater using different procedures in order to optimize the method conditions.

CHAPTER 1

GENERAL BACKGROUND ON POPS

This chapter focuses on the well-known POPs, including PAHs, PCBs and OCPs, pointing out their proprieties, their source and fate in the marine environment and their adverse effects on human health. Even though the use and production of many POPs have been banned, they are globally distributed through the air-water exchange and environmental cycles due to their high persistence, causing great concerns for human health and for environment.

1.1 INTRODUCTION

Persistent organic pollutants (POPs), such as PCBs, OCPs, and PAHs are well known environmental pollutants which are of great concern for their effects on human health and environment. They are included in priority pollutant list of the European Union and US Environmental Protection Agency (EPA) due to their mutagenic and carcinogenic properties. POPs show some common features, such as:

- long half-lives which give rise to the high persistence in the environment for years or decades;
- bioaccumulation and biomagnification, entering the food chain – i.e., they concentrate at higher trophic levels, thus polluting and exposing all living beings, including humans;
- they are globally dispersed, being present in air, in water currents and in living organisms;
- they have high fat solubility and thus accumulate in fatty tissues of living organisms with serious health effects.[8]

Due to their high lipophilicity, POPs bioaccumulate in the adipose tissues of animals. In fact, several studies report not negligible levels of POPs measured in living organisms as well as in foodstuffs, including fish, meat, eggs and milks and, thus, traces of POPs may be found in human body blood, body fat and breast milk.[8]

The Stockholm Convention on Persistent Organic Pollutants was adopted in May 2001 with the aim of protecting human health and the environment from POPs and initially targets 12 POPs (known as the "Dirty Dozen"). Nine new chemicals and the pesticide endosulfan were added in 2009 and 2011, respectively. Several other regional and global conventions have been established in order to identify hazardous substances and to protect humans and the environment by taking global actions (i.e. reducing or eliminating their production and use). These include the Aarhus Protocol on Persistent Organic Pollutants to the Long-Range Transboundary Air Pollution Convention (LRTAP), the Rotterdam Convention on the Prior Informed Consent (PIC) Procedure for Certain Hazardous Chemicals and Pesticides in International Trade, the Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and their Disposal, and the OSPAR (Oslo-Paris Convention for the Protection of the Marine Environment of the North-East Atlantic).

As regarding PAHs, this class of pollutants is referred to as Persistent Toxic Substances (PTS),[9] sharing some proprieties with POPs. The list of PTS is not fully defined, and several expert groups are working to establish criteria for adding new chemicals with similar features to POPs.

Even though the use and production of many POPs have been banned, they are globally distributed through the air-water exchange and environmental cycles, thus they are found in places far away from the sites where they are still used.

Therefore, the monitoring of these pollutants has been carried out for several decades and still continues today in local, regional, and national-scale programs.[10] Major long-term monitoring programs, such as the Arctic Monitoring and Assessment Program (AMAP), the Baltic Marine Environment Protection Commission (Helsinki Commission) and the National Status and Trends Program (US), Oslo and Paris Commission (OSPAR), focus on organic pollutants in marine biota and sediment as part of their strategies to assess marine environmental quality. In this contest, the development of analytical methods to detect POPs, which provide reliable and accurate data, plays an important role to study their environmental distribution, their fate and their potential sources.

1.2 POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

Polycyclic Aromatic Hydrocarbons (PAHs) are organic ubiquitous compounds, consisting of only carbon and hydrogen atoms with two or more condensed aromatic rings. Although there are several hundred chemically related compounds, which show high persistency in the environment and varied toxicity, most regulations, analyses and data reporting focus on only a limited number of PAHs, typically between 14 and 20 individual PAH compounds. The US Environmental Protection Agency (EPA) has identified 16 PAHs as priority pollutant due to their toxicity to mammals and aquatic organisms (Figure 1). PAHs are characterised by high melting and boiling points, low vapor pressure, and very low aqueous solubility, which decrease with increasing molecular weight, whereas their resistance to oxidation and reduction increases. Solubility and vapor pressure are the major physical/chemical characteristics that set their distribution between the soluble and particle components of the atmosphere, hydrosphere, and biosphere.

Due to the aromatic rings, PAHs show characteristic UV and fluorescent spectra, which are largely employed in many analytical techniques for their detection and identification.

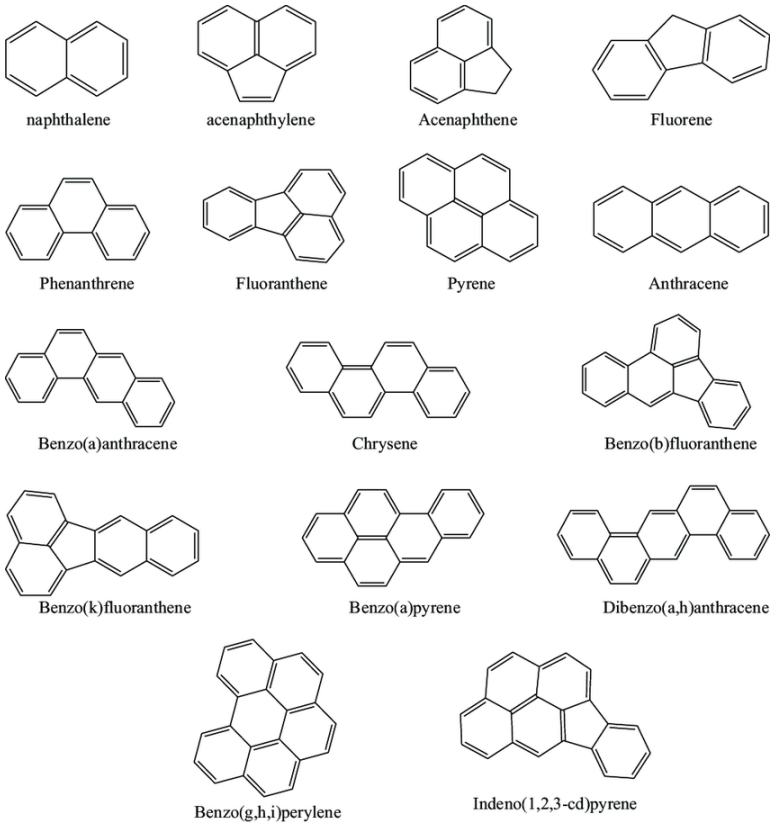

Figure 1: structure of the 16 priority pollutant PAHs

PAHs generally form by incomplete combustion of organic matters either from natural or anthropogenic sources. Some examples of natural sources of PAHs formation include forest and brush fires, volcanoes, bacterial and algal synthesis, and petroleum seeps, whereas PAHs anthropogenic sources include the incomplete combustion of incinerators and some industrial processes, car emissions, jet exhausts, smoke and cigarette, petroleum product spills, sewage sludge, and tarry or creosote waste materials.

As regarding the sources, PAHs may originate from petrogenic,[11] pyrogenic [12] or biological processes.[13] Pyrogenic PAHs are formed during combustion of organic matters under low or no oxygen conditions. The distillation of coal into coke and coal tar, the thermal cracking of petroleum residuals in addition to the incomplete combustion of motor fuels in cars and trucks or the incomplete combustion of wood in forest fires are examples of pyrolytic process occurring at high temperature (350°C-1200°C). It should be stressed that the incomplete combustion has been identified as the single largest contributor of PAHs to the environment.[14]

Petrogenic PAHs are formed during crude oil maturation and similar processes. They are widespread due to the common processes linked to the activities of crude oil and crude oil products. Some examples of petrogenic PAHs sources include oceanic and fresh- water oil spills, underground and above ground storage tank leaks, and the accumulation of vast numbers of small releases of gasoline, motor oil, and related substances associated with transportation.

On the other hand, the processes that lead to the formation of biological PAHs are not well understood. They can be originated by the synthesis processes that occur in several plants or during the degradation of vegetative matter.[15]

As previously reported, there are numerous and generally well-known sources for PAHs, though the identification of the PAHs source is often difficult, and it is based mainly on real environmental sample. The formation temperature of PAHs is one of the factors that could be useful in the identification of PAHs origin. In fact, higher temperatures contribute to produce PAHs with fewer alkylated chains than that formed under lower temperatures, leading to a different pattern.[13] Moreover, the number of five-member hydrocarbon rings in the PAHs pattern could be used as another method to distinguish pyrogenic PAHs from petrogenic PAHs. Five-member rings are more abundant in PAHs from petroleum origin, because the long-time of their formation promotes the ring condensation. Whereas, during pyrolytic processes, owing to the higher temperatures, the five-rings PAHs are rapidly converted into more stable six-member rings.[16]

1.2.1 The Sources, Transport, and Fate of PAHs in the Marine Environment

Figure 2 shows the distribution and dispersion of PAHs into the environment due to the different processes involved in addition to the possible routes of exposure (inhalation, ingestion, or direct dermal contact) for humans. The introduction of PAHs in the marine environment occurs primarily from two processes: 1) the movement of water containing dissolved and particulate constituents derived from watersheds; 2) the atmospheric deposition both by dry or wet (precipitation) processes.[17] It has been shown that estuaries and coastal areas contain large amount of PAHs due to the most localized inputs of the watersheds and airsheds. Urban runoff, wastewater effluents, industrial outfalls, atmospheric deposition, and spills and leaks during the transport and production of fossil fuels are the major sources of PAHs to the coastal marine environment. Among these different inputs that contribute to the dispersion of PAHs into the marine environment, it is possible to distinguish between point and non-point-sources. The point sources include the discharges of identifiable pipes or outfalls, for example the municipal wastewater facilities and industrial outfalls. On the other hand, the non-point sources include urban runoff and other surface runoff coming either from industrial and agricultural sites as well as atmospheric inputs.[17] The atmosphere is the most important means of PAHs dispersion. PAHs enter the atmosphere primarily from incomplete combustion of organic matter either from natural or anthropogenic sources. According to their physical/chemical proprieties, PAHs, released into the atmosphere, are distributed between the two phase, a vapor phase and a solid phase.[18,19]

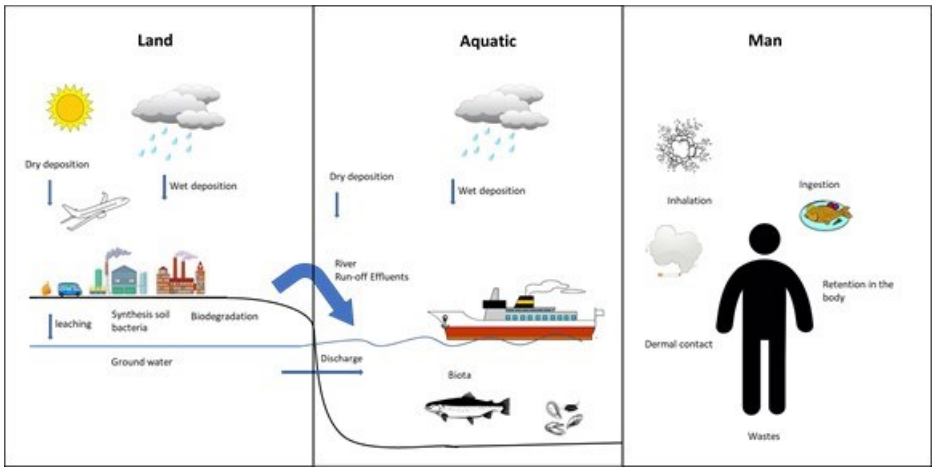

Figure 2: Dispersion of PAHs through the air, the terrestrial and aquatic environments.

PAHs with low vapor pressures, such as benzo(a)pyrene, are absorbed onto atmospheric particulates, whereas PAHs with higher vapor pressure, e.g. naphthalene, tend to be associated with the vapor phase. A significant correlation was also found between the concentration values of PAHs in the gas phases and the seasonal climate changes. In summer or in general in tropical regions, the PAHs amount increases in the gas phase, while during winter or in Artic regions the PAHs amount in particulate phase increases.[19]

Moreover, it has been shown that the PAH adsorption also depends on the types and sizes of the suspended particulates (e.g., soot, dust, fly-ash, pyrogenic metal oxides, pollens, etc.).[14]

The atmospheric PAHs contribute to the levels discharged into the coastal marine environment via dry and wet deposition on the surface of lakes, streams, and oceans. Once PAHs enter the marine environment, their distribution is largely controlled by their solubility and hydrophobicity, making sediments their primary repository. PAHs in water distribute between two phases, the dissolved and particulates fractions, according to the solubility of individual PAHs and the availability of suspended particulates. On the other hand, non-polar PAHs are immobilized into sediments and could be dissolved in small amounts when including in the pore water organic colloids.[20] As regarding the distribution pattern of PAHs in sediments, the 4-, 5-, and 6-ring PAHs are the predominant species, while in seawater the 2- and 3-ring PAHs are the most abundant species.

Furthermore, the specific isomeric ratios of PAHs are an useful tool to determine the dominance of petrogenic vs. pyrogenic sources.[21–23] Since PAHs are always emitted as a mixture, their relative molecular concentration ratios are characteristic of a given emission source. Most diagnostic ratios involve pairs of PAHs with the same molar mass and similar physicochemical properties,[24] that should undergo similar environmental fate processes and, thus, as a consequence, PAH diagnostic ratios could be a very useful tool for the identification of pollution emission sources.

The metabolic and photooxidation processes occurring in seawater affect the toxicity towards aquatic organisms. In fact, the ultraviolet light tends to enhance their toxicity. PAHs exert moderate to high acute toxicity towards aquatic organisms, causing adverse effects including tumours and reproduction and development disorders. Several studies have shown that PAHs are organic persistent environmental pollutants and they can be bioaccumulated.[25] Even though they are quickly metabolized and do not show biomagnifications[26,27] and bioaccumulation in fish muscle,[28,29] the study of the toxicity, environmental persistence, bioaccumulation, and trophic transfer of PAHs in aquatic ecosystem is in any case crucial to assess the ecological risk of contaminants.[13]

The occurrence of PAHs in marine organisms is influenced by their physicochemical proprieties that determine also their absorption and distribution in man. For example, their low solubility in water leads to a different distribution in the environmental compartments and therefore to a different uptake and accumulation in living organisms. Their volatility influences their dispersion ad transportation into atmosphere. Therefore, all these factors contribute to the high persistence in the environment and to the bioaccumulation in the food chain.[30] Since PAHs are highly lipophilic, they accumulate in lipid tissue of plants and animals. Although most organisms quickly metabolize PAHs resulting in no significant biomagnifications in the aquatic food chain, bivalves (e.g. mussel and oysters) may accumulate PAHs due to the large volumes of filter water in addition to their slow PAH metabolic pathway. Therefore, the main route of human exposure to marine PAHs is through marine living organisms that enter human diet. The benthic organisms could uptake PAHs from contaminated sediments and colloidal water and they could be directly consumed by humans or by higher predators.[31]

1.2.2 Effects on human health

IARC (International Agency for Research on Cancer) has classified sixteen PAHs as probable or possible (Group 1, 2A or 2B) human carcinogens able to cause mutagenic effects in humans and others animal species.[32–34] Benzo[a]pyrene is classified as carcinogenic (Group 1), while naphthalene, chrysene, benz[a]anthracene, benzo[k]fluoranthene and benzo[b]fluoranthene as possible carcinogenic (Group 2B). The major routes of exposure to PAHs include ingestion, inhalation and dermal contact (e.g. breathing ambient and indoor air, eating food containing PAHs, smoking cigarettes, or breathing smoke from open fireplaces). The inhalation exposure of PAHs could lead to the development of lung cancer.[35] Since PAHs are always emitted as a mixture, understanding the metabolism of individual PAHs, the possible toxic effect and the possible interaction mechanism, that can co-occur, is of particular interest. In fact, it has been shown that the metabolism of some PAHs (Phenanthrene, Fluoranthene and Benzo (a)pyrene) in single experiment differ from that in binary and ternary mixtures because of enzyme

competition.[36] Once PAHs enter the organisms, they undergo to metabolic pathway by the inducible enzymes of the cytochrome P450 family,[37] in particular CYP1A, that catalyse the addition of an oxygen atom to the PAH molecules. The PAHs epoxides can be conjugated with glutathione, or converted into phenols and diols, and excreted into the urine or the bile for rapid rejection over the gastrointestinal tract.[36]

Several factors contribute to the human health effects of PAHs, such as the duration and route of exposure, the amounts of PAHs one is exposed to, in addition to their relative toxicity.

The short-term health effects (acute) are not enough clear; study of exposures to high levels of PAHs have shown several symptoms, such as eye irritation, nausea, vomiting, diarrhoea and confusion,[38] even though it is not known which components caused these effects. Anthracene, benzo(a)pyrene and naphthalene are also known to cause skin irritation, while anthracene and benzo(a)pyrene are reported to be skin sensitizers. Health effects from chronic exposure largely depend on the way of exposure and generally include decreased immune functions, cataracts, kidney and liver damage (e.g. jaundice), breathing problems, asthma-like symptoms, lung function abnormalities and skin inflammation.[39,40]

The epoxide and dihydrodiol metabolites show more toxic effects than the relative unmetabolized PAHs, because of their ability to bind proteins and DNA.[41] These binding lead to biochemical disruptions and cell damage causing mutations, development disorder and cancer.

Studies of long-term exposure to mixtures containing PAHs have shown an increased risk of skin, lung, bladder and gastrointestinal cancers development.[41,42] In vivo studies have reported that Benzo(a)pyrene, the first carcinogen to be discovered, in addition to other PAHs, cause cancer in animals.[43]

Generally, the initial mechanism of cancer induction involves the immune suppression which lead to inflammation. It has been reported that the immune suppression was observed as the most frequent effect after exposure to PAHs.[44] Nevertheless, the immune-potentiation was also observed after atmospheric, topic exposure, or during in vitro experiments, by chancing some parameters in the design of the protocol used to study the effects of PAHs on the immune system. Experimental studies, conducted on the immune-toxicity after oral intake of food contaminated with PAHs, have shown the formation of DNA adducts in the lungs.[44]

Several studies report that the genotoxic effects, occurring in rodents and in vitro tests using mammalian (including human) cell lines after PAHs exposure, are due to the diol epoxide metabolite which react with DNA, inducing genotoxic damage.[45]

Benzo[a]pyrene and benzo[a]pyrene-7,8-diol-9,10-epoxide (BaPDE) were used as model compounds to investigate the mechanism of PAH mutagenicity.[46,47] The mechanism of interaction between benzo[a]pyrene, BaPDE and the nucleic acids mainly involves the exocyclic amino group of purines, leading to a prevalence of G>T transversions in mammalian cells in vitro,[48] in

vivo in transgenic mice[49] as well as in lung cancers of smokers,[50] probably reflecting the contribution of PAH to tobacco smoke carcinogenesis. Moreover, the formation of adducts between PAHs and DNA bases can induce frameshift mutations, deletions, S-phase arrest, strand breakage and several chromosomal alterations.[51]

As regarding the teratogenicity, it has been reported that ingestion of high levels of Benzo[a]pyrene during pregnancy in mice lead to birth defects and decrease body weight.[52] Also in human the exposure to PAH pollution during pregnancy is related to adverse birth effects, premature delivery, heart malformations as well as lower IQ and behaviour problems in child.[53]

1.3 POLYCHLORINATED BIPHENYLS (PCBS)

Polychlorinated Biphenyls (PCBs) are synthetic organic compounds consisting of carbon, hydrogen and chlorine atoms with the formula $C_{12}H_{10-x}Cl_x$. They were synthetized by catalytic chlorination of biphenyls leading to the formation of 209 congeners.[54] A subset of PCBs are classified as "dioxin-like PCBs" with a toxicity equivalent factors (TEFs) relative to 2,3,7,8-tetrachlorodibenzopara-dioxin(TCDD).

The manufacture of PCBs began in 1929 in the US and 1930 in Germany, and expanded to other countries after the world war II. Thanks to their excellent physical and chemical properties, they were employed in a wide range of industrial area, such as the electrical industry as heat exchange fluids, electric transformers and capacitors, and also as additives in paint, carbonless copy paper, sealants and plastics.[55] PCBs show high resistant to degradation and they persist in soil and waters for many years, bioaccumulating in cells due to their lipophilicity.

Therefore, due to their high environmental persistence, PCBs production, use and importation were banned by United States federal law in 1978 and by the Stockholm Convention on Persistent Organic Pollutants in 2001.

In Figure 3 the basic structure of PCBs and some congeners are reported. PCB molecular weight depend on the number of chlorine atoms attached to the biphenyl ring occurring during the manufacturing processes. The most toxic congeners carry the chlorine atoms at the 3,4-ortho positions and generally toxic congeners bear between 5 and 10 chlorine atoms.[54]

The degree of chlorination influences the proprieties of each PCB compound. In fact, PCBs are colourless highly mobile liquids or more viscous dark liquids.

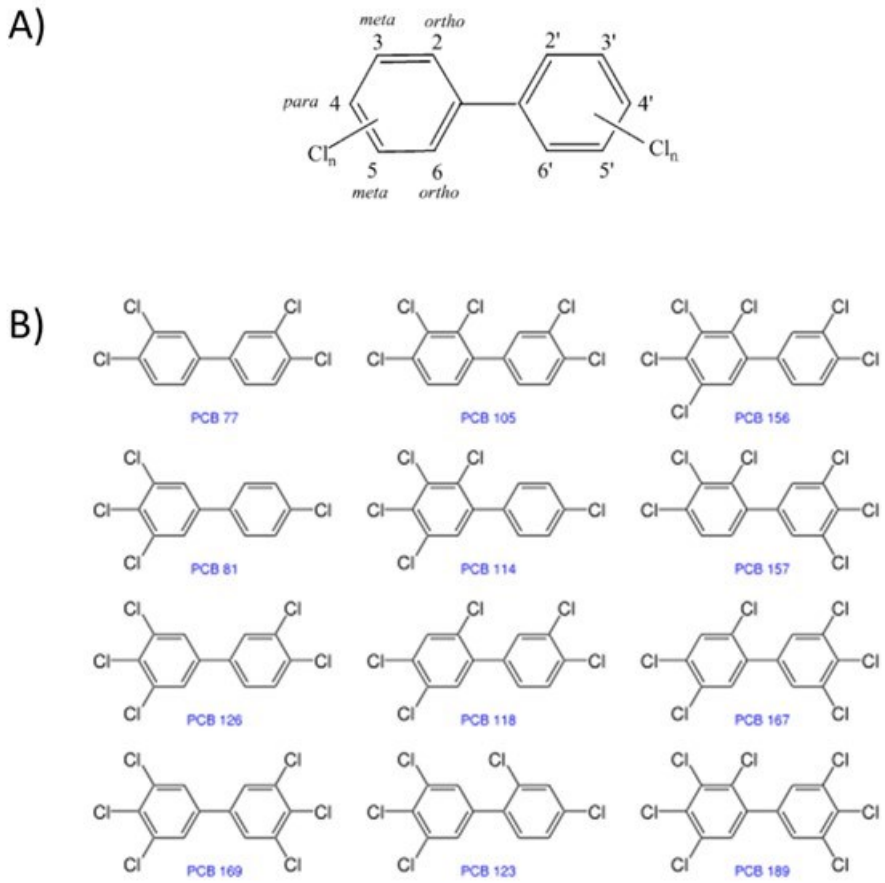

Figure 3: A) the basic structure of PCBs; B) some PCB congeners.

the mono-, di-, tri-, and tetra-chlorinated PCBs are colourless oily liquids, while pentachlorobiphenyls are heavy oils. Moreover, the number of chlorine atoms influences the flash point, that can be low, high or absent,[56] and the solubility in water. Usually, PCBs have poor solubility in water, which decreases on increasing the degree of chlorination.[57]

Their properties, such as thermal stability, chemical inertness, non-flammability, high electrical resistivity or high dielectric constant, make them useful for industrial applications.[54]

Unlike PAHs that could be of natural origin, PCBs are only of anthropogenic sources. As previously reported, they show high persistence in the environment and, thus, they could be found in air, water, soil and food. PCBs entered environment (air, water and soil) during their production, use and disposal, in addition to the accidental spills and leaks during their transport, hazardous waste incineration, steel and iron reclamation facilities.[58,59] PCBs have been detected mainly in the soil, where they can last for several years, with environmental cycling being the most source of these congeners.[58] Other sources of PCBs may be due to the repair and maintenance of PCB transformers, accidents, fires, spills, or disposal of PCB- containing material. Moreover, PCB levels indoor are always higher than outdoor levels due to the old appliances and electrical equipment containing PCBs.

1.3.1 Sources and Fate of PCBs in the Marine Environment

PCBs enter marine environment mainly by effluents (principally from paper factories), or as a result of local emergencies (e.g., leakage from equipment containing PCBs), whereas the atmospheric deposition of PCBs only rarely occurs.[60] Higher concentration values of PCBs are generally detected near urban and industrial area, while environmental cycling from sediment, air and land is the main source of PCBs in surface water. Once PCBs enter marine environment, they could distribute between dissolved and absorbed phases (e.g. sediments and colloidal organic matters), which can act as a reservoir gradually releasing PCBs into water. Studies on the octanol-water partition coefficient show that the congeners with the lower number of chlorine atoms less strongly adsorb than those with higher chlorine content. These last bind to organic matter, showing a slower degradation rate and extraction from sediments to water.[61,62] Moreover, the seasonal changes influence the transport of PCBs from sediments to water; for example in summer more PCBs are released into the water.[63]

The vapour pressure also influences the distribution of PCBs through the different environmental compartments; PCBs with a higher tendency to volatilize are transported from warmer regions to the colder ones such as the Arctic and Antarctic regions.[64,65]

The literature does not report any index or markers that could be used to define the sources of PCBs emission. This is because PCBs are synthetic compound that derive from many sources (e.g. emergencies and leakage from industrial installations, effluent discharges, and combustion) as a mixture of various congeners. Thus, unlike PAHs, the molecular ratios cannot be used to determine their origin.

In general, the number of chlorine atoms and the planarity of the molecule influence their distribution, defining their environmental fate. As previously reported, the increase in the number of chlorine atoms lead to high persistence and stability of PCB. Mono-, di- and tri-chloro derivatives undergo microbiological biodegradation faster than tetra-chlorobiphenyls. Thus, the highly chlorinated compounds show less desorption and remobilization from sediment. Moreover, it has been reported that non-ortho-substituted PCBs are characterized by partition coefficients that are higher than those of ortho-substituted compounds,[61] probably because the non-planar compounds weakly interact with the adsorption surface. Additionally, their large shape prevents their entry into the nanopores. The processes of biodegradation and transformation, volatilization, sorption and desorption to organic matter thus affect the environmental fate of PCBs and eventually their bioavailability and bioaccumulation. These processes give rise to mixtures of PCB congeners that differ to the ones originally deposited into the environment. In particular, biodegradation by bacteria and other microorganisms, which is the only known process able to degrade PCBs in the aquatic environment, occurs either aerobically (oxidative dichlorination) or anaerobically (reductive dichlorination) and by hydrolytic dehalogenation.[66] Theoretically, the

degradation processes involve the removal of chlorine atoms from biphenyl ring followed by ring cleavage and oxidation, producing CO_2, chlorine, and water. It has been reported that the ortho-substituted congeners are not abundant in aged environmental samples because they are readily degradable. On the other hand, the coplanar (non-ortho) "dioxin-like" and mono-ortho PCBs resist biodegradation due to their higher stability. As regards the biotransformation, some studies reported that fish have poor capability to biotransform PCBs.[67,68] However, hydroxylated PCBs have been detected in fish species,[69] producing major new concerns because these products may be more toxic than the parent compounds.[70]

After dechlorination, PCBs can be subjected to volatilization. The rate of PCB volatilization is influenced by several factors such as heat, air-flow coarse grain size, high water content, and enrichment in lighter ortho-chlorinated congeners.[66] As previously reported, the partitioning of PCBs between aqueous and solid phases is driven by the combination of binding processes (sorption) and mass transport processes (diffusion). The sorption of PCBs to dissolved organic matter makes them less bioavailable, even though PCBs could be available through the fraction sorbed to particulate organic matter, which is an important detrital food source. In addition, the fate of adsorbed PBCs depend on their structure, planar PCBs being less bioavailable because they strongly bind to particulate organic matter.[66]

Thus, either PCBs in water or in sediments are filtered by fish, crustaceans and molluscs. Since PCBs are highly lipophilic, they accumulate in fish, showing concentration values higher than in water, and they enter food chain. This phenomenon leads to bioconcentration, bioaccumulation and biomagnification. Bioaccumulation is the process that results from uptake by all exposure routes including transport across respiratory surfaces, dermal absorption (bioconcentration), and dietary absorption (biomagnification), and leads to concentrations of pollutant chemicals in organisms larger than those in the surrounding medium. The bioaccumulation factor (BAF) in fish is calculated as the ratio between the concentration values of pollutants in the organism and those found in water, similarly to the bioconcentration factor (BCF). Generally, the bioconcentration factor is obtained under laboratory conditions as a result of the uptake of chemicals from water through the respiratory surface and/or skin. Biomagnification occurs when residues of pollutants are transferred from smaller organisms by larger ones along the food chain, producing higher concentration values in organisms at higher trophic levels. The biomagnification factor (BMF) can be calculated as the ratio between the concentration values of pollutants in the organism and those found in the organism's diet. However, the mechanisms of biomagnification are not simple because many factors influence the uptake and elimination of a chemical after ingestion of contaminated food in addition to the different source of contamination that can co-occur.

Several studies report PCBs biomagnification with less ortho-substituted congeners being the most abundant up the food chain.[66,71] On the other hand, at higher trophic levels no enrichment of mono- and non- ortho -substituted congeners was observed. However, many coplanar congeners (i.e. PCB 77) are not detected with increasing trophic levels, probably because there are metabolizated.[64]

1.3.2 Effects on human health

Recently, the International Agency for Research on Cancer (IARC) has classified PCBs as carcinogenic to humans (Group 1).[72] In the last decade several studies have provided evidence for the PCB carcinogenicity, such the capacity to induce malignant melanoma. In addition, some associations between PCBs and non-Hodgkin lymphoma (NHL) and breast cancer were reported.[72]

Nowadays, the main route of exposure for humans is the ingestion of PCB contaminated food, especially due to the biomagnification.[58] Once inside the body, PCBs are transported through the blood stream to liver, and they accumulate in adipose tissue. The first step of biotransformation is carried out by cytochrome P450-dependent monooxygenation. Also in this case, the number and the position of chlorine atoms influence the metabolism rates of individual congeners. PCBs with four or fewer chlorines and those with adjacent unsubstituted meta, para positions show higher metabolism rates than five chlorines and meta, para substituted congeners. PCB metabolites can be transformed to potentially electrophilic and genotoxic compounds such as arene oxides and quinones. Despite these metabolism pathways, highly chlorinated congeners persist in the body for years.[72]

Several epidemiological studies were carried out considering different groups of workers exposed to PCBs and of not directly exposed population. Capacitor and transformer-manufacturing workers and in general workers exposed to PCBs were found to have increased risk of melanoma. However, other studies show that the development of adverse health effects are strictly influenced by the route of exposure, age, sex, and area of the body where PCBs are concentrated. In addition to NHL and breast cancer, other cancers, with sporadic positive correlation between PCBs exposure and cancer incidence risk, were found such as those of liver and biliary tract, extrahepatic biliary tract, lung and respiratory tract, thyroid, stomach, pancreas, colon and rectum, urothelial organs, uterus and ovary combined, as well as childhood acute lymphatic leukaemia, and multiple myeloma. As regarding the genetic and related effects, few studies are available with a limited information about the route of exposure, the concentration values of PCBs and in general of the protocol design used, making elusive the finding and without a clear conclusion.

The twelve “dioxin-like PCBs” show a strong affinity to bind the aryl hydrocarbon receptor. This binding is one of the key events that lead to carcinogenesis as well as to deregulation of cell-cycle control and cell proliferation, inhibition of apoptosis, suppression of cell–cell communication and adhesion. The most toxic agonist of the aryl-hydrocarbon receptor is the PCB-126, followed by PCB-169. On the other hand, non-dioxin-like PCBs induce the activation of the constitutive androstane or pregnane X receptor, perturbations in cell–cell communication and cell adhesion, as well as production of reactive oxygen species and activation of many pathway that play important role in tumor proliferation and progression.

Moreover, PCBs are well-known endocrine disruptors. In fact, several studies have shown an inverse correlation between the dioxin-like PCBs concentration values and circulating hormones (both estrogen and androgen hormones), suggesting an anti-estrogenic activity. On the other hand, less chlorinated PCBs and their hydroxylated metabolites show estrogenic activities, with different potency depending upon ortho chlorination and para hydroxylation.[73] Since PCBs are always present in mixture, the effects of PCBs on the hormone pathway are various and include the effects on reproductive function and behaviour, and on the neuroendocrine systems.

Furthermore, the exposure to high concentration values of PCBs lead to short-term (acute) effects such as skin disease (e.g. chloracne), liver damage, body weight loss, impaired immune function as well as damage of central nervous system.[58]

1.4 ORGANOCHLORINE PESTICIDES (OCPS)

Organochlorine pesticides (OCPs) are semi volatile chlorinated hydrocarbons that were extensively used from the 1940s through the 1960s in agriculture and for mosquito control. Dichlorodiphenyltrichloroethane (DDT), the best known OCP, has been widely used during the War World II in controlling human and agriculture pest. OCPs include several compounds with different structures and chemical proprieties that could be divided in five major groups reported in Figure 4: DDT and its analogues, hexachlorocyclohexane (HCH), cyclodienes and similar compounds, the caged structure mirex and chlordecone, toxaphene and related compounds.[74] DDT was banned from manufacture in 1972 by EPA after 4 decades of extensive global use because of its adverse effects on nontarget organisms. Nowadays, the Stockholm Convention allows DDT to be used in public health situations for disease vector control as recommended by and under the guidance of the World Health Organization (WHO).[9] Moreover, in 2001 the Stockholm Convention included some organochlorine pesticides such as aldrin, chlordane (CHL), dieldrin, endrin, heptachlor, dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), mirex and toxaphene in the list of the “dirty dozen” agents, banning their production and use. Additionally, in 2009 hexachlorocyclohexane (HCH) isomers (e.g. α-HCH, β-HCH and γ-HCH)

were added to this list due to their potential adverse effects on humans and ecosystems.[8] Even though almost all organochlorines have been banned, they are still detected in the environment due to their high persistence.

As reported previously, OCPs can be divided in five classes based on their structures. Among these, toxaphene is made up of approximately 200 chemicals, technical-grade chlordane is primarily made up of cis- and trans-chlordane, heptachlor, and cis- and trans- nonachlor,[75] in addition to more than 120 structurally related chemical pesticides.

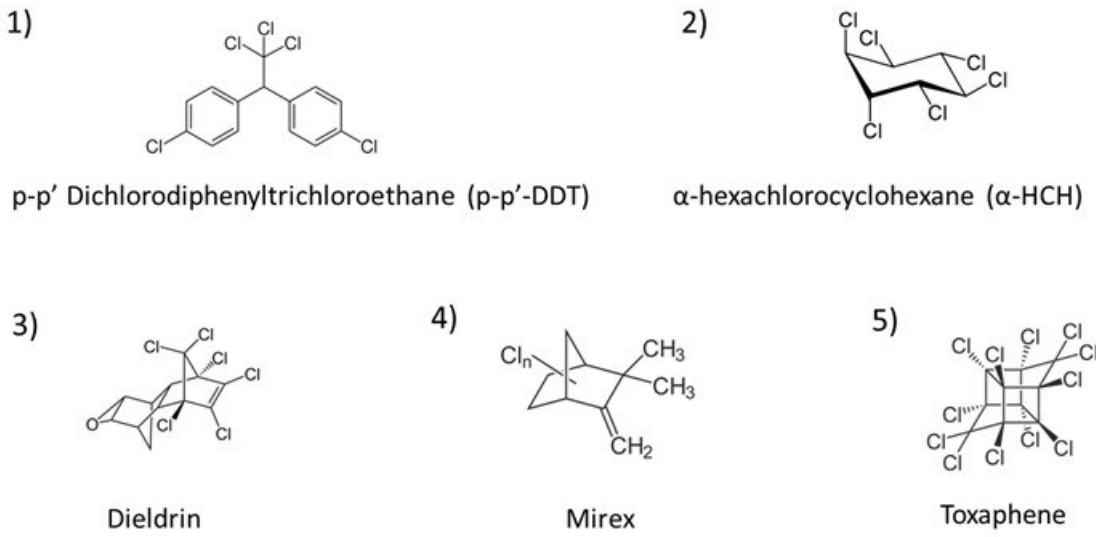

Figure 4: Organochlorine pesticides representative of the five classes

Furthermore, many OCPs are made up of mixtures of enantiomers or diastereomers. As example, the DDT family is made up of enantiomeric ortho- and para-isomers including DDT and its metabolites DDD and DDE. While cis-chlordane and trans- chlordane or α-endosulfan or β-endosulfan are examples of diastereomers.

OCPs generally show low solubility in water and high lipophilicity with a relative high octanol-water partition coefficient. Therefore, the highest concentrations of OCPs are found in lipids. The stored residues of OCPs could be mobilized under some conditions (e.g. stress or loss of body weight) and transported to the brain or other sites inducing serious adverse health effects.[74] Residues of organochlorines are detected in the fat tissues of biota throughout the world.

The main feature that distinguishes OCPs is their high persistence in the environment, which is based on their long half-life both in the environment and in the living organisms. Residues of OCPs may persist for years, decades and centuries in the environment.[76] However, either physical and biological factors affect their persistence such as temperature, light, pH, moisture as well as microbiological degradation, hibernation and reproductive activity.[74] Therefore, each OCP shows different persistence according to existing physical and biological conditions. Some bacteria

strains could break down the covalent C-Cl bond, leading to microbial degradation of OCPs. Even though DDT and its derivates are very stable, some earthworms can enhance the degradation of DDT in soil.[77] However, DDT is mainly metabolized into DDE, which shows the higher concentration values in organisms because of bioaccumulation. For example, Okay et al. (2011)[78] detected that the ratio of DDE/DDT in sediment and mussels in Turkey was 3.5 and 7.1, respectively.

Once inside the organism, several pesticides are rapidly metabolized into their metabolites that could exert the same, or higher toxicity of the parent compounds. Heptachlor is metabolized into heptachlor epoxide, which is more persistent and as toxic as the parent compound, [79] while aldrin breaks down into dieldrin, which is not toxic to insects, and its stereoisomer, endrin which instead has greater toxicity.[80]

Since OCPs volatize from soil and disperse into the atmosphere, travelling long distance, countries that still use the OCPs could be considered as a "new" source of these pollutants to the rest of the world.[81]

1.4.1 Sources and Fate of OCPs in the Marine Environment

OCPs can enter the aquatic environment, in the area where they are still used, through several processes, including deposition and air water exchange processes, ponding irrigation, leaching disposal of pesticide containers, and agricultural runoff.[82] From these sites, the pollutants can be recycled along various parts of the worlds. Since OCPs can volatilize from soil, they travel through atmosphere to different areas and can return to water by wet or dry precipitation. Once discharged into the marine environment, these pollutants distribute between the water, sediment and marine organisms, according to their physical-chemical proprieties. OCPs with high water solubility, such as HCHs, distribute primarily in water more than those with high lipophilicity. On the other hand, those that have high Kow values are adsorbed by the sediments or by colloidal organic particles, where they remain for many years. Additionally, some environmental factors (pH, temperature UV rays, wind etc) and some proprieties of living organisms of the area subjected to these anthropic pressures influence the distribution of OCPs.

Since OCPs have high persistence in the marine environment and long lifetime, they raise many concerns for marine organisms. Thus, the distribution of OCPs in the marine environment has been reported by several studies.[78,82–85]

Sediments constitute the environmental compartment with the highest concentration values of OCPs, and in general of POPs,[86] being the best matrix for pollutants monitoring. Sediments are heterogeneous media made up of detritus, organic and inorganic particles, which accumulate OCPs through several sources, including surface runoff, leaching and vapor phase, for long time. One of the major sources of POPs are glaciers, which contribute during the water run-off to the

transport of rock debris and sediments rich in POPs. Several factors affect the transport of OCPs from one environmental compartment to another, including leaching, precipitation, surface erosion, solubilization, volatilization, sorption, complication and above all, the water-solubility.[84] In fact, OCPs that are more water soluble have greater mobility towards long routs in sediment.
Due to their high lipophilicity and high persistence, OCPs bioaccumulate in marine organisms. Bioaccumulation process involves both bioconcentration, as the result of OCPs intake from water through gill, epidermis and similar way, and biomagnification only due to food intake. Several studies were carried out on bioaccumulation and bioconcentration of OCPs; for example, it has been reported that mirex residues are bioaccumulated at high rate from water to bird eggs,[87] and DDT and its metabolites may be bioconcentrated from surrounding water to America oysters (*Crassostrea virginica*) up to 700,000 times.[88] Biomagnification is related to the increase of concentration values of OCPs on increasing the trophic levels, through the food chain. For instance, OCPs in water are presented in concentration range of parts per billions and they may be assimilated by zooplankton entering the food chain ("big fish eat small fish") up to the highest trophic level, where they reach concentration values 10 million times those in water. This biomagnification process was well manifested during the 1980s and 1990s, when the population declines of many fish-eating raptors occurred. Concentration values of OCPS about 30- to 100-fold from prey to tissues and eggs of fish-eating birds were detected.[89] OCPs, especially DDT, were capable of affect eggshell thickness, causing severe depletions of many populations of fish-eating birds such as pelicans American bald eagles, peregrine falcons, and ospreys.[80]

1.4.2 Effects on human health

The effects on human health linked to the exposure of OCPs mainly derive from extrapolation of animal studies, which should be carefully evaluated, and from epidemiological studies. These animal tests are carried out in laboratory using OCP concentration values that sometimes did not respect the real environmental conditions. Thus, they should be carefully used for the human health risk assessment.
The predominant route of human exposure to OCPs is through the consumption of foods from areas of the world where they are still used as well as from those that have the potential of bioaccumulated OCP residues.
Several OCPs are classified as probable or potential carcinogens in humans. The International Agency for Research on Cancer (IARC) has classified DTT as a possible human carcinogen. Even though the effects of DDT at low environmental doses are unknown, this pollutant at higher dose induces vomiting, tremors or shakiness and seizures.[90] In addition, the US EPA lists DDT and its derivatives as probable human carcinogen with the following human health hazards: damage to

the liver, temporarily damage to nervous systems, reduced reproductive success, and damaged reproductive system.[91] Moreover, it has been reported that the amounts of DDT used during malaria control might cause preterm birth and early weaning. These findings in addition to several toxicological data[91] show that DDT and its derivatives have endocrine-disrupting properties, they can act as possible disruption in semen quality, menstruation, gestational length, and duration of lactation. Several acute effects of DDT exposure are reported by the study of Velbinger et al. (summarized by ATSDR, 2019),[91] in which volunteers were exposed to oral doses of DDT ranging from 250 to 1500 mg DDT. After six hours of exposure, the volunteers shown several adverse effects, including numbness of the lower part of the face, uncertain gait, malaise, cold moist skin, and hypersensitivity to contact. While higher exposed doses of DDT (1500 mg) have resulted in more severe adverse effects, such as loss of balance, dizziness, confusion, tremors, malaise (nausea), headache, fatigue, and severe vomiting, but in any case, resolvable 24 hours after exposure.

Since DDT was banned, its environmental concentrations have been decreasing. In fact, the daily intakes of total DDT, reported by the US Food and Drug Administration (FDA) total Diet Studies, have fallen averaging 6.51, 2.38, 1.49, and 0.97 μg/person/day for 1978–1979, 1979–1980, 1984–1986, and 1986–1991, respectively. Nevertheless, DDT and its derivates are still detected in some foodstuff, with p,p'-DDE being the most frequently detected isomer in studies of U.S. food samples.[91] In 2015, Wattigney et al. have found that the serum levels of p,p'-DDE have been decreased 5-fold in samples collected from 1976 to 2004.[92]

The *Minimal Risk Level* (MRL), i.e. "the estimated daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse, noncancer health effects over a specified duration of exposure", for DDT was set at 0.0005 mg/kg/day for oral acute exposure.[91] While the *Human health ambient water quality criteria* in drinking water determined by the US EPA is 0.000030 μg/L.[93] The directive 98/83/EC on the quality of water intended for human consumption set the pesticides values at 0.1 μg/L. As regarding the DDT concentration values in atmosphere, they should not exceed OSHA's Permissible Exposure Limit (PEL) of 1 mg/m^3.

Among the other classes of OCPs, toxaphene induces neurological effects, including convulsive seizures. Inhalation exposure of toxaphene in humans causes reversible respiratory toxicity, while oral exposure in animals causes damage to the liver, kidneys, spleen, adrenal and thyroid glands, central nervous system, and the immune system. Other toxicological studies carried out on animals report an increased incidence of thyroid gland cancer and liver cancer; thus toxaphene was set as a probable human carcinogen by the US EPA.[93]

In addition to cancer, the exposure to OPCs may causes several chronic effects, including disruption of hormones, enzymes, growth factors, and neurotransmitters as well as induction of key genes involved in steroid metabolism and xenobiotics. These chronic effects lead to complex

and not well-known changes such as alteration of the cell homeostatic condition that lead to oxidative stress and apoptosis or cell death related to several diseases conditions (immunodeficiency, autoimmune diseases, cancer, and reproductive problems).[94] OCPs may affect the DNA functioning and gene transcription by suppressing or reactivating some genes. These actions can lead to uncontrolled cell proliferation and cancer. These findings are objects of the ongoing research on animal models and thus should be carefully evaluated. [94] In fact, under laboratory conditions, it is possible to determine the effects of the individual OCPs and to distinguish them from each other, whereas, under field conditions, the wildlife population is seldom exposed to only one pollutant. During the last decade, many studies provide some evidence about the correlation effects among OCPs, PCBs and heavy metals. For example, Finkelstein et al. (2007)[95] analysed the blood of adult birds on Midway Island in order to detect PCBs, OCPs and heavy metals. They found all studied pollutants and a high correlation between OCPs and mercury concentration values that led to an increase in immune system responses, highlighting the difficulties in identifying the effect of individual pollutants.

Similarly to PCBs, OCPs can act as endocrine disruptors, interfering with the endocrine system at several points. Ensosulfan, lindano, DDT and its derivatives can bind the specialized receptors, inducing the effects normally produced by the hormones. In vitro studies show that DDT, lindane and endosulfan interfere with the biotransformation of estrogen, enhancing the level of estrogen. It has been reported that DDT and its derivatives affect the androgenic and estrogenic pathway, for example, o,p′-DDT mimics 17-β-estradiol, which is the most common estrogen in mammals;[96] the exposure to p,p′-DDT lead to lower testosterone concentration values in rats[97] and p,p′-DDE shows antiandrogenic proprieties, counteracting the effects of testosterone.[98] In addition, under the same conditions an increase in the blood concentration values of thyroid hormone T3, a decrease in the 4 and the induction of hypothyroidism were observed.[99]

CHAPTER 2

DEVELOPMENT OF ANALYTICAL METHODS USING GC-MS TO DETECT POPS IN BIOTA

This chapter focus on the development of analytical methods to detect POPs in marine biota using gas chromatography mass spectrometry (GC-MS). After a brief review of the analytical methods commonly used to detect POPs in complex environmental matrices, including the extraction, the clean-up and the instrumental analysis, the development of modified QuEChERS extraction GC-MS methodologies implemented for the quantification of 16 priority PAHs, 29 PCB and 23 OCPs in muscle fish was reported in the experimental section. Furthermore, the developed methods were validated according to ISO 17025, and the results are also shown.

2.1 INTRODUCTION

The analysis of organic pollutants in environmental matrices requires complex analytical chemistry procedures, primarily due to the extreme complexity of the environmental matrices and to the low detection limits that are often required by the environmental legislation (ppb to ppq). During the last decades, several analytical methods have been developed in order to detect POPs in different environmental matrices, including biota and foodstuffs. These methods have been published in academic articles as well as in standard operational procedures developed by several authorities such as the United States Environmental Protection Agency (USEPA) and the European Environment Agency (EU/CEN methods).[100]

It has to be considered that, since POPs are constituted by different classes of pollutants and since some classes are made up of numerous compounds, the chemical analysis of these pollutants is commonly addressed to the most important congeners, leading to a loss of information on the distribution of other pollutants. For example, the analysis of PCB mainly refers to the sum of seven indicators (PCB-28, 52, 101, 118, 138, 153 and 180), while PAHs analysis includes benzo(a)pyrene and the sum of 4 congeners, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene and indeno(1,2,3-cd)pyrene.

Furthermore, since marine biota is a complex environmental matrix, the commonly analytical procedures used to detect POPs in fish are generally made up of three phases: extraction, clean-up and instrumental analysis (Figure 5).

Figure 5: Flow chart of the analytical procedures commonly used to detect POPs in fish

The extraction of POPs from fish samples usually involves Soxhlet extraction,[101] pressurized liquid extraction (PLE), microwave assisted extraction[102] or sonication.[103] Marine fishes have a high lipid content that is often co-extracted during sample preparations, interfering with the detection and quantification of the target analytes. Therefore, several clean-up procedures have been reported to eliminate these matrix interferences, such as gel permeation chromatography (GPC)[104] or column clean-up with silica gel, alumina, florisil or combinations of the above sorbents.[105] However, these sample preparation techniques are typically time-consuming and require large amounts of solvents and multiple evaporation steps. Recently, there have been numerous attempts to find alternative multi-class and multi-residual procedures that are less time-consuming, use less solvent and/or enable miniaturisation. In 2003, the QuEChERs (Quick, Easy, Cheap, Effective, Rugged and Safe) method was first developed for the extraction of multi-class pesticide residues from fruits and vegetables.[106] The technique has hence been extended to other analytes including PAHs in a wide range of edible samples. Recently, several QuEChERs-based methods for the extraction of OCPs, PCBs, PBDEs and PAHs from fish and shrimp samples were reported.[107,108]

2.1.1 Extraction

During the samples preparation, one of the critical steps is exactly the extraction that allows the separation of the target analytes from the bulk matrix. The internal standards are generally added to the sample before the extraction in order to allow the quantification of the analytes and to calculate the extraction recovery. The internal standards should possess the same physical chemical proprieties of the native compounds, showing the same behaviour. Therefore, the isotopically labelled analogues of the target compounds are the best choice as internal standards, and they give rise to the most accurate and precise data. However, these isotope labelled standards are very expensive and sometimes not available for all compounds. In these cases, a representative compound, which share similar proprieties with the target compound, could be used. The accuracy and precision of the method can be strongly influenced by the number of internal standards used. Obviously, the higher the number of internal standards used, the better the method performance.
Soxhlet extraction, or an automated version called Soxtec, is the oldest method used for solid matrices, including biota. As previously indicated, pressurised liquid extraction (PLE), also known as accelerated solvent extraction (ASE),[104,109,110]microwave assisted extraction (MAE),[111,112] and sonication have also been used. PLE and MAE are more efficient than Soxhlet and sonication at the high temperatures and pressures used during the automated extraction process. Furthermore, supercritical fluid extraction (SFE),[113,114] based on the use of supercritical carbon dioxide, has been employed for the analysis of POPs in solid samples. The advantage of this last technique is the use of very little quantities of solvent in order to elute the analytes. The acid or basic digestion can be also used for solid environmental samples, followed by extraction with SPE or liquid/liquid extraction.[115,116] However, these procedures should be carefully employed, since the acid or basic conditions could degrade the compounds of interest.
However, many of the techniques discussed in this section, besides requiring advanced analytical expertise and lab equipment and relying on the use of chlorinated extraction solvents, are often hard-working, as well as time and solvent intensive. In 2003 the development of QuEChERS (quick, easy, cheap, effective, rugged and safe) sample processing procedures allowed to overcome these problems,[106] giving rise to a method that would be mainly used for the rapid (<15 min/batch) determination of pesticide residues in fruits and vegetables.[117] This method allows the sample extraction by the addition of acetonitrile and the subsequent liquid-liquid partitioning of the residues through the addition of magnesium sulphate, sodium chloride and various pH buffering agents specific for pesticides, such as sodium acetate or sodium citrate, and gives rise to a relatively clean extract. However, additional clean up steps are often required, that can be achieved by using various dispersive solid-phase extraction materials and magnesium sulphate to remove polar matrix components and water.[118] Although initially developed for the extraction of

pesticides from fruits and vegetables, the method has been modified to detect non-polar analytes like PAHs and PCBs and, in general, POPs in environmental matrices.[119,120]

2.1.2 Clean-up

During the extraction procedures, some organic matrix components can be co-extracted interfering with the detection of the target compounds and thus leading to more intense noise in the chromatograms. Therefore, extract clean up procedures are highly needed to remove the potential organic interfering compounds. These clean-up procedures should be highly selective in removing the possible interfering matrix compounds, without influencing the recovery yields of the target compounds. Some commonly used clean up techniques involve combinations of different sorbents such as silica, alumina, Florisil and carbon, or size exclusion materials (e.g. gel permeation chromatography).[104,105,121] Sometimes, prior to adsorption columns, an alkaline treatment or a treatment with sulphuric acid is required to remove the co-extracted lipids. Based on these techniques, EPA methods (3630C, 3160B and 3620 B) have been published and applied for the purification of organic extracts from solid environmental samples. Furthermore, some automated high throughput on-line clean-up systems have also been used to provide alternative and rapid techniques.[122–124] Gel permeation chromatography (GPC) is based on a size exclusion separation mechanism that involves the fractionation and removal of lipids (>500Å). These interfering components are not retained by the gels and therefore they elute first from the column, when biota matrices are treated. The most used gels are Bio-Beads S-X3 (200–400 mesh).[124] Unlike other methods employed for lipid removing, including saponification or sulphuric acid treatment, GPC is a non-destructive technique, which allows handling larger masses of lipids in each sample. However, GPC has the disadvantage of a not complete removal of lipids, requiring further clean-up steps generally based on liquid adsorption chromatography columns.[124] For example, the clean-up of PCBs and OCPs in extracts of salmon were carried out using Bio-Beads S-X3 column followed by a Florisil column.[125]

Solid phase microextraction (SPME), which is based on the partition of target analytes between a polymeric stationary phase coating a fused silica fiber and the sample, is commonly employed for the analysis of volatile compounds in the environmental matrices. Since this technique eliminates the use of organic solvents, nowadays it has many applications, for the detection of organic pollutant in different matrices, including water, soil, food and biological fluids.[126] However, the application of SPME to more complex matrices is not so effective, and therefore it can be used as clean-up/enrichment technique after different extraction procedures. For example, a clean-up procedure for the detection of OCPs in fish tissues after Soxhlet extraction was developed by Fidalgo-Used et al.[127]

Recently, the development of QuEChERS extraction and its widespread application to different matrices have led to new and faster sample clean-up techniques.[118] After the extraction of POPs from complex matrices, including biota samples, the lipid removing is achieved using different sorbent and a combination of these, such as C18, PSA (primary secondary amine), Florisil, graphitized carbon black (GCB) and zirconia- based sorbent (Z-Sep and Z-Sep Plus).[108,128]

2.1.3 Instrumental Analysis

Gas chromatography (GC) and high performance liquid chromatography (HPLC) are the most widely used techniques for environmental analyses. Since POPs are a group made of several compounds, the chromatography separation techniques should achieve accurate quantification, resolving all the target compounds. Moreover, the separation and detection techniques should allow to perform multi-class and multi-residual analysis. As regarding LC, reverse phase liquid chromatography is the most widely used separation technique, which usually employs C18 and C8 bonded silica columns. The advantages of LC include the possibility to analyse polar or ionic, non-volatile, and thermally labile analytes, as well as short run times. Generally LC systems are coupled with high sensitivity/selectivity tandem mass spectrometry detection systems, which makes them the first choice for polar pesticides detection.[105] However, many POPs have physicochemical properties that make them suitable for GC analysis, even though, since hundreds of halogenated compounds occur in complex matrices, the chromatographic separation is still a challenge. In fact, the use of a general GC column (e.g. 5% diphenyl dimethylpolysiloxane) could be a good choice for screening purposes, but it can't resolve all the congeners of toxaphene, PCBs and PCDD/PCDFs. Thus, the use of two different capillary columns can lead to better results, representing the best solution to resolve all target congeners.[129] Comprehensive two dimensional gas chromatography (GCxGC) was developed by John Philips in 20th century.[130] This technique can increase peak selectivity and sensitivity, reducing the times of analysis. The best chromatography separation is given by the use of two different columns containing different stationary phases, employing different physicochemical properties, e.g. boiling point and polarity, to resolve the analytes. This mechanism of separation allows the identification of several group of POPs during the same analytical run, reducing in many cases matrix interferences. Several studies report the application of GCxGC for environmental application and this technique can also be used for routine analysis of PCBs, organochlorines and chlorobenzenes as reported by the Ontario Ministry of the Environment and the Climate Change method MOE E3487.[129]

As regards the detection and quantification, the chromatographic systems can be coupled with several detectors that have different performances referred to sensitivity, selectivity and speed of analysis. The Electron Capture Detector (ECD) detectors are still employed for PCB and OCPs analysis, since these detectors show high sensitivity to halogenated compounds, providing

detection limits comparable to those of many MS systems. The development of MS techniques and their recent advances have resulted in commercially available instruments that are highly sensitive, selective and automated. Thus, MS has been widely used for POPs analysis, becoming the most commonly used detectors and replacing in many cases FID and ECD detectors. Furthermore, due to the mass separation of MS detectors, the co-elution problems between different analytes can be resolved (e.g. PCB-180 and BDE-47) and the application of the isotope dilution technique for POPs analysis can be carried out using MS.[131] In fact, a wide range of (^{13}C and ^{2}H) labelled POPs standards are now available. Nowadays, Gas Chromatographs coupled with single quadrupole Mass spectrometers have become the most used instruments in many laboratories due to their ability to achieve the lower concentration levels (ppb) for PCB and OCPs, employing the selected ion monitoring (SIM) mode. Another GC-MS technique that provide high sensitivity and specificity is the ion trap MS in the MS/MS mode by the monitoring of fragment ions.[100] However, the instrumental analysis of POPs in environmental complex matrices has been detailed in several reviews.[132–134] GC could be coupled with sophisticated tandem MS and high resolution MS (HRMS) instruments, which provide much higher specificity for individual congeners compared with GC-ECD and GC-MS. As an example, the determination of ultra-trace levels of PCDD/PCDFs and DL-PCBs was achieved by HRMS as reported by standard analytical protocols of USEPA.[135] Moreover, HRMS is mainly applied for qualitative analysis, since it can provide structure information on compounds present at only few pictograms levels.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and Reagents

Acetonitrile (purity > 99.9%), isooctane (purity > 99.0%), hexane (purity > 99.0%), sodium chloride (NaCl), magnesium sulphate ($MgSO_4$), sodium acetate (NaAC) and anhydrous calcium chloride ($CaCl_2$) were obtained from Sigma-Aldrich. SPE columns (BOND ELUT - C_{18}, 100MG 3ML, 50/PK) were purchased from Agilent Technologies.

Supel^{TM} QuEChERs products were prepackaged in anhydrous packages for EN 15662 containing 4 g magnesium sulfate ($MgSO_4$), 1 g sodium chloride (NaCl), 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate and 5 g sodium bicarbonate ($NaHCO_3$).

dSPE tube, containing 900 mg $MgSO_4$, 150 mg primary secondary amine (PSA) and 150 mg C18, were purchased from Sigma-Aldrich.

A 200 μg/mL standard stock solution of 16 PAHs includes Naphthalene (NA), Acenaphthylene (ACY), Acenaphthene (AC), Fluorene (FL), Phenanthrene (PHE), Anthracene (AN), Fluoranthene (FLA), Pyrene (PY), Benzo(a)Anthracene (BaA), Chrysene (CH),

Benzo(b)Fluoranthene (BbF), Benzo(k)Fluoranthene (BkF), Benzo(a)Pyrene (BaP), DiBenzo(a,h)Anthracene (DBahA), Benzo(g,h,i)Perylene (BghiP), Indeno (1,2,3-cd)Pyrene (IP), were obtained from Absolute Standards, Inc. As internal standards (IS), the naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, perylene-d12 standard mix (Absolute Standards, Inc.) was used, each component at 2000 μg/mL in methylene chloride.

A PCB standard mixture (Absolute Standards, Inc.) containing PCB-028, PCB-052, PCB-077, PCB-081, PCB-095, PCB-099, PCB-101, PCB-105, PCB-110, PCB-114, PCB-118, PCB-123, PCB-126, PCB-128, PCB-138, PCB-146, PCB-149, PCB-151, PCB-153, PCB-156, PCB-157, PCB-167, PCB-169, PCB-170, PCB-177, PCB-180, PCB-183, PCB-187, PCB-189 was used in this study (concentration 100 μg/mL). A 10 μg/mL mixture containing twelve isotopically labelled PCB (Wellington Laboratories), namely PCB 77L, PCB 81L, PCB 105L, PCB 114L, PCB 118L, PCB 123L, PCB 126L, PCB 156L, PCB 157L, PCB 167L, PCB 169L, PCB 189L was used as internal standard.

A customized pesticide standard solution, containing 23 OCPs (Aldrin, α-hexachlorocyclohexane, β- hexachlorocyclohexane, γ- hexachlorocyclohexane, δ-hexacholorocyclohexane, cis-Chlordane, trans-Chlordane, Chlorpyrifos-methyl, Dieldrin, 2,4'-DDE, 2,4'-DDD, 4,4'-DDD, 4,4'-DDE, 2-4'-DDT, 4,4'DDT, Dieldrin, α-Endosulfan, β-Endosulfan, Endosulfan sulfate, Endrin, Heptachlor, Heptachlor epoxide, isodrin, hexachlorobenzene), at the concentration of 10 μg/ml was prepared by appropriate dilution from stock solution of single (Sigma Aldrich) and mixed pesticides (Absolute Standard, Inc).

2.2.2 Standard and sample preparation

Working standard solutions including all the target compounds were prepared at various concentrations. A 1 μg/mL working solution of all 16 PAHs was prepared in isooctane. Calibration mixtures in the concentration ranges of 5, 10, 25, 50, and with 25 ng/mL of internal standards were prepared by successive dilutions of the working solution in hexane.

PCB working solution (1 μg/mL) was prepared by diluting original stock solutions in isooctane. The solutions for the Calibration curve (50, 75, 100, 125 and 150 ng/mL) with IS 100 ng/ml were prepared from the working solution.

OCP working standard solutions (1 μg/mL) were prepared by diluting the customized pesticide standard solution and the calibration curve was obtained, by using solutions opportunely diluted of the working solution (1 μg/mL) in the range 10-100 ng/mL.

A standard mixture of PAHs, PCB and OCPs at 1 μg/mL in acetonitrile was used as a spiking solution for 10 ng/g spiking level in the recovery experiments.

All the solutions were stored in closed vials at -20°C in the dark.

Samples of frozen codfish were obtained from local markets. The sample tissues (fresh or partially frozen) were homogenized using a mixer.

2.2.3 Optimization of sample extraction and clean-up

Homogenized fish fillets (5 g) in a 50 mL centrifuge tube were spiked with the standard analyte mix (OCPs, PAHs and PCBs) at 10 µg/Kg and were extracted in separate batches using three different methods in order to optimize the extraction procedure:

1) 10 mL of acetonitrile were added (in presence of 2 g NaCl), followed by vortexing for 1 minute and centrifugation at 5000 rpm for 5 minutes. The supernatant was transferred into a 15 ml tube and 5 ml of hexane were added. After vortexing and centrifugation at 5000 rpm for 5 minutes, the hexane layer was pipetted out, while the acetonitrile layer was evaporated using rotavapor. The residue was re-dissolved in 5 ml of methanol/H_2O (1:9) and was loaded onto a C18 solid phase extraction column, preconditioned with 3 ml of methanol and 3 ml of H_2O. Then, the analytes were eluted with 3 ml of methanol, evaporated to dryness and redissolved in 250 µl of isooctane.
2) 10 mL of acetonitrile were added (in presence of 2 g NaCl), followed by vortexing for 1 minute and centrifugation at 5000 rpm for 5 minutes. The supernatant was transferred into a 15 ml tube and 5 ml of hexane were added. After vortexing and centrifugation at 5000 rpm for 5 minutes, the hexane layer was pipetted out, while the extract was loaded onto a C18 solid phase extraction column, preconditioned with 5 ml of acetonitrile. The eluate was collected and was evaporated to dryness under a gentle stream of nitrogen, and the residue was redissolved in 250 µl of isooctane.
3) 10 mL of acetonitrile were added (in presence of 2 g NaCl), followed by vortexing for 1 minute and centrifugation at 5000 rpm for 5 minutes. The supernatant was transferred into a 15 ml tube and kept at -20 °C for 2 h. Then, the extract was filtered with nylon filter 0.45 µm and was loaded onto a C18 solid phase extraction column as reported in Method 2.

2.2.4 Final sample preparation methods

The optimized methods are here described:

1) PAHs and PCBs extraction: 5 g of homogenate codfish fillet were added to 10 mL of acetonitrile and mixed by vortex. Then, 4 g of anhydrous $MgSO_4$, 1 g NaCl, 5 g $NaHCO_3$ and 0.5 g $C_6H_6Na_2O_7$ 1,5 H_2O (Supel^{TM} QuEChERs) were added and the resulting mixture was mixed again. The extract was then centrifuged for 5 min at 5,000 rpm and, subsequently, 5 mL of the acetonitrile layer was transferred into a tube and freezed at -20°C for 3 h to remove

fatty acids. The extract was centrifuged for 5 min at 5,000 rpm, and the supernatant was transferred into a tube containing 1 g of $CaCl_2$ for the removal of free fatty acids co-extracted. The last clean up step was carried out using dSPE (900 mg $MgSO_4$, 150 mg PSA and 150 mg C18). The 15 ml dSPE tubes were vigorously vortex for 30 s and centrifuged for 5 min at 5,000 rpm.

Finally, the supernatant was evaporated to dryness under a gentle stream of nitrogen, and the residue was redissolved in 250 µl of isooctane

2) Pesticides extraction: 5 g of homogenate codfish fillet were added to buffered acetonitrile (1% acid acetic in presence of 4 g of anhydrous MgSO4, 1 g NaCl and 1.5 g NaAC). The clean-up step, consisting of remove of lipid by freezing the extract and dSPE, was carried out in similar way as described above.

2.2.5 GC-MS Analysis

The analyses of the extracted samples were performed using a GC coupled with a single quadrupole mass spectrometer (Thermo Finnigan Trace GC Ultra DSQ) managed by the Xcalibur software (ver 1.4 SR1). The analytical separations were performed using a Restek Rtx-5MS, 15 m, 25 mm ID, 0.25 µm capillary column. The carrier gas (Helium) flow was set at a constant rate of 2 mL/min and the injector, the ion source, and the MS transfer line temperatures were set at 280°C. The injected volumes were 1 µL in splitless mode. The analyses of OCPs, PCBs and PAHS were performed using three different GC-MS methods with the following oven temperature programs: for PAHs, the GC oven temperature program was set at initial temperature of 70 °C (1 minute hold), ramped to 300 °C at 6 °C/minute (5 minute hold), resulting in a total run time of 44 minutes; for PCBs, the GC oven temperature was set at initial temperature of 75°C (2 minute hold), ramped to 150°C at 25°C/minute and then to 200°C at 3°C/minute; for OCPs, the GC oven temperature was initially set to 80°C (1 minute hold), ramped to 150°C at 50°C/minute and to 300°C at 5°C/minute (8 minute hold).

The mass spectrometer operated in Selected Ion Monitoring (SIM) mode. SIM parameters (target and qualifier ions) and the average retention time for PAHs, PCB and OCPs are specified in Table 1, Table 2 and Table 3, respectively.

Table 1: Retention time, target and qualifier ions for PAHs. IS: Internal Standard

Analyte	RT (min)	Target ion (m/z)	Qualifier ions (m/z)
Naphtalene-d8 (IS)	8.85	136	68 - 108
Naphthalene	8.89	128	51 - 102
Acenaphthylene	14.60	152	76 - 126
Acenaphthene-d10 (IS)	15.16	162	164 - 80
Acenaphthene	15.27	153	76 - 126
Fluorene	17.27	165	82 - 139
Phenanthrene-d10 (IS)	20.87	188	94 - 80
Phenanthrene	20.93	178	152 - 89
Anthracene	21.14	178	152 - 89
Fluoranthene	25.58	202	174 - 101
Pyrene	26.39	202	174 - 101
Benzo(a)anthracene	31.14	228	200 - 114
Chrysene-d12 (IS)	31.18	240	236 - 120
Chrysene	31.26	228	202 - 114
Benzo(b)fluoranthene	35.05	252	126 - 113
Benzo(k)fluoranthene	35.16	252	126 - 113
Benzo(a)pyrene	36.11	252	126 - 112
Perylene-d12 (IS)	36.33	264	260 - 132
Dibenzo(a,h)anthracene	39.34	278	139 - 125
Indeno(1,2,3-cd)pyrene	39.35	276	138 - 125
Benzo(g,h,i)perylene	40.29	276	138 - 124

Table 2: Retention time, target and qualifier ions for PCBs. L: labelled

IUPAC NAME	CONGENERE	RT (min)	Target ion (m/z)	Qualifier ions (m/z)
PCB-028	2,4,4'-trichlorobiphenyl	10.35	256	186 - 256
PCB-052	2,2',5,5'-tetrachlorobiphenyl	11.66	292	220 - 290
PCB-095	2,2',3,5',6-pentachlorobiphenyl	14.32	326	254 - 328
PCB-101	2,2',4,5,5-pentachlorobiphenyl	15.40	326	254 - 328
PCB-099	2,2',4,4',5-pentachlorobiphenyl	15.62	326	254 - 328
PCB-081L	3,4,4',5-tetrachlorobiphenyl $^{13}C_{12}$	16.56	304	232 - 302
PCB-081	3,4,4',5-tetrachlorobiphenyl	16.58	292	220 - 290
PCB-077L	3,3',4,4'-tetrachlorobiphenyl $^{13}C_{12}$	17.02	304	232 - 302
PCB-077	3,3',4,4'-tetrachlorobiphenyl	17.04	292	220 - 290
PCB-110	2,3,3',4',6-pentachlorobiphenyl	17.06	326	254 - 328
PCB-151	2,2',3,5,5',6-hexachlorobiphenyl	17.68	360	290 - 325
PCB-123L	2',3,4,4',5-pentachlorobiphenyl $^{13}C_{12}$	18.23	338	264 - 336
PCB-123	2',3,4,4',5-pentachlorobiphenyl	18.25	326	254 - 328
PCB-149	2,2',3,4',5',6-hexachlorobiphenyl	18.27	360	290 - 325
PCB-118L	2,3',4,4',5-pentachlorobiphenyl $^{13}C_{12}$	18.35	338	264 - 336
PCB-118	2,3',4,4',5-pentachlorobiphenyl	18.37	326	254 - 328
PCB-114L	2,3,4,4',5-pentachlorobiphenyl $^{13}C_{12}$	18.85	338	264 - 336
PCB-114	2,3,4,4',5-pentachlorobiphenyl	18.89	326	254 - 328
PCB-146	2,2',3,4',5,5'-hexachlorobiphenyl	19.26	360	290 - 325
PCB-153	2,2',4,4',5,5'-hexachlorobiphenyl	19.55	360	290 - 325
PCB-105L	2,3,3',4,4'-pentachlorobiphenyl $^{13}C_{12}$	19.64	338	264 - 336
PCB-105	2,3,3',4,4'-pentachlorobiphenyl	19.65	326	254 - 328
PCB-138	2,2',3,4,4',5'-hexachlorobiphenyl	20.90	360	290 - 325
PCB-126L	3,3',4,4',5-pentachlorobiphenyl $^{13}C_{12}$	21.31	338	264 - 336
PCB-126	3,3',4,4',5-pentachlorobiphenyl E	21.34	326	254 - 328
PCB-187	2,2',3,4',5,5',6-heptachlorobiphenyl	21.77	394	324 - 359
PCB-183	2,2',3,4,4',5',6-heptachlorobiphenyl	22.00	394	324 - 359
PCB-167L	2,3',4,4',5,5'-hexachlorobiphenyl $^{13}C_{12}$	22.17	372	151- 302
PCB-167	2,3',4,4',5,5'-hexachlorobiphenyl	22.18	360	290 - 325
PCB-128	2,2',3,3',4,4'-hexachlorobiphenyl	22.32	360	290 - 325
PCB-177	2,2',3,3',4,5',6'-heptachlorobiphenyl	22.95	394	324 - 359
PCB-156L	2,3,3',4,4',5-hexachlorobiphenyl $^{13}C_{12}$	23.14	372	151- 302
PCB-156	2,3,3',4,4',5-hexachlorobiphenyl	23.16	360	290 - 325
PCB-157L	2,3,3',4,4',5'-hexachlorobiphenyl $^{13}C_{12}$	23.34	372	151- 302
PCB-157	2,3,3',4,4',5'-hexachlorobiphenyl	23.35	360	290 - 325
PCB-180	2,2',3,4,4',5,5'-heptachlorobiphenyl	23.77	394	324 -359
PCB-169L	3,3',4,4',5,5'-hexachlorobiphenyl $^{13}C_{12}$	24.35	372	151 - 302

IUPAC NAME	CONGENERE	RT (min)	Target ion (m/z)	Qualifier ions (m/z)
PCB-169	3,3',4,4',5,5'-hexachlorobiphenyl	24.36	360	290 - 325
PCB-170	2,2',3,3',4,4',5-heptachlorobiphenyl	24.60	394	324 - 359
PCB-189L	2,3,3',4,4',5,5'-heptachlorobiphenyl $^{13}C_{12}$	25.36	336	168 - 406
PCB-189	2,3,3',4,4',5,5'-heptachlorobiphenyl	25.38	394	324 - 359

Table 3: Retention time, target and qualifier ions for OCPs. L: labelled

Analyte	RT (min)	Target ion (m/z)	Qualifier ions (m/z)
α - Hexachlorocyclohexane (HCH)	5.17	183	181 - 219
Hexachlorobenzene	5.30	284	142 - 286
β -HCH	5.75	219	183 - 181
γ -HCH	5.84	219	181 - 183
δ -HCH	6.41	219	181 - 183
Chlorpyrifos-methyl	7.41	286	47 - 288
Heptachloro	7.48	100	272 - 274
Aldrin	8.37	66	79 - 263
Isodrin	9.17	193	66 - 263
Heptachlor epoxide	9.55	353	351 - 355
Cis-chlordane	10.25	373	375 - 377
2,4'-DDE	10.53	246	248 - 318
β -Endosulfan	10.59	241	237 - 239
Trans-Chlordane	10.72	375	65 - 237
Dieldrin	11.39	79	81 - 263
4,4'-DDE	11.53	246	176 - 318
2,4'-DDD	11.79	235	199 - 237
Endrin	12.02	79	67 - 263
α -Endosulfan	12.37	241	237 - 239
4,4'-DDD	12.88	235	165 - 237
2,4'-DDT	12.92	235	165 - 236
Endosulfan-Solfato	13.78	387	272 - 229
4,4'-DDT	14.06	235	165 - 236

2.2.6 Validation

The developed methods were validated according to the UNI CEI EN ISO 17025 guideline. The evaluated parameters were precision, accuracy, trueness, linear dynamic range, limit of detection (LOD), limit of quantification (LOQ) and recoveries.

2.2.6.1 Precision

The precision is generally expressed as an absolute or relative standard deviation from the mean of repeated determinations. The repeatability of the methods, representing the precision under the same operating conditions by the same operator, over a short interval of time, in the same laboratory, was evaluated at two concentration levels (CV%) as percent coefficient of variation (CV%) from the mean values.

2.2.6.2 Accuracy and Trueness

The accuracy represents the percent deviation from the accepted reference value. The accuracy was calculated by comparing the concentration values determined in the spiked samples used for the precision tests with their nominal concentration, while the trueness was calculated by comparing the mean values of the spiked samples with their nominal concentration.

2.2.6.3 Linearity

Five calibration solutions were used to spike the codfish homogenates before the extraction procedure. Concentration ranges were 10-100 μg/L (10, 25, 50, 75, 100 μg/L) for PAHs, 50-150 μg/L (50, 75, 100, 125, 150 μg/L) for PCBs and pesticides, each concentration was determined in duplicate. Calibration curves were obtained by plotting the peak area ratio of the compound signal to the IS versus nominal concentration. The measurements for the quantification of pesticides were carried out without IS, thus calibration curves were obtained by plotting the peak area of the compound versus nominal concentration.

2.2.6.4 Limit of Detection (LOD)

The limit of detection (LOD) is the minimum concentration value that provides a statistically distinguishable signal from the background noise, with a specified significance level (α=5%). Thus, at LOD value, the analyte can be detected but not quantified with reliable accuracy and precision. The LOD must be calculated in sample matrix and several useful approaches has been reported.[136] In this study, the LOD was evaluated using the criteria “3.3s standard deviation” (e.g.

3.3 times the standard deviation of the blank divided by the slope of the calibration curve), by following formula:

$$\text{LOD} = \frac{Ksb}{m}$$

where k=confidence factor (equal to 3.3); sb=standard deviation of the blank, calculated at the time window in which the analyte is expected (n=10); m=slope of the calibration curve.

2.2.6.5 Limit of Quantification (LOQ)

The LOQ is the lowest concentration value that can be quantified with accuracy and precision under the fixed acceptance criteria (normally 10–20%). As for LOD, LOQ was determined in matrix sample, using the "10s Standard deviation" criteria (e.g. 10 times the standard deviation of the blank (sb) divided by the slope of the calibration curve):

$$\text{LOQ} = \frac{Ksb}{m}$$

where k=confidence factor (equal to 10); sb=standard deviation of the blank; m=slope of the calibration curve.

2.2.6.6 Recovery

Recovery represents the percentage of the analyte still present after sample workup, as compared with the amount of the analyte initially contained in the sample. Recovery was determined within the same analytical session by preparing 3 sets of samples, each including 5 replicates. In the first set named "spiked", blank codfish sample were spiked with the standard solutions of target analytes at two concentration levels (20 and 80 µg/Kg for PAHs, 60 and 130 µg/Kg for PCBs and OCPs) before the extraction step; in the second set named "post-spiked", working solutions of the analytes were added (at the same concentrations) on the blank codfish extracts; the third set "matrix" was represented by the codfish sample extracted. The recovery of the analytes was calculated by the ratio between the peak area ratio of the compound signal to the IS determined after its extraction (spiked) and that determined on the spiked extract (post-spiked).

$$\text{Recovery} = [(\text{Area S/Area IS})_{\text{spiked}}/(\text{Area S/Area IS})_{\text{post-spiked}}]*100$$

2.3 RESULTS AND DISCUSSION

2.3.1 Development of GC-MS analytical methods

Three different GC-MS methods were developed for determining 29 polychlorinated biphenyls (PCBs), including 13 dioxin like (DL) congeners (PCB 77, PCB 81, PCB 105, PCB 114, PCB 118, PCB 123, PCB 126, PCB 156, PCB 157, PCB 167, PCB 169, PCB 170), 23 organochlorine pesticides (OCPs) and 16 polycyclic aromatic hydrocarbons (PAHs) in fish muscle tissues. In order to improve the resolution of the analytes, several methods were tested. In particular, GC oven, transfer line and inlet temperatures were optimized for each class of analytes. In this first step, the acquisition was carried out in full scan mode. Once the GC parameters have been optimized, the acquisition was carried out in Selected Ion Monitoring (SIM) mode. Figure 6, Figure 7, Figure 8 show chromatographic profiles for 16 PAHs, 29 PCBs and 23 OCPs, respectively.

Since a GC column that can resolve at the same time all the congeners of PCBs, OCPs and PAHs is not available, the three developed methods provide anyway good resolution for most of the target analytes. However, the used GC columns have a general low polarity stationary phase (e.g. 5% diphenyl dimethyl polysiloxane), leading to the co-elution of some PCBs and PAHs. As shown in Figure 7, the PCB chromatogram peaks are overlapped between 21.35 min and 24.38 min, however the acquired SIM ions are different for most of the co-eluted congeners and therefore these compounds can be adequately detected. Furthermore, slight interferences occur between the hexachlorobiphenyl PCB-167 and PCB-128, which have the same target and qualifier ions (m/z) and close retention times.

As regards PAHs separation, all analytes were adequately resolved except some isomeric compounds, such as Benzo(b)fluoranthene, Benzo(k)fluoranthene, Chrysene and Benzo(a)anthracene. These isomeric compounds require specific GC capillary column, since they show close retention times and the same selected ions monitored.

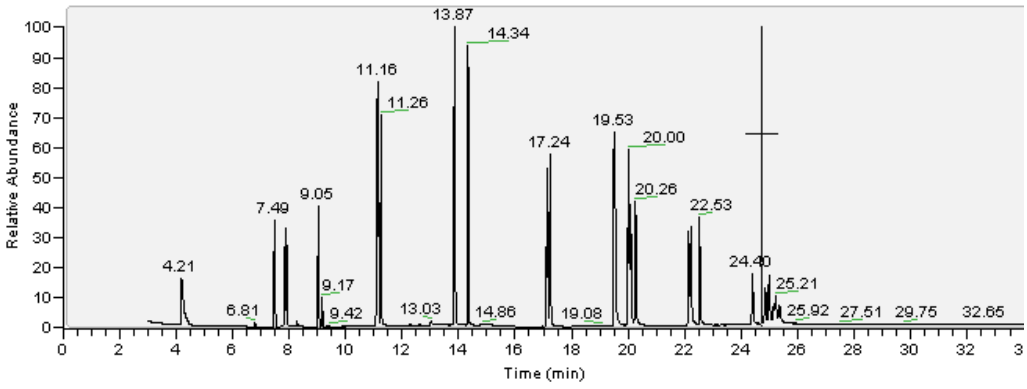

Figure 6: chromatogram of PAHs standard solution (100 µg/L) acquired in SIM mode

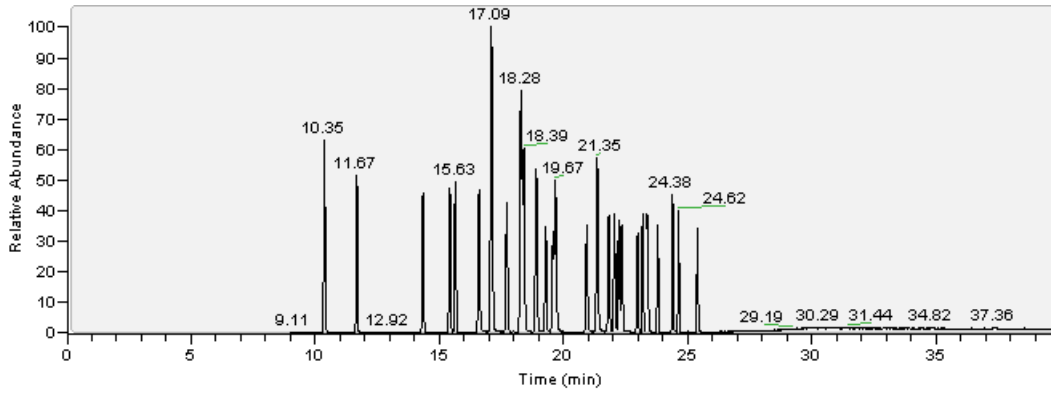

Figure 7: Chromatogram of PCBs standard solution (100 µg/L) in SIM mode

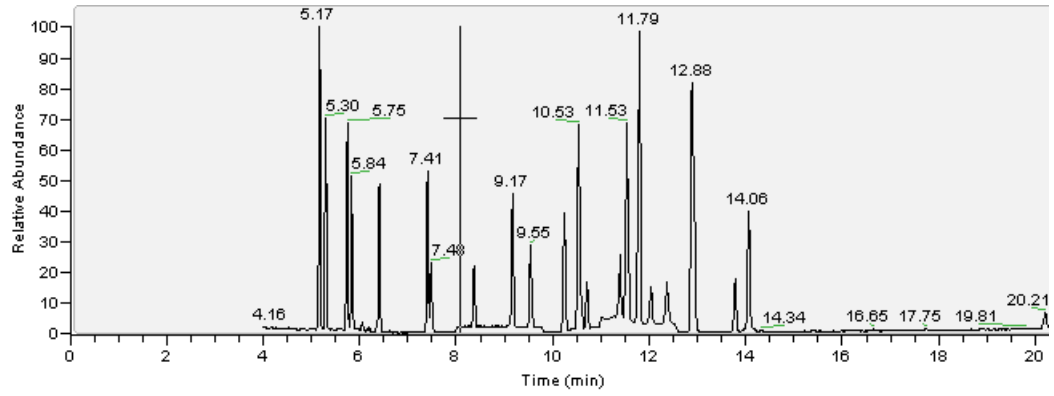

Figure 8: Chromatogram of OCPs standard solution (100 µg/L) in SIM mode

2.3.2 Extraction methods

Regarding the analytical methodologies for the quantification and unequivocal identification of these organic compounds, one of the golden keys has always been the sample pre-treatment improvement, through interference reduction techniques in order to obtain reproducible and accurate data, which are especially challenging for complex matrices. Different extraction and clean-up methods, based on solid phase extraction (SPE) and QuEChERS method, were investigated to enhance the recoveries of analytes. Considering the high fat fish content, a small sample size of 5 g was used for extraction to minimize the co-extractives (fats/lipids) without compromising the method performance.

The extraction methods 1 and 2 gave low recovery (<40%) for several test analytes, probably because a high amount of hexane (e.g. 5 mL) did not reduce the lipid co-extractives in the acetonitrile layer any further, but instead reduced the recovery of POCs, PAHs and PCBs. Method 3, freezing the extract in order to remove lipid co-extractives at low temperature, gave better

recovery (50-60%) than methods 1 and 2, but it was still not satisfactory. Based on these preliminary results, two other methods that involve the addition of a further clean-up step with $CaCl_2$ to remove the fatty acid co-extractives were evaluated, as described in the experimental part.

The best methods were based on a modified QuEChERS extraction, as reported in section 2.2.4, with slight difference compared to similar existing extraction and clean-up techniques.[108,137] A comparison of extracted ion chromatograms (Phenanthrene and Antracene, 178 m/z) of spiked fish matrix (20 µg/Kg) extracted with the three different approaches and with the final optimized methods is reported in Figure 9. Considering signal intensities and the corresponding areas, the final method shows better recovery than the other methods tested and a higher Signal to Noise (S/N) ratio for the tested analytes.

Pesticides extraction was carried out using a different QuEChERS methods because the final method used for PAHs and PCBs gave low recovery (<40%) for several test analytes. Figure 10 shows the overlaid Total Ion Chromatogram (TIC) of fish extracts obtained with these different extraction and clean-up methods for pesticides analysis. Black TIC corresponding to the final optimized method showed significant removal of co-extractives matrix interferences.

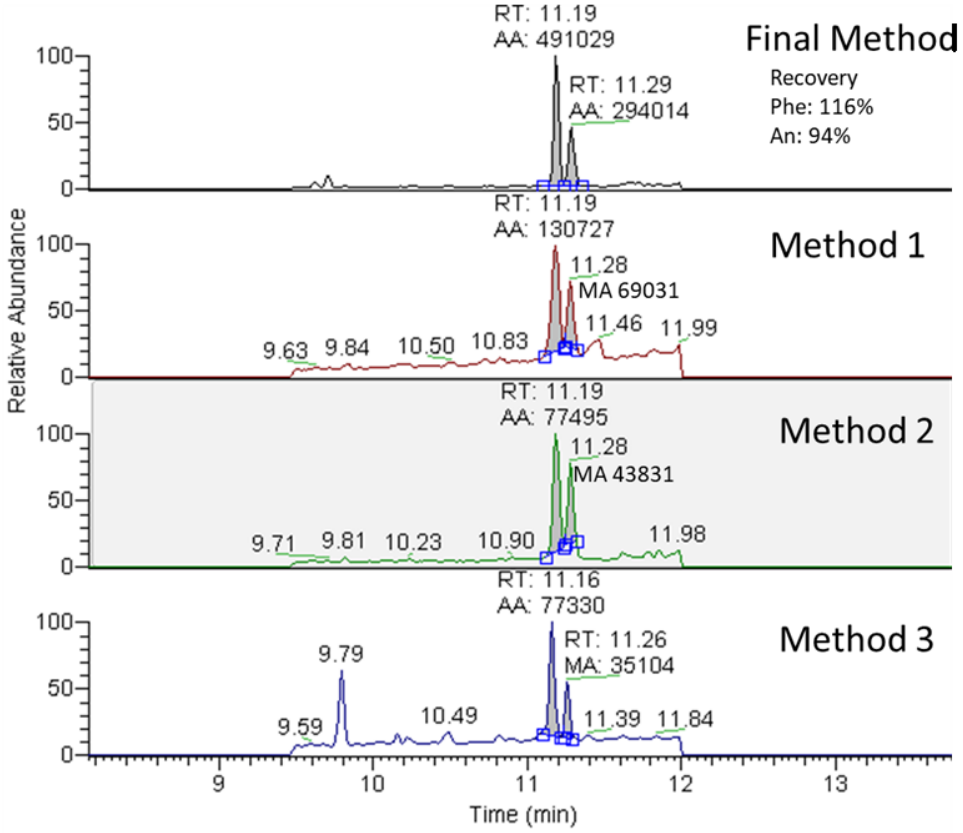

*Figure 9: Comparison of extracted ion chromatogram (178 m/z) of spiked fish matrix with PAH standard solution (10 µg/Kg). RT 11.16 min: Phenanthrene (Phe); RT 11.26: Anthracene (An); recovery: [(Area S/Area IS)spiked/(Area S/Area IS)post-spiked]*100*

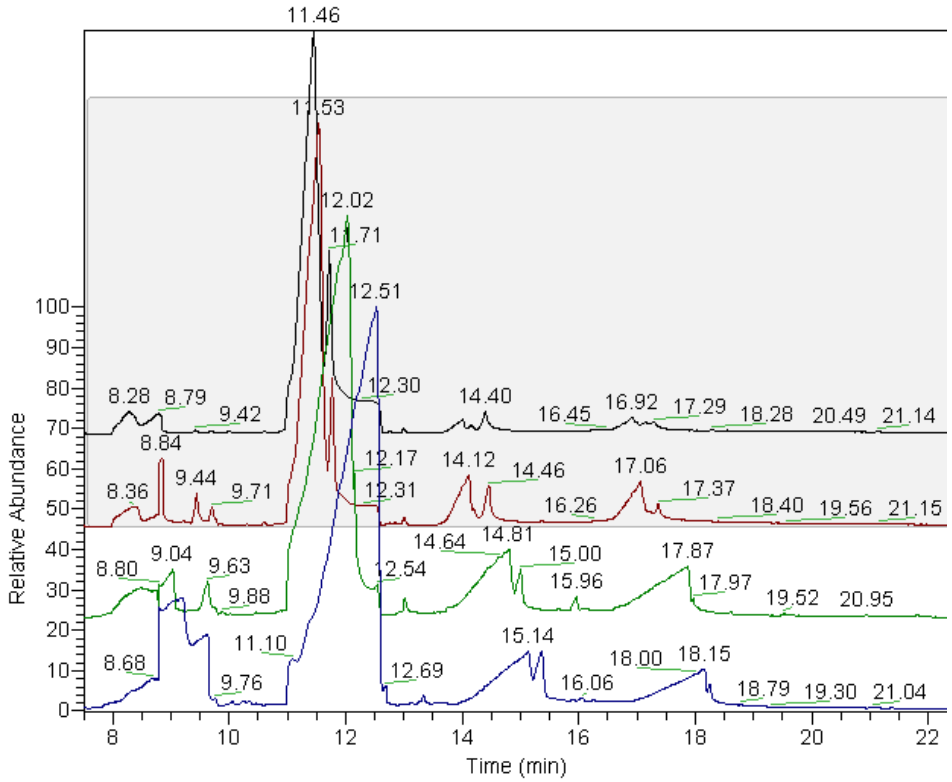

Figure 10: Comparison of different extraction and clean-up approaches for pesticide analysis showing effect of clean-up on removal of matrix co-extractives where BLUE: TIC of control fish matrix with method 3. GREEN: TIC of control fish matrix with method 2; RED: TIC of control fish matrix with methods 1; BLACK: Total Ion Chromatogram (TIC) of fish matrix with the final optimized method.

2.3.3 Validation

Validation means confirmation by examination and provision of objective evidence that the requirements for a specific intended use are fulfilled. After the optimization of extraction procedures, the methods were validated following the UNI CEI EN ISO 17025 guidelines, checking the criteria set out in the Environmental Chemistry Methods and Associated Independent Laboratory Validation OCSPP 850.6100 (US-EPA).[138]

The validation parameters generally required for quantitative procedures were determined, including, linearity, accuracy, precision, limit of quantification (LOQ), limit of detection (LOD) and recovery.

Table 4 report the R^2, LOD, LOQ and recoveries for PAHs. Calibration curves were obtained by plotting the peak area ratio of the compound signal to the IS versus nominal concentration. Calibration curves obtained for this class of pollutants well fit and showed a good linearity over the range of interest (1, 2.5, 5, 7.5 and 10 µg/kg) for almost all analytes. As an example, some calibration curves are reported in Figure 1. Unfortunately, the resulting R^2 values for some analytes are lower than 0.980, especially for some isomeric PAHs, e.g. Benzo(a)anthracene,

Chrysene, Benzo(b)fluoranthene Benzo(k)fluoranthene, that have close retention times resulting in poor resolution.

The LOD and LOQ values reported in Table 4 are in the range of 0.25–0.50 and of 0.75-1.50 μg/kg, respectively. The higher molecular weight PAHs showed higher LOD and LOQ values than the lower molecular PAHs because of the matrix interferences lead to a lower S/N ratio at low concentration values.

Recoveries, calculated at two concentration levels (2 μg/kg and 8 μg/kg), are also reported in Table 4 and range from 75% to 116%, complying the criteria of 70 -120% set out by US-EPA.[138]

PCBs validation parameters, including R^2, LOD, LOQ and recoveries are reported in Table 5. Calibration curves were obtained by spiking codfish tissues with the appropriate volume of the spiking standard solution, covering the range 5-15 μg/kg (5, 7.5, 10, 12.5 and 15 μg/kg). The developed methods provide good fit and linearity for PCBs and the coefficient of variation R^2 ranges from 0.980 to 0.998. The LOD and LOQ values are in the range of 1.00-1.40 μg/kg and of 3.30-4.70 μg/kg, respectively (Table 5). Recoveries, reported in Table 5 and calculated at two concentration levels (8 and 14 μg/kg), ranged from 62 to 89 %, show a lower extraction efficiency for this class of pollutants. The incomplete recovery for some analytes and IS from fish matrix could be probably attributed to a partial adsorption of these organic lipophilic compounds with the fat and lipid components, removed during the sample clean-up.

Accuracy, trueness and precision (CV%) values for PAHs and PCBs are reported in Table 6 and in Table 7, respectively. For both class of pollutants, experimental trueness values proved satisfactory, yielding percent deviations within the range 100 ± 15%. For PAHs, the accuracy evaluation proved satisfactory, even though a few single determinations exhibited biases slightly higher than 15%, but always within the range of 20%. While for PCBs, few accuracy values are higher than 20% (e.g. PCB 105, PCB 187, PCB 170 and PCB 189 at low level and PCB 149 and PCB118 at high level).

All experimental CV% are lower than 15% for PAHs and PCBs (Table 6 and in Table 7), proving that the methods have good within-run precisions. Only Chrysene and Benzo(b)fluoranthene show CV% higher than 15 % (e.g. 15.8 and 15.4 %, respectively).

As regarding OCPs validation, some problems occurred since the quantification of pesticides was carried out without IS. Initially, deuterated PAHs were evaluated as internal standard for pesticide analysis as reported in EPA METHOD 8270E, revision 6, 2018, but the validation process did not provide satisfactory results. Thus, for this class of pollutants, only recoveries were evaluated by comparing the peak area of the compound obtained after its extraction (spiked) and that determined on the spiked extract (post-spiked). The recovery data are shown in Table 8 and ranged from 70-120 % for many analytes. Unfortunately, some pesticides, including aldrin, isodrin, endrin, β-endosulfan, α-endosulfan and endosulfan sulfato, show lower recoveries

(<50%), probably due to some matrix interferences. In fact, the chromatogram of blank extract shows at the retention times of the expected peaks higher noise.

Therefore, for quantitative purposes, the recovery data were used to quantify the concentrations of OCPs in quality control (CQ) samples by using an external standard calibration curve.

Considering the validation results, the three developed methods show different performances. The PAHs method show good linearity and recoveries for almost all analytes, except for some isomeric compounds (Benzo(a)anthracene, Chrysene, Benzo(b)fluoranthene Benzo(k)fluoranthene); the PCBs method provides good linearity but shows lower recoveries (range 62-89%) and the OCPs methods could be used only with external calibration and manly for qualitative purposes, considering the recovery of analytes. Anyway, the validation process of PAHs and PCBs methods proved satisfactory, with some exceptions, and these methods could be employed both for qualitative and quantitative purposes.

Table 4: Validation parameters, including R^2, LOD, LOQ and Recovery (n=5, mean ± standard deviation) for PAHs.

PAHs	Internal Standard (IS)				Recovery (%)	
		R^2	LOD (µg/kg)	LOQ (µg/kg)	C_{PAHs} 2 µg/kg	C_{PAHs} 8 µg/kg
Naphthalene	Naphtalene-d8	0.978	0.25	0.75	79 ± 3	80 ± 4
Acenaphthylene	Acenaphthene-d10	0.988	0.25	0.75	111 ± 7	82 ± 3
Acenaphthene		0.983	0.25	0.75	96 ± 2	103 ± 7
Fluorene	Phenanthrene-d10	0.992	0.25	0.75	108 ± 3	95 ± 5
Phenanthrene		0.998	0.25	0.75	116 ± 2	98 ± 3
Anthracene		0.999	0.25	0.75	86 ± 4	87 ± 4
Fluoranthene	Chrysene-d12	0.993	0.40	1.20	102 ± 5	95 ± 5
Pyrene		0.999	0.40	1.20	96 ± 2	89 ± 6
Benzo(a)anthracene		0.978	0.40	1.20	102 ± 7	110 ± 7
Chrysene		0.972	0.40	1.20	93 ± 4	106 ± 8
Benzo(b)fluoranthene	Perylene-d12	0.978	0.40	1.20	87 ± 3	81 ± 6
Benzo(k)fluoranthene		0.976	0.40	1.20	85 ± 4	87 ± 7
Benzo(a)pyrene		0.996	0.25	0.75	92 ± 2	95 ± 5
Dibenzo(a,h)anthracene		0.983	0.50	1.50	81 ± 2	87 ± 6
Indeno(1,2,3-cd)pyrene		0.982	0.50	1.50	75 ± 5	80 ± 3
Benzo(g,h,i)perylene		0.972	0.50	1.50	78 ± 6	83 ± 4

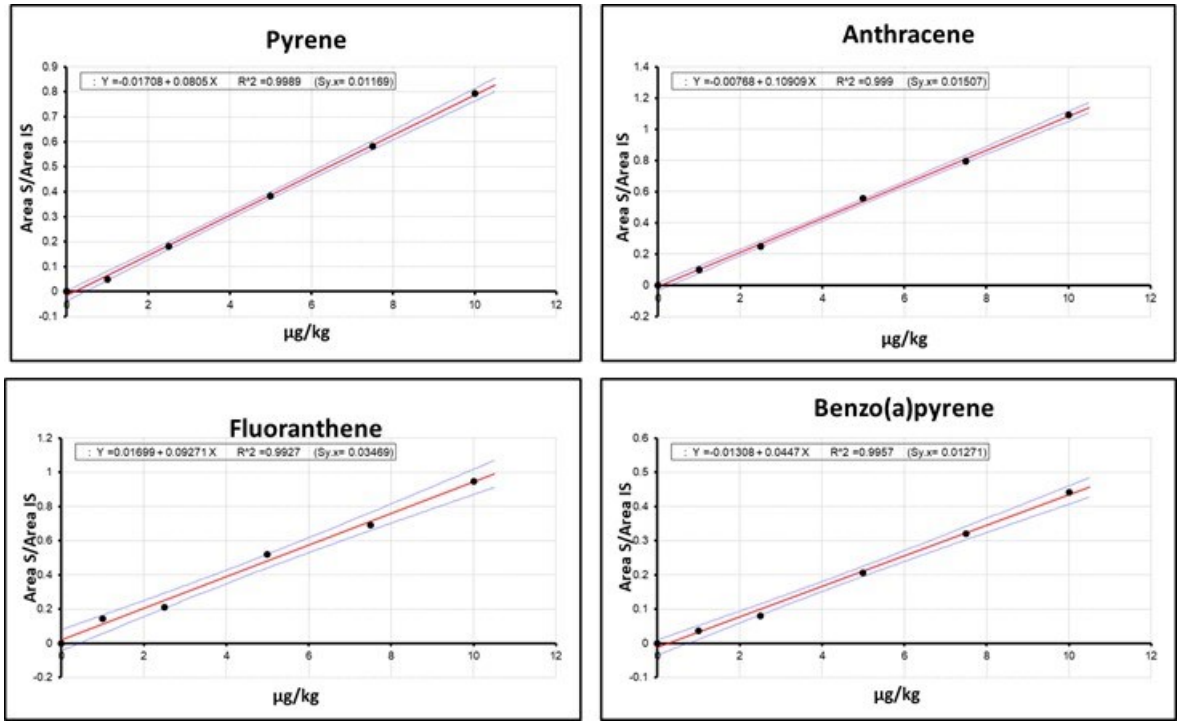

Figure 11: Matrix calibration curve for Pyrene, Anthracene, Fluoranthene and Benzo(a)pyrene; each level was determined in duplicate; regression line with 95% confidence bands. Sy.x= standard error of the estimate.

Table 5: Validation parameters, including R^2, LOD, LOQ and Recovery (n=5, mean ± standard deviation) for PCBs. Internal standard used as single area or mean area.

PCBs	Internal Standard (IS)				Recovery (%)	
		R^2	LOD (µg/kg)	LOQ (µg/kg)	C_{PCBs} 8 µg/kg	C_{PCBs} 14 µg/kg
PCB 28	PCB 77L, PCB 81L	0.997	1.00	3.30	80 ± 5	85 ± 4
PCB 52		0.993	1.25	4.16	85 ± 4	87 ± 3
PCB 77		0.992	1.25	4.16	87 ± 3	73 ± 5
PCB 81		0.998	1.00	3.30	78 ± 6	68 ±5
PCB 95	PCB 105L, PCB 114L, 118L, PCB 123L, PCB 126L	0.991	1.15	3.45	73 ± 4	79 ± 6
PCB 99		0.988	1.15	3.45	70 ± 5	82 ± 4
PCB 101		0.982	1.15	3.45	65 ± 3	88 ± 6
PCB 110		0.980	1.15	3.45	69 ± 2	87 ± 7
PCB 105	PCB 105L	0.994	1.20	3.60	73 ± 5	72 ± 4
PCB 114	PCB 114L	0.998	1.20	3.60	81 ± 6	68 ± 5
PCB 118	PCB 118L	0.997	1.30	4.30	62 ± 5	63 ± 4
PCB 123	PCB 123L	0.993	1.30	4.30	72 ± 4	79 ± 2
PCB 126	PCB 126L	0.990	1.20	3.60	83 ± 4	82 ± 4
PCB 128	PCB 156L, 157L, 167L, 169L	0.982	1.25	4.16	85 ± 6	87 ± 5
PCB 138		0.985	1.30	4.30	78 ± 7	76 ± 3
PCB 146		0.995	1.25	4.16	63 ± 5	64 ±4
PCB 149		0.990	1.25	4.16	68 ± 3	78 ± 7
PCB 151		0.994	1.30	4.30	82 ± 4	65 ± 3
PCB 153		0.989	1.30	4.30	73 ± 5	83 ± 4
PCB 156	PCB 156L	0.997	1.20	3.60	65 ± 2	88 ± 3
PCB 157	PCB 157L	0.996	1.25	4.16	68 ± 7	89 ± 4
PCB 167	PCB 167L	0.992	1.15	3.45	75 ± 4	70 ± 6
PCB 169	PCB 169L	0.991	1.15	3.45	89 ± 3	76 ± 3
PCB 170	PCB 189L	0.987	1.40	4.70	87 ± 4	71 ± 2
PCB 177		0.991	1.40	4.70	85 ± 6	88 ± 4
PCB 180		0.990	1.40	4.70	75 ± 8	67 ± 7
PCB 183		0.986	1.40	4.70	81 ± 4	73 ± 4
PCB 187		0.993	1.40	4.70	85 ± 6	78 ± 5
PCB 189		0.995	1.15	3.45	68 ± 5	64 ± 3

Table 6: Validation parameters, including accuracy, trueness and precision (n=5) for PAHs. CV= coefficient of variations

PAHs	C_{PAHs} 2 µg/kg			C_{PAHs} 8 µg/kg		
	Accuracy % (min-max)	**Trueness %**	**CV %**	**Accuracy % (min-max)**	**Trueness %**	**CV %**
Naphthalene	75 - 115	99	15.1	84 - 109	101	10.8
Acenaphthylene	80 - 100	88	8.6	85 - 120	102	13.5
Acenaphthene	80 - 110	99	12.2	89 - 124	105	12.7
Fluorene	90 - 120	105	11.6	90 - 114	101	10.4
Phenanthrene	80 - 100	91	8.1	84 - 120	107	11.8
Anthracene	85 -105	93	8.1	81 - 119	103	11.5
Fluoranthene	90 - 110	102	8.9	91 - 112	101	11.4
Pyrene	90 - 110	98	9.3	81 - 106	96	11.2
Benzo(a)anthracene	80 - 110	103	14.8	82 - 100	90	9.6
Chrysene	80 - 120	96	15.8	85 - 115	101	13.2
Benzo(b)fluoranthene	80 - 115	102	15.4	85 - 118	98	15.4
Benzo(k)fluoranthene	85 - 120	96	14.4	82 - 108	96	7.6
Benzo(a)pyrene	80 - 105	90	10.4	92 - 109	99	9.3
Dibenzo(a,h)anthracene	80 - 115	98	13.8	80 - 100	92	12.1
Indeno(1,2,3-cd)pyrene	90 - 120	101	11.2	86 - 95	91	6.7
Benzo(g,h,i)perylene	80 - 105	95	12.3	86 - 105	94	7.3

Table 7: Validation parameters, including accuracy, trueness and precision (n=5) for PCBs. CV= coefficient of variations

PCB	C_{PCBs} 8 µg/kg			C_{PCBs} 14 µg/kg		
	Accuracy % (min-max)	Trueness %	CV %	Accuracy % (min-max)	Trueness %	CV %
PCB-028	87 -112	105	10.6	80 - 95	82	7.7
PCB-052	85 - 100	92	7.4	90 -120	105	12.4
PCB-095	86 - 112	99	8.8	85 - 121	103	12.6
PCB-101	87 - 125	102	13.2	80 -120	98	14.0
PCB-099	88 - 97	85	11.3	85 - 100	94	8.3
PCB-081	80 - 100	90	10.8	80 - 115	90	10.7
PCB-077	83 – 106	90	9.9	92 - 121	111	10.1
PCB-110	81 – 96	85	9.6	85 - 114	99	13.3
PCB-151	87 - 112	92	12.1	80 - 100	90	10.6
PCB-123	81 - 106	92	10.1	80 - 114	97	14.3
PCB-149	87 - 112	95	10.5	78 - 121	105	14.3
PCB-118	85 - 125	115	13.7	78 - 114	100	14.6
PCB-114	87 - 112	97	11.9	85 - 117	103	13.3
PCB-146	83 – 115	102	11.6	80 - 100	90	10.6
PCB-153	86 - 106	94	8.1	80 -114	97	14.3
PCB-105	75 - 100	82	13.5	80 -100	87	10.7
PCB-138	83 - 112	94	12.6	92 - 121	106	11.1
PCB-126	86 - 112	95	11.6	85 - 121	108	14.2
PCB-187	75 - 112	85	14.1	80 - 107	97	12.3
PCB-183	87 - 111	99	11.6	100 - 121	108	8.6
PCB-167	85 - 120	97	14.4	78 - 112	95	13.7
PCB-128	90 - 125	115	13.4	80 - 104	91	13.1
PCB-177	99 - 125	115	9.1	93 - 121	107	10.5
PCB-156	83 - 108	91	11.0	85 - 114	103	11.6
PCB-157	81 - 120	103	13.2	91 - 117	105	9.1
PCB-180	86 - 121	107	13.3	80 - 112	101	13.9
PCB-169	85 - 111	92	12.5	93 - 121	99	13.0
PCB-170	75 - 106	93	13.2	85 - 117	98	13.9
PCB-189	77 - 100	88	10.1	80 - 112	96	14.7

Table 8: OCPs recoveries at 10 ug/Kg, n=5, mean ± standard deviation.

Analyte	**Recovery (%)** (n=5, C_{OCPs}=10 µg/kg)
α - Hexachlorocyclohexane (HCH)	90 ± 5
Hexachlorobenzene	115 < 50 4
β -HCH	73 ± 6
γ -HCH	112 ± 7
δ -HCH	95 ± 4
Chlorpyrifos-methyl	98 ± 5
Heptachloro	97 ± 4
Aldrin	< 50
Isodrin	< 50
Heptachlor epoxide	110 ± 6
Cis-chlordane	85 ± 4
2,4'-DDE	70 ± 5
β -Endosulfan	< 50
Trans-Chlordane	120 ± 6
Dieldrin	98 ± 4
4,4'-DDE	115 ± 4
2,4'-DDD	110 ± 7
Endrin	< 50
α -Endosulfan	< 50
4,4'-DDD	75 ± 5
2,4'-DDT	82 ± 7
Endosulfan-Solfato	< 50
4,4'-DDT	72 ± 4

CHAPTER 3

BIOMONITORING OF POPS IN *MULLUS SURMULETUS* FROM THE CATANIA GULF: DISTRIBUTION AND POTENTIAL HUMAN HEALTH RISKS

This chapter focus on the biomonitoring of POPs in *M. surmuletus* caught in the Catania Gulf, which has been carried out using the developed methodologies discussed in Chapter 2.

After a brief review of the monitoring programs commonly used under local, regional, and national-scale, the general information required to design the monitoring program, considering the objectives, were also reported.

Regarding the human health risks resulting from dietary intake of POPs, a brief review of the different methods used to evaluate the risks associate to fish consumption have been carried out.

The experimental section focuses on the detection of POPs (PAHs, PCBs and OCPs) in muscle fillet of *M. surmuletus*, caught in the Catania Gulf (Sicily, Italy), providing an overview of the distribution and sources of these pollutants in the investigated area as well as of the potential health risk of human consumption of *M. surmuletus* for local population.

3.1 POLICY FRAMEWORK

In 1977 the term "monitoring" was defined by the International Council of Scientific Union's Scientific Committee on Problems of the Environment (SCOPE) as:

the collection, for a predetermined purpose, of systematic, inter-comparable measurements or observations in a space-time series, of any environmental variables or attributes which provide synoptic view or a representative sample of the environment (global, regional, national or local). Such a sample may be used to assess existing and past states, and to predict likely future trends in environmental features.[139]

Basic reasoning for monitoring changed over the years. In the past, the first concern was to avoid hazards to human health, while today, a more holistic approach is applied. The concern is reaching Good Environmental Status (GES), as indicated in Water Framework Directive and in MSFD, and the focus is now on the environment.

Organic microcontaminants have been monitored in the marine environment for several decades and this continues today in local, regional, and national-scale programs.[10] Marine monitoring is more specifically configured in the framework of international conventions with major long-term monitoring programme, such as:

- London Dumping Convention: signed in 1972 intended to cover the world's oceans and seas. It follows a "black list/grey list" approach to regulate ocean dumping; black list (annex I) chemicals banned, grey list (annex II) chemicals for which dumping was restricted.
- Oslo and Paris Conventions, OSPAR Convention: started in 1972 with the Oslo Convention against dumping and covered the North-East Atlantic and part of the Arctic Ocean, excluding the Baltic seas. In 1974 Oslo Convention was broadened to cover land-based sources of marine pollution and the offshore industry by the Paris Convention. The current legislation is the OSPAR Convention (1992, in force 1998), which combines the Oslo Convention and the Paris Convention.
- Helsinki Convention: adopted in 1974, employed the black and grey list of hazardous substances. In the light of political changes, a new Helsinki Convention was signed in 1992. The Convention covers the whole of the Baltic Sea areas and aims at the regulation of inputs.
- Bonn Agreement: started in 1969, focus on discharges of oil and other substances into the North Sea region. Now parties were required to jointly develop and stablish guidelines for joint action and to provide information on pollution incidents.
- MARPOL: signed in 1973, but in force only in 1983, The International Convention for the Prevention of Pollution from Ships aims to preserve the marine environment through

the complete elimination of pollution by oil and other harmful substances and to minimize the accidental discharge of such substances.

- Other convention: based on the concept of Oslo and Paris Convention, the Convention for the Protection of the Mediterranean Sea against Pollution and the Barcelona Convention were signed in 1976. Moreover, in 1992 the Convention on the Protection of the Black Sea against Pollution was signed.

Biomonitoring is associated with different fields of investigation, including chemical analysis and other biological parameters related to biodiversity. A more recent kind of biomonitoring is the investigation of biological effect, using cellular and molecular tests, in order to detect effects of natural and anthropogenic impacts on the health status of biota.[140,141]

Biota chemical analysis has become a useful instrument to detect and quantify the presence and the concentration of hazardous substances, especially in the case of those substances that accumulate in organisms and are difficult to determine in other matrices (water and sediments), due to their large dilution. Moreover, the accumulation period is age-correlated, allowing the evaluation of political environmental effects or of the consequences of chemicals that accidently enter the seas.

In the Water Framework Directive (WFD) 2013/39/EU eleven different substances group have been identified for which the assessment of compliance with environmental quality standards (EQSs) is required in biota (Table 9). Furthermore, this Table reports the matrix and the protection goal used in the derivation of EQSs.

The EQS were established considering several different protection goals such as the pelagic and benthic communities in freshwater, brackish and marine ecosystems, the predators of these ecosystems, and humans.

Health EQS_{biota} have two protection goals: the protection of the top predators from the risks of secondary poisoning through consumption of contaminated prey and the protection of human health from deleterious effects resulting from the consumption of fish contaminated by chemicals.[142]

Table 9: Analytes for which there is currently an EQS_{biota} (µg/Kg ww), matrices to which the EQS apply, and protection goals (Guidance Document No. 32 ON BIOTA MONITORING);

Analytes	EQS_{biota} (µg/kg wet weight (ww))	Matrix	Protection goal
Brominated diphenyl ethers	0.0085	Fish	Human health via consumption of fishery products
Fluoranthene	30	Crustaceans and molluscs	Human health via consumption of fishery products
Hexachlorobenzene	10	Fish	Human health via consumption of fishery products
Hexachlorobutadiene	55	Fish	Secondary poisoning
Mercury and its compounds	20	Fish	Secondary poisoning
PAHs Benzo(a)Pyrene	5	Crustaceans and molluscs	Human health via consumption of fishery products
Dicofol	33	Fish	Secondary poisoning
PFOS	9.1	Fish	Human health via consumption of fishery products
Dioxins and sioxin-like compounds	0.0065 TEQ_{2005}	Fish, crustaceans and molluscs	Human health via consumption of fishery products
HBCDD	167	Fish	Secondary poisoning
Hepatchlor and heptachlor epoxide	6.7×10^{-3}	Fish	Human health via consumption of fishery products

Notably, as underling by the limited number of chemicals reported in Table 9, only a very small selection of the thousands of compounds entering the sea[143] is subjected to the authority controls, as expressed in the regular programmes; therefore, current monitoring efforts do not describe the actual presence of organic contaminants. Based on this evidence, there is a need of improvement in monitoring programs. In many cases the improvement of the analytical methods and instruments has enabled the quantification of substances that are produced and used in high quantities for many years, for example the perfluorinated compounds.

3.2 BIOTA MONITORING PROGRAMME

General information about the objectives of a monitoring programme affects sampling of marine biota. When a biota monitoring programme is building up, the typical questions are "what fish species to choose, what size of fish to target, what matrix to analyse (e.g., fillet or whole fish), whether to pool samples or analyse individual fish, how to convert data from a matrix or species to another, and how to assess compliance with target values". Often this information is included in the available monitoring programmes.

Regarding the sampling frequency for long-term temporal trend assessments, at least one sampling period per year is common. As the condition of fish periodically changes over the year, sampling typically takes place in a period stable for the environmental conditions and the physiological processes and outside the spawning period.[144]

3.2.1 Choice of species

The choice of species depends on the specific objectives of the monitoring programme; the species should be representative and sufficiently abundant with a slow tendency to migrate, in order to relate the results with the surrounding environment. The most commonly used Mediterranean species include: the red mullet, *Mullus barbatus* or *Mullus surmuletus*, the gilthead seabream *Sparus aurata,* various gobiid species, the seabass *Dicentrarchus lab* and *Zosterissessor ophiocephalus*.[7]

Several studies report the accumulation of pollutants in fish species across Europe.[145,146,147,148] Furthermore, the distribution of pollutants in fish is influenced by several factors such as the feeding and habitat preferences of different species and it is also possible to observe different temporal trends for the same substance in different species sampled in the same locations.[149,150] The guidelines suggest to sample multiple species from different trophic levels and/or habitat types, at a single location.[142]

During the selection of samples, the natural variability within biota samples should be reduced. Since age, size, sex and sexual maturity status can affect the concentration values of the contaminants,[148,150,151] sampling strategies should be designed to minimise the impact of these factors.

Trophic level, which defines the position of the species within the food chain, is the most important biological variable;[152] the species used for the monitoring of substances with SQA_{biota} can be considered appropriate if they are at a level between 3.5 and 4.5. In fact, considering the human consumption of fish, it is estimated that fishes at the trophic level 4 are the species mainly consumed by humans.

Contaminant levels have been shown to be related to the age, and therefore size of the fish sampled.[145, 151,153] Since age and size are correlated for most species,[154] the length of the fish can be used as a selection criteria. The guidance N.32[142] suggests a fish age between 3-5 years. For the length-stratified sampling, Quevauviller et al. (2011)[144] indicate that the minimum number of fish sample is 25, which results in at least five pooled analytical samples.

3.2.2 Choice of the tissue

Several factors influence the choice of the tissue, i.e. the purpose of monitoring, the chemical proprieties of the investigated contaminants and the availability of the tissue. Contamination is generally evaluated by analysing the muscle fillets in relation to the protection of human health, or the whole fish in relation to the protection of predators. Obviously, pollutants are not distributed uniformly in the fishes due to their chemical properties and their metabolic pathway.

For example, hydrophobic compounds are more concentrated in fat tissues than in muscles. Muscle tissues and viscera preferentially concentrate other contaminants, such as mercury which binds to muscle proteins.[145,155] Also lipophilic chemicals mainly accumulate in fatty tissues, including subcutaneous and dorsal fat, gills, eyes, brain, and internal organs; for example concentration of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and DDTs in the deep-sea fish *Mora moro* followed the trend muscle < gills ~digestive tube < liver, according to the lipid content of the organs.[156] Thus, it is usually assumed that the concentrations of pollutants in fillet are lower than the corresponding concentrations in the whole fish, since the whole fish have a greater percentage of lipids. Based on this knowledge, use of fillet data may underestimate the risks to secondary poisoning, while whole-body pollutant concentrations in fish would overestimate the risk associated to people who generally consume the fillet portion of the fish.

3.2.3 Data normalization

For hydrophobic substances that accumulate into the adipose tissues of organisms, detected concentrations in biota can be normalised to fish with a lipid content of 5%,[157] as this standard value was used in the derivation of the SQA_{biota} referred to fish. For other substances that do not accumulate into lipids, the normalisation could be carried out against another parameter, such as dry weight. The percent value dry weight content for fish is 26%.

When monitoring aims to local risk assessment, it is always preferable to use the exact lipid content or the dry weight for biota samples compared to a generic value for the species.[142]

3.3 HUMAN HEALTH RISK ASSESSMENT

The main route of human exposure to marine pollutants is through the marine living organisms that enter the human diet. In fact, these pollutants are released into the marine environment from point sources, including industrial and municipal discharges and from nonpoint sources such as agricultural runoff and atmospheric deposition. Since many POPs are highly lipophilic compounds, they accumulate in fatty tissues of fish and shellfish according to their marine distribution and dietary exposure.[158] Therefore, the bioaccumulation of these highly lipophilic pollutants could pose health risks to fish consumers.

The distribution of POPs in marine organisms strongly depends on the lipid content of the organisms themselves, on the feedings habitat and the trophic level. For example, it has been reported that the distribution of PAHs compounds differs from species to species[159] and their accumulation in fish can negatively affect the health of fish[160,161] as well as the health of human populations who consume these fish.

In this context, the investigated area, the Catania Gulf, is of particular interest being characterized either by anthropogenic pressures, including industrial and municipal impacts and high maritime traffic,[162,163] and by natural pressures, such as the Etna volcanic activity,[164,165] that differently contribute to the dispersion of some POPs into marine environment.

In the last few years, the human health risks resulting from dietary intake of PAHs have been assessed using different equations that properly combine different factors.[21,166–171] Many of these are based on the benzo(a)pyrene equivalent (BaPeq) concept, e.g. the toxicity of PAHs in food is calculated from the concentration values of benzo(a)pyrene and their toxicity equivalency factors (TEF). Using the BaPeq, age, body weight and carcinogenic potency of benzo(a)pyrene, the incremental lifetime cancer risk (ILCR) (also referred to as the excess cancer risk) associated to ingestion of food contaminated with these PAHs can be estimated.[167,172–175]

Generally, the first stage of risk assessment methodology involves the extraction of selected contaminates from fish tissues using validated methods, followed by basic statistical analysis (mean, standard deviations and analysis of variance) and other statistical test to check the normality. The US-EPA (2000a) established standardized methodologies to evaluate the risks of both cancer and non-cancer endpoints due to fish consumption. It is possible to estimate the "potential" risk assessment assuming standardized parameters such as ingestion of 227 g (meal size) and body weight (BW) of 70 kg. On the other hand, the "real" risk assessment should be carried out by administering a validate questionnaire to local population to obtain real data on the fish consumption, the ingestion rate, the body weight and the age classes.

Additionally, according to the US-EPA guidance[177], the ingestion dose is assumed to be equal to the absorbed contaminant dose with cooking having no effect on the contaminants.

Initially, preliminary investigations are based on "the estimated daily intake" (EDI) that is calculated from the concentration values of pollutants, the ingestion rate and the body weight or "the dietary daily intake (DDI)" arising only from the concentration values of pollutants and the ingestion rate. These calculated values can be compared with daily and weekly tolerable intake (TI) levels suggested by WHO. Moreover, according to US-EPA guidelines, other risk factors could be calculated, including the Target Hazard Quotient (THQ). THQ, which is mainly used to assess the potential health risk of metals in fish, indicates the ratio between the exposure and the reference dose (e.g. the level at which no adverse effects are expected). If the calculated THQ value is below 1, then adverse effects are not likely to occur, it is assumed as an acceptable risk for chronic systemic effects.

The evaluation of the potential carcinogen risk associated with the exposure at the detected doses of pollutants could be based on the calculation of the Incremental lifetime cancer risk (ILCR), using the Cancer Slope Factor (CSF). According to US EPA, the acceptable risk range for ILCR is 1×10^{-6} to 1×10^{-4}, whereas an ILCR value of 1×10^{-4} or greater is considered to be serious.[178]

As regards the carcinogenic effects, another useful index is the "maximum allowable fish consumption rate (*CRmwc*) meals/week", which is calculated based on US-EPA equations,[176,179] and represents the amounts of fish (kg) expected to generate a risk no greater than the maximum acceptable individual lifetime risk (ARL).

Since there is not a regulatory limit for all hazard substances in fish, the risk assessment resulting from the consumption of fish is essential for risk prevention and management plan.

3.4 AIMS

To assess the environmental status of coastal area, the Catania Gulf, and the potential toxic effects due to POPs exposure, information on the distributions of these pollutants in edible fishes is essential. In the present PhD thesis, the fish *M. surmuletus* has been selected to monitor POPs pollution in Catania Gulf, since this species is widely distributed in the Mediterranean Sea. It has great ecological importance and has been suggested as bioindicator for assessing the contamination status and ecological risks by the European monitoring program,[7] in addition to being highly consumed from the local population. The determination of the PAHs concentration values in the fish was carried out on properly selected tissues, and by using the QuEChERS method that is still widely used for the determination of PAHs in marine organisms.[180,181]

Furthermore, the PAH isomeric diagnostic ratios have been evaluated to identify the pollution emission sources[21–23] In fact, the biomass burning, the diagenetic as well as the volcanic activity phenomena[182] can form PAHs; however, in the regions subjected to a high human pressure, where coal and wood burning, as well as petrol and diesel oil combustion, and industrial processes are largely present, they are mainly of anthropogenic origin.[183] PAHs are always emitted as a mixture

and thus the ratios of their relative molecular concentrations, (considering pairs of PAHs with the same molar mass and slightly different physicochemical properties,[24] that should then suffer a similar environmental fate), could be characteristic of specific pollution emission sources, constituting a very useful tool for their identification.

To the best of my knowledge, this is the first study that provides information of POPs distribution in muscle tissues of *M. surmuletus* collected from Catania Gulf, Sicily in order to (1) to evaluate the levels of POPs in *M. surmuletus* collected from Catania Gulf, as well as the pollution emission sources of PAHs and (2) to assess possible human health risks arising from the consumption of POPs contaminated fishes.

3.5 MATERIALS AND METHODS

3.5.1 Sampling

Specimens of *M. surmuletus* (n=40) were sampled at the local fish market in Catania. The sampling took place in April 2019 before the spawning season. All information about the habitat and the characteristics of this species were collected from the online database www.fishbase.org. Consulting the growth curve of Von Bertalanffy (age-size relation),[184] fishes of 3 years were sampled (length cm, 18.5 ± 1.8) and biometric data (length and weight) were recorded for every fish (Table 10). Dissection was performed after arriving in the laboratory with a stainless-steel knife, the internal organs were removed and muscle tissues with skin were isolated from the mid-body dorsal area (Figure 12). After that, samples were homogenized using a mixer and pooled, each pooled sample being made up of five specimens and a total of eight composite samples were stored at -20°C until the extraction process for PAH analysis.

Table 10: Biometric data, habitat, feeding mode and number of pooled individuals during sample processing of the investigated fish species collected from the Catania Gulf, Sicily, Italy

Fish Species	**Red Mullet – *Mullus Surmuletus***
Habitat	demersal
Sedentary	medium
Trophic level	3.5
Bathymetric distribution	5 - 409 m
Medium lipid content	5%
Length [cm]	18.5 ± 1.8
Weight [g]	75 ± 13.8
Total number of samples	40
Total pooled samples analysed	8

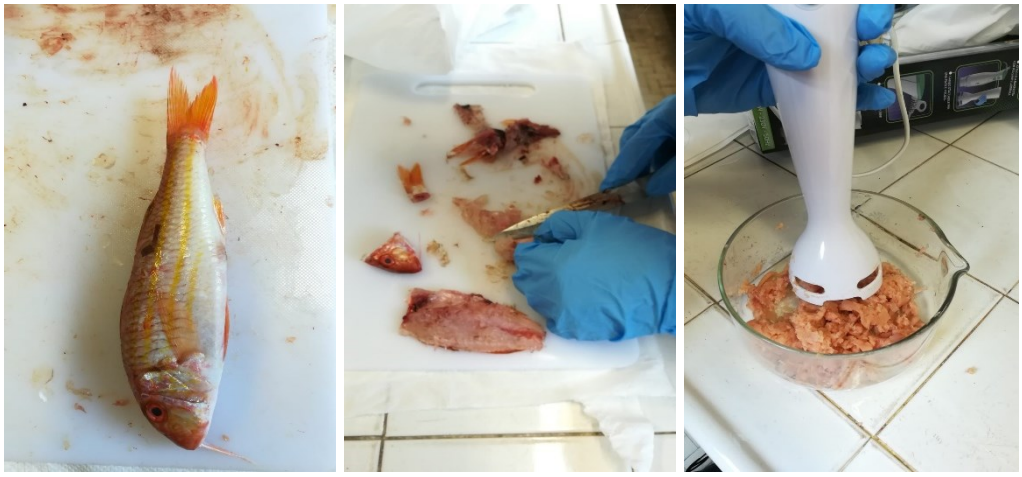

Figure 12: Pre-treatment of M. surmuletus samples: dissection, removing of internal organs, isolation of muscle tissues and homogenization.

3.5.2 Sample Extraction and GC-MS Analysis

The pooled muscle samples of *M. surmuletus* fishes were extracted with the method reported in 2.2.4 section. Briefly, 5 g of homogenized fillet were spiked with perdeuterated PAHs surrogate standard solution described in the Materials Section "2.2.2 Standard and sample preparation", added to 10 mL of acetonitrile and mixed by vortex. Then, 4 g of anhydrous $MgSO_4$, 1 g NaCl, 5 g $NaHCO_3$ and 0.5 g $C_6H_6Na_2O_7$ 1,5 H_2O (Supel^{TM} QuEChERs) were added and the resulting mixture was mixed again. The extract was then centrifuged for 5 min at 5,000 rpm and, subsequently, 5 mL of the acetonitrile layer was transferred into a tube and freezed at -20°C for 3 h to remove fatty acids. The extract was centrifuged for 5 min at 5,000 rpm, and the supernatant was transferred into a tube containing 1 g of $CaCl_2$ for the removal of free fatty acids co-extracted. The last clean up step was carried out using dSPE (900 mg $MgSO_4$, 150 mg PSA and 150 mg C18). The 15 ml dSPE tubes were vigorously vortex for 30 s and centrifuged for 5 min at 5,000 rpm. Finally, the supernatant was evaporated under nitrogen, and the residue was redissolved in 250 µl of isooctane.

The analyses of the samples were performed using a GC coupled with a single quadrupole mass spectrometer (Thermo Finnigan Trace GC Ultra DSQ managed by the Xcalibur software ver 1.4 SR1). The analytical separation was performed using a Restek Rtx-5MS, 15 m, 25 mm ID, 0.25 µm capillary column. The carrier gas (Helium) flow was set at a constant rate of 2 mL/min. The injector, ion source, and MS transfer line temperatures were set at 280°C. The injected volume was 1 µL in splitless mode. The mass spectrometer operated in SIM mode.

Analysis of OCPs, PCBs and PAHS was performed using the three different GC-MS methods reported in 2.2.5 paragraph.

3.5.3 Quality assurance and quality control

In order to ensure quality control, blanks samples, subjected to the same procedure of extraction previously described but without adding fish tissue, were regularly analysed. The linearity and recovery were checked using homogenized muscle tissues of codfish, previously analysed as blank matrix (2.2.6).

The recovery data were used to quantify the concentrations of POPs in the samples of *M. surmuletus* by using an external standard calibration curve. Quality controls (CQ) were performed by monitoring the recovery of spiked codfish within the same analytical sessions. Concentrations were calculated as ng/g (ppb) wet weight (ww).

3.5.4 Data analysis

Basic descriptive statistical analyses were carried out using Microsoft Excel (vs 1908) and the distribution of the total PAH levels (∑PAHs, the sum of the 16 individual PAHs analysed) were checked for normality using the Shapiro–Wilk test. Analytes with a detection frequency below 30% in all the examined samples were excluded from statistics (sums and relative abundances), whereas for analytes with a higher detection frequency, the values below the method quantification limits (LOQ) were set to half their respective LOQ.

In order to identify the pollution emission sources, PAHs were grouped into three different classes according to the number of the rings: low molecular weight PAHs (LMW-PAHs) including 2–3 ring PAHs (NA, ACY, AC, FL, PHE, AN,), medium molecular weight PAHs (MMW-PAH) including 4 ring PAHs (FLA, PY, BaA, CH), and high molecular weight PAHs (HMW-PAH) including 5-6 ring PAHs (BbF, BkF, BaP, DBahA, BghiP, IP). Moreover, the isomeric ratio of AN/(AN+PHE), FLA/(FLA+PY), (FLA/PY) and (PHE/AN) were also calculated.

Furthermore, considering the IARC classification, the sum (∑cPAHs) of the carcinogenic (Group 1), probable (Group 2A) and possible (Group 2B) carcinogenic PAHs, was evaluated.

3.5.5 Human Health risk assessment

According to US-EPA (2000a), the toxicity equivalent factor (TEF) was used to calculate the total toxic equivalent (TEQ) in order to convert individual PAH component to an equivalent concentration of BaP, as follows:

$$\text{TEQ} = \sum TEFi \times Ci$$

where Ci is the concentration of the individual PAH in fish muscle (ng/g ww), and TEFi is the corresponding toxic equivalency factor related to BaP.[185]

The daily dietary intake (DDI) due to fish consumption was evaluated according to the following equation:

$$\mathrm{DDI} = C \times IR$$

where C is the concentration of PAHs (∑16PAHs) and IR is the ingestion rate of fish (19.25 g/day), as the mean ingestion rate for the Italian population specific for demersal fish species supplied by FAOSTAT "Food Supply - Livestock and Fish Primary Equivalent" database online. The incremental lifetime cancer risk (ILCR) associated with lifetime dietary exposure to the PAHs was calculated using the following equation:[173,174]

$$\mathrm{ILCR} = \frac{TEQ \times IR \times ED \times EF \times SF \times CF}{BW \times AT}$$

where TEQ represents the BaP-equivalent concentration, IR is the ingestion rate (g/day), ED is the exposure duration (year), EF the exposure frequency (day/year), SF is the cancer oral slope factor of BaP, CF is the conversion factor of mg to ng, BW is the body weight (kg) and AT is the averaging time (day).

The values used in the ILCR calculation were set as follows: BW = 70 kg; IR = 19.25 g/day; AT = 25,550 day (70 year × 365 day/year); ED = 30 year for adults; EF = 365 day/year (US EPA 1989); SF = 7.3 $(\mathrm{mg/kg\text{-}d})^{-1}$ for BaP; CF = 10^{-6}.

3.6 RESULTS AND DISCUSSION

M. surmuletus was chosen for this study, since this species is representative and sufficiently abundant in the investigated area with a slow tendency to migrate, and it is thus possible to relate the obtained results with the surrounding environment. *M. surmuletus* is a sedentary benthic species with a high lipid content, which promotes bioaccumulation of compounds such as PAHs. Biometrical data and all general information about habitat and properties of this species are reported in Table 10.

Several factors influence the choice of tissue as reported in the Choice of the tissue section and, thus, considering the purpose of this monitoring and the chemical proprieties of investigated pollutants, muscle fillet was selected for this study.

3.6.1 PAHs analysis

PAHs levels, composition and sources

The concentration values of PAHs found in the *M. surmuletus* analysed samples, as well as the total PAHs content ($\sum_{16}$PAHs) are reported in Table 11. The concentration values ranged from 0.25 to 6.10 µg/kg ww, whereas the concentration value of $\sum_{16}$PAHs was 19.49 µg/kg.

Table 11: Concentration values (µg/Kg ww) of PAHs, frequency of the presence of individual PAH (%) in the pooled samples, TEFs values adopted from Nisbet and LaGoy (1992) and toxicity equivalent of BaP (BaPeq, µg/Kg ww) in pooled fish samples of M. surmuletus from Catania Gulf, Sicily.

	Rings	Mean ± SD	Frequency (%)	TEFs	BaPeq
Naphthalene	2	4.51 ± 0.21	100	0.001	0.0045
Acenaphthylene	3	0.26 ± 0.16	66	0.001	0.0003
Acenaphtene	3	0.65 ± 0.10	100	0.001	0.0007
Fluorene	3	3.22 ± 0.48	100	0.001	0.0032
Phenanthrene	3	6.10 ± 0.60	100	0.001	0.0061
Anthracene	3	1.19 ± 0.62	100	0.01	0.0119
Fluoranthene	4	1.42 ± 0.60	100	0.001	0.0014
Pyrene	4	0.90 ± 0.20	100	0.001	0.0009
Benzo[a]Anthracene	4	0.38 ± 0.25	50	0.1	0.0380
Chrysene	4	0.35 ± 0.15	50	0.01	0.0035
Benzo[b]Fluoranthene	5	0.25 ± 0.18	37.5	0.1	0.0250
Benzo[k]Fluoranthene	5	<LOQ	-	0.1	
Benzo[a]Pyrene	5	0.25 ± 0.17	37.5	1	0.25
DiBenzo[a,h]Anthracene	5	<LOQ	-	5	
Benzo[g,h,i]Perylene	6	<LOQ	-	0.01	
Indeno[1,2,3-cd]Pyrene	6	<LOQ	-	0.1	
LMW-PAHs		15.94			
MMW-PAHs		3.05			
HMW-PAHs		0.50			
$\sum_{16}$PAHs		19.49			
$\sum$cPAHs		5.74			
TEQ (PAHs)		0.3455			

Benzo[k]fluoranthene, DiBenzo[a,h]Anthracene, Benzo[g,h,i]Perylene and Indeno[1,2,3-cd]Pyrene were never detected, whereas seven of the 16 target PAHs were found in all muscle pool samples (Table 11). Interestingly, Benzo(a)anthracene and Chrysene were detected in over 30% of the samples, while Benzo(b)fluoranthene and Benzo(a)pyrene, this last recognized as the most carcinogenic PAHs, were found in only 3 of the 8 analysed pools and always at concentrations near the LOQ (0.25 µg/kg).

Since several factors contribute to the levels of pollutants in fish, such as the uptake from the water column, the diet, the presence of different sediments in a given habitat, the metabolism, as well as the partitioning and the elimination mechanisms, the comparison among the data obtained by different studies carried out at different sites is not straightforward. However, several studies were carried out in Sicily: Oliveri et al. (2012) analysed muscle tissue of *Mullus barbatus* sampled in Lampedusa, Saija et al. (2016) investigated the accumulation of POPs in *Thunnus thynnus* from the Straits of Messina, Di Bella et al. (2018) detected persistent organic pollutants in farmed Sicilian sea bass and Ferrante et al. (2018) detected PAHs in three species (*Sardina pilchardus*, *Solea solea* and *Donax trunculus*) caught in the Catania Gulf. In particular, in the paper of Oliveri et al the muscle tissue of a species similar to *M. surmuletus*, *M. barbatus*, sampled in Lampedusa was analysed, and it is thus possible to properly compare the two sets of data. Interestingly, the mean PAHs concentrations found in this study (Table 11) are lower than those found in Lampedusa (∑PAHs 26.47 $\mu g \cdot kg^{-1}$) [187]. Furthermore, these authors found higher levels of naphthalene (3.52 ± 6.13 $\mu g\ kg^{-1}$ w.w.) and acenaphthylene (13.16 ± 3.44 $\mu g\ kg^{-1}$ w.w.), whereas in this study acenaphthylene was detected in traces and the most abundant PAHs are Phenanthrene, Naphthalene and Fluorene, as shown in Figure 13. This different PAHs distribution probably relies on the different anthropogenic inputs to which marine environment is subjected. In fact, the intense maritime traffic is the primary PAHs source of contamination of the Sicilian Channel area, that originate, not only from the partial combustion of the fossil fuels, the combustion of biomass, the municipal waste, the accidental spills and the decomposition of the organic matter, but also from the vessel traffic.[187]

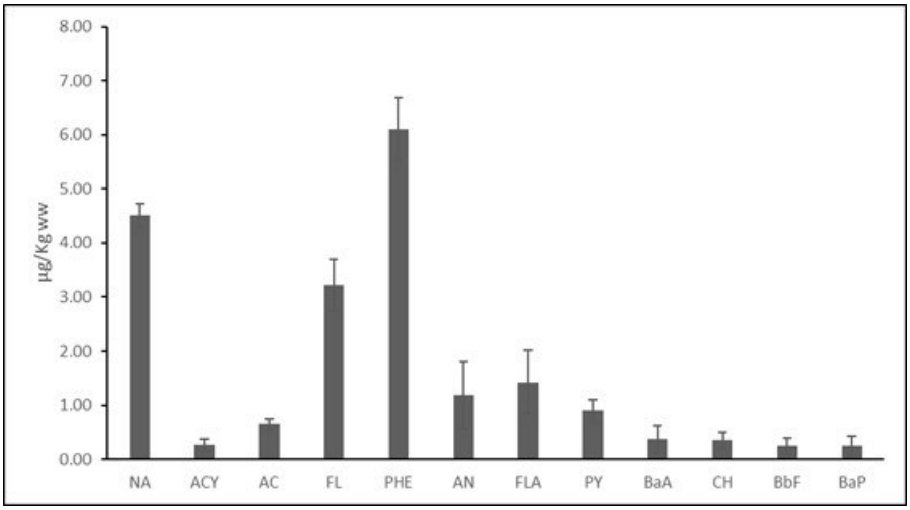

Figure 13: Distribution pattern of PAH congeners in muscle tissues of M. surmuletus, where the error bars correspond to the standard deviation SD.

The composition profile (%) based on the number of rings of PAHs identified in the pooled fishes is reported in Figure 14. The most abundant detected compounds are the low molecular weight PAHs (LMW-PAHs), accounting for the 81.8 % of total PAHs with percentages of 23.1% and 58.6% for the two rings and for the three rings compounds, respectively. Instead, four rings MMW-PAHs and five to six-ring HMW-PAHs only accounted for 15.7% and 2.5% of total PAHs, respectively. Among the detected PAHs, phenanthrene was the most abundant congener reflecting the distribution patterns of PAHs in seawater reported in some studies conducted in Mediterranean Sea.[191,192] Phenanthrene, which is one of the main compounds of crude oil, is listed in Group 3-Unclassifiable as carcinogenic in humans by IARC.[193] The second most abundant congener detected was naphthalene, which is classified as possible carcinogen to humans (Group 2B).[193]

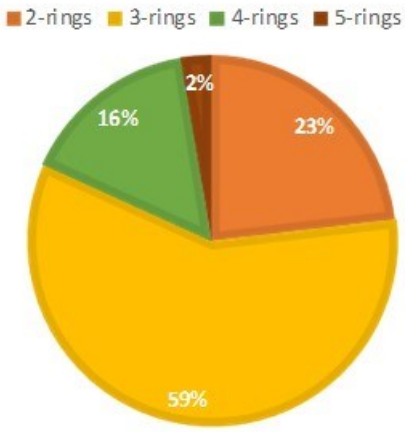

Figure 14: Percentage Distribution (%) of PAHs based on the number of rings in pooled muscle samples of M. surmuletus from the Catania Gulf, Sicily

These results are in agreement with those of several studies that report that PAHs found in living organisms are generally those containing two or three rings.[175,194–199] In fact, the accumulation of PAHs in fish is strongly influenced by several factors, including the PAHs solubility and bioavailability, molecular weight, and exposure route. 2 to 3 rings PAHs show a greater solubility in water and a relatively slow metabolism, suggesting that the abundance of these compounds in the fish muscles is likely due to their uptake through the gills.[200] Noteworthy, several studies have shown a negative correlation between the accumulation of PAHs in several species and their log K_{ow}, which leads to a higher bioavailability of low molecular weight PAHs.[167,197,201] On the other hand, higher molecular weight PAHs bind to the sediments and to organic colloidal particles. Thus, they show lower concentration values in water which make them less bioavailable for their uptake through the gills, even though they could be absorbed from the sediments and from the organic particles present in the water columns via the digestive tract. Furthermore, in fish high molecular weight PAHs have a higher metabolism rate than the lower molecular weight ones (e.g. up to 99% of HMW-PAHs can be converted to metabolites within 24 h of uptake) and thus no accumulation of HMW-PAHs is usually observed[202] in these organisms. In fact, even though

Frenna et al. (2013) reported high concentration values of 4 to 5-rings PAHs in Mediterranean sea sediments sampled in different sites of Sicily, in the present study these compounds accounted for only 15.7% and 2.5% of the total PAHs detected in the investigated benthic fishes from the Catania Gulf, Sicily. This trend is also in agreement with the results reported in a study conducted on samples pooled in the Adriatic Sea, that reports the proportion of low molecular weight PAHs, middle molecular weight PAHs, and high molecular weight PAHs residuals in fish to be of 62%, 37%, and 1%, respectively[204]. This percentage composition seems to indicate a petrogenic origin. In order to better clarify the pollution sources, the ratio LMW/HMW and the isomeric ratios of AN/(AN+PHE), FLA/(FLA+PY), (FLA/PY) and (PHE/AN) were calculated for the samples analysed in this work and the results are reported in Table 12. In fact, these ratios are commonly used to assess the petrogenic or the pyrogenic origin of PAHs:[205] PAHs of pyrogenic origin consist of HMW (four to six rings), whereas PAHs of petrogenic origin are mainly LMW (two to three rings). In the present study, the predominance of LMW-PAHs with the LMW/HMW value >1 (Table 12) seems to indicate a petrogenic origin, whereas the isomer pair data [AN/(AN+PHE), FLA/(FLA+PY), (FLA/PY) and (PHE/AN)] would indicate a pyrogenic origin. Thus, these results suggested combined sources, either petrogenic and pyrogenic, as oil spill, petroleum combustion and burning of coal and wood. However, due to the higher metabolism speed and consequent biotransformation of HMW-PAHs in fish, the pattern and distribution of PAHs themselves could be altered in fish tissues. Therefore, the accumulation of LMW-PAHs in fish muscle could be an artefact indeed due to the faster metabolic rate of HMW-PAHs.[167,202] Although the use of diagnostic ratios seems to be not closely applicable for source identification in fish, the data reported in literature on Mediterranean Sea sediments sampled in different sites of Sicily mainly indicate a pyrogenic sources[203] of PAHs, in agreement with the data reported in this paper. However, to confirm this trend, further investigations are highly needed.

Table 12: Selected PAHs diagnostic ratios identified in fish samples collected from Ionian Sea as compared to literature data

PAHs Potential Diagnostic Ratios	Pollution Emission Sources	Calculated ratios	Reference
LMW/HMW	>1 Petrogenic <1 Pyrogenic	31.9	206
AN/(AN + PHE)	<0.1 Petrogenic >0.1 Pyrogenic	0.2	207
FLA/(FLA + PY)	<0.4 Petrogenic 0.4–0.5 Fossil fuel combustion >0.5 Biomass and coal combustion	0.6	208
(FLA/PY)	>1 pyrogenic <1 petrogenic	1.6	209
(PHE/AN)	>10 petrogenic origin <10 combustion	5.1	210

The environmental legislation, directive 2013/39/UE,[211] establishes the biota EQS only for Fluoranthene and Benzo(a)pyrene, this last being considered as a marker for the other PAHs. For these classes of pollutants, the EQS_{biota} refers to crustaceans and molluscs with concentration limits of 5.0 μg kg^{-1} and 30 μg kg^{-1} for Benzo(a)pyrene and Fluoranthene, respectively. Even though, for the purpose of assessing the chemical status of water, monitoring of Fluoranthene and PAHs is usually carried out on molluscs and crustaceans, the determination of the concentrations of these pollutants in fish is also widely used.[10,156,159,212] In fact, even if fishes metabolize PAHs faster than invertebrates, the detected values are not negligible, since the distribution of PAHs in marine organism strongly depends on the lipid content of organisms, feedings habitat and trophic level. Indeed, it has been reported that the distribution of PAHs compounds differs from species to species [159]. Hence, the accumulation of PAHs and their metabolites in fish can negatively affect the health of fish [160,161] as well as the health of human populations who consume these fish.
As regards the foodstuff legislation, Regulation (EU) No 835/2011, which modifies Regulation (EC) No 1881/2006 regarding maximum levels for polycyclic aromatic hydrocarbons in foodstuff, establishes that maintaining a maximum level for PAHs (expressed as Benzo(a)pyrene) in fresh fish is no longer appropriate, because it has been shown that PAHs are quickly metabolised in fresh fish and do not accumulate in the muscle meat. Therefore, the previous limit of 2 μg kg^{-1} for Benzo(a)pyrene in fresh fish has been repealed. The results of the present work show no accumulation of higher molecular weight PAHs in fish muscle, even though Benzo(a)pyrene was detected in three pooled samples at a concentration below the previous food limit of 2 μg kg^{-1}. On the other hand, for the lower molecular weight PAHs, such as Naphthalene, Fluorene and Phenanthrene, no recommendations for foodstuff are reported. Therefore, these compounds are not subjected to biota monitoring programme and authority control, leaving anyway a gap in the knowledge of their distribution and of their effects on human health.

Human Health risks assessment

The main route of human exposure to marine PAHs is through the marine living organisms that enter the human diet. Considering the IARC classification, the sum (∑c PAHs) of the carcinogenic (Group 1), probable (Group 2A) and possible (Group 2B) carcinogenic PAHs, was evaluated and it accounts for 29.5% of total PAHs. NA, classified as possible human carcinogenic (Group 2B), was the most abundant compound, while BaP (carcinogenic to human, Group 1), was detected only in three of the analysed fish muscle samples.
The toxicity equivalent of BaP (TEQ BaP) value was 0.3455 ng/g ww, which is lower than the screening values for the BaPeq (0.677 ng/g ww) suggested by USEPA for human fish consumption.[178]

The Human Health Risk linked to fish consumption was evaluated using the concept of the daily dietary intake (DDI) and results to be 375.19 ng/day ww, that is slightly lower than those reported by Ferrante et al. (2018) for other demersal fishes collected in the same study area, the Catania Gulf. Analogously, the calculated ILCR index value is quite low (2.97×10^{-7}), considering that an ILCR of 1×10^{-5} is regarded as the "maximum acceptable risk level" (ARL).[178]

Thus, the preliminary results reported in this paper suggest a potential low carcinogenic risk linked to the consumption of *M. surmuletus* for local population. However, the assessment of carcinogenic risks posed by fish consumption requires deeper investigation and should be carried out by taking into account the multiple pathways of the biotransformation of PAHs[213] and the potential presence of other pollutants.[214,215]

3.6.2 PCB and pesticides analysis

The results of PCB analysis only show the presence of PCB 028, PCB 138 and PCB 153 with a detection frequency above 30% but always at concentrations near LOQ (0.5 μg kg^{-1}). According to other studies,[216,217,218] PCB 138 and PCB 153 are the most frequently PCB detected in fish and, due to their chemical properties, they are also those that accumulate more along the food chain. In fact, being less hydrophobic, they weakly bind to the sediments and, as a consequence, show a major availability to the water organisms. In addition, their stereochemical characteristics, such as non-vicinal hydrogens in meta-para or ortho-meta position, lead to a greater resistance towards the metabolic pathways and to a more difficult elimination from the organisms with a consequent longer half-life.[217]

Among organochlorine pesticide (OCPs) only 4,4′-DDE residues were detected (detection frequency below 30%). Although these pollutants are detected in traces with concentration near LOQ, their presence confirms their high persistence in the environment despite their use has been banned many years ago.

CHAPTER 4

DEVELOPMENT OF ANALYTICAL METHODS TO DETECT PAHS IN ETNA VOLCANIC ASHES

This short chapter focuses on the development of analytical methods to detect PAHs in Etna volcanic ashes. Since the area investigated in this Thesis, the Catania Gulf, is of particular interest being characterized either by anthropogenic pressures, including industrial and municipal impacts and high maritime traffic, and by natural pressures, such as the Etna volcanic activity, a preliminary study was carried out in order to evaluate the potential impact of volcanic ash falls in the aquatic ecosystem. After a brief review of the literature, the common diagnostic ratios used to distinguish the sources (pyrogenic or petrogenic) of PAHs were also reported. The experimental section focuses on the development of extraction methods to detect PAHs in volcanic ashes. Finally, preliminary results of volcanic ashes sampled at high altitude (~3000 m) during the intense eruption of November 2013 were shown.

4.1 IMPACT OF VOLCANIC ERUPTIONS IN THE AQUATIC ECOSYSTEM

As one of the most intensive natural phenomena, volcanic eruptions release huge amounts of gases and solid particles into the atmosphere, impacting the environment and the living species with several effects. Volcanic ashes travel hundreds of kilometres downwind from the volcano generating the characteristic ash falls. The ash fall causes several complications to human life and to economic activity by covering roads and airport runways with expensive disposal activities. Moreover, intense eruptions contribute to the pollution of atmosphere,[219] and they may also alter the characteristics of clouds, trigger the greenhouse effect, and introduce climate changes.[219]

Although several studies investigate the impact of volcano eruptions in the atmospheric chemistry,[220,221] including the climate changes,[222] there is a lack of knowledge related to the emission of organic compounds, such as PAHs, during geological activities. Furthermore, many potential sources of pollutants may co-occur, including natural ones (e.g., fires of forests), as well as anthropogenic emissions, making difficult the identification of the sources themselves.

PAHs enter the aquatic environment mainly through air dispersion as a result of various processes.[223] Generally, natural sources of PAHs emission are regarded as insignificant and treated as background.[224] In their work, Kozak ed al. (2017)[225] discuss about the source of PAHs that have been detected in the Arctic tundra environment, studying the hypothesis regarding the impact of Eyjafjallajökull volcano eruption (Iceland, 63.63_ N, 19.62_ W), from 14 April to 22 May 2010, of the Grímsvötn volcano eruption and of the related pollutants emission. In particular, the authors analysed the concentration of PAHs detected in water samples collected from the Fuglebekken catchment area using the diagnostic ratios and compared these data with that provided in the study by Polkowska.[226] The results show an increase in PAHs concentration preceded by the 2010 and 2011 volcanic eruptions in Iceland; however, using only the diagnostic ratios it was not possible to identify natural or anthropogenic sources of these compounds. Despite these results do not allow an unequivocal correlation between the increased PAHs concentrations and the volcanic eruptions, as there were other likely sources of these compounds, the authors disagree with the claim that natural sources of emission are negligible, and therefore concluded that the contribution of volcanic eruptions should be estimate case by case.

Considering these results, the Sicilian marine environment could be subjected to several sources of PAHs emissions, including the Etna volcano eruptions. In literature, some works report the emission of pollutants from Etna volcano[227,228,229] and few studies, at the best of my knowledge, focus the attention on PAHs.[230,231] As regards PAHs concentration in Etna volcanic ashes, Stracquadanio et al. (2003)[231] examined the presence of PAHs in volcanic ash deposited, in 2001 and 2002 during eruptive activity of Mount Etna, at increasing distances from the vent, in order to identify the possible origin, natural vs. anthropogenic, of these pollutants and to obtain

information about possible removal processes of these pollutants from the atmosphere. The authors place particular emphasis on the distribution sizes of the volcanic ashes, questioning the role of volcanic ashes as scavenger of PAHs from troposphere and giving further evidence about the major anthropogenic origin of these pollutants.

4.1.1 PAHs diagnostic ratios

Some of the diagnostic ratios commonly used are reported in Table 13. Diagnostic ratios are a useful tool to identify the sources of PAHs emission in water, soil and air,[232] allowing to distinguish between PAHs pollution originating from petrogenic (liquid fuels spills), pyrogenic (combustion of fuels) and burning biomass or coal sources.[21–23] In fact, the biomass burning, the diagenetic as well as volcanic activity phenomena[182] can form PAHs; however, in the regions subjected to a high human pressure, where coal and wood burning, as well as petrol and diesel oil combustion, and industrial processes are largely present, they are mainly of anthropogenic origin.[183] PAHs are always emitted as a mixture and thus the ratios of their relative molecular concentrations, (considering pairs of PAHs with the same molar mass and slightly different physicochemical properties,[24] that should then suffer a similar environmental fate), could be characteristic of specific pollution emission sources, constituting a very useful tool for their identification.

As regarding PAHs emission from volcanic eruptions, it should be noted that no coefficient can unequivocally verify natural sources of PAHs emission into the environment. Moreover, the relative concentration of PAHs may be affected by temperature differences that occur during a volcano eruption and by different magma composition.[233] Nevertheless, considering the thermodynamics similarities between the process of combustion and volcanic eruption, the volcanic source of PAHs emission can be regarded as combustion. Podkletnov and Markhinin (1981)[234] demonstrated that heating at 340–1000 °C a H_2O:CO_2:NH_3 mixture in the 90:90:1 v/v ratio (simulating the composition of volcanic gas) in a reactor containing volcanic ash, light PAHs (mainly 2–3 ring) are formed together with nitrogen containing organic compounds.[234]

Table 13: PAHs diagnostic ratios commonly used. ANT: Anthracene; PHE: phenanthrene; BaA: Benzo(a)Anthracene; CHR: chrysene; FLA: fluoranthene; PYR: pyrene; IcdP: Indeno(1,2,3-c)pyrene; BghiP: Benzo(ghi)perylene; Fl: fluorene; BaP: benzo(a)pyrene.

PAHs Diagnostic Ratios Potential	Pollution Emission Sources	Reference
LMW/HMW	>1 Petrogenic <1 Pyrogenic	206
ANT/(ANT + PHE)	<0.1 Petrogenic >0.1 Pyrogenic	207
BaA/(BaA + CHR)	0.2–0.35 Coal combustion <0.2 Petrogenic >0.35 Pyrogenic	235 236
FLA/(FLA + PYR)	<0.4 Petrogenic 0.4–0.5 Fossil fuel combustion >0.5 Grass, wood, coal	208
IcdP/(IcdP + BghiP)	<0.2 Petrogenic 0.2–0.5 Petroleum combustion >0.5 Grass, wood	236
(PHE/ANT)	>10 petrogenic origin <10 whereas combustion	210
(Fl/PYR)	~1 indicate pyrolytic origins >1 attributed to petrogenic source	210
BaP/BghiP	<0.6 Non-traffic emissions >0.6 Traffic emissions	237
FL/(FL + PYR)	<0.5 Petrol emissions >0.5 Diesel emissions	238

4.2 MATERIALS AND METHODS

4.2.1 Sample Extraction

Etna volcanic ashes were sampled at high altitude (~3000 m) during the intense eruption of November 2013. 5 g of ashes (size <1 mm) (Figure 15) were spiked with deuterated internal standard (IS) solution (naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, perylene-d12) at 5 µg/kg and were then extracted with an acetone:hexane (1:1) mixture using an ultrasonic bath (15 minute x 3 times); the extract was filtered (0,45 µm Nylon) and evaporated to dryness and the residue was redissolved in 2 ml of hexane. The last clean up step was carried out using silica gel SPE cartridge preconditioned with 4 ml of hexane. The analytes were eluted with 5 ml of hexane and 5 ml di hexane:ethyl acetate 1:1. Then, the eluate was evaporated to dryness under a gentle stream of nitrogen, and the residue was redissolved in 250 µl of hexane.

Figure 15: Ash samples analysed (Volcanic eruption of November 2013)

4.2.2 GC-MS Analysis

The analyses of samples were performed using the same GC-MS conditions described previously (2.2.5 GC-MS Analysis) with the following oven temperature program (Table 14):

Table 14: Oven temperature program for PAH analysis

	Rate **°C/min**	**Value** **°C**	**Hold Time** **min**
Initial	-	70°C	1
Ramp 1:	12°C/min	160°C	1
Ramp 2:	15°C/min	300°C	8

4.2.3 Recovery

Recovery was determined within the same analytical session by preparing 3 sets of samples. In the first set named "spiked", ash samples were spiked with the analytes (PAHs) at 5 µg/Kg before the extraction step; in the second set named "post-spiked", working solutions of the analytes were added (at the same concentrations) on the blank ash extracts; the third set "matrix" was represented by the ash samples extracted. The recovery of the analytes was calculated by the ratio between the peak area ratio of the compound signal to the IS determined after its extraction (spiked) and that determined on the spiked extract (post-spiked).

$$\text{Recovery} = [(\text{Area S/Area IS})_{\text{spiked}}/(\text{Area S/Area IS})_{\text{post-spiked}}]*100$$

S= Standard; IS= Internal Standard

4.2.4 Data Analysis

In order to identify the pollution emission sources, PAHs were grouped into three different classes according to the number of the rings: low molecular weight PAHs (LMW-PAHs) including 2–3 ring PAHs (NA, ACY, AC, FL, PHE, AN,), medium molecular weight PAHs (MMW-PAH) including 4 ring PAHs (FLA, PY, BaA, CH), and high molecular weight PAHs (HMW-PAH) including 5-6 ring PAHs (BbF, BkF, BaP, DBahA, BghiP, IP). Moreover, the ratio between LMW-PAHs and HMW-PAH was calculated.

4.3 RESULTS AND DISCUSSION

A GC-MS method was developed for determining 16 polycyclic aromatic hydrocarbons (PAHs) in Etna volcanic ashes. As regards the analytical methodologies for the quantification and the unequivocal identification of these organic compounds, one of the golden keys has always been the sample pre-treatment improvement, through the interferences reduction in order to reach reproducible and accurate data, which are especially challenging for complex matrices. Different extraction and clean-up procedures, based on solid phase extraction (SPE), were tested. The best method was based on ultrasonic extraction and silica gel SPE clean-up step, as reported in the Sample Extraction section, with slight differences compared to similar existing extraction and clean-up methods.[239,240]

An example of Etna volcanic ashes SIM chromatograms is shown in Figure 16.

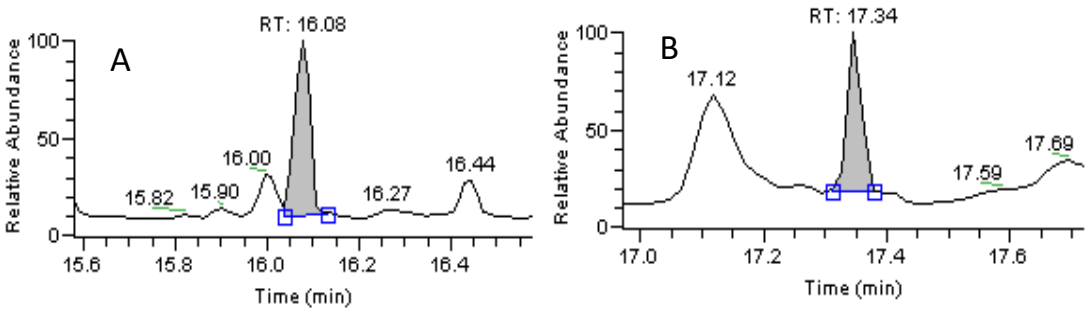

Figure 16: SIM chromatograms of Etna volcanic ashes: A) Perylene (m/z 252); B) Indeno(1,2,3-cd)pyrene (m/z 276)

The recovery was within 70-120% for almost every analyte, except for acenaphthylene which shows a recovery < 50% (Table 15).

Table 15: recovery at 5 µg/kg of PAHs in volcanic ashes

PAHs	Internal Standard (IS)	Recovery (%)
		C_{PAHs} 5 µg/kg
Naphthalene	Naphtalene-d8	82 ± 4
Acenaphthylene	Acenaphthene-d10	<50
Acenaphthene		118 ± 2
Fluorene	Phenanthrene-d10	70 ± 3
Phenanthrene		90 ± 2
Anthracene		116 ± 4
Fluoranthene	Chrysene-d12	100 ± 5
Pyrene		104 ± 4
Benzo(a)anthracene		88 ± 7
Chrysene		86 ± 4
Benzo(b)fluoranthene	Perylene-d12	93 ± 3
Benzo(k)fluoranthene		80 ± 4
Benzo(a)pyrene		76 ± 2
Dibenzo(a,h)anthracene		124 ± 2
Indeno(1,2,3-cd)pyrene		76 ± 5
Benzo(g,h,i)perylene		91 ± 6

The data obtained by the analysis of Etna volcanic ashes show the presence of the three- to six-ring PAHs: Phenanthrene, Anthracene, Fluoranthene, Pyrene, Chrysene, Benzo(b)Fluoranthene, Benzo(k)Fluoranthene, Benzo(j)Fluoranthene, Perylene, Indeno(1-2-3-cd)pyrene, Dibenzo(a,h)antracene.

The percentage distribution based on the number of ring of individual PAHs detected in the samples is reported in Figure 17. The most abundant detected PAHs are the high molecular weight PAHs (HMW-PAHs) accounting for 55% of total PAHs with percentages of 37% and 18% for the 5 rings and for the 6 rings, respectively. Instead, four rings MMW-PAHs and three rings LMW-PAHs accounted for only 27% and 18%, respectively. This percentage composition, seems to indicate a pyrogenic origin, supported by the LMW/HMW ratio value <1.[206]

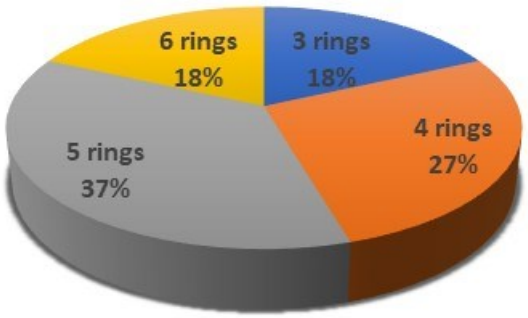

Figure 17: Percentage distribution (%) based on the number of rings of individual PAHs detected in Etna volcanic ashes.

Although these data are only qualitative, and further quantitative analysis are required, the presence of the three- to six-ring PAHs suggest a mixture of petrogenic and pyrogenic sources.
Considering the study carried out by Podkletnov (1981),[234] it is expected that Etna volcanic ashes sampled at high altitude, are rich in 2-3 ring PAHs, as natural sources. However, the data reported in literature are very few and controversial, Stracquadanio et al. (2003)[231] reported that the presence of PAHs found in Etna volcanic ashes are related to the distance travelled from ash, since the ashes adsorb pollutants from the troposphere. They analysed volcanic ashes, sampled at three locations in Sicily, namely in Catania, Augusta and Siracusa, after a flank eruption with intense explosive activity occurred in Mount Etna in 2001 and 2002. They found the highest concentrations of PAHs in Augusta samples, which may be likely related to the activity of the petrochemical district and they emphasised on the size of volcanic dust fraction as a transport medium for individual PAHs compounds. Based on these results, the authors suggest the role of volcanic ash as scavenger of PAHs and claim that the origin of these pollutants is mainly anthropogenic, according to other study that considered PAHs natural sources as negligible.[224]
In contrast to this study, Kozak et al. (2017)[1] reported an increase of PAHs concentrations in the Artic aquatic environment after the eruption of Eyjafjallajökull volcano and of Grímsvötn volcano. Although they could not unequivocally relate the increased PAHs concentration values to the volcanic eruptions, as there were other likely sources of these compounds, the authors disagree with the claim that natural sources of emission are negligible in comparison to anthropogenic emission. Very recently, Guiñez et al. (2020)[241] detected nitro-PAHs and oxy-PAHs in the volcanic ash samples collected from Puyehue, Copahue, and Calvuco volcanoes ranged from 3.48 to 15.90 ng/g and 1.89 to 20.66 ng/g, respectively. Once again, the source of these PAHs cannot be easily attributed to anthropogenic or natural activity.
Thus, the data obtained by this preliminary study clearly indicate the need of additional and in depth investigations in order to evaluate the impact of Etna volcanic activity in the aquatic environment, as many pollutants such as PAHs enter the seas, the lakes and the rivers mainly through air dispersion.

CHAPTER 5

DEVELOPMENT OF FLUORESCENCE-BASED TECHNIQUES FOR THE DETECTION OF PAHS

This chapter focuses on the development of fluorescence-based analytical techniques to detect PAHs in seawater. In the introduction section, the application of fluorescence spectroscopy, the recent advances in the development of fluorescent chemosensors and the photophysical phenomena involved are discussed. The experimental section reports the synthesis and characterisation of novel fluorescent nanoparticles (AgNPs) capped with fluorescein (Fluo), and the development of rapid sensitive fluorescence-based techniques to detect PAHs in seawater. For this purpose, several fluorescence experiments were carried out using Fluo-AgNPs, the free ligand Fluo and pyrene as model compounds.

5.1 INTRODUCTION

Several analytical methods, including high performance liquid chromatography and gas chromatography coupled with mass spectrometry,[242,243,244,245] have been used to detect PAHs in water samples and in other environmental matrices. These methods require preliminary extraction and clean up steps before the chromatographic quantification. The PAH detection limits of these techniques are in the ppb range, and, since the concentration values of PAHs in seawater are lower, preconcentration treatments (for example, using solid phase extraction) of the samples are required and, thus, despite their high accuracy and sensitivity, these methods are time-consuming. To overcome these problems, novel analytical methods that can provide accurate data in a fast way are highly needed. Fluorescence spectroscopy has been shown to be a valuable technique to characterize and monitor water pollutants, since it is a zero-background technique and has the advantage of high sensitivity and simplicity. In particular, the highly fluorescent nature of PAHs has made this spectroscopy a commonly used technique for the analysis of this class of pollutants.[246] However, the complexity of environmental samples, which could lead to broad emission spectra and background fluorescence, interferes with the fluorescence analysis of PAHs, often requiring a preliminary chromatographic separation. Additionally, due to the high sensitivity of this technique, other interferences may occur due to the presence of external quencher molecules and photophysical processes (e.g. ground state complex formation, excimer formation, exciplex formation, self-quenching etc) that could affect the fluorescence properties of the target analytes. Therefore, the analysis of complex multicomponent PAHs mixtures without preliminary separation steps did not find wide application.

The recent developments on the synthesis of new fluorophores and on the respective photophysical phenomena involved for the selective detection of water pollutants, including PAHs, have led to the reassessment of the fluorescence techniques and to advances in the development of fluorescent sensors for the rapid and on-site detection of water pollutants.[247]

5.2 FLUORESCENT CHEMOSENSORS

Usually, the basic building blocks of fluorescent chemosensors are the fluorophore (signaling moiety) and the receptor (recognition moiety).[248] The emission of fluorescence light is influenced by the interaction of fluorophore with the medium and could be tuned from visible to NIR wavelengths depending on the energy gap of the individual fluorophores.

The sensing applications are based on the changes of the photophysical properties of a fluorophore, such as emission wavelength, lifetime, quantum yield and quenching, that occur

during the interaction with the target analyte. This interaction could involve various mechanisms such as: Förster resonance energy transfer (FRET), photoinduced electron transfer (PET), electron exchange (EE), aggregation caused quenching (ACQ) and aggregation induced emission (AIE).[249–253] The transfers are characterized by different movements of the electrons between the lowest unoccupied molecular orbital (LUMO) and highest occupied molecular orbital (HOMO) of the participating molecules (donor and acceptor) resulting in changes in the emission profile, whereas ACQ and AIE refer to the aggregation of molecules, often nanoaggregates, in the aqueous medium resulting in reduced or enhanced emission.

These mechanisms, which require the interaction between the fluorophore and the analyte, are used to design turn-on/-off fluorescent chemosensors.[247] On the other hand, other fluorescent chemosensors are based on different mechanisms such as the inner filter effect (IFE), wherein the emission of a donor is absorbed by an energy acceptor, which prevents the excitation energy from reaching the luminophore, resulting in a filtering of the excited state. The fluorescence chemosensors using IFE generally involve nanomaterials, that, owing to their high absorbing capacity and extinction coefficients, offer several advantages, and do not require any specific complexation and covalent linking of the fluorophore and the analyte.[254,255]

Recently, several papers have been published on the fluorometric determination of PAHs, as well as of PCB and OCPs.[256–259] Besides the direct measurements of the quenching or the enhancement of the analytes fluorescence intensities, Energy Transfer mechanisms, either FRET and non-FRET have been exploited to this aim and a schematic diagram of these mechanisms is reported in Figure 18. In these phenomena, typically, a donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through non-radiative dipole–dipole coupling (FRET)[260] or through electron exchange (Dexter).[261]

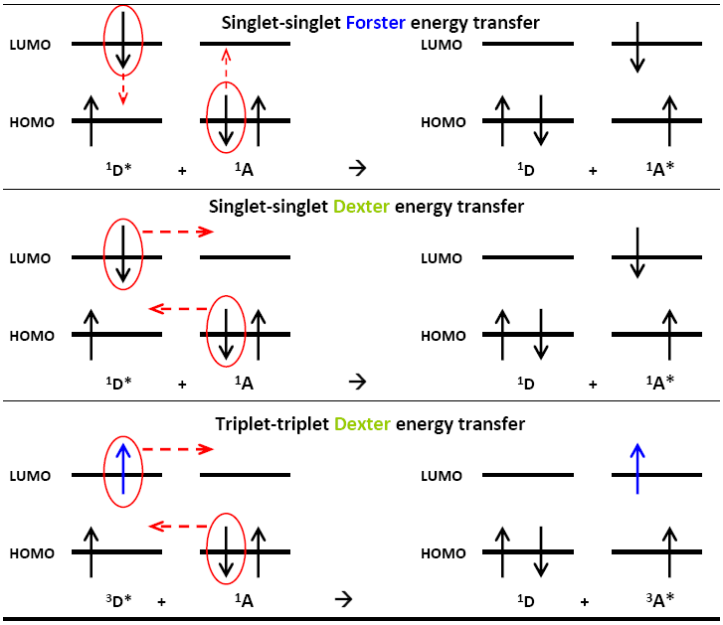

Figure 18: Schematic diagram for Förster and Dexter energy transfer

The donor, by absorbing light, changes to an electronic excited state and, instead to emit, transfers energy to an acceptor chromophore through non-radiative mechanisms. This non-radiative process is referred to as "resonance" and it happens only in the case that the fluorescence spectrum of the donor and the absorption spectrum of the acceptor significantly overlap. After excitation, the excited acceptor emits a photon and returns to the ground state, giving rise to an enhancement of its fluorescence.[261,262]

In both cases, the efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making the phenomena extremely sensitive to small changes in distance.[262] Hence these mechanisms have been exploited to study many biological systems by covalently link a donor and an acceptor on the molecules skeleton and evaluating their conformational changes in solution. However, if the ET is supposed to be due to non-linked Donor and Acceptor, the scenario is quite different. In fact, in this case, the close proximity between the two species is not obvious and mainly is not dictated by the conformational rearrangements of large macromolecules, but it is rather due to the possible weak interactions and the non-covalent bonds between the Donor and the Acceptor themselves, that should give rise to the formation of Supramolecular aggregates and complexes.[250,252,263,264] Among non-covalent interactions, π-π interactions play a crucial role, either as stacking between aromatic isolated systems and as the driving force responsible for the formation of many host-guest systems in which the host cavity and the guest are aromatic in character.[252] Obviously the formation of inclusion complexes represents a "non-covalent" way to get in close proximity a D and an A and several examples are present in the literature where this effect has been exploited.[252,256–259,265]

Considering that PAHs are very hydrophobic and thus, as a consequence, avoid, if possible, any interaction with water, probably they could strongly interact with aromatic molecules, mainly if these last are also highly soluble in water. In fact, in this case, they could be present in solution at a high concentration, becoming very efficient in stacking the PAHs.

5.3 NANOMATERIAL-BASED FLUOROPHORES FOR THE DETECTION OF POLLUTANTS IN WATER

Several fluorophores are reported in the literature including organic dyes, inorganic complexes, metal organic frameworks, quantum dots and noble metal nanoclusters for the detection of water pollutants.[266–268] Among these, metal nanoparticles (NPs) are promising and suitable materials to detect environmental pollutants,[269] thanks to their unique physical and chemical properties. These characteristics are influenced by the surrounding chemical environment, making these systems good sensors for pollutants sensing. Furthermore, NPs can be also capped with a wide range of small molecules or polymers allowing researchers to fine tune their characteristics and to obtain

novel detection systems.[270] Due to their optical properties, nanoparticles, especially silver nanoparticles (AgNPs), have been used as substrates in Surface-Enhanced Raman Scattering (SERS) to enhance the Raman signals of the pollutants. Organochlorine pesticide endosulfan was detected using bis-acridiminium lucigenin functionalized AgNPs,[271] while Sanchez-Cortes et al. (2015)[272] synthetized alkyl dithiol-functionalized AgNPs to detect other organochlorine pesticide (aldrin, dieldrin, lindane, and α-endosulfan). As regards the detection of hydrophobic compounds, such as PAHs, AgNPs functionalized with viologen,[273] dithiocarbamate calix[4]arene[274] and cyclodextrin derivatives[275] were reported as SERS substrates.

Also metal-enhanced fluorescence (MEF) is a promising tool for applications in fluorometric assays, rendering these last more rapid and sensitive.[276] MEF has been extensively used in the biosensing field, while few studies report environmental applications for pollutants detection.[269,277,278] As an example, the PCB trace detection was realized using the fluorescence of quantum dots (QDs) and the surface molecular imprinting effect.[279] The fluorescence signal was amplified using the room-temperature phosphorescence (RTP) emitted by Mn-doped ZnS QDs in order to detect pentachlorophenol (PCP). Moreover, quenching fluorescence of QDs can be used to detect heavy metals. Jackie Y. Ying's group synthesized several kinds of quantum dots,[280,281,282] in order to study the fluorescence quenching of AuNPs entrapped by proteins due to the interaction of Au quantum dots with metallophilic Hg^{2+} ions. They found that the fluorescence of the Au nanoparticles decreases with increasing Hg^{2+} concentration. However, the application of noble metal nanoparticles in fluorescence assays to enhance their sensitivity often requires time consuming procedures such as the surface functionalization of the nanoparticles and the modification of the fluorophore which could affect the inherent properties of the fluorophore itself. In this context, recent advances have been carried out in the design of IFE based systems.[255] Metal nanoparticles are suitable as potential absorbers in IFE based sensing systems because of their unique properties, allowing the changes in spectral overlap of the fluorophore and absorber by fine tuning the localized surface plasmon resonance peak. The sensing mechanism is based on the changes in the absorbance of the metal nanoparticles which exponentially influence the fluorescence of the fluorophores. As an example, the detection of cyanide in drinking water has been carried out using a "turn on" IEF based fluorescence chemosensor.[283]

Based on these previous evidences, fluorescence techniques based on nanomaterial as well as on organic chemosensors could be a promising tool for the detection of water pollutants.

With the above in mind, in this chapter a new method for the determination of pyrene in ultrapure water and in seawater has been developed by using an aromatic anion highly fluorescent, i. e fluorescein, either as it is and as silver nanoparticles capping agent.

5.4 MATERIALS AND METHODS

5.4.1 Synthesis of fluorescent silver nanoparticles

Fluorescent nanoparticles were synthesized at ice-cold temperature, as previously described for L-Tyr AgNPs[284,285]. The syntheses were carried out by dropwise adding 30.0 ml of a silver nitrate solution (4 x 10^{-4} M) to 27 ml of a freshly prepared solution of $NaBH_4$ (4.4 x 10^{-3} M) put in an ice bath under stirring. 3 ml of a 1 x 10^{-3} M of Fluorescein solutions were added to the silver sol to obtain Fluo AgNPs. The stirring was stopped immediately after the addition of the capping agent solutions.

5.4.2 Characterization of Fluo AgNPs

TEM analysis were carried out using a TEM JEOL JEM 2010 F, as previously reported.[285] The average sizes were obtained using the following formula: $= \sum (n_i d_i)/n$, where n_i is the number of AgNPs of diameter d_i and n is the total number of Ag particles.

UV–Vis spectra were carried out at room temperature, using a diode-array Agilent 8453 spectrophotometer in the 200–600 nm wavelength range. The Fluorescein colloids were opportunely diluted with borate buffer. The concentration of the AgNPs solutions was calculated accordingly to Mulfinger et al.(2007) study.[286]

5.4.3 Fluorescence measurements

5.4.3.1 Apparatus, reagents and standards

Fluorescence measurements were recorded at room temperature on a Varian Cary Eclipse Fluorescence Spectrophotometer using a standard 1 cm cell in the range 325-650 nm. All fluorescence measurements were carried out under the same experimental conditions, setting the excitation wavelength at 315 nm, the excitation and the emission slits width at 10 and 5 nm, respectively. The scan speed was 600 nm min^{-1}, and the PMT voltage was set at 600 V. The Cary Eclipse and the SpectraGryph software (vs 1.2.12) were used to acquire and process the spectral data, respectively.

Pyrene (PY, purity >99%), fluorescein sodium salt (Fluo, purity >99%), NaCl (purity >99%) and sodium tetraborate pentahydrate (purity >99%) were obtained from Sigma Aldrich. The stock solutions of PY (1000 ppm) and Fluo (1×10^{-3} M) were prepared by dissolving the compounds in ethanol and in ultrapure water, respectively. The intermediate solutions were obtained by serial dilutions of the stock solutions in ultrapure water. All the solutions were stored in brown vials at 4 °C and wrapped with aluminum foil to avoid possible photodegradation and the working solutions were freshly prepared before use.

5.4.3.2 Fluorescence titrations in aqueous solution

Different fluorescence titrations were carried out with different procedures in order to optimize the titration conditions:

(1) by adding to water or to different pyrene solutions (0.025, 0.625, 2.5 and 5 ppb) increasing amounts of the colloid, or of a fluorescein solution (2.5×10^{-5} M);

(2) by adding to different solutions of pyrene (from 0.01 to 5 ppb) the same amounts of fluorescein solution (2.5×10^{-5} M);

(3) by adding to water and to the more dilute pyrene solution (0.01 ppb, 4.95×10^{-11} M) increasing amounts of different fluorescein solutions (1×10^{-8} M, 1×10^{-7} M, 1×10^{-5} M).

The calibration curve for the determination of pyrene in aqueous solution (pH=6.5) was obtained by adding to different solutions of pyrene (0, 1.65×10^{-11}, 3.30×10^{-11}, 4.95×10^{-11} and 6.60×10^{-11} M) the same volume (100 µl) of a fluorescein solution (1×10^{-5} M). Each concentration was checked in triplicate.

5.4.3.3 Fluorescence titrations in seawater

Seawater samples were collected in amber glass bottles and stored at 4°C until fluorescence measurements. Two different protocols were used:

(1) The seawater samples were filtered before the experiments using cellulose acetate membrane filter (0.45 µm) and diluted (1:1) with 0.1 M borate buffer pH=9. Considering the different pH and salinity of seawater from those of ultrapure aqueous solution, the working solutions of PY and Fluo were prepared using an 80 mM NaCl 0.05 M borate buffer (pH 9). The fluorescence titrations were carried out by adding to different solutions of pyrene (dissolved in 80 mM NaCl 0.05 M borate buffer), or seawater samples spiked with the same concentrations of pyrene, the same amounts of fluorescein solution (1×10^{-5} M).

(2) The seawater sample was divided into two aliquots (100 ml) named A and B; the first one was spiked with pyrene at a final concentration of 0.5 ppm. 25 ml of the two aliquots were subjected to liquid-liquid extraction with 15 ml of dichloromethane. The extracts were evaporated under nitrogen and the residues were redissolved in 25 ml of ultrapure water. The fluorescence titrations were carried out by adding to the two aliquots (A and B) and to ultrapure water increasing volumes (100, 200 and 300 µL) of a fluorescein solution (1×10^{-5} M). Then, in order to quantify the possible amount of pyrene dissolved in the seawater sample, the method of standard addition was performed by diluting the aliquot B with water (ratio 1:3) and by adding increasing amounts of a pyrene solution (0.1 ppb) with a final concentration range from 0 to 0.013 ppb.

5.5 RESULTS AND DISCUSSION

5.5.1 Synthesis and characterisation of fluorescent silver nanoparticles

The AgNPs were synthesized at low temperature to obtain small particles with a narrow distribution range. In Figure 19 the TEM image for the Fluo AgNPs is reported. The microphotograph shows the presence of an abundant population of small nanoparticles with an average size distribution of 4 ± 2 nm and a very few particles of larger size.[287] The concentration values of AgNPs-Fluo solution was estimated according to the methods reported by Mulfinger et al. (2007)[286]. For particles of 4 nm we obtained a volume of 33 nm^3 and considering that each Silver atom occupies the volume of a cube with an edge of 0.3 nm, in a 4 nm AgNP there are almost 1241 atoms. Assuming that all the silver ion is reduced, the concentration of Ag in solution is 2 x 10^{-4} M, that corresponds to a concentration of 1.61 x 10^{-7} M for the AgNPs.

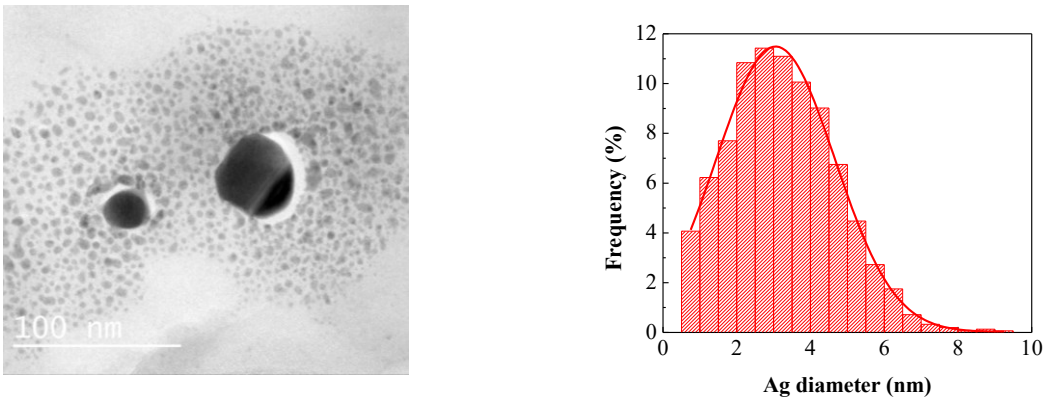

Figure 19: TEM microphotographs and related particle size distribution for Fluo AgNPs

The UV-Vis spectrum for Fluo AgNPs (Figure 20) shows the typical absorption peak of the fluorescein at λ= 495 nm and the Surface Plasmon Resonance of the AgNPs at 399 nm.
This spectrum confirms the results obtained by the theoretical calculations for the diameter sizes for dilute dispersions of silver nanoparticles in water reported in the literature,[288,289] indicating the value of 4 nm accordingly to the TEM size distributions.

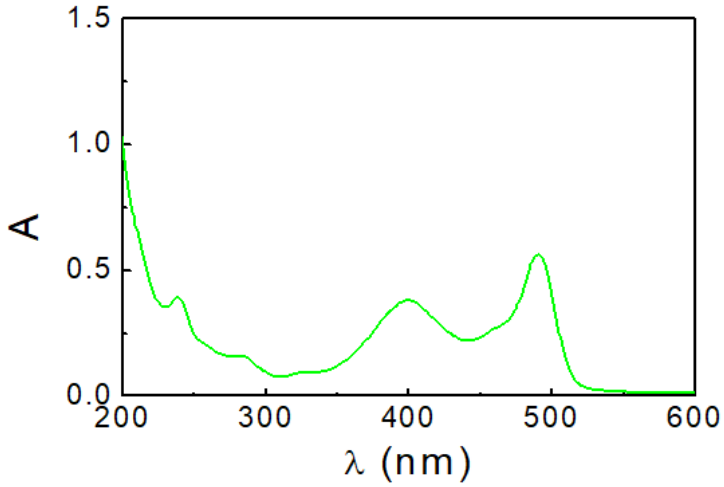

Figure 20: UV-Vis Spectra of AgNPs-Fluo

5.5.2 Fluorescence measurements

5.5.2.1 Fluorescence titrations with Fluo

The fluorescence spectra of a pyrene solution (5 ppb) and of the Fluo solution are reported in Figure 21 and Figure 22, respectively.

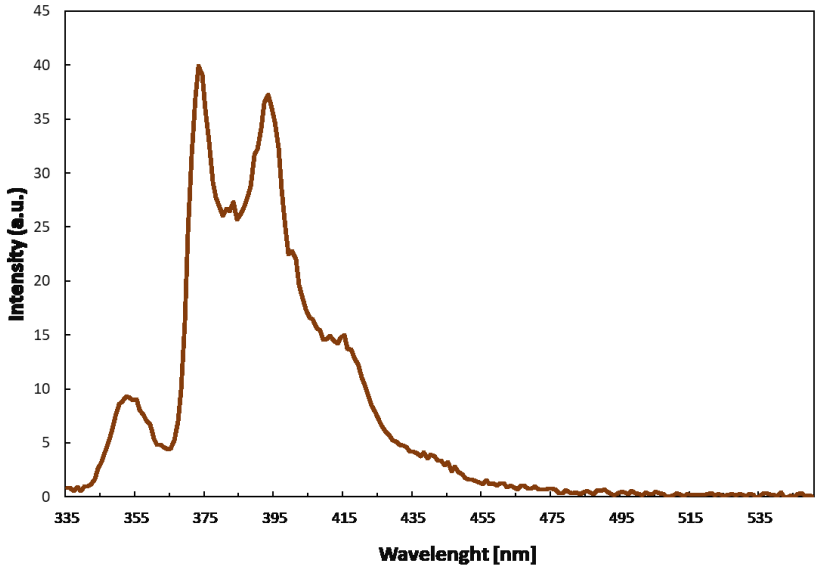

Figure 21: Emission spectra of Pyrene solution (5 ppb) excited at 315 nm

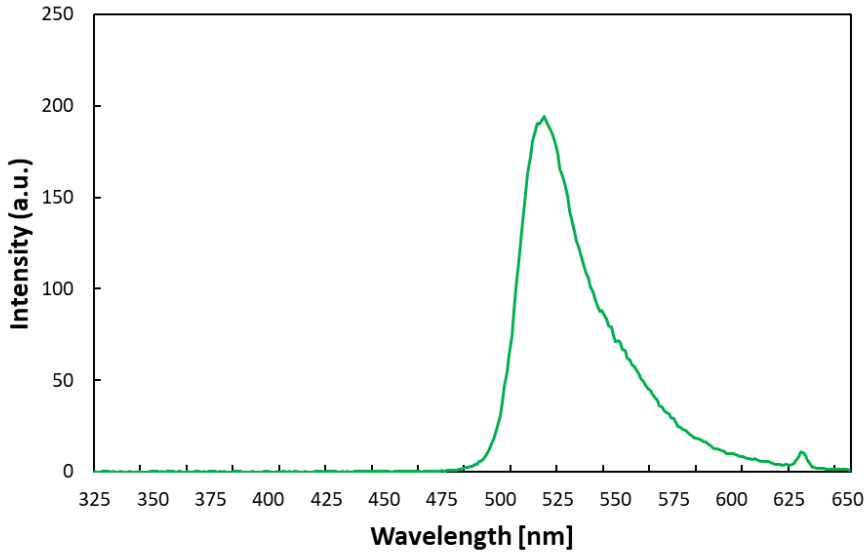

Figure 22: Emission spectra of AgNps-Fluo solution excited at 315 nm

The effects of Fluo on the fluorescence behaviours of pyrene at 5 ppb was investigated, by titrating the pyrene solution with the Fluo one and the results are reported in Figure 23. The Figure shows a quenching of the fluorescence of pyrene, on increasing the amount of added Fluo. The emission intensities of pyrene at 373 nm, corrected by taking into account the dilution effect and those experimentally recorded as a function of the various volumes added of a 5×10^{-5} M Fluo solution (from 0 to 50 µl) are reported in the Figure 24.

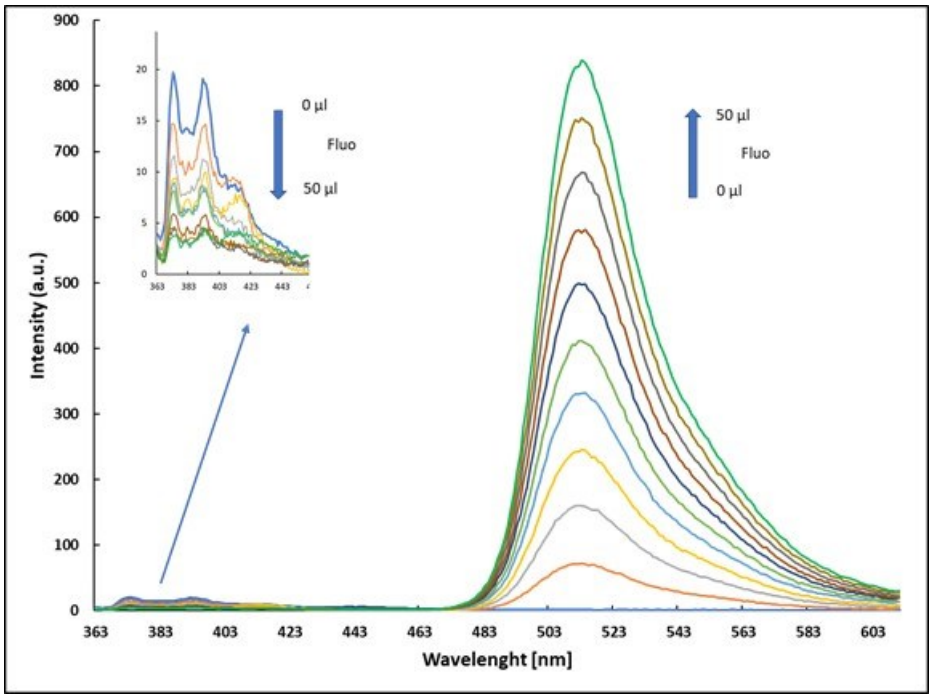

Figure 23: Fluorescence spectral changes of a 5 ppb Pyrene solution on addition of various volumes of Fluo solution (0-50 µL)

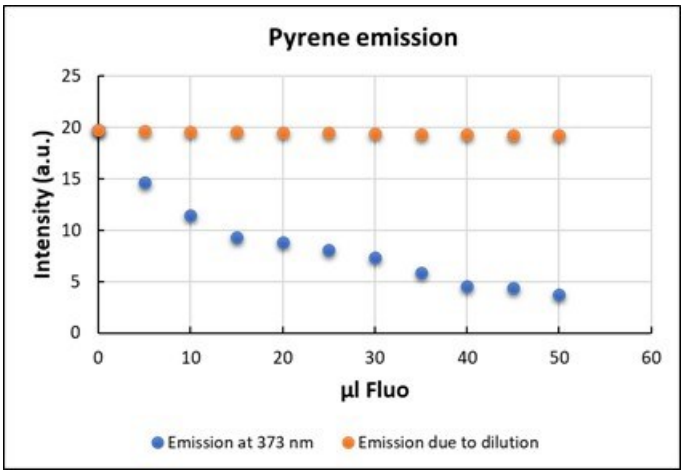

Figure 24: Comparison of pyrene emission at 373 nm; orange: theoretical emission corrected for the dilution; blue: experimental emission recorded by adding increasing volumes (0-50 µL) of the Fluo solution (5× 10^{-5} M).

The addition of the Fluo solution dramatically quenches the fluorescence of the pyrene solution, perhaps suggesting a not negligible interaction between the pollutant and the aromatic anion. At the same time, an enhancement in the fluorescence emission of fluorescein was observed. In order to better understand the nature of the phenomena involved, the same amount of Fluo solution was added to a solution of water and to a solution of pyrene (5 ppb) and the resulting fluorescence spectra are reported in Figure 25a, whereas in Figure 25b the emission values (λ 513 nm) of water and of a 5 ppb pyrene solution as a function of increasing fluorescein concentrations are reported. Interestingly, the emission of fluorescein is greater in the presence of the pyrene solution, probably suggesting a Förster Resonance Energy Transfer (FRET), where the donor (pyrene), by absorbing light, changes to an electronic excited state and, instead to emit, transfers energy to an acceptor chromophore (fluorescein) through non-radiative mechanisms. This non-radiative process is referred to as "resonance" and it usually happens only in the case that the fluorescence spectrum of the donor and the absorption spectrum of the acceptor significantly overlap. After excitation, the excited acceptor emits a photon and returns to the ground state, giving rise to an enhancement of its fluorescence. This phenomenon is probably promoted by the stacking interactions of the two aromatic systems that result to be an important driving force in solution, especially in the case of very hydrophobic analytes, as pyrene, that avoid, if possible, any interaction with water and are thus prone to strongly interact with aromatic molecules, mainly if these last are also highly soluble in water. In fact, aromatic anions can be present in solution at a high concentration, becoming very efficient in stacking the PAHs.

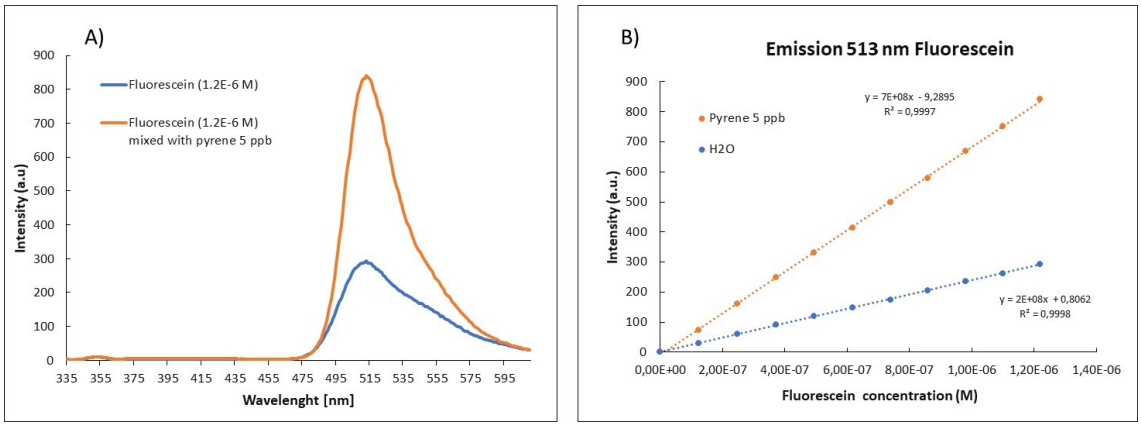

Figure 25: a) Fluorescence spectra of Fluo in water (blue) and in 5 ppb pyrene solution (orange) b) fluorescein emission values (λ 513 nm) in water (blue) and in 5 ppb pyrene solution (orange) as a function of increasing fluorescein concentrations;

In order to evaluate if the presence of a metal core could enhance this effect, ultrapure water and a 5 ppb pyrene solution were also titrated with a solution of the synthesized Fluo-AgNPs and the results are reported in Figure 26. Unfortunately, even if an enhancement of the capping fluorescein emission intensities is observed, this is less dramatic than that observed with the free ligand. In fact, also in this case, the intensities at 513 nm follow a linear trend, however the slope of the straight-line is less steep than that reported for the data obtained for the free ligand. Probably, in this case, silver NPs can adsorb pyrene molecules from diluted solutions quenching the pyrene fluorescence itself,[290,291,292] and rendering the resonance process less efficient.

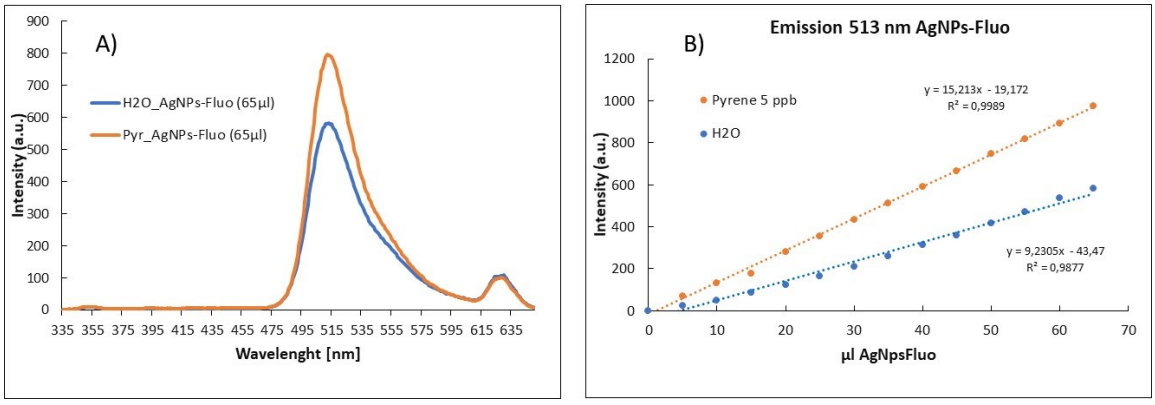

Figure 26: a) Fluorescence spectra of AgNps-Fluo in water (blue) and in 5 ppb pyrene solution (orange) b) fluorescein emission values (λ 513 nm) in water (blue) and in 5 ppb pyrene solution (orange) as a function of the different volumes of added AgNPs-Fluo solution;

Based on these evidences, further experiments were carried out using pyrene solutions at different concentrations in order to evaluate a possible linear correlation between the fluorescence intensity of fluorescein and the pyrene concentration.

5.5.2.2 Fluorescence titrations with Fluo

Optimization of the method

The enhancement of the fluorescence intensities of fluorescein mixed with the pyrene aqueous solutions, stronger than that observed for the fluorescein capping the nanoparticles, is quite puzzling and was thus further investigated. On the other side, even though AgNPs are promising fluorescence sensors for the detection of water pollutants[247] at trace level, the possible use of an even simpler system is interesting and allows to limit their impact on the environment, that is not fully investigated and could also present potential human health risks.[293]

The fluorescence spectra of fluorescein in the presence of different concentrations of pyrene are reported in Figure 27a. The Figure clearly shows that the fluorescein emission is enhanced by pyrene in a way depending on the concentration of the pollutant. The emission intensities of Fluo (λ=513 nm) obtained by titrating different solutions of pyrene (0.025, 0.625 and 2.5 ppb) with fluorescein (2.5×10^{-5} M) are reported in Figure 27b. In all cases the emission intensities of Fluo (λ=513 nm) increase, following a linear trend, and the presence of pyrene at different concentration values gives rise to different slopes of the straight lines. This trend seems to indicate a linear relationship between the enhancement of the fluorescence intensities of fluorescein and the increasing concentration values of pyrene solutions. These results are very promising. In fact, the pyrene solutions with concentration values below 5 ppb do not give any emission signal at 373 nm (Figure 27a), and, thus, the direct fluorimetric detection of pyrene in water is not possible at these concentration values.

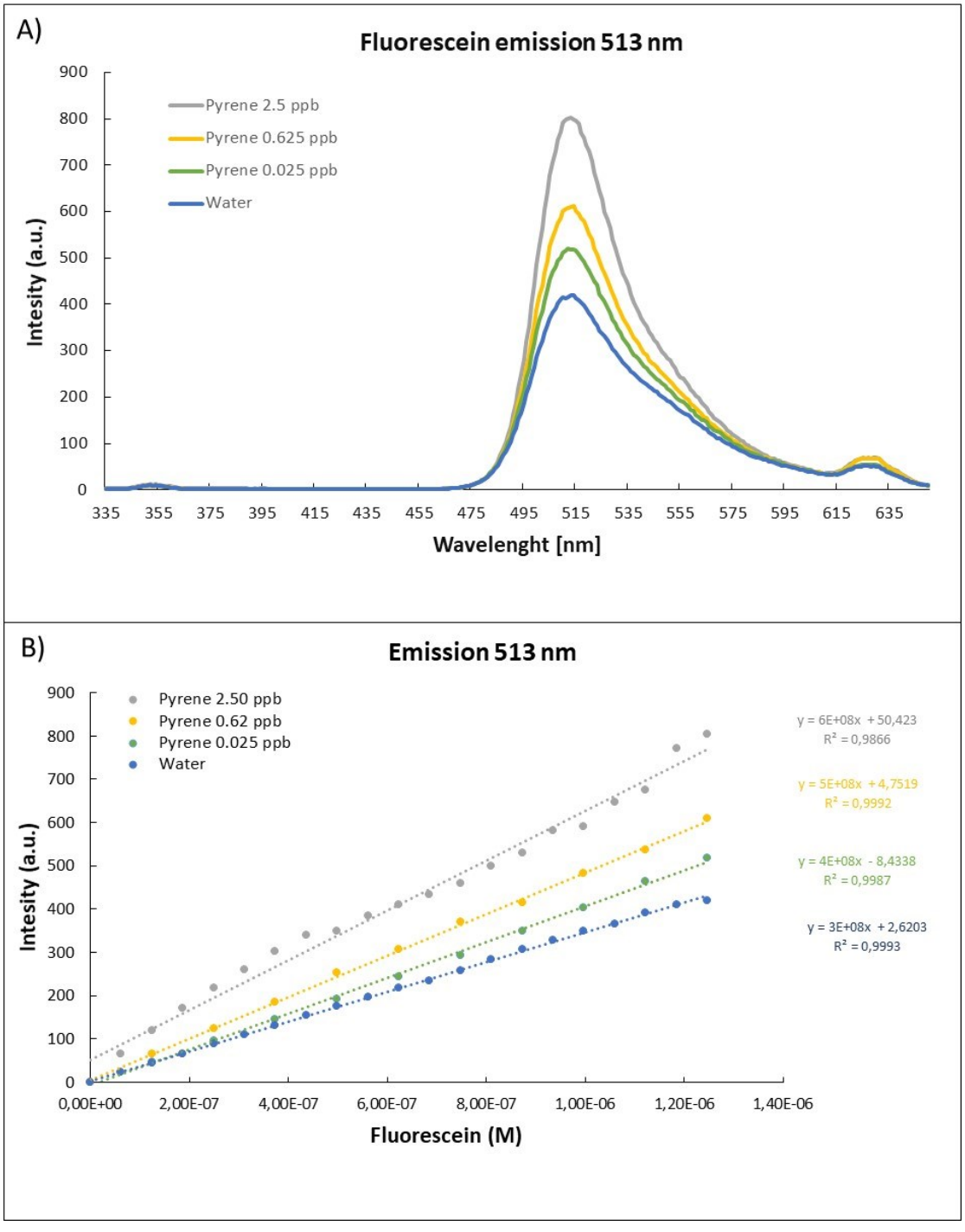

Figure 27: A) Fluorescence spectra of Fluorescein (1.25 x 10^{-6} M) in water (blue line) and in the different pyrene solutions b) fluorescence titrations of water and of different solutions of pyrene (0.025, 0.625, 2.5, 5 ppb) with Fluorescein (2.5 x 10^{-5} M).

Therefore, considering these preliminary results, several fluorescence titrations were performed adding the same amount (100 µL) of a fluorescein solution (2.50 x 10^{-5} M) to different solutions of pyrene and in Figure 28, as an example, one of these titrations is reported. Unfortunately, even though the presence of pyrene gives rise to fluorescein emission intensities greater than the fluorescein emission in water (at the same concentration value), no linear correlation was obtained between the fluorescence enhancement and the pyrene concentration values.

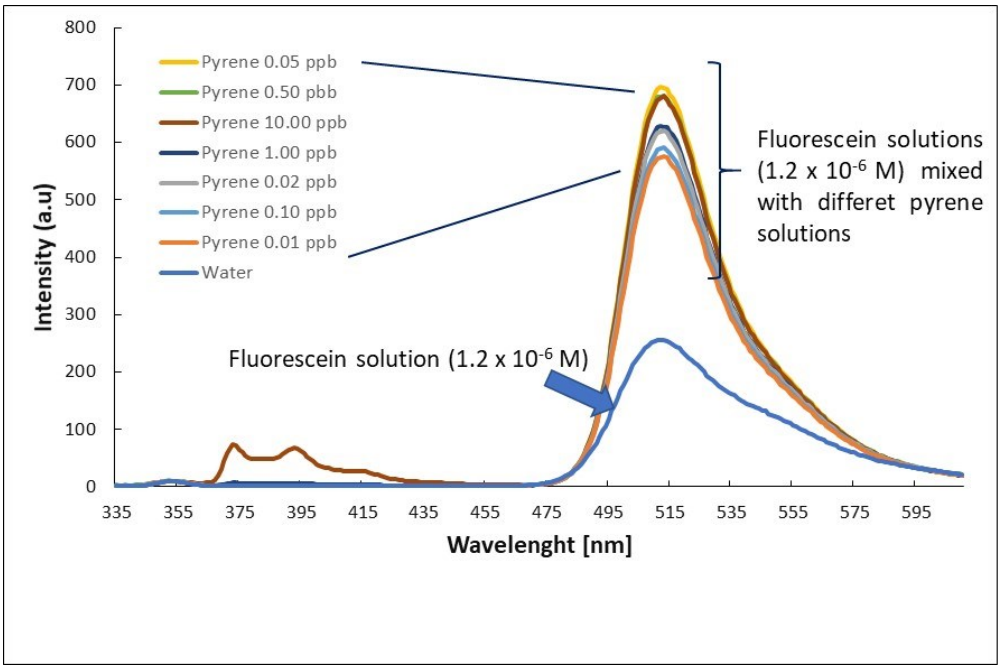

Figure 28: Fluorescence spectra of different pyrene solutions (0.01, 0.025, 0.05, 0.1, 0.5, 1 and 10 ppb) titrated by adding the same amount (100 µL) of a 2.5 x 10^{-5} M fluorescein solution (cuvette final concentration 1.2 x 10^{-6}M).

On the other hand, it is well acknowledged that the ratio between the molar concentration values of donor and acceptor is crucial in FRET.[260] Therefore, the optimization of the fluorescein concentration was carried out by titrating the more diluted solution of pyrene (4.95×10^{-11} M) with different fluorescein solutions (1.00×10^{-8} M, 1.00×10^{-7} M, 1.00×10^{-5} M) and the emission spectra obtained for the second and the third solutions are reported in Figure 29. The lowest concentration of fluorescein that gives rise to statistically different signals if it is added to water or to the 4.95×10^{-11} M pyrene solution is the 1×10^{-5} M with a final concentration value in the cuvette of 2.6×10^{-7} M, confirming that the concentration of the acceptor has to be much greater than that of the donor to obtain an efficient process.

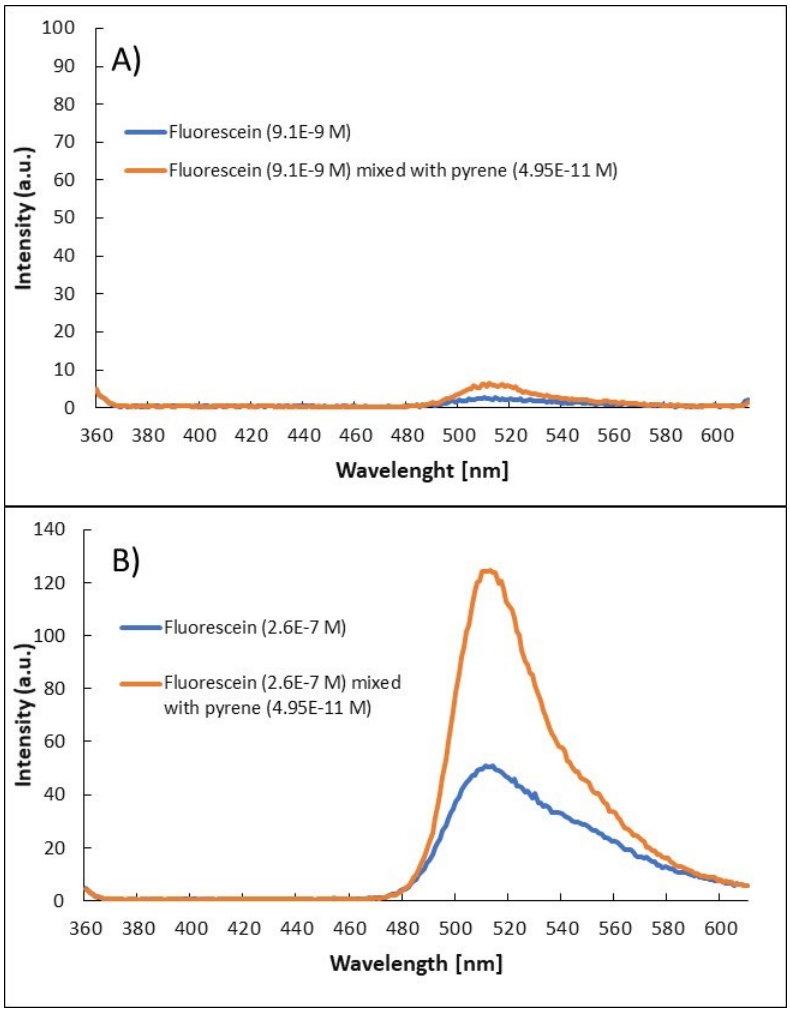

Figure 29: Optimization of the fluorescein concentration: emission spectra a) of the fluorescein solution (1×10^{-7} M) and of the fluorescein solution (1×10^{-7} M) with pyrene (4.95×10^{-11} M) and b) of the fluorescein solution (1×10^{-5} M) mixed with pyrene (4.95×10^{-11} M).

Quantitative determination of pyrene in aqueous solution

After the optimization of the molar concentrations ratio between fluorescein and pyrene, the calibration curve for the quantitative determination of pyrene in aqueous solution was obtained by adding to different diluted solutions of pyrene the same amount of the 1×10^{-5} M fluorescein solution and the concerning fluorescence spectra are reported in Figure 30. In the inset the calibration curve obtained by plotting the normalized emission intensity values ($I-I_0/I_0$) versus the pyrene concentration values is reported. Each calibration point was triplicated and the calibration curves obtained in different days show the same slope, suggesting a satisfactory inter-day repeatability of the method. The present method allows to detect pyrene in aqueous solutions at ppt level without additional preconcentration of the samples. In fact, the investigated concentrations (in the range from 0.003 to 0.01 ppb) are lower than those usually determined by the common analytical methods where the reported limits of detection are at ppb level.[243,294,295]

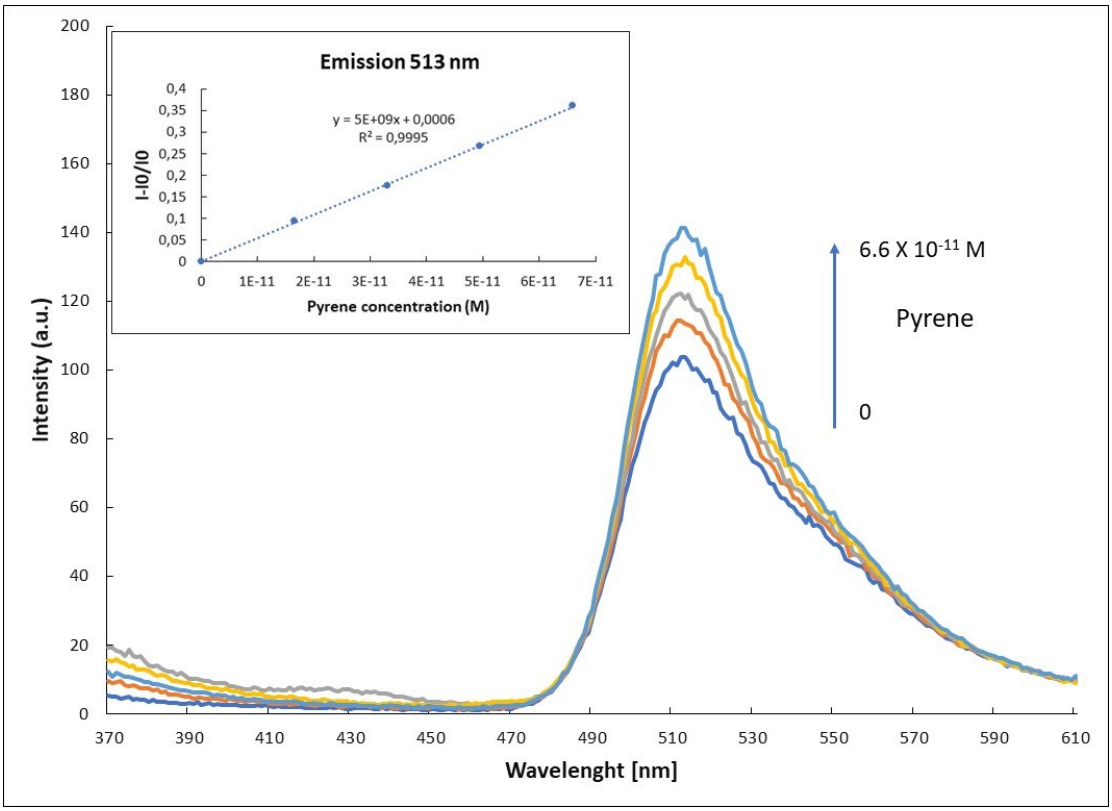

Figure 30: Fluorescence spectra (λ_{max}= 513 nm) obtained by titrating different pyrene solutions (0 - 6.6×10^{-11} M) with the same amount of a 1 x 10^{-5} M fluorescein solution. In the inset, the calibration curve obtained by plotting the normalized emission intensity values ($I-I_0/I_0$) versus the pyrene concentration values is reported.

Since the fluorescence emission intensities of fluorescein increase linearly on increasing the concentration values of pyrene at trace level, this method could be a smart and promising tool for the detection of pyrene in environmental samples, such as the seawater.

Quantitative determination of pyrene in seawater

The method for the quantitative determination of pyrene in seawater is still in progress; the preliminary data here reported were obtained using two different procedures, as described in the Fluorescence measurement section. In the first protocol, taking into account that the pH of seawater (~ 8.0) is greater than that of ultrapure water (~ 6.0) and that the fluorescence of fluorescein is pH dependent,[296] the calibration curve for the direct quantitative determination of pyrene in seawater was performed using buffered solution (pH=9). Unfortunately, no variation in the emission intensities of fluorescein on increasing the concentration values of pyrene was observed. Several factors could influence the fluorescence behaviour of fluorescein, such as pH, ionic strength and the presence of possible matrix interferences that could alter the interactions between pyrene and fluorescein. Therefore, in order to remove the possible matrix interferences, in the second procedure preliminary liquid-liquid extractions of two different aliquots of seawater, A (spiked with pyrene) and B (as it is) were carried out as described in the 5.4.3.3 section of

Materials and Methods. In Figure 31 the fluorescence spectra obtained by titrating the aliquot A with increasing volumes (100, 200, 300 and 400 µL) of a 1×10^{-5} M fluorescein solution are reported.

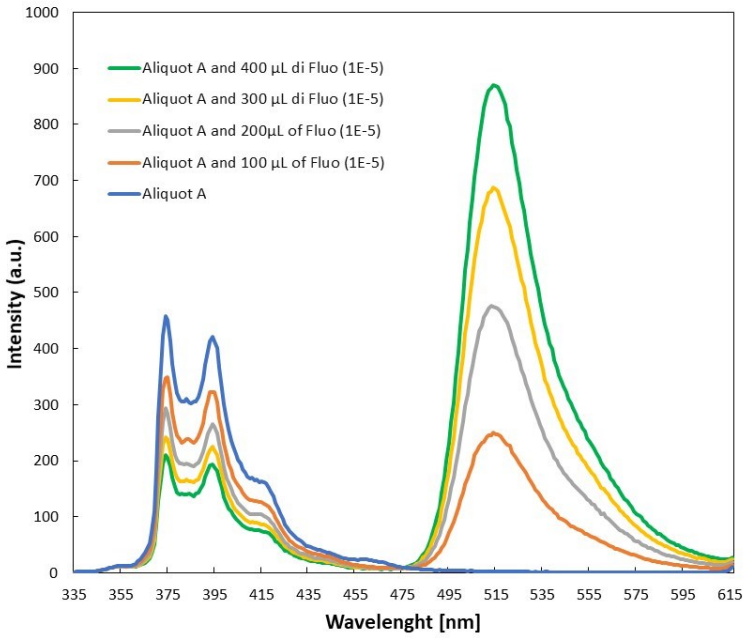

Figure 31: Fluorescence spectra (λ max= 513 nm) obtained by titrating aliquot A (spiked with pyrene 0.5 ppm) with increasing amounts of a 1 x 10^{-5} M fluorescein solution (from 0 to 3.03x10^{-7}M).

Once again, the fluorescence intensities of pyrene decrease increasing the fluorescein concentration, while an enhancement in the fluorescence emission intensities of fluorescein was observed.

The emission intensities of pyrene at 373 nm, corrected by taking into account the dilution effect and those experimentally recorded as a function of the various volumes added of Fluo solution (from 0 to 400 µl) are reported in Figure 32.

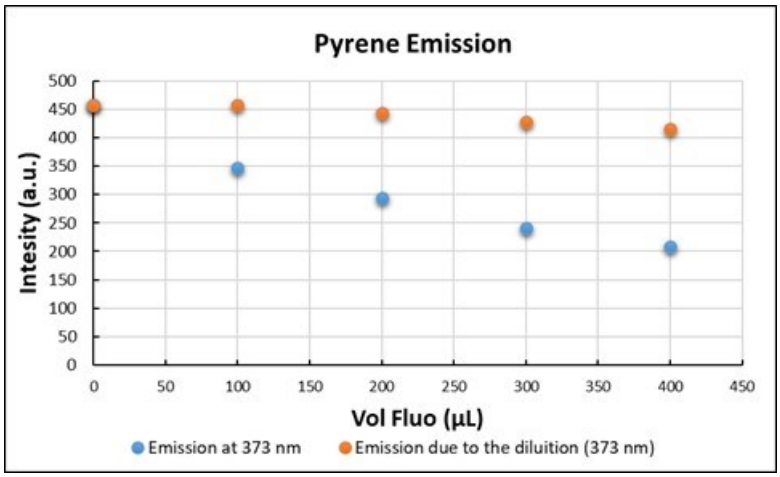

Figure 32: Comparison of pyrene emission at 373 nm; orange: theoretical emission corrected for the dilution; blu: experimental emission recorded by adding increasing volumes (0-400 µL) of the Fluo solution (1 x 10^{-5} M).

The addition of fluorescein quenches the fluorescence of the pyrene solution and corresponds to an enhancement of the fluorescein emission intensities themselves, confirming a not negligible interaction between the pollutant and the fluorophore and suggesting a FRET phenomenon.
In Figure 33 the fluorescence spectra of aliquot B obtained by adding increasing amounts of a 1×10^{-5} M fluorescein solution are reported. The aliquot B was subjected to the same sample pre-treatment of aliquot A without adding pyrene and a magnification of its fluorescence spectra is reported in the inset of Figure 33. In the region from 364 to 540 nm, the spectra of aliquot B show a noisy emission with a small intensity which shape suggests the presence of pyrene.

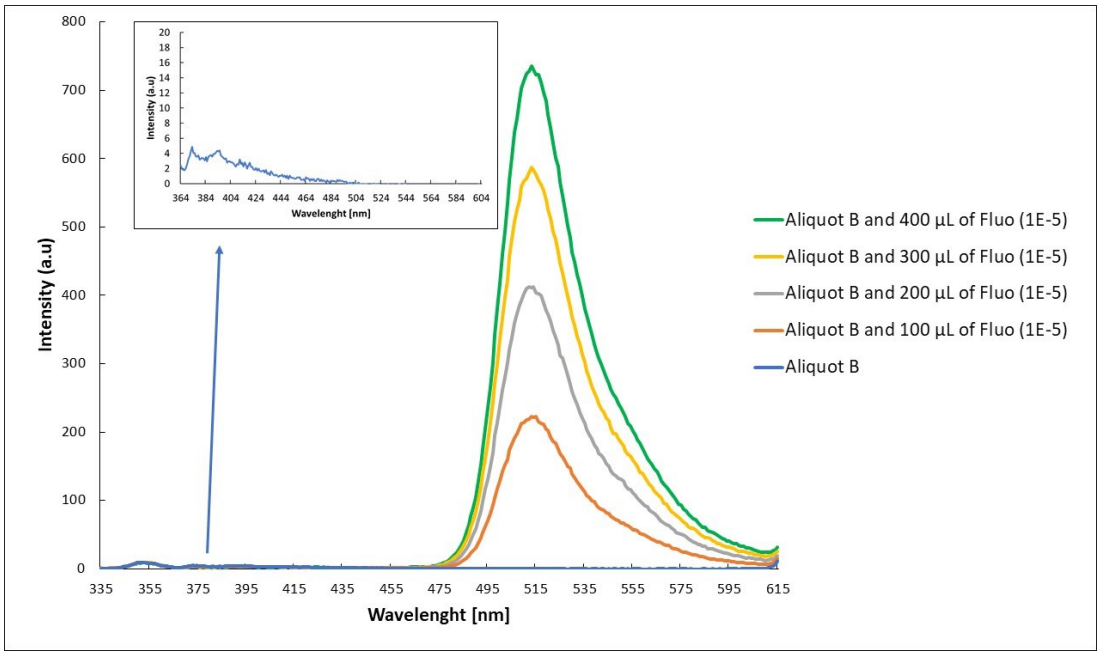

Figure 33: Fluorescence spectra (λ max= 513 nm) obtained by titrating the aliquot B of seawater sample with increasing amounts of a 1 x 10-5 M fluorescein solution (from 0 to 3.03x10^{-7}M).

In order to verify if the seawater indeed contains pyrene, an aliquot of the ultrapure water, used to re-dissolve the aliquots A and B after the L-L extractions, was analogously titrated with increasing amounts of a fluorescein solution (1×10^{-5} M). The fluorescence spectra of fluorescein (9.38×10^{-7} M) in ultrapure water, in aliquot B and in aliquot A are reported in Figure 34a, whereas the emission values (λ 513 nm) of water, of aliquot B and of aliquot A as a function of increasing fluorescein concentrations are reported in Figure 34b, respectively. Also in this case the increases in the emission intensities of fluorescein are in all cases linear, and the presence of pyrene gives rise to an even steeper slope of the straight line.

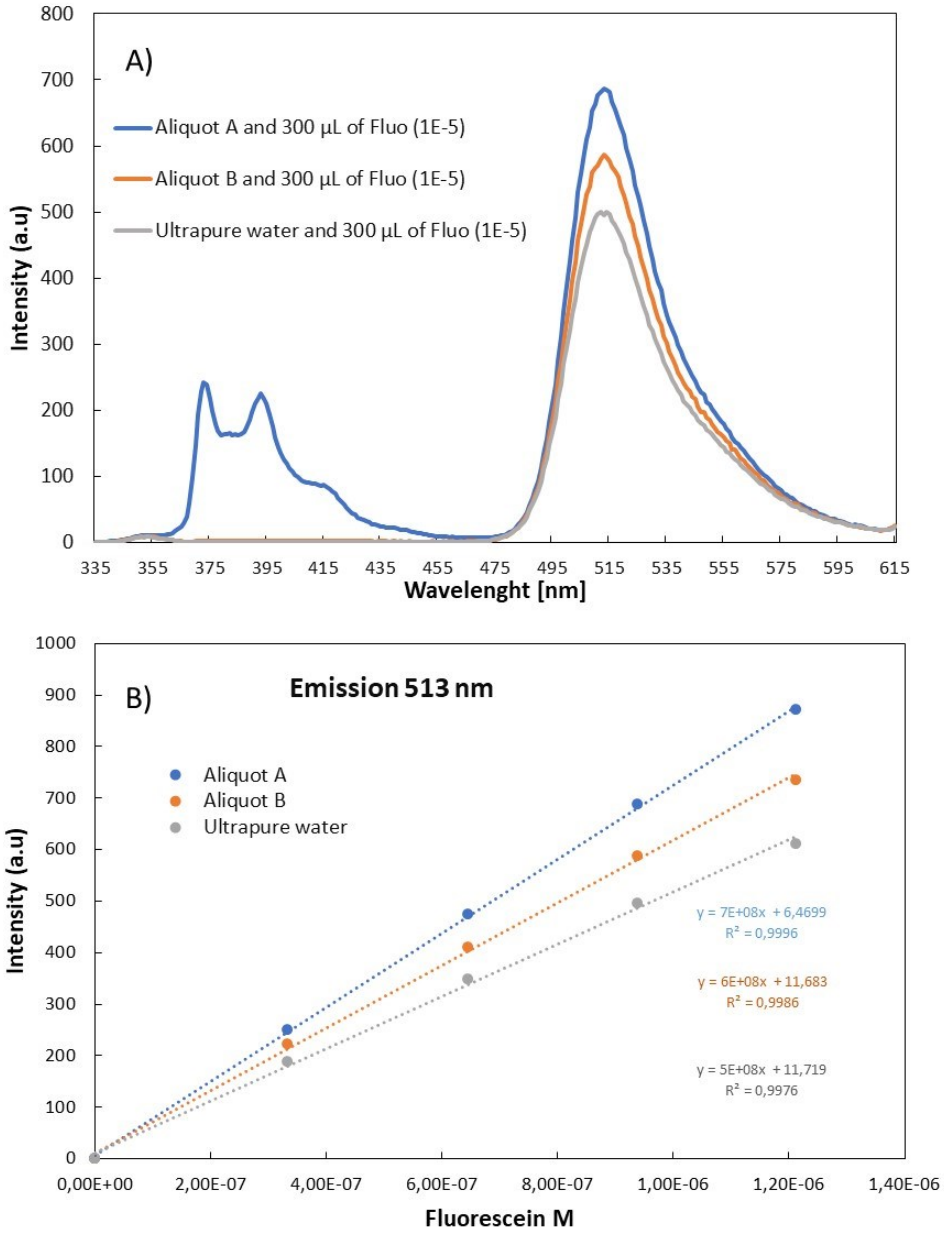

Figure 34: a) Fluorescence spectra of Fluo (9.38×10^{-7} M) in aliquot A (blue), in aliquot B (orange) and in water (grey); b) fluorescein emission values (λ 513 nm) in aliquot A (blue), in aliquot B (orange) and in water (grey) as a function of the various concentration values of Fluo.

Interestingly, aliquot B, that seems to contain pyrene at an unknown and very low concentration value, (Figure 33) shows a slope steeper than that of ultrapure water (Figure 34) and less steep than that of aliquot A. In order to determine the "true" concentration of pyrene in seawater, the standard addition method was performed by adding to aliquot B increasing amounts of a pyrene solution (0.1 ppb) with a final concentration range from 0 to 0.013 ppb, as reported in 5.4.3.2 of Materials and Methods section. Unfortunately, it was not possible to detect significant changes in the emission intensities of fluorescein (data not shown), probably due to the different molar

concentration ratio between fluorescein and pyrene, that in seawater seems to be very low. However, the optimization of this method is still ongoing and the quantitative determination of pyrene in seawater could still be performed using the standard additions method, opportunely optimized.

Once again, the reported data seem to indicate a FRET mechanism between pyrene (donor) and fluorescein (acceptor), since a significant decrease of the donor emission is related to a strong increase of the acceptor fluorescence. In order to explain why in the untreated seawater (Protocol 1), no significant variations in the emission spectra were obtained, we have to take into account the factors that influence the efficiency of the transfer in FRET, i.e. the quantum yield of the donor, the refractive index of the solution, the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor and their concentration values.[261,262]

To this aim, the normalized emission spectra of pyrene and absorption spectra of fluorescein at pH=6 and pH=9 are reported in Figure 35.

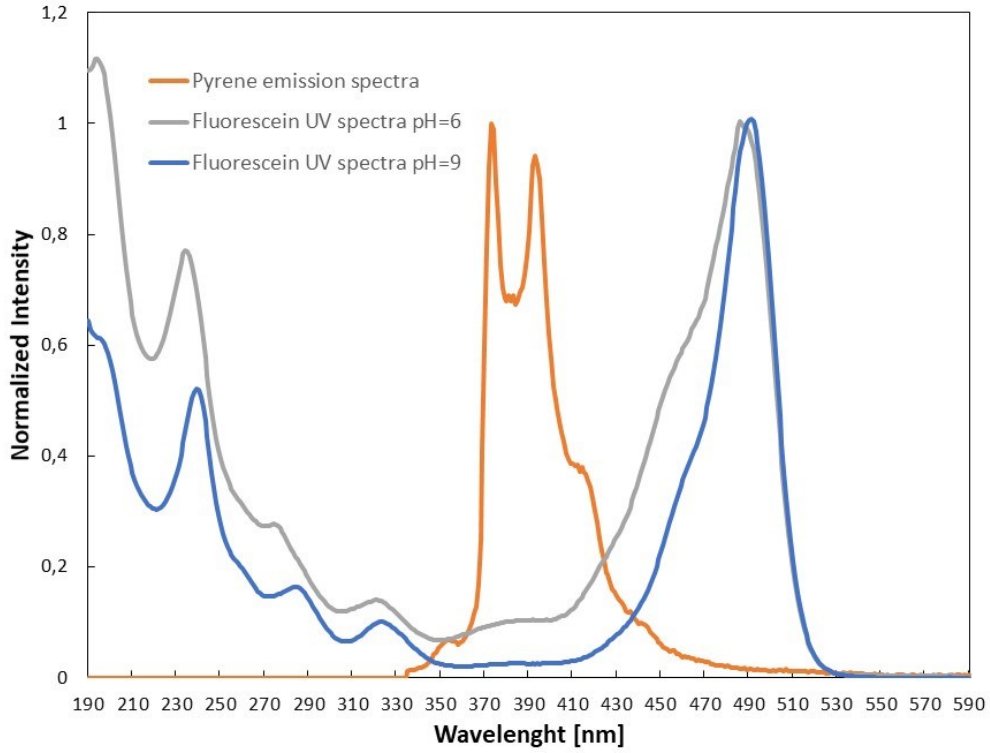

Figure 35: Normalized emission spectra of pyrene (orange) and absorption spectra of fluorescein at pH=6 (grey) and pH=9 (blu)

The Figure clearly shows that the emission spectrum of pyrene displays a better overlap with the absorption spectrum of fluorescein at pH 6 than with that of fluorescein at pH 9. This different overlap could be responsible of the lack of variation in the emission intensities of fluorescein on increasing the concentration values of pyrene observed during the experiments carried out in the seawater samples at pH 9, even though recently, it has been demonstrated a superior energy

transfer with decreased spectral overlap between the donor and the acceptor in the case in which supramolecular aggregates are present.[252,256–259,265] In our case, the π-π interactions between pyrene and anionic fluorescein is so effective that allows the detection of the analyte at 10 pM, obtaining a sensitivity much better of the direct fluorescence methods in pure water. However, at pH 8-9 this mechanism is no more effective probably due to the lower spectral overlap. Obviously, to properly settle this issue further investigations, among which fluorescence lifetime measurements and thermodynamic investigations in order to assess the nature and the entity of the non-covalent binding are highly needed.

CONCLUSIONS

The work described in this PhD thesis reports the development of new analytical strategies, using either the common GC-MS methodologies and the fluorescence detection, to assess the distribution of different classes of POPs, including PCBs, OCPs and PAHs, in environmental matrices (biota, volcanic ashes and water), as well as the monitoring of these pollutants in *M. surmuletus* of the Catania Gulf (Sicily, Italy) and the potential health risks linked to the consumption of fish for the local population.

Four main research activities were investigated: (1) the development and validation of analytical methods using GC-MS to detect POPs in biota; (2) the biomonitoring of POPs in *M. surmuletus* from Catania Gulf; (3) the development of analytical methods to detect PAHs in Etna volcanic ashes and (4) the development of new sensitivity fluorescence-based techniques to assess the presence of PAHs in seawater.

As regards each research activity, the conclusions of this PhD thesis are the following:

1) Different extraction and clean-up methods, based on solid phase extraction (SPE) and QuEChERS method, were investigated to enhance the recoveries of the analytes under study, minimizing the interferences. In this respect, a modified QuEChERS extraction and gas-chromatography mass spectrometry (GC-MS) methodologies were developed and implemented for the quantification of 16 priority PAHs, 29 PCB and 23 OCPs in muscle fish. The developed multiresidue method showed high sample clean-up efficiency due to the additional step with $CaCl_2$, followed by dSPE (900 mg $MgSO_4$, 150 mg PSA and 150 mg C18). The methods were validated according to UNI ENI ISO 17025, and the validation parameters tested were linearity, LOD, LOQ, recoveries, precision, accuracy and trueness. Calibration curves obtained for PAHs and PCBs well fit and showed a good linearity over the range of interest, expect for some isomeric compounds. Recoveries for PAHs range from 75% to 116%, complying the criteria of 70 -120% set out by US-EPA, whereas PCBs show a lower extraction efficiency (recoveries range from 62 to 89%), probably due to a partial adsorption of these organic lipophilic compounds with the fat and lipid components, removed during the sample clean-up. For both class of pollutants, experimental trueness values proved satisfactory, yielding percent deviations within the range 100 ± 15%, and the accuracy evaluation proved satisfactory. All experimental CV% are lower than 15% for PAHs and PCBs, proving that the methods have good within-run precisions. As regards OCPs validation, the recoveries range from 70-120 % for many analytes. Therefore, the developed methods show different performances, the OCPs methods being able to be used only with external calibration and mainly for qualitative purposes, whereas the PAHs and PCBs

methods, being their validation process satisfactory in almost all cases, can be used both for qualitative and quantitative purposes.

2) Considering all the factors that can affect biota monitoring, *M. surmuletus* was chosen for the monitoring of POPs in marine ecosystems of Sicily. The methods developed in the research activity 1) were used to detect and to determine the concentration values of 16 PAHs, 29 PCB and 23 OCPs in muscle fillet of *M. surmuletus*, caught in the Catania Gulf. 7 of the 16 target PAHs were found in all pooled muscle samples of *M. surmuletus*, with Phenanthrene, Naphthalene and Fluorene being the most abundant 2 to 3 rings PAHs. Interestingly, Benzo(a)pyrene, which is recognized as the most carcinogenic PAH, was found in only 3 of the 8 pooled samples analysed and always at concentrations near the LOQ. The concentration values of PAHs in muscle fillet from *M. surmuletus* were lower than those detected in other studies conducted on similar species living in the Mediterranean Sea.

   In agreement with the data reported in the literature, the analysis of the composition profile, based on the number of rings, shows a greater occurrence of LMW-PAHs than HMW-PAHs. This trend is due to the higher solubility, bioavailability, and lower metabolism rate of the 2-3 rings compounds. PCB 028, PCB 138 and PCB 153 were detected with a detection frequency above 30% and at concentration near LOQ (0.5 μg kg^{-1}). Among organochlorine pesticide (OCPs) only 4,4′-DDE residues were detected (detection frequency below 30%). Although the use of these pollutant has been banned many years ago, their presence at trace levels confirms their high persistence in the environment. The data shown in the present PhD thesis, even though preliminary, contribute to obtain a more comprehensive picture of the distribution of PAHs in the Catania Gulf and provides as well as an overview of the potential health risk due to the human consumption of *M. surmuletus* through the calculation of the ILCR index value that falls well below the "maximum acceptable risk level", suggesting low potential human health risks through consumption of *M. surmuletus*. Since the concentration values of LMW PAHs are not negligible, continuous monitoring biota programmes, including also species having similar characteristics as bioindicators, should be carried out in order to shed light on the PAHs distribution and to evaluate the risk for human health associated to fish consumption in the Sicilian region. In this context, this study being the first report on the distribution of PAHs in *M. surmuletus* caught in the Catania Gulf could represent a baseline for the monitoring of this area.

3) As regards the development of analytical methods to detect PAHs in Etna Volcanic ashes, different extraction and clean-up techniques were investigated in order to remove potential interferences and to clarify the final extracts prior to GC analysis, enhancing the recoveries of analytes. The developed method provides a satisfactory recovery (70-120%) for almost analytes. Preliminarily qualitative analysis of Etna volcanic ashes, sampled at high altitude,

show the presence of the three- to six-ring PAHs suggesting a mixture of petrogenic and pyrogenic sources. As the Sicilian marine environment is subject to several sources of PAHs emission, including Etna volcanic ashes, further studies are required in order to identify the possible origin, natural vs. anthropogenic, of these pollutants and to evaluate the impact of Etna volcanic activity in the aquatic environment.

4) The preliminary results obtained for the development of fluorescence-based techniques to detect PAHs in seawater suggest an efficient energy transfer from a donor, i.e. pyrene, to an acceptor, i. e. the highly fluorescent aromatic anion, fluorescein, which probably occur via the stacking interactions of the two aromatic systems that result to be an important driving force in solution. The presence of a metal core (AgNPs) did not enhance this effect, probably because silver NPs can adsorb pyrene molecules from diluted solutions, quenching the pyrene fluorescence itself and rendering the resonance process less efficient. The developed method allows to detect pyrene in aqueous solutions at ppt level, using a very simple system, without pre-concentration of the samples and obtaining a sensitivity much better than that reported for the direct fluorescence methods. Thus, this method could provide a smart and promising tool for the detection of pyrene in environmental samples, such as in seawater and some preliminary data are here reported. However, this last method is still ongoing and the obtained results show that a pre-treatment of the seawater sample is required to remove potential interferences and to optimize the efficiency of the energy transfer between the donor and the acceptor, which is, in this case, probably influenced by the pH. In this context, further investigations, among which fluorescence lifetime measurements and thermodynamic investigations in order to assess the nature and the entity of the non-covalent binding, are highly needed.

The present PhD thesis would like to make a significant contribution to the development of more sensitive analytical methods, either chromatographic and spectroscopic, to detect POPs in environmental matrices, to obtain a better picture of the distribution of POPs in the Catania Gulf and providing as well an overview of the potential health risks due to the human consumption of *M. surmuletus* from the investigated area.

BIBLIOGRAPHY

1. Kozak K, Ruman M, Kosek K, Karasinski G, Stachnik Ł, Polkowska Z. Impact of Volcanic Eruptions on the Occurrence of PAHs Compounds in the Aquatic Ecosystem of the Southern Part of West Spitsbergen. *Water*. 2017; 9:42. doi:10.3390/w9010042
2. Jensen S, Johnels AG, Olsson M OG. DDT and PCB in marine animals from Swedish waters. *Nature*. 1969;224:224-247. doi:10.1038/224488a0
3. Zahn D, Neuwald IJ, Knepper TP. Analysis of mobile chemicals in the aquatic environment—current capabilities, limitations and future perspectives. *Anal Bioanal Chem*. 2020:4763-4784. doi:10.1007/s00216-020-02520-z
4. AMAP. *Arctic Pollution 2009 Artic Monitoring and Assessment Progamme, Oslo*.; 2009.
5. Fernández-González R, Yebra-Pimentel I, Martínez-Carballo E, Simal-Gándara J. Decontamination solutions for polychlorinated biphenyls (PCBs) in raw fish oils from environmentally contaminated seafishes. *Sci Total Environ*. 2014;468-469:1007-1013. doi:10.1016/j.scitotenv.2013.09.036
6. Ferna ÆD, Garcı MS. Sorption of PAHs to Colloid Dispersions of Humic Substances in Water. *Bull Environ Contam Toxicol*. 2007;79:251-254. doi:10.1007/s00128-007-9022-0
7. EU. *Common Implementation Strategy for the Water Framework Directive (2000/60/EC), Guidance Document No . 25, Guidance on Chemical Monitoring of Sediment and Biota under the Water Framework Directive*.; 2010. doi:10.2779/43586
8. United Nations Environment Programme (UNEP). *Ridding the World of POPs: A Guide to the Stockholm Convention*.; 2010.
9. Wordl Health Organization. *World Health Organization. "Persistent Organic Pollutants: Impact on Child Health*.; 2010.
10. Roose P, Brinkman UAT. Monitoring organic microcontaminants in the marine environment: Principles, programmes and progress. *TrAC - Trends Anal Chem*. 2005;24(11):897-926. doi:10.1016/j.trac.2005.10.007
11. Pampanin D. Introduction to Petrogenic Polycyclic Aromatic Hydrocarbons (PAHs) in the Aquatic Environment. *Petrogenic Polycycl Aromat Hydrocarb Aquat Environ Anal Synth Toxic Environ Impact*. 2017;17(15):3-17. doi:10.2174/9781681084275117010004
12. Balmer JE, Hung H, Yu Y, Letcher RJ, Muir DCG. Sources and environmental fate of pyrogenic polycyclic aromatic hydrocarbons (PAHs) in the Arctic. *Emerg Contam*. 2019;5:128-142. doi:10.1016/j.emcon.2019.04.002
13. Abdel-shafy HI, Mansour MSM. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egypt J Pet*. 2016;25(1):107-123. doi:10.1016/j.ejpe.2015.03.011
14. Zhang Y, Tao S. Global atmospheric emission inventory of polycyclic aromatic

hydrocarbons (PAHs) for 2004. *Atmos Environ*. 2009;43(4):812-819. doi:10.1016/j.atmosenv.2008.10.050

15. Krauss M, Wilcke W, Martius C, Bandeira AG, Garcia MVB, Amelung W. Atmospheric versus biological sources of polycyclic aromatic hydrocarbons (PAHs) in a tropical rain forest environment. *Environ Pollut*. 2005;135(1):143-154. doi:10.1016/j.envpol.2004.09.012
16. Tolosa I, Bayona JM, Albaigés J. Aliphatic and polycyclic aromatic hydrocarbons and sulfur/oxygen derivatives in Northwestern Mediterranean sediments: Spatial and temporal variability, fluxes, and budgets. *Environ Sci Technol*. 1996;30(8):2495-2503. doi:10.1021/es950647x
17. Peter E.T. Douben. *PAHs: An Ecotoxicological Perspective*.; 2003. doi:10.1002/0470867132.ch16
18. Ravindra K, Sokhi R, Grieken R Van. Atmospheric polycyclic aromatic hydrocarbons: Source attribution, emission factors and regulation. *Atmos Environ*. 2008;42:2895-2921. doi:10.1016/j.atmosenv.2007.12.010
19. Wang Z, Ren P, Sun Y, et al. Gas/particle partitioning of polycyclic aromatic hydrocarbons in coastal atmosphere of the north Yellow Sea, China. *Environ Sci Pollut Res*. 2013;20(8):5753-5763. doi:10.1007/s11356-013-1588-y
20. Chiou CT, Mcgroddy SE, Kile DE, Survey USG, Federal D. Partition Characteristics of Polycyclic Aromatic Hydrocarbons on Soils and Sediments. *Environ Sci Technol*. 1998;(32):264-269.
21. Yu Z, Lin Q, Gu Y, et al. Bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in wild marine fish from the coastal waters of the northern South China Sea: Risk assessment for human health. *Ecotoxicol Environ Saf*. 2019;180(March):742-748. doi:10.1016/j.ecoenv.2019.05.065
22. Tobiszewski M, Namie J. PAH diagnostic ratios for the identification of pollution emission sources. *Environ Pollut*. 2012;162:110-119. doi:10.1016/j.envpol.2011.10.025
23. Evans M, Davies M, Janzen K, et al. PAH distributions in sediments in the oil sands monitoring area and western Lake Athabasca: Concentration, composition and diagnostic ratios. *Environ Pollut*. 2016;213:671-687. doi:10.1016/j.envpol.2016.03.014
24. Mackay D, Shiu WY, Ma K, Lee SC. *Properties and Environmental Fate Second Edition Introduction and Hydrocarbons*. Vol I.; 2006.
25. Domingo JL, Bocio A, Falcó G, Llobet JM. Benefits and risks of fish consumption. Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants. *Toxicology*. 2007;230(2-3):219-226. doi:10.1016/j.tox.2006.11.054
26. Inomata Y, Kajino M, Sato K, et al. Emission and atmospheric transport of particulate

PAHs in Northeast Asia. *Environ Sci Technol*. 2012;46(9):4941-4949. doi:10.1021/es300391w

27. Zhao ZY, Chu YL, Gu JD. Distribution and sources of polycyclic aromatic hydrocarbons in sediments of the Mai Po Inner Deep Bay Ramsar Site in Hong Kong. *Ecotoxicology*. 2012;21(6):1743-1752. doi:10.1007/s10646-012-0948-6
28. Baali A, Yahyaoui A. Polycyclic Aromatic Hydrocarbons (PAHs) and Their Influence to Some Aquatic Species. *IntechOpen*. 2019. doi:10.5772/intechopen.86213
29. Franco ME, Lavado R. Applicability of in vitro methods in evaluating the biotransformation of polycyclic aromatic hydrocarbons (PAHs) in fish: Advances and challenges. *Sci Total Environ*. 2019;671:685-695. doi:10.1016/j.scitotenv.2019.03.394
30. Jiang Y, Wang X, Wu M, Sheng G, Fu J. Contamination, source identification, and risk assessment of polycyclic aromatic hydrocarbons in agricultural soil of Shanghai, China. *Environ Monit Assess*. 2011;183(1-4):139-150. doi:10.1007/s10661-011-1913-1
31. Chen H yang, Teng Y guo, Wang J sheng. Source apportionment of polycyclic aromatic hydrocarbons (PAHs) in surface sediments of the Rizhao coastal area (China) using diagnostic ratios and factor analysis with nonnegative constraints. *Sci Total Environ*. 2012;414:293-300. doi:10.1016/j.scitotenv.2011.10.057
32. IARC. *Bitumens and Bitumen Emissions , and Some N - and S -Heterocyclic Polycyclic Aromatic Hydrocarbons Bitumens and Bitumen Emissions, and Some N- and S-Heterocyclic Polycyclic Aromatic Hydrocarbons*. Vol 103.; 2014.
33. Idowu O, Semple KT, Ramadass K, Connor WO, Hansbro P. Beyond the obvious: Environmental health implications of polar polycyclic aromatic hydrocarbons. *Environ Int*. 2019;123(October 2018):543-557. doi:10.1016/j.envint.2018.12.051
34. Savchenko YA, Minina VI, Bakanova ML, Glushkov AN. Genotoxic and Carcinogenic Effects of Industrial Factors in Coal Mining and Coal-Processing Industry (Review). *Russ J Genet*. 2019;55(6):681-691. doi:10.1134/S1022795419060140
35. Kim K, Ara S, Kabir E, Brown RJC. A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. *Environ Int*. 2013;60:71-80. doi:10.1016/j.envint.2013.07.019
36. Tarantini A, Maître A, Lefèbvre E, Marques M, Rajhi A, Douki T. Polycyclic aromatic hydrocarbons in binary mixtures modulate the efficiency of benzo[a]pyrene to form DNA adducts in human cells. *Toxicology*. 2011;279(1-3):36-44. doi:10.1016/j.tox.2010.09.002
37. Frasco MF, Guilhermino L. Effects of dimethoate and beta-naphthoflavone on selected biomarkers of Poecilia reticulata. *Fish Physiol Biochem*. 2002;26(2):149-156. doi:10.1023/A:1025457831923
38. Unwin J, Cocker J, Scobbie E, Chambers H. An assessment of occupational exposure to

polycyclic aromatic hydrocarbons in the UK. *Ann Occup Hyg*. 2006;50(4):395-403. doi:10.1093/annhyg/mel010

39. Diggs DL, Huderson AC, Harris KL, et al. Polycyclic aromatic hydrocarbons and digestive tract cancers: A perspective. *J Environ Sci Heal - Part C Environ Carcinog Ecotoxicol Rev*. 2011;29(4):324-357. doi:10.1080/10590501.2011.629974
40. Olsson AC, Fevotte J, Fletcher T, et al. Occupational exposure to polycyclic aromatic hydrocarbons and lung cancer risk: A multicenter study in Europe. *Occup Environ Med*. 2010;67(2):98-103. doi:10.1136/oem.2009.046680
41. Armstrong B, Hutchinson E, Unwin J, Fletcher T. Lung Cancer Risk after Exposure to Polycyclic Aromatic Hydrocarbons: A Review and Meta-Analysis. *Environ Health Perspect*. 2004;112(9):970-978. doi:10.1289/ehp.6895
42. Boffetta P, Jourenkova N, Gustavsson P. Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. *Cancer causes Control*. 1997;8(3):444-472.
43. US-EPA. Polycyclic Aromatic Hydrocarbons (PAHs) Factsheet. *JRC Tech Notes*. 2010;3:1-25. doi:10.1016/S0167-7799(02)01943-1
44. Burchiel SW, Luster MI. Signaling by environmental polycyclic aromatic hydrocarbons in human lymphocytes. *Clin Immunol*. 2001;98(1):2-10. doi:10.1006/clim.2000.4934
45. Gamboa RT, Gamboa AR, Bravo AH, Ostrosky WP. Genotoxicity in child populations exposed to Polycyclic Aromatic Hydrocarbons (PAHs) in the air from Tabasco, Mexico. *Int J Environ Res Public Health*. 2008;5(5):349-355. doi:10.3390/ijerph5050349
46. Tarantini A, Maitre A, Lefebvre E, et al. Relative contribution of DNA strand breaks and DNA adducts to the genotoxicity of benzo[a]pyrene as a pure compound and in complex mixtures. *Mutat Res - Fundam Mol Mech Mutagen*. 2009;671(1-2):67-75. doi:10.1016/j.mrfmmm.2009.08.014
47. Spink DC, Wu SJ, Spink BC, et al. Induction of CYP1A1 and CYP1B1 by benzo(k)fluoranthene and benzo(a)pyrene in T-47D human breast cancer cells: Roles of PAH interactions and PAH metabolites. *Toxicol Appl Pharmacol*. 2008;226(3):213-224. doi:10.1016/j.taap.2007.08.024
48. Yang H, Mazur-Melnyk M, De Boer JG, Glickman BW. A comparison of mutational specificity of mutations induced by S9-activated B[a]P and benzo[a]pyrene-7,8-diol-9,10-epoxide at the endogenous aprt gene in CHO cells. *Mutat Res - Fundam Mol Mech Mutagen*. 1999;423(1-2):23-32. doi:10.1016/S0027-5107(98)00221-8
49. Miller KP, Ramos KS. Impact of cellular metabolism on the biological effects of benzo[a] pyrene and related hydrocarbons. *Drug Metab Rev*. 2001;33(1):1-35. doi:10.1081/DMR-100000138

50. Hainaut P, Pfeifer GP. Patterns of p53 G → T transversions in lung cancers reflect the primary mutagenic signature of DNA-damage by tobacco smoke. *Carcinogenesis*. 2001;22(3):367-374.
51. Hwa K, Kyu J, Heon J, et al. Characteristic molecular signature for the early detection and prediction of polycyclic aromatic hydrocarbons in rat liver. *Toxicol Lett*. 2013;216(1):1-8. doi:10.1016/j.toxlet.2012.11.001
52. Kristensen P, Eilertsen E, Einarsdottir E, Haugen A, Skaug V, Ovrebo S. Fertility in Mice after Prenatal Exposure to Benzo[a]pyrene and Inorganic Lead. 1995;103(6):588-590.
53. Perera F, Tang D, Whyatt R, Lederman SA. DNA Damage from Polycyclic Aromatic Hydrocarbons Measured by Benzo[a] pyrene-DNA Adducts in Mothers and Newborns from Northern Manhattan, The World Trade Center Area, Poland, and China. *Cancer Epidemiol Biomarkers Prev*. 2005;14(March).
54. UNEP Chemicals. Guidelines for the Identification of PCBs and Materials Containing PCBs. *First issue, Inter-organization Progr sound Manag Chem*. 1999;(August).
55. Borja J, Taleon DM, Auresenia J, Gallardo S. Polychlorinated biphenyls and their biodegradation. *Process Biochem*. 2005;40:1999-2013. doi:10.1016/j.procbio.2004.08.006
56. Department of Health Wellington. *Safe Management of PCBs: Code of Practice*.; 2008.
57. Waid JS. *PCBs and the Environment.* Vol 19.; 1986.
58. Agency for toxic substances and disease registry. *TOXICOLOGICAL PROFILE FOR POLYCHLORINATED BIPHENYLS (PCBs)*; 2000.
59. US-EPA. *PCBs: Cancer Dose-Response Assessment and Application to Environmental Mixtures EPA/600/P- 96/001F*.; 1996.
60. Wolska L, Mechlinska A, Rogowska J, Namiesnik J. Sources and Fate of PAHs and PCBs in the Marine Environment. *Crit Rev Environ Sci Technol*. 2012;42:1172-1189. doi:10.1080/10643389.2011.556546
61. Wolska L, Namies J. Physical speciation of polychlorinated biphenyls in the aquatic environment. *Trends Anal Chem*. 2007;26(10):1005-1012. doi:10.1016/j.trac.2007.08.006
62. Moret I, Gambaro A, Piazza R, Ferrari S, Mnodori L. Determination of polychlorobiphenyl congeners (PCBs) in the surface water of the Venice lagoon. *Mar Pollut Bull*. 2005;50:167-174. doi:10.1016/j.marpolbul.2004.10.005
63. Vilanova RM, Fernández P, Grimalt JO. Polychlorinated biphenyl partitioning in the waters of a remote mountain lake. *Sci Total Environ*. 2001;279(1-3):51-62. doi:10.1016/S0048-9697(01)00725-2
64. Borghini F, Grimalt JO, Sanchez-Hernandez JC, Barra R, García CJT, Focardi S. Organochlorine compounds in soils and sediments of the mountain Andean Lakes.

Environ Pollut. 2005;136(2):253-266. doi:10.1016/j.envpol.2005.01.007

65. Schulz-Bull DE, Petrick G, Bruhn R, Duinker JC. Chlorobiphenyls (PCB) and PAHs in water masses of the northern North Atlantic. *Mar Chem*. 1998;61(1-2):101-114. doi:10.1016/S0304-4203(98)00010-3
66. Beyer A, Biziuk M. Environmental Fate and Global Distribution of Polychlorinated Biphenyls. *Rev Environ Contam Toxicol*. 2009;201(137-158). doi:10.1007/978-1-4419-0032-6
67. Wong CS, Lau F, Clark M, Muir DCG. Rainbow Trout (Oncorhynchus mykiss) Can Eliminate Chiral Organochlorine Compounds Enantioselectively. *Environ Sci Technol*. 2002;36:1257-1262.
68. Whittle DM, Backus SM, Teixeira C, Devault DS. Organochlorine Compounds in Lake Superior: Chiral Polychlorinated Biphenyls and Biotransformation in the Aquatic Food Web. *Environ Sci Technol*. 2004;38(1):84-92.
69. Campbell LM, Muir DCG, Whittle DM, Backus S, Norstrom RJ, Fisk AT. Hydroxylated PCBs and other chlorinated phenolic compounds in lake trout (Salvelinus namaycush) blood plasma from the Great Lakes region. *Environ Sci Technol*. 2003;37(9):1720-1725. doi:10.1021/es026225m
70. Purkey HE, Palaninathan SK, Kent KC, et al. Hydroxylated polychlorinated biphenyls selectively bind transthyretin in blood and inhibit amyloidogenesis: Rationalizing rodent PCB toxicity. *Chem Biol*. 2004;11(12):1719-1728. doi:10.1016/j.chembiol.2004.10.009
71. Campfens JAN, Mackay D. Fugacity-Based Model of PCB Bioaccumulation in Complex Aquatic Food Webs. *Environ Sci Technol*. 1997;31(2):577-583.
72. IARC. *POLYCHLORINATED BIPHENYLS AND POLYBROMINATED MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS*. Vol 107.; 2015.
73. Bell MR. Endocrine-disruptiong acrions of PCBs on brain development and social and reproductive behaviors. *Curr Opin Pharmacol*. 2014;19:134-144. doi:10.1016/j.coph.2014.09.020.Endocrine-disrupting
74. Blus LJ. *Organochlorine Pesticides Chapter 13*; 2002. doi:10.1201/9781420032505.sec2
75. Bondy GS, Newsome WH, Armstrong CL, et al. Trans-nonachlor and cis-nonachlor toxicity in sprague-dawley rats: Comparison with technical chlordane. *Toxicol Sci*. 2000;58(2):386-398. doi:10.1093/toxsci/58.2.386
76. Beyer WN, Gish CD. PERSISTENCE IN EARTHWORMS AND POTENTIAL HAZARDS TO BIRDS OF SOIL APPLIED DDT, DIELDRIN AND HEPTACHLOR. *Jorunal Appl Ecol*. 1980;17(2):295-307.
77. Lin Z, Li XM, Li YT, Huang DY, Dong J, Li FB. Enhancement effect of two ecological earthworm species (Eisenia foetida and Amynthas robustus E. Perrier) on removal and

degradation processes of soil DDT. *J Environ Monit*. 2012;14(6):1551-1558. doi:10.1039/c2em30160a

78. Okay OS, Karacık B, Henkelmann B, Schramm KW. Distribution of organochlorine pesticides in sediments and mussels from the Istanbul Strait. *Environ Monit Assess*. 2011;176(1-4):51-65. doi:10.1007/s10661-010-1566-5
79. ATSDR (Agency for Toxic Substances and Disease Registry). Toxicological Profile for Heptachlor and Heptachlor Epoxide. *US Dep Heal Hum Serv Public Heal Serv*. 2007;3(200):203.
80. W. Sparling D. *Organochlorine Pesticides Chapter 4*; 2016. doi:10.1201/9781351069656-1
81. Bidleman TF, Kurt-Karakus PB, Wong F, Alegria HA, Jantunen LM, Hung H. Is there still "new" DDT in North America? An investigation using proportions of DDT compounds. *ACS Symp Ser*. 2013;1149:153-181. doi:10.1021/bk-2013-1149.ch008
82. Pandit GG, Sahu SK, Sharma S, Puranik VD. Distribution and fate of persistent organochlorine pesticides in coastal marine environment of Mumbai. *Environ Int*. 2006;32:240-243. doi:10.1016/j.envint.2005.08.018
83. Phillips DJH. The Chemistries and Environmental Fates of Trace Metals and Organochlorines in Aquatic Ecosystems. *Mar Pollut Bull*. 1995;31(95):193-200.
84. Ali U, Hussain J, Naseem R, et al. Science of the Total Environment Organochlorine pesticides (OCPs) in South Asian region: A review. *Sci Total Environ*. 2014;477:705-717. doi:10.1016/j.scitotenv.2013.12.107
85. Sarkar SK, Bhattacharya BD, Bhattacharya A, et al. Occurrence, distribution and possible sources of organochlorine pesticide residues in tropical coastal environment of India: An overview. *Enviromental Int*. 2008;34:1062-1071. doi:10.1016/j.envint.2008.02.010
86. Guzzella L, Roscioli C, Vigano L. Evaluation of the concentration of HCH, DDT, HCB, PCB and PAH in the sediments along the lower stretch of Hugli estuary, West Bengal, northeast India. *Enviromental Int*. 2005;31:523-534. doi:10.1016/j.envint.2004.10.014
87. Norstrom RJ, Hallett DJ. Coho Salmon (Oncorhynchus kisutch) and Herring Gulls (Larus argentatus) as Indicators of Organochlorine Contamination in Lake Ontario. *J Fish Res Board Canada*. 1978;35:1401-109.
88. Butler BYPA. Pesticides in the Marine Environment. *J Appl Ecol*. 1966;3:253-259.
89. Nygård T. Long Term Trends in Pollutant Levels and Shell Thickness in Eggs of Merlin in Norway, in Relation to its Migration Pattern and Numbers. *Ecotoxicology*. 1999;8:23-31. doi:10.1023/A
90. Agency for toxic substances and disease registry. *PUBLIC HEALTH STATEMENT DDT, DDE, and DDD*.; 2002.

91. Agency for toxic substances and disease registry. *Toxicological Profile for DDT, DDE and DDD*.; 2019.
92. Wattigney WA, Irvin-barnwell E, Pavuk M, Ragin-wilson A. Regional Variation in Human Exposure to Persistent Organic Pollutants in the United States, NHANES. *J Environ Public Health*. 2015;1-12.
93. US-EPA. *Final Updated Ambient Water Quality Criteria for the Protection of Human Health*. Vol 80.; 2015.
94. Mrema EJ, Rubino FM, Brambilla G, Moretto A, Tsatsakis AM, Colosio C. Persistent organochlorinated pesticides and mechanisms of their toxicity. *Toxicology*. 2013;307:74-88. doi:10.1016/j.tox.2012.11.015
95. Finkelstein ME, Grasman KA, Croll DA, et al. Contaminant-associated alteration of immune function in black-footed albatross (Phoebastria nigripes), a North Pacific predator. *Environ Toxicol Chem*. 2007;26(9):1896-1903. doi:10.1897/06-505R.1
96. Bradlow HL, Davis DL, Lin G, Sepkovic D, Tiwari R. Effects of pesticides on the ratio of 16α/2-hydroxyestrone: A biologic marker of breast cancer risk. *Environ Health Perspect*. 1995;103:147-150. doi:10.2307/3432525
97. Rhouma K Ben, Te´bourbi O, Krichah R, Sakly M. Reproductive toxicity of DDT in adult male rats. *Hum Exp Toxicol*. 2001;20(8):393-397. doi:10.1191/096032701682692946
98. WR K, CR S, SC L, LE G, JA K, EM. W. Persistent DDT metabolite p,p'–DDE is a potent androgen receptor antagonist. *Nature*. 1995;375:581-585.
99. Tebourbi O, Hallègue D, Yacoubi MT, Sakly M, Rhouma K Ben. Subacute toxicity of p,p′-DDT on rat thyroid: Hormonal and histopathological changes. *Environ Toxicol Pharmacol*. 2010;29(3):271-279. doi:10.1016/j.etap.2010.03.002
100. Xu W, Wang X, Cai Z. Analytical chemistry of the persistent organic pollutants identified in the Stockholm Convention: A review. *Anal Chim Acta*. 2013:1-13. doi:10.1016/j.aca.2013.04.026
101. Manirakiza P, Covaci A, Nizigiymana L, Ntakimazi G, Schepens P. Persistent chlorinated pesticides and polychlorinated biphenyls in selected fish species from Lake Tanganyika, Burundi, Africa. *Environ Pollut*. 2002;117(3):447-455. doi:10.1016/S0269-7491(01)00188-9
102. Wang P, Zhang Q, Wang Y, et al. Evaluation of Soxhlet extraction, accelerated solvent extraction and microwave-assisted extraction for the determination of polychlorinated biphenyls and polybrominated diphenyl ethers in soil and fish samples. *Anal Chim Acta*. 2010;663(1):43-48. doi:10.1016/j.aca.2010.01.035
103. Sapozhnikova Y, Bawardi O, Schlenk D. Pesticides and PCBs in sediments and fish from the Salton Sea, California, USA. *Chemosphere*. 2004;55(6):797-809.

doi:10.1016/j.chemosphere.2003.12.009

104. Sandau CD, Davis MD, Saito K, et al. Development of a accelerated solvent extraction and gel permeation chromatography analytical method for measuring persistent organohalogen compounds in adipose and organ tissue analysis. *Chemosphere*. 2004;57:373-381. doi:10.1016/j.chemosphere.2004.04.050
105. LeDoux M. Analytical methods applied to the determination of pesticide residues in foods of animal origin. A review of the past two decades. *J Chromatogr A*. 2011;1218(8):1021-1036. doi:10.1016/j.chroma.2010.12.097
106. Anastassiades M, Lehotay SJ, Štajnbaher D, Schenck FJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and. *J AOAC Int*. 2003;86:412-431. doi:10.2478/s11687-011-0011-9
107. Sapozhnikova Y, Lehotay SJ. Multi-class, multi-residue analysis of pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers and novel flame retardants in fish using fast, low-pressure gas chromatography-tandem mass spectrometry. *Anal Chim Acta*. 2013;758:80-92. doi:10.1016/j.aca.2012.10.034
108. Chatterjee NS, Utture S, Banerjee K, et al. Multiresidue analysis of multiclass pesticides and polyaromatic hydrocarbons in fatty fish by gas chromatography tandem mass spectrometry and evaluation of matrix effect. *Food Chem*. 2016;196:1-8. doi:10.1016/j.foodchem.2015.09.014
109. Antunes P, Viana P, Vinhas T, Capelo JL, Rivera J, Gaspar EMSM. Optimization of pressurized liquid extraction (PLE) of dioxin-furans and dioxin-like PCBs from environmental samples. *Talanta*. 2008;75:916-925. doi:10.1016/j.talanta.2007.12.042
110. Richter BE, Jones BA, Ezzell JL, et al. Accelerated Solvent Extraction: A Technique for Sample Preparation. *Anal Chem*. 1996;68(6):1033-1039. doi:10.1021/ac9508199
111. Srogi K. A Review: Application of Microwave Techniques for Environmental Analytical Chemistry. *Anal Lett*. 2006;39:1261-1288. doi:10.1080/00032710600666289
112. Sun Y, Takaoka M, Takeda N, Matsumoto T, Oshita K. Application of water as a solvent in microwave-assisted extraction for analysis of PCBs and CBzs in fly ash. *J Sep Sci*. 2005;28(6):585-588. doi:10.1002/jssc.200401860
113. Miyawaki T, Kawashima A, Honda K. Development of supercritical carbon dioxide extraction with a solid phase trap for dioxins in soils and sediments. *Chemosphere*. 2008;70(4):648-655. doi:10.1016/j.chemosphere.2007.06.068
114. García-Rodríguez D, Carro-Díaz AM, Lorenzo-Ferreira RA. Supercritical fluid extraction of polyhalogenated pollutants from aquaculture and marine environmental samples: A review. *J Sep Sci*. 2008;31(8):1333-1345. doi:10.1002/jssc.200700637

115. Megson D, Kalin R, Worsfold PJ, et al. Fingerprinting polychlorinated biphenyls in environmental samples using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry. *J Chromatogr A*. 2013;1318:276-283. doi:10.1016/j.chroma.2013.10.016
116. Megson D, Focant JF, Patterson DG, et al. Can polychlorinated biphenyl (PCB) signatures and enantiomer fractions be used for source identification and to age date occupational exposure? *Environ Int*. 2015;81:56-63. doi:10.1016/j.envint.2015.04.006
117. Fenik J, Tankiewicz M, Biziuk M. Properties and determination of pesticides in fruits and vegetables. *TrAC - Trends Anal Chem*. 2011;30(6):814-826. doi:10.1016/j.trac.2011.02.008
118. Rejczak T, Tuzimski T. A review of recent developments and trends in the QuEChERS sample preparation approach. *Open Chem*. 2015;13(June):980-1010. doi:10.1515/chem-2015-0109
119. Bruzzoniti MC, Checchini L, De Carlo RM, Orlandini S, Rivoira L, Del Bubba M. QuEChERS sample preparation for the determination of pesticides and other organic residues in environmental matrices: A critical review. *Anal Bioanal Chem*. 2014;406(17):4089-4116. doi:10.1007/s00216-014-7798-4
120. González-Curbelo M, Socas-Rodríguez B, Herrera-Herrera A V., González-Sálamo J, Hernández-Borges J, Rodríguez-Delgado M. Evolution and applications of the QuEChERS method. *TrAC - Trends Anal Chem*. 2015;71:169-185. doi:10.1016/j.trac.2015.04.012
121. Megson D, Reiner EJ, Jobst KJ, Dorman FL, Robson M, Focant J. A review of the determination of persistent organic pollutants for environmental forensics investigations. *Anal Chim Acta*. 2016. doi:10.1016/j.aca.2016.08.027
122. Wittsiepe J, Nestola M, Kohne M, Zinn P, Wilhelm M. Determination of polychlorinated biphenyls and organochlorine pesticides in small volumes of human blood by high-throughput on-line SPE-LVI-GC-HRMS. *J Chromatogr B Anal Technol Biomed Life Sci*. 2014;945-946:217-224. doi:10.1016/j.jchromb.2013.11.059
123. Kole PL, Venkatesh G, Kotecha J, Sheshala R. Recent advances in sample preparation techniques for effective bioanalytical methods. *Biomed Chromatogr*. 2011;25(1):199-217. doi:10.1002/bmc.1560
124. Fidalgo-Used N, Blanco-González E, Sanz-Medel A. Sample handling strategies for the determination of persistent trace organic contaminants from biota samples. *Anal Chim Acta*. 2007;590(1):1-16. doi:10.1016/j.aca.2007.03.004
125. Smith KEC, Northcott GL, Jones KC. Influence of the extraction methodology on the analysis of polycyclic aromatic hydrocarbons in pasture vegetation. *J Chromatogr A*.

2006;1116:20-30. doi:10.1016/j.chroma.2006.03.040

126. Easton MDL, Luszniak D, Geest E Von der. Preliminary examination of contaminant loadings in farmed salmon, wild salmon and commercial salmon feed. *Chemosphere*. 2015;46(2002):1053-1074.

127. Fidalgo-used N, Centineo G, Blanco-gonzález E, Sanz-medel A. Solid-phase microextraction as a clean-up and preconcentration procedure for organochlorine pesticides determination in fish tissue by gas chromatography with electron capture detection. *J Chromatogr A*. 2003;1017:35-44. doi:10.1016/S0021-9673(03)01321-9

128. Kim L, Lee D, Cho HK, Choi SD. Review of the QuEChERS method for the analysis of organic pollutants: Persistent organic pollutants, polycyclic aromatic hydrocarbons, and pharmaceuticals. *Trends Environ Anal Chem*. 2019;22:e00063. doi:10.1016/j.teac.2019.e00063

129. Muscalu AM, Reiner EJ, Liss SN, Chen T, Ladwig G, Morse D. A routine accredited method for the analysis of polychlorinated biphenyls, organochlorine pesticides, chlorobenzenes and screening of other halogenated organics in soil, sediment and sludge by GCxGC- μECD. *Anal Bioanal Chem*. 2011;401:2403-2413. doi:10.1007/s00216-011-5114-0

130. Liu Z, Phillips JB. Comprehensive two-dimensional gas chromatography using an on-column thermal modulator interface. *J Chromatogr Sci*. 1991;29(5):227-231. doi:10.1093/chromsci/29.6.227

131. Mechlińska A, Wolska L, Namieśnik J, Wolska L. Isotope-labeled substances in analysis of persistent organic pollutants in environmental samples. *TrAC - Trends Anal Chem*. 2010;29(8):820-831. doi:10.1016/j.trac.2010.04.011

132. Leeuwen SPJ Van, Boer J De. Advances in the gas chromatographic determination of persistent organic pollutants in the aquatic environment. *J Chromatogr A*. 2008;1186:161-182. doi:10.1016/j.chroma.2008.01.044

133. Guo Y, Kannan K. *Analytical Methods for the Measurement of Legacy and Emerging Persistent Organic Pollutants in Complex Sample Matrices*. Vol 67. Elsevier; 2015. doi:10.1016/B978-0-444-63299-9.00001-6

134. Petrovic M, Farré M, de Alda ML, et al. Recent trends in the liquid chromatography-mass spectrometry analysis of organic contaminants in environmental samples. *J Chromatogr A*. 2010;1217(25):4004-4017. doi:10.1016/j.chroma.2010.02.059

135. US-EPA. *Method 1668B - Chlorinated Biphenyl Congeners In Water, Soil, Sediment, Biosolids, And Tissue By HRGC/HRMS*.; 2008. http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/2009_01_07_methods_method_1668.pdf.

136. Peris-vicente J, Esteve-romero J, Carda-broch S. Validation of Analytical Methods Based on Chromatographic Techniques: An Overview. *Anal Sep Sci*. 2015:1757-1808.
137. Norli RH, Christiansen A, Deribe E. Application of QuEChERS method for extraction of selected persistent organic pollutants in fish tissue and analysis by gas chromatography mass spectrometry. *J Chromatogr A*. 2011;1218(41):7234-7241. doi:10.1016/j.chroma.2011.08.050
138. US-EPA. *Ecological Effects Test Guidelines Environmental Chemistry Methods and Associated Independent Laboratory Validation*.; 2012.
139. Holdgate MW, White G. *Scientific Committee on Problems of the Environment SCOPE 10: Environmental Lssues,John Wiley & Sons, London*.; 1977.
140. Wepener V, van Vuren JHJ, Chatiza FP, Mbizi Z, Slabbert L, Masola B. Active biomonitoring in freshwater environments: Early warning signals from biomarkers in assessing biological effects of diffuse sources of pollutants. *Phys Chem Earth*. 2005;30(11-16 SPEC. ISS.):751-761. doi:10.1016/j.pce.2005.08.018
141. Viarengo A, Burlando B, Dondero F, Marro A, Fabbri R. Metallothionein as a tool in biomonitoring programmes. *Biomarkers*. 1999;4(6):455-466.
142. European Union. *CIS-WFD Guidance Document No. 32 On Biota Monitoring (the Implementation of EQSbiota) under the Water Framework Directive Technical Report 2014-083*.; 2014. doi:10.2779/833200
143. Tornero V, Hanke G. Chemical contaminants entering the marine environment from sea-based sources: A review with a focus on European seas. *Mar Pollut Bull*. 2016;112(1-2):17-38. doi:10.1016/j.marpolbul.2016.06.091
144. *Chemical Marine Monitoring – Policy Framework and Analytical Trends. Quevauviller P., Roose P., Verreet G., 2011. Water Quality Measurements Series, Wiley Edition*.; 2011.
145. Vykusova B, Jarkovsky J, Dus L, Svobodova Z. Bioaccumulation of mercury in muscle tissue of fish in the Elbe River (Czech Republic): multispecies monitoring study 1991 – 1996. *Ecotoxicol Environ Saf*. 2005;61:256-267. doi:10.1016/j.ecoenv.2004.11.007
146. Erdogrul O, Covaci A, Schepens P. Levels of organochlorine pesticides , polychlorinated biphenyls and polybrominated diphenyl ethers in fish species from Kahramanmaras, Turkey. *Enviromental Int*. 2005;31:703-711. doi:10.1016/j.envint.2005.01.002
147. Hajs J, Poustka J, Pulkrabova J. Brominated flame retardants and related chlorinated persistent organic pollutants in fish from river Elbe and its main tributary Vltava. *Chemosphere*. 2007;69:1195-1203. doi:10.1016/j.chemosphere.2007.06.030
148. Pulkrabová J, Hajs J, Poustka J, Kazda R. Monograph Fish as Biomonitors of Polybrominated Diphenyl Ethers and Hexabromocyclododecane in Czech Aquatic Ecosystems: Pollution of the Elbe River Basin. *Environ Health Perspect*. 2007;115(May

2006):28-34. doi:10.1289/ehp.9354

149. Mcgoldrick DJ, Keir MJ, Backus SM. Changes in Mercury Levels in Great Lakes Fish Between 1970s and. *Environ Sci Technol*. 2010;44(9):3273-3279.

150. Brázová T, Hanzelová V. Biomonitoring of polychlorinated biphenyls (PCBs) in heavily polluted aquatic environment in different fish species. *Enviromental Monit Assess*. 2012;184:6553-6561. doi:10.1007/s10661-011-2440-9

151. Gewurtz SB, Backus SM, Bhavsar SP, Mcgoldrick DJ, Solla SR De, Murphy EW. Contaminant biomonitoring programs in the Great Lakes region: Review of approaches and critical factors. *Environ Rev*. 2014;19(May 2011). doi:10.1139/a11-005

152. Mcintyre JK, Beauchamp DA. Age and trophic position dominate bioaccumulation of mercury and organochlorines in the food web of Lake Washington. *Sci Total Environ*. 2007;372:571-584. doi:10.1016/j.scitotenv.2006.10.035

153. Burger J, Gaines KF, Boring CS, Stephens WL, Snodgrass J, Gochfeld M. Mercury and Selenium in Fish from the Savannah River: Species, Trophic Level, and Locational Differences. *Environ Res Sect A*. 2001;87:108-118. doi:10.1006/enrs.2001.4294

154. Britton BJR. Reference data for evaluating the growth of common riverine fishes in the UK. *J Appl Ichthyol*. 2007;23:555-560. doi:10.1111/j.1439-0426.2007.00845.x

155. Rice KM, Jr EMW, Wu M, Gillette C, Blough ER. Environmental Mercury and Its Toxic Effects. *J Prev Med Public Heal*. 2014:74-83.

156. Porte C, Albaige J. Hydrocarbons, PCBs and DDT in the NW Mediterranean deep-sea fish Mora moro. *Deep Res Part I*. 2001;48:495-513.

157. EU. *Common Implementation Strategy for the Water Framework Directive (2000/60/EC) Guidance Document No. 27 Technical Guidance For Deriving Environmental Quality Standards*; 2011.

158. Tomasello B, Copat C, Pulvirenti V, et al. Ecotoxicology and Environmental Safety Biochemical and bioaccumulation approaches for investigating marine pollution using Mediterranean rainbow wrasse, Coris julis (Linneaus 1798). *Ecotoxicol Environ Saf*. 2012;86:168-175. doi:10.1016/j.ecoenv.2012.09.012

159. Rose A, Ken D, Kehinde O. Bioaccumulation of Polycyclic Aromatic Hydrocarbons in Fish and Invertebrates of Lagos Lagoon, Nigeria Bioaccumulation of Polycyclic Aromatic Hydrocarbons in Fish and Invertebrates of Lagos Lagoon, Nigeria Corresponding Author: Olayinka Kehinde. *J Emerg Trends Eng Appl Sci*. 2012;3(2):287-296.

160. Balk L, Hylland K, Hansson T, et al. Biomarkers in Natural Fish Populations Indicate Adverse Biological Effects of Offshore Oil Production. *PLoS One*. 2011;6(5):e19735. doi:10.1371/journal.pone.0019735

161. Logan DT. Perspective on ecotoxicology of PAHs to fish. *Hum Ecol Risk Assess*.

2007;13(2):302-316. doi:10.1080/10807030701226749

162. Astiaso Garcia D, Bruschi D, Cumo F, Gugliermetti F. The Oil Spill Hazard Index (OSHI) elaboration. An oil spill hazard assessment concerning Italian hydrocarbons maritime traffic. *Ocean Coast Manag*. 2013;80:1-11. doi:10.1016/j.ocecoaman.2013.03.016

163. Viola S, Grammauta R, Sciacca V, et al. Continuous monitoring of noise levels in the Gulf of Catania (Ionian Sea). Study of correlation with ship traffic. *Mar Pollut Bull*. 2017;121(1-2):97-103. doi:10.1016/j.marpolbul.2017.05.040

164. Conte F, Copat C, Longo S, et al. First data on trace elements in Haliotis tuberculata (Linnaeus, 1758) from southern Italy: Safety issues. *Food Chem Toxicol*. 2015;81:143-150. doi:10.1016/j.fct.2015.04.020

165. Conte F, Copat C, Longo S, et al. Polycyclic aromatic hydrocarbons in Haliotis tuberculata (Linnaeus, 1758) (Mollusca, Gastropoda): Considerations on food safety and source investigation. *Food Chem Toxicol*. 2016;94:57-63. doi:10.1016/j.fct.2016.05.016

166. Zhao Z, Zhang L, Cai Y, Chen Y. Distribution of polycyclic aromatic hydrocarbon (PAH) residues in several tissues of edible fishes from the largest freshwater lake in China, Poyang Lake, and associated human health risk assessment. *Ecotoxicol Environ Saf*. 2014;104(1):323-331. doi:10.1016/j.ecoenv.2014.01.037

167. Oliva AL, La Colla NS, Arias AH, Blasina GE, Lopez Cazorla A, Marcovecchio JE. Distribution and human health risk assessment of PAHs in four fish species from a SW Atlantic estuary. *Environ Sci Pollut Res*. 2017;24(23):18979-18990. doi:10.1007/s11356-017-9394-6

168. Barhoumi B, El Megdiche Y, Clérandeau C, et al. Occurrence of polycyclic aromatic hydrocarbons (PAHs) in mussel (Mytilus galloprovincialis) and eel (Anguilla anguilla) from Bizerte lagoon, Tunisia, and associated human health risk assessment. *Cont Shelf Res*. 2016;124:104-116. doi:10.1016/j.csr.2016.05.012

169. Moslen M, Miebaka CA, Boisa N. Bioaccumulation of Polycyclic Aromatic Hydrocarbon (PAH) in a bivalve (Arca senilis- blood cockles) and health risk assessment. *Toxicol Reports*. 2019;6(September):990-997. doi:10.1016/j.toxrep.2019.09.006

170. Apiratikul R, Pongpiachan S, Hashmi MZ. Health risk assessment of polycyclic aromatic hydrocarbons in coastal soils of Koh Samed Island (Thailand) after the oil spill incident in 2013. *Mar Pollut Bull*. 2020;150:110736. doi:10.1016/j.marpolbul.2019.110736

171. Soltani N, Moore F, Keshavarzi B, Sorooshian A, Javid R. Potentially toxic elements (PTEs) and polycyclic aromatic hydrocarbons (PAHs) in fish and prawn in the Persian Gulf, Iran. *Ecotoxicol Environ Saf*. 2019;173(February):251-265. doi:10.1016/j.ecoenv.2019.02.005

172. Xia Z, Duan X, Qiu W, et al. Health risk assessment on dietary exposure to polycyclic

aromatic hydrocarbons (PAHs) in Taiyuan, China. *Sci Total Environ*. 2010;408(22):5331-5337. doi:10.1016/j.scitotenv.2010.08.008

173. Wang X, Celander MC, Yin X, Zhang Z, Chen Y. PAHs and PCBs residues and consumption risk assessment in farmed yellow croaker (Larimichthys crocea) from the East China Sea, China. *Mar Pollut Bull*. 2019;140(January):294-300. doi:10.1016/j.marpolbul.2019.01.062
174. Bandowe BAM, Bigalke M, Boamah L, Nyarko E, Saalia FK, Wilcke W. Polycyclic aromatic compounds (PAHs and oxygenated PAHs) and trace metals in fish species from Ghana (West Africa): Bioaccumulation and health risk assessment. *Environ Int*. 2014;65:135-146. doi:10.1016/j.envint.2013.12.018
175. Fang C, Bo J, Zheng R, et al. Biomonitoring of aromatic hydrocarbons in clam Meretrix meretrix from an emerging urbanization area, and implications for human health. *Ecotoxicol Environ Saf*. 2020;192(December 2019):110271. doi:10.1016/j.ecoenv.2020.110271
176. US-EPA. Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories. *Fish Sampl Anal*. 2000;1(4305):1-485.
177. US-EPA. Risk Characterization Handbook, US Environmental Protection Agency, Washington DC,. 2000.
178. US-EPA. Guidance for assessing chemical contaminant data for use in fish advisories Risk assessment and fish consumption limits, 3rd edition. *Risk Assess fish Consum limits, 3rd Ed*. 2000;2:1-383.
179. Copat C, Conti GO, Signorelli C, et al. Risk Assessment for Metals and PAHs by Mediterranean Seafood. *Food Nutr Sci*. 2013;04(07):10-13. doi:10.4236/fns.2013.47a002
180. Man YB, Mo WY, Zhang F, Wong MH. Health risk assessments based on polycyclic aromatic hydrocarbons in freshwater fish cultured using food waste-based diets. *Environ Pollut*. 2020;256:113380. doi:10.1016/j.envpol.2019.113380
181. Duedahl-Olesen L, Iversen NM, Kelmo C, Jensen LK. Validation of QuEChERS for screening of 4 marker polycyclic aromatic hydrocarbons in fish and malt. *Food Control*. 2020;108(October 2018):106434. doi:10.1016/j.foodcont.2018.12.010
182. Wang Z, Chen J, Yang P, Qiao X, Tian F. Polycyclic aromatic hydrocarbons in Dalian soils: Distribution and toxicity assessment. *J Environ Monit*. 2007;9(2):199-204. doi:10.1039/b617338c
183. Mostert MMR, Ayoko GA, Kokot S. Application of chemometrics to analysis of soil pollutants. *TrAC - Trends Anal Chem*. 2010;29(5):430-445. doi:10.1016/j.trac.2010.02.009
184. Mukadder Arslan Aİ. Age, growth and reproduction of Mullus surmuletus (Linnaeus,

1758) in Saros Bay (Northern Aegean Sea). *J Black Sea / Mediterr Environ*. 2013;19(2):217-233.

185. Nisbet ICT, LaGoy PK. Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). *Regul Toxicol Pharmacol*. 1992;16(3):290-300. doi:10.1016/0273-2300(92)90009-X
186. EPA. Risk Assessment Guidance for Superfund Volume I Human Health Evaluation Manual (Part A). 1989;I.
187. Oliveri G, Chiara C, Caterina C. Evaluation of Heavy Metals and Polycyclic Aromatic Hydrocarbons (PAHs) in Mullus barbatus from Sicily Channel and Risk-Based Consumption Limits. *Bull Environ Contam Toxicol*. 2012;88:946-950. doi:10.1007/s00128-012-0611-1
188. Saija E, Mangano V, Casale KE, et al. Data in Brief Determination and quantification of PCBs, POCs and PAHs in Thunnus thynnus from the Straits of Messina (Italy). *Data Br*. 2016;7:129-134. doi:10.1016/j.dib.2016.02.027
189. Di Bella G Di, Russo E, Potortì AG, et al. Persistent organic pollutants in farmed European sea bass (Dicentrarchus labrax , Linnaeus , 1758) from Sicily (Italy). *Food Addit Contam Part A*. 2018;35(2):282-291. doi:10.1080/19440049.2017.1372642
190. Ferrante M, Zanghì G, Cristaldi A, et al. PAHs in seafood from the Mediterranean Sea: An exposure risk assessment. *Food Chem Toxicol*. 2018;115:385-390. doi:10.1016/j.fct.2018.03.024
191. Berrojalbiz N, Dachs J, Ojeda MJ, et al. Biogeochemical and physical controls on concentrations of polycyclic aromatic hydrocarbons in water and plankton of the Mediterranean and Black Seas. *Global Biogeochem Cycles*. 2011;25:1-14. doi:10.1029/2010GB003775
192. Vecchiato M, Turetta C, Patti B, et al. Distribution of fragrances and PAHs in the surface seawater of the Sicily Channel, Central Mediterranean. *Sci Total Environ*. 2018;634:983-989. doi:10.1016/j.scitotenv.2018.04.080
193. IARC. *Some Nonheterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Exposures*. Vol 92.; 2010. http://monographs.iarc.fr/ENG/Monographs/%0Avol92/mono92.pdf.
194. Binelli AÃ, Provini A. Risk for human health of some POPs due to fish from Lake Iseo. *Ecotoxicol Environ Saf*. 2004;58:139-145. doi:10.1016/j.ecoenv.2003.09.014
195. Pointet K, Milliet A. PAHs analysis of fish whole gall bladders and livers from the Natural Reserve of Camargue by GC/MS. *Chemosphere*. 2000;40:293-299.
196. Mashroofeh A, Riyahi A, Pourkazemi M. Distribution and composition pattern of polycyclic aromatic hydrocarbons in different tissues of sturgeons collected from Iranian

coastline of the Caspian Sea. *Chemosphere*. 2015;120:575-583. doi:10.1016/j.chemosphere.2014.09.071

197. Dsikowitzky L, Nordhaus I, Andarwulan N, et al. Accumulation patterns of lipophilic organic contaminants in surface sediments and in economic important mussel and fish species from Jakarta Bay, Indonesia. *Mar Pollut Bull*. 2016;110(2):767-777. doi:10.1016/j.marpolbul.2016.01.034
198. Wang Q, Peng F, Chen Y, et al. Heavy metals and PAHs in an open fishing area of the East China Sea: Multimedia distribution, source diagnosis, and dietary risk assessment. *Environ Sci Pollut Res*. 2019;26(21):21140-21150. doi:10.1007/s11356-019-05355-z
199. Ranjbar Jafarabadi A, Riyahi Bakhtiari A, Yaghoobi Z, Kong Yap C, Maisano M, Cappello T. Distributions and compositional patterns of polycyclic aromatic hydrocarbons (PAHs) and their derivatives in three edible fishes from Kharg coral Island, Persian Gulf, Iran. *Chemosphere*. 2019;215:835-845. doi:10.1016/j.chemosphere.2018.10.092
200. Bautista NM, Pothini T, Meng K, Burggren WW. Behavioral consequences of dietary exposure to crude oil extracts in the Siamese fighting fish (Betta splendens). *Aquat Pollut*. 2019;207(November 2018):34-42. doi:10.1016/j.aquatox.2018.11.025
201. Honda M, Suzuki N. Toxicities of Polycyclic Aromatic Hydrocarbons for Aquatic Animals. *Int J Environ Res Public Health*. 2020;17(1363).
202. Varanasi U, Stein J, Nishimoto M. *Biotransformation and Disposition of Polycyclic Aromatic Hydrocarbons (PAH) in Fish.*; 1989.
203. Frenna S, Mazzola A, Orecchio S, Tuzzolino N. Comparison of different methods for extraction of polycyclic aromatic hydrocarbons (PAHs) from Sicilian (Italy) coastal area sediments. *Environ Monit Assess*. 2013;185(7):5551-5562. doi:10.1007/s10661-012-2966-5
204. Perugini M, Visciano P, Giammarino A, Manera M, Nardo W Di, Amorena M. Polycyclic aromatic hydrocarbons in marine organisms from the Adriatic Sea , Italy. *Chemosphere*. 2007;66:1904-1910. doi:10.1016/j.chemosphere.2006.07.079
205. Sette CB, Pedrete TDA, Felizzola J, Nudi AH, Sco ADL, Wagener ADLR. Formation and identification of PAHs metabolites in marine organisms. *Mar Environ Res*. 2013;91(March):2-13. doi:10.1016/j.marenvres.2013.02.004
206. Zhang W, Zhang S, Wan C, Yue D, Ye Y, Wang X. Source diagnostics of polycyclic aromatic hydrocarbons in urban road runoff, dust, rain and canopy throughfall. *Environ Pollut*. 2008;153:594-601. doi:10.1016/j.envpol.2007.09.004
207. Pies C, Hoffmann B, Petrowsky J, Yang Y, Ternes TA, Hofmann T. Characterization and source identification of polycyclic aromatic hydrocarbons (PAHs) in river bank soils. *Chemosphere*. 2008;72:1594-1601. doi:10.1016/j.chemosphere.2008.04.021

208. Torre-roche RJD La, Lee W, Campos-díaz SI. Soil-borne polycyclic aromatic hydrocarbons in El Paso, Texas: Analysis of a potential problem in the United States/Mexico border region. *J Hazard Mater*. 2009;163:946-958. doi:10.1016/j.jhazmat.2008.07.089

209. Shi Z, Tao S, Pan B, Liu WX, Shen WR. Partitioning and source diagnostics of polycyclic aromatic hydrocarbons in rivers in Tianjin, China. *Environ Pollut*. 2007;146:492-500. doi:10.1016/j.envpol.2006.07.009

210. Qiu Y, Zhang G, Liu G, Guo L, Li X, Wai O. Estuarine, Coastal and Shelf Science Polycyclic aromatic hydrocarbons (PAHs) in the water column and sediment core of Deep Bay, South China. *Estuar Coast Shelf Sci*. 2009;83(1):60-66. doi:10.1016/j.ecss.2009.03.018

211. EU. *DIRECTIVE 2013/39/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 12 August 2013 Amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy*. Vol 2013.; 2013:1-17.

212. Dhananjayan V, Muralidharan S. Polycyclic Aromatic Hydrocarbons in Various Species of Fishes from Mumbai Harbour, India, and Their Dietary Intake Concentration to Human. *Int J Oceanogr*. 2012;2012:1-6. doi:10.1155/2012/645178

213. Johnson-Restrepo B, Olivero-Verbel J, Lu S, et al. Polycyclic aromatic hydrocarbons and their hydroxylated metabolites in fish bile and sediments from coastal waters of Colombia. *Enviromental Pollut*. 2008;151:452-459. doi:10.1016/j.envpol.2007.04.011

214. Pompa G, Caloni F, Fracchiolla ML. Dioxin and PCB Contamination of Fish and Shellfish: Assessment of Human Exposure . Review of the International Situation. *Vet Res Commun*. 2003;1:159-167.

215. Wei X, Huang Y, Wong MH, Giesy JP, Wong CKC. Assessment of risk to humans of bisphenol A in marine and freshwater fish from Pearl River Delta, China. *Chemosphere*. 2011;85(1):122-128. doi:10.1016/j.chemosphere.2011.05.038

216. Nie XP, Lan CY, An TC, Li KB, Wong MH. Distributions and Congener Patterns of PCBs in Fish from Major Aquaculture Areas in the Pearl River Delta, South China. *Hum Ecol Risk Assess*. 2006;12:363-373. doi:10.1080/10807030500537908

217. Brázová T, Hanzelová V, Miklisová D. Bioaccumulation of six PCB indicator congeners in a heavily polluted water reservoir in Eastern Slovakia: tissue-specific distribution in fish and their parasites. *Parasitol Res*. 2012;111:779-786. doi:10.1007/s00436-012-2900-3

218. Batang ZB, Alikunhi N, Gochfeld M, et al. Science of the Total Environment Congener-speci fi c levels and patterns of polychlorinated biphenyls in edible fi sh tissue from the central Red Sea coast of Saudi Arabia. *Sci Total Environ*. 2016;572:915-925.

doi:10.1016/j.scitotenv.2016.07.207

219. Robock A. Volcanic eruptions and climate. *Rev Geophys*. 2000;38:191-219.

220. von Glasow R, Bobrowski N, Kern C. The effects of volcanic eruptions on atmospheric chemistry. *Chem Geol*. 2009;263:131-142. doi:10.1016/j.chemgeo.2008.08.020

221. Mather TA, Pyle DM. Tropospheric Volcanic Aerosol. *Volcanism Earth's Atmos Geophys Monogr*. 2003;139:189-212.

222. Halmer MM, Schmincke HU, Graf HF. The annual volcanic gas input into the atmosphere, in particular into the stratosphere: A global data set for the past 100 years. *J Volcanol Geotherm Res*. 2002;115(3-4):511-528. doi:10.1016/S0377-0273(01)00318-3

223. Abdel-shafy HI, Mansour MSM. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egypt J Pet*. 2016;25(1):107-123. doi:10.1016/j.ejpe.2015.03.011

224. Wild SR, Jones KC. Polynuclear aromatic hydrocarbons in the United Kingdom environment: A preliminary source inventory and budget. *Environ Pollut*. 1995;88(1):91-108. doi:10.1016/0269-7491(95)91052-M

225. Thiombane M, Albanese S, Di Bonito M, et al. Source patterns and contamination level of polycyclic aromatic hydrocarbons (PAHs) in urban and rural areas of Southern Italian soils. *Environ Geochem Health*. 2019;41(2):507-528. doi:10.1007/s10653-018-0147-3

226. Polkowska Z. Organic Pollution in Surface Waters from the Fuglebekken Basin in Svalbard, Norwegian Arctic. *Sensors*. 2011;11:8910-8929. doi:10.3390/s110908910

227. Varrica D, Aiuppa A, Dongarrà G. Volcanic and anthropogenic contribution to heavy metal content in lichens from Mt. Etna and Vulcano island (Sicily). *Environ Pollut*. 2000;108(2):153-162. doi:10.1016/S0269-7491(99)00246-8

228. Pyle DM, Mather TA. The importance of volcanic emissions for the global atmospheric mercury cycle. *Atmos Environ*. 2003;37(36):5115-5124. doi:10.1016/j.atmosenv.2003.07.011

229. Cimino G, Toscano G. Dissolution of trace metals from lava ash: Influence on the composition of rainwater in the Mount Etna volcanic area. *Environ Pollut*. 1998;99(3):389-393. doi:10.1016/S0269-7491(98)00004-9

230. Tassi F, Capecchiacci F, Cabassi J, et al. Geogenic and atmospheric sources for volatile organic compounds in fumarolic emissions from Mt. Etna and Vulcano Island (Sicily, Italy). *J Geophys Res Atmos*. 2012;117:1-20. doi:10.1029/2012JD017642

231. Stracquadanio M, Trombini C, Donato PS. Role of volcanic dust in the atmospheric transport and deposition of polycyclic aromatic hydrocarbons and mercury. *J Enviromental Monit*. 2003;5:984-988. doi:10.1039/b308587b

232. Tobiszewski M, Namie J. PAH diagnostic ratios for the identification of pollution

emission sources. *Environ Pollut*. 2012;162:110-119. doi:10.1016/j.envpol.2011.10.025

233. Zolotov MY, Shock EL. A thermodynamic assessment of the potential synthesis of condensed hydrocarbons during cooling and dilution of volcanic gases. *J Geophys Res*. 2000;105:539-559.
234. Podkletnov NE, E.K M. New data on Abiogenic Synthesis of Prebiological compounds in volcanic porcesses. *Orig Life*. 1981;11:303-315.
235. Akyüz M, Çabuk H. Science of the Total Environment Gas – particle partitioning and seasonal variation of polycyclic aromatic hydrocarbons in the atmosphere of Zonguldak, Turkey. *Sci Total Environ*. 2010;408(22):5550-5558. doi:10.1016/j.scitotenv.2010.07.063
236. Yunker MB, Macdonald RW, Vingarzan R, Mitchell H, Goyette D, Sylvestre S. PAHs in the Fraser River basin: a critical appraisal of PAH ratios as indicators of PAH source and composition. *Org Geochem*. 2002;33:489-515.
237. Katsoyiannis A, Terzi E, Cai Q. On the use of PAH molecular diagnostic ratios in sewage sludge for the understanding of the PAH sources. Is this use appropriate? *Chemosphere*. 2007;69:1337-1339. doi:10.1016/j.chemosphere.2007.05.084
238. Ravindra K, Wauters E, Grieken R Van. Variation in particulate PAHs levels and their relation with the transboundary movement of the air masses. *Sci Total Environ*. 2008;3966:100-110. doi:10.1016/j.scitotenv.2008.02.018
239. EPA. *METHOD 3500C - ORGANIC EXTRACTION AND SAMPLE PREPARATION;* 2007.
240. EPA. *METHOD 3600C CleanUP*; 1996.
241. Guiñez M, Escudero L, Mandelli A, Martinez LD, Cerutti S. Volcanic ashes as a source for nitrated and oxygenated polycyclic aromatic hydrocarbon pollution. *Environ Sci Pollut Res*. 2020:1-11.
242. Wang H, Campiglia AD. Determination of polycyclic aromatic hydrocarbons in drinking water samples by solid-phase nanoextraction and high-performance liquid chromatography. *Anal Chem*. 2008;80(21):8202-8209. doi:10.1021/ac8014824
243. Poster DL, Schantz MM, Sander LC, Wise SA. Analysis of polycyclic aromatic hydrocarbons (PAHs) in environmental samples: A critical review of gas chromatographic (GC) methods. *Anal Bioanal Chem*. 2006;386(4):859-881. doi:10.1007/s00216-006-0771-0
244. Wenzl T, Simon R, Anklam E, Kleiner J. Analytical methods for polycyclic aromatic hydrocarbons (PAHs) in food and the environment needed for new food legislation in the European Union. *TrAC - Trends Anal Chem*. 2006;25(7):716-725. doi:10.1016/j.trac.2006.05.010
245. Wise SA, Sander LC, Schantz MM. Analytical Methods for Determination of Polycyclic

Aromatic Hydrocarbons (PAHs) — A Historical Perspective on the 16 U.S. EPA Priority Pollutant PAHs. *Polycycl Aromat Compd*. 2015;35(2-4):187-247. doi:10.1080/10406638.2014.970291

246. Patra D. Applications and New Developments in Fluorescence Spectroscopic Techniques for the Analysis of Polycyclic Aromatic Hydrocarbons. *Appl Spectrosc Rev*. 2003;38(2):155-185. doi:10.1081/ASR-120021166
247. Pooja D, Kumar P, Singh P, Patil S. *Sensors in Water Pollutants Monitoring: Role of Material*. Springer; 2020.
248. Wasswa J, Mladenov N, Pearce W. Assessing the potential of fluorescence spectroscopy to monitor contaminants in source waters and water reuse systems. *Environ Sci Water Res Technol*. 2019;5(2):370-382. doi:10.1039/c8ew00472b
249. Mei J, Leung NLC, Kwok RTK, Lam JWY, Tang BZ. Aggregation-Induced Emission: Together We Shine, United We Soar! *Chem Rev*. 2015;115(21):11718-11940. doi:10.1021/acs.chemrev.5b00263
250. Das AK, Goswami S. 2-Hydroxy-1-naphthaldehyde: A versatile building block for the development of sensors in supramolecular chemistry and molecular recognition. *Sensors Actuators, B Chem*. 2017;245:1062-1125. doi:10.1016/j.snb.2017.01.068
251. He L, Dong B, Liu Y, Lin W. Fluorescent chemosensors manipulated by dual/triple interplaying sensing mechanisms. *Chem Soc Rev*. 2016;45(23):6449-6461. doi:10.1039/c6cs00413j
252. Mako TL, Racicot JM, Levine M. Supramolecular Luminescent Sensors. *Chem Rev*. 2019;119:322-477. doi:10.1021/acs.chemrev.8b00260
253. De Silva AP, Moody TS, Wright GD. Fluorescent PET (Photoinduced Electron Transfer) sensors as potent analytical tools. *Analyst*. 2009;134(12):2385-2393. doi:10.1039/b912527m
254. Gowri A, Vignesh R, Kathiravan A. Anthracene based AIEgen for picric acid detection in real water samples. *Spectrochim Acta Part A Mol Biomol Spectrosc*. 2019;220:117144. doi:10.1016/j.saa.2019.117144
255. Chen S, Yu Y, Wang J. Inner filter effect-based fluorescent sensing systems: A review. *Anal Chim Acta*. 2018;999:13-26. doi:10.1016/j.aca.2017.10.026
256. Tamgho I, Chaudhuri S, Verderame M, Discenza DJ, Levine M. A highly versatile fluorenone-based macrocycle for the sensitive detection of polycyclic aromatic hydrocarbons and fluoride anions. *RSC Adv*. 2017;7:28489-28493. doi:10.1039/C7RA05404A
257. Serio N, Moyano DF, Rotello VM, Levine M. Array-based detection of persistent organic pollutants via cyclodextrin promoted energy transfer. *Chem Commun*.

2015;51(58):11615-11618. doi:10.1039/c5cc04153h

258. Serio N, Miller K, Levine M. Efficient detection of polycyclic aromatic hydrocarbons and polychlorinated biphenyls via three-component energy transfer. *Chem Commun*. 2013;49(42):4821-4823. doi:10.1039/c3cc40534f

259. Mako T, Marks P, Cook N, Levine M. Fluorescent detection of polycyclic aromatic hydrocarbons in ternary cyclodextrin complexes. *Supramol Chem*. 2012;24(10):743-747. doi:10.1080/10610278.2012.703325

260. Förster T. Energy migration and fluorescence. *J Biomed Opt*. 2012;17(1):011002. doi:10.1117/1.jbo.17.1.011002

261. Parkesh R, Veale EB, Gunnlaugsson T. Fluorescent Detection Principles and Strategies. *Chemosens Princ Strateg Appl*. 2011:229-252. doi:10.1002/9781118019580.ch12

262. Lakowicz JR. *Principles of Fluorescence Spectroscopy*.; 2006. doi:10.1007/978-0-387-46312-4

263. Tyson DS, Gryczynski I, Castellano FN. Long-range resonance energy transfer to [Ru(bpy)3]2+. *J Phys Chem A*. 2000;104(13):2919-2924. doi:10.1021/jp994023f

264. Menting R, Ng DKP, Röder B, Ermilov EA. Sequential energy and charge transfer processes in mixed host-guest complexes of subphthalocyanine, porphyrin and phthalocyanine chromophores. *Phys Chem Chem Phys*. 2012;14(42):14573-14584. doi:10.1039/c2cp42416a

265. Levine M, Song I, Andrew TL, Kooi SE, Swager TM. Photoluminescent energy transfer from poly(phenyleneethynylene)s to near-infrared emitting fluorophores. *J Polym Sci Part A Polym Chem*. 2010;48(15):3382-3391. doi:10.1002/pola.24123

266. Kreno LE, Leong K, Farha OK, Allendorf M, Van Duyne RP, Hupp JT. Metal-organic framework materials as chemical sensors. *Chem Rev*. 2012;112(2):1105-1125. doi:10.1021/cr200324t

267. Chen LY, Wang CW, Yuan Z, Chang HT. Fluorescent gold nanoclusters: Recent advances in sensing and imaging. *Anal Chem*. 2015;87(1):216-229. doi:10.1021/ac503636j

268. Formica M, Fusi V, Giorgi L, Micheloni M. New fluorescent chemosensors for metal ions in solution. *Coord Chem Rev*. 2012;256(1-2):170-192. doi:10.1016/j.ccr.2011.09.010

269. Zhang L, Fang M. Nanomaterials in pollution trace detection and environmental improvement. *Nano Today*. 2010;5(2):128-142. doi:10.1016/j.nantod.2010.03.002

270. Liu Y. Nanoparticle-based strategies for detection and remediation of environmental. *Analyst*. 2011;136:872-877. doi:10.1039/c0an00905a

271. Guerrini L, Aliaga AE. Functionalization of Ag nanoparticles with the bis-acridinium lucigenin as a chemical assembler in the detection of persistent organic pollutants by surface-enhanced Raman scattering. *Anal Chim Acta*. 2008;4(624):286-293.

doi:10.1016/j.aca.2008.06.038

272. Sanchez-cortes S. Sensitive Surface-Enhanced Raman Spectroscopy (SERS) Detection of Organochlorine Pesticides by Alkyl Dithiol-Functionalized Metal Nanoparticles-Induced Plasmonic Hot Spots. *Anal Chem*. 2015;87:663-669. doi:10.1021/ac503672f
273. Guerrini L, Sanchez-cortes S. Building Highly Selective Hot Spots in Ag Nanoparticles Using Bifunctional Viologens: Application to the SERS Detection of PAHs. *J Phys Chem C Lett*. 2008;112:7527-7530.
274. Guerrini L, Sanchez-cortes S. Sensing Polycyclic Aromatic Hydrocarbons with Dithiocarbamate-Functionalized Ag Nanoparticles by Surface-Enhanced Raman Scattering. *Anal Chem*. 2009;81(3):953-960.
275. Hahm E, Jeong D, Cha MG, et al. β - CD Dimer-immobilized Ag Assembly Embedded Silica Nanoparticles for Sensitive Detection of Polycyclic Aromatic Hydrocarbons. *Nat Publ Gr*. 2016;6(April):1-7. doi:10.1038/srep26082
276. Jeong Y, Kook Y, Lee K, Koh W. Biosensors and Bioelectronics Metal enhanced fluorescence (MEF) for biosensors: General approaches and a review of recent developments. *Biosens Bioelectron*. 2018;111(April):102-116. doi:10.1016/j.bios.2018.04.007
277. Liu Q, Zhou Q, Jiang G. Nanomaterials for analysis and monitoring of emerging chemical pollutants. *Trends Anal Chem*. 2014;58:10-22. doi:10.1016/j.trac.2014.02.014
278. Walekar L, Dutta T, Kumar P, et al. Functionalized fluorescent nanomaterials for sensing pollutants in the environment: A critical review. *Trends Anal Chem*. 2017;97:458-467. doi:10.1016/j.trac.2017.10.012
279. Wang HF, He Y, Ji TR, Yan XP. Surface molecular imprinting on Mn-doped ZnS quantum dots for room-temperature phosphorescence optosensing of pentachlorophenol in water. *Anal Chem*. 2009;81(4):1615-1621. doi:10.1021/ac802375a
280. Xie J, Zheng Y, Ying JY. Highly selective and ultrasensitive detection of Hg2+ based on fluorescence quenching of Au nanoclusters by Hg2+-Au+ interactions. *Chem Commun*. 2010;46(6):961-963. doi:10.1039/b920748a
281. Xie J, Zheng Y, Ying JY. Protein-directed synthesis of highly fluorescent gold nanoclusters. *J Am Chem Soc*. 2009;131(3):888-889. doi:10.1021/ja806804u
282. Zheng Y, Yang Z, Ying JY. Aqueous synthesis of glutathione-capped ZnSe and Zn1-xCd xSe alloyed quantum dots. *Adv Mater*. 2007;19(11):1475-1479. doi:10.1002/adma.200601939
283. Shang L, Dong S. Design of fluorescent assays for cyanide and hydrogen peroxide based on the inner filter effect of metal nanoparticles. *Anal Chem*. 2009;81(4):1465-1470. doi:10.1021/ac802281x

284. Contino A, Maccarrone G, Zimbone M, Musumeci P, Calcagno L, Pannitteri S. Fine tuning the pH triggers the enantiorecognition of underivatized amino acids by silver nanoparticles: A novel approach based on the focused use of solution equilibria. *J Colloid Interface Sci*. 2015;443:30-35. doi:10.1016/j.jcis.2014.11.067

285. Contino A, Maccarrone G, Zimbone M, et al. Tyrosine capped silver nanoparticles: A new fluorescent sensor for the quantitative determination of copper(II) and cobalt(II) ions. *J Colloid Interface Sci*. 2016;462(Ii):216-222. doi:10.1016/j.jcis.2015.10.008

286. Mulfinger L, Solomon SD, Bahadory M, Jeyarajasingam A V., Rutkowsky SA, Boritz C. Synthesis and Study of Silver Nanoparticles. *J Chem Educ*. 2007;84(2):322. doi:10.1021/ed084p322

287. Scollo F, Seggio M, Torrisi RL, et al. New fluorescent-labelled nanoparticles: synthesis, characterization and interactions with cysteine and homocysteine to evaluate their stability in aqueous solution. *Appl Nanosci*. 2020;10(4):1157-1172. doi:10.1007/s13204-019-01241-9

288. Slistan-Grijalva A, Herrera-Urbina R, Rivas-Silva JF, Ávalos-Borja M, Castillón-Barraza FF, Posada-Amarillas A. Classical theoretical characterization of the surface plasmon absorption band for silver spherical nanoparticles suspended in water and ethylene glycol. *Phys E Low-Dimensional Syst Nanostructures*. 2005;27(1-2):104-112. doi:10.1016/j.physe.2004.10.014

289. Paramelle D, Sadovoy A, Gorelik S, Free P, Hobley J, Fernig DG. A rapid method to estimate the concentration of citrate capped silver nanoparticles from UV-visible light spectra. *Analyst*. 2014;139(19):4855-4861. doi:10.1039/c4an00978a

290. Romanovskaya GI, Koroleva M V, Bronskii VS, Zuev BK. New Nanomaterials for Control of the Luminescence of Polycyclic Aromatic Hydrocarbons. *Chemistry (Easton)*. 2016;467(3):296-299. doi:10.1134/S0012500816030095

291. Ncube S, Madikizela L, Cukrowska E, Chimuka L. Recent advances in the adsorbents for isolation of polycyclic aromatic hydrocarbons (PAHs) from environmental sample solutions. *Trends Anal Chem*. 2018;99:101-116. doi:10.1016/j.trac.2017.12.007

292. Romanovskaya GI, Olenin AY, Vasil'Eva SY. Concentration of polycyclic aromatic hydrocarbons by chemically modified silver nanoparticles. *Russ J Phys Chem A*. 2011;85(2):274-278. doi:10.1134/S0036024411010237

293. Ferdous Z, Nemmar A. Health Impact of Silver Nanoparticles: A Review of the Biodistribution and Toxicity Following Various Routes of Exposure. *Int J Mol Sci*. 2020:1-31.

294. Driskill AK, Alvey J, Dotson AD, Tomco PL. Monitoring polycyclic aromatic hydrocarbon (PAH) attenuation in Arctic waters using fluorescence spectroscopy. *Cold*

Reg Sci Technol. 2018;145(September 2017):76-85. doi:10.1016/j.coldregions.2017.09.014

295. US-EPA. Method 550.1 *Determination of polycyclic aromatic hydrocarbons in drinking water by liquid-solid extraction and hplc with coupled ultraviolet and fluorescence detection*; 1990. http://w.ultrasci.com/docs/analyticalmethod/method_550_1.pdf.

296. Doughty MJ. pH dependent spectral properties of sodium fluorescein ophthalmic solutions revisited. *Ophthalmic Physiol Opt*. 2010;30:167-174. doi:10.1111/j.1475-1313.2009.00703.x

LIST OF PUBLICATIONS AND PROCEEDINGS

Publications:

New fluorescent-labelled nanoparticles: synthesis, characterization and interactions with cysteine and homocysteine to evaluate their stability in aqueous solution.
Scollo, F., Seggio, M., Torrisi, R. L., **Bua, R. O**., Zimbone, M., Contino, A., & Maccarrone, G. *Applied Nanoscience*, **2020**, 1-16.

Polycyclic aromatic hydrocarbons in *Mullus surmuletus* from the Catania Gulf (Sicily, Italy): distribution and potential health risks
Bua, R. O.,* Contino, A., Maccarrone, G., Giuffrida A.
Environmental Science and Pollution Research, **2020**, 1-10.

A new method for the determination of pyrene in ultrapure water and in seawater using an aromatic anion highly fluorescent. (in preparation)

Oral Presentations:

Inquinanti emergenti: analisi di screening delle acque superficiali e delle acque reflue.
Bua, R.O., Antoci, M.L., Giuffrida A.
1° Workshop "La Chimica Analitica Forense", Catania, 20th June 2018

The role of mass spectrometry in environmental crimes prevention
Bua, R.O., Giuffrida A.
1^ Workshop of Forensic Analytical Chemistry, *Forensic investigations and the contribution of mass spectrometry,* Roma 23 March 2018

Co-authored Presentations:

Analisi Ambientali degli ecosistemi acquatici
Antoci, M.L, **Bua, R.O.**, Campo, D., Rapisarda, M.
Convegno Azioni di diversificazione della pesca, sviluppo delle potenzialità del settore acquacoltura, sostenibilità ambientale, Ragusa, 28 September 2018

Poster Presentations:

Determination by GC-MS of organic pollutants in etna volcanic ashes

Bua, R.O., Galizia, B., Giuffrida, A.

XXVIII Congress of the Analytical Chemistry Division, 22-26 September 2019, Bari

Determination by GC-MS/MS and GCxGC-TOFMS techniques of persistent organic pollutants in biota: development and validation of a modified QuEChERS extraction

Bua, R.O., Antoci M. L., Campo, D., Giuffrida, A.

XXVII Congress of the Analytical Chemistry Division, 16-20 September 2018, Bologna

Determinazione di microinquinanti organici nel biota mediante GC-MS/MS: Sviluppo e implementazione della metodica QuEChERS

Bua, R.O., Antoci, M. L., Campo, D., Giudice, R. C., Giuffrida, A.

Congresso Congiunto delle Sezioni Sicilia e Calabria SCI 2018, 9-10 February 2018, Catania