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# METHYLMERCURY AND POLYCYCLIC AROMATIC HYDROCARBONS IN MEDITERRANEAN SEAFOOD: A MOLECULAR ANTHROPOLOGICAL PERSPECTIVE

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## Abstract

Seafood carries several contaminants, among which mercury and polycyclic aromatic hydrocarbons are those that cause major concern. Evidence exists that human populations are exposed to these environmental chemicals since ancient times, which may have driven the positive selection of specific genetic polymorphisms related to chemicals toxicokinetic. Both mercury and polycyclic aromatic hydrocarbons are able to cause DNA methylation changes in humans. Some Mediterranean populations may be particularly exposed to these contaminants, being the Mediterranean Sea at a high-risk for contamination by toxic compounds, and because of their traditionally high consumption of locally caught seafood. Starting from these premises the present thesis aims to contribute to the understanding of the molecular impact of seafood consumption on the biology of the Mediterranean population. To this end the work has been divided into four main parts: (1) the development and meta-analysis of a georeferenced database on polycyclic aromatic hydrocarbons in Mediterranean seafood aimed at identifying geographical patterns of contamination and trends that could be related to the biology of the marine organisms, to the physico-chemical properties of each hydrocarbon and to the oceanographic characteristic of the Mediterranean; (2) the development and validation of a food frequency questionnaire to estimate the intake of mercury through seafood consumption among a population living in a geographic area that is usually considered a contamination hotspot; (3) the creation of a biobank made of biological samples from members of several Italian communities together with information on their dietary habits, lifestyle and general health; (4) a review of the literature on the genetic component of individual susceptibility to methylmercury and polycyclic aromatic hydrocarbons exposure in humans, to the effects that these pollutants have on human DNA methylation, and to the evidence that Mediterranean coastal communities represent an informative case study to investigate the potential molecular impact of these chemicals.

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## 1. General introduction

Today, seafood consumption is increasing worldwide: per capita global fish consumption has grown from 9.0 kg in 1961 to 20.2 kg in 2015 [1]. European countries bordering the Mediterranean, in particular, are among the world's highest consumers, with Spain, Italy and France accounting for more than half of the European expenditure on fish and fishery products despite having only around a third of the EU's population. In 2019, per capita apparent consumption of fish and seafood products amounted to 31.21 kg live weight/capita/year , a share significantly higher than the EU average in the same year (23.97 kg live weight/capita/year) [2].

Seafood is an important part of human diet, providing nutrients essential to human brain development and functions [3], and having played a crucial role in the evolution of our species [4]. Seafood consumption leads to epigenetic changes of human DNA [5], and has driven the selection of specific genetic variants in some populations. In Inuit populations, for example, high long-chain polyunsaturated fatty-acids led to the positive selection of alleles of fatty acid desaturase (FADS) genes correlated with less efficient endogenous synthesis of these nutrients [6].

Fish is a good source of a lot of nutrients that are known to positively affect human health, but, at the same time, it is a source of chemicals that could counteract its health benefits. Fish is filled with omega-3 fatty acids and vitamins such as D and B2, it is rich in calcium and phosphorus and it is a great source of minerals, such as iron, zinc, iodine, magnesium, and potassium [7]. On the other hand, among the main seafood contaminants there are organochlorine pesticides, organotin compounds, phthalates, brominated flame retardants, polyfluorinated compounds, polycyclic aromatic hydrocarbons (PAHs), dioxins, dioxin-like PCBs and non-dioxin-like PCBs, heavy metals—e.g., mercury (Hg), cadmium (Cd), and lead (Pb)—, radionuclides and arsenic (As) [8].

Some authors tried to determine the balance between pros and cons of eating seafood, showing that, for major health outcomes among adults, the benefits of fish intake exceed the potential risks [9,10]. Unfortunately, these studies, as the many indexes that have been developed to measure the risk connected to eating contaminated seafood, almost never take into account neither the genetic and epigenetic background of the exposed population, nor the varying traditional consumption rate that characterized the different populations. On the other hand, as we will see, the need for the inclusion of these information in studies addressing health outcomes of eating seafood and in decision-making processes, is made apparent by several recent findings.

## 1.1 Past and present exposure to mercury and polycyclic aromatic hydrocarbons

Evidence exist that environmental Hg and PAHs could have been posing a risk to human health since ancient times.

Analysing the mercury record of a *Posidonia oceanica* mat in the northwest Mediterranean, Serrano and colleagues [11] confirmed the ability of *P. oceanica* meadows to accurately record a high number of historic contamination phases, probably because the bioaccumulating capacity of this species magnifies environmental changes in Hg concentrations. Moreover, the authors discovered several episodes of increasing anthropogenic inputs to the Mediterranean. A first small but significant increase occurred around 2500 years before present, coinciding with the beginning of intense mining in Spain. Then, four major periods of anthropogenic Hg pollution inputs took place: the first during the Roman Empire (2100–1800 years before present); the second in the Late Middle Ages (970–650 years before present); the third in the modern historical era (530–380 years before present); and the fourth in the industrial period (in the last 250 years), with Hg concentrations respectively two-, four-, five-, and tenfold higher than concentrations older than 2800 calibrated years before present. These dates are in agreement with records from other parts of the Iberian Peninsula and France, and they are temporally consistent with important changes in impacting human activities. In Roman times, cinnabar refining and the greatest mining activities took place in Iberia; many ancient mines in Central Europe were reopened around 1000 years before present; finally, the exponential increase in Hg concentration during the last 250 years is attributable to the impact of agricultural expansion, forest exploitation, high grazing pressure, burning of coal, intense mining activities in the Iberian pyrite belt in southwest Spain, metallurgical activities, gold and silver extraction from sediments through amalgamation in Europe, and the utilization of Hg-based pesticides.

Direct evidence of Hg exposure in ancient communities come from a study carried out on human bones recovered at the archaeological necropolis of A Lanzada, in the North western Spain [12]. The authors measured Hg and Pb concentrations and the ratio between Pb isotopes in bones dating to Roman and Early Medieval time. What emerged from this study is that Roman inhabitants of this settlement incorporated two times more Hg and Pb into their bones than post-Romans inhabiting the same site, independent of sex or age.

Anyway, in this case the authors argue that the most suitable explanation for the observed results is inhalation of atmospheric Pb and Hg, as suggested by the parallel increase of the two metals in bones, and by the correlation between Pb and Hg concentrations and Pb isotopic composition of both peat and bone. Pb isotopic composition is deemed to mirror the atmospheric contribution to Pb deposition, and, in our case, it shows a decreasing trend from the local Early Iron Age, to reach a

minimum by the 1st-2nd centuries CE, reflecting the addition of Pb due to increased mining and metallurgy. With the decline of the Roman Empire by the 5th century CE, the isotopic ratios return to values similar to those seen in the Early Iron Age, pointing to a decrease in atmospheric metal pollution.

Another interesting witness from the past comes from the study of ancient marine fauna remains from Norway [13]. In this study, the authors measured Cd, Pb and Hg concentrations in cod and harp seal bones dating to the Younger Stone Age of the Varanger Area, in the Arctic Norway. People living in the area at that time mostly relied on seafood, including whales and dolphins. The analysis revealed highly elevated levels of Cd and Pb, and elevated levels of Hg. On average, the levels of Cd and Pb contamination in cod were up to 22 and 3–4 times, respectively, higher than today's recommended limits in soft tissue. The corresponding figures for seal were 15 and 3–4 times, respectively. Instead, the levels of Hg were generally below today's recommended limit in soft tissue, but still of considerable magnitude. What the authors concluded from these results is that people relying on local marine resources at that time could had been suffering the harmful effects of these toxins. As regards the cause of the high levels of heavy metals measured in this study, the authors hypothesized that climate change might have been responsible. In particular, the mid-Holocene transgression, culminating at around 6.6 thousand calibrated years before present, is supposed to have caused the release of huge quantities of heavy metals by erosion of landmasses.

Despite direct evidence of ancient human exposure to PAHs are scant [14], natural sources of these compounds—e.g., forest fires and volcanic activity—have been acting for billions of years. Moreover, as we will see, the use of controlled fires in narrow places like caves may have driven the evolution of specific genetic adaptations to PAHs exposure in *H. sapiens* and *neanderthalensis* [15].

Nowadays, among the largest anthropogenic Hg emission sources in the world are human activities, such as the burning of coal, oil and wood, mining, artisanal and small-scale gold mining in developing countries, while the combustion of fossil fuels, oil and wood are among the main anthropogenic sources of PAHs.

## 1.2 An ecogenetic approach to the study of Mediterranean communities

Mediterranean fishing communities could represent an informative case study to gain insight into the potential impact of seafood consumption on the human genome, particularly in terms of seafood pollutants, like Hg and PAHs.

Mediterranean Sea is a semi-enclosed basin, surrounded by countries highly industrialized and with high agricultural development, and, as such, is of particular concern with respect to

contamination by toxic compounds. Moreover, the Mediterranean Sea is generally considered a geological hot spot for Hg, as it is characterised by large deposits of cinnabar (HgS) that account for about 65% of the global Hg reserves [16]. Finally, Mediterranean coastal populations are characterized by food habits based on local seafood consumption [17], and, as we have already seen, European countries bordering the Mediterranean are among the world's highest seafood consumers.

A potential way to address this matter would be to adopt what Basu and colleagues call an "ecogenetic approach" [18]. In practice, this approach could encompass the following main steps: selection of the communities that are suitable to be included in the study because of the traditionally high seafood consumption, and selection of communities that would represent the control group, for example inland communities, characterized by a very low fish intake; collection of genetic and epigenetic data and collection of information on diet, lifestyle and family history; analysis of the genetic variability and methylation profiles of those genes implicated in susceptibility to environmental chemicals. Such an approach would enable to answer different questions: are there any biological differences between fishing and non-fishing communities that could have been caused by the different seafood intake? Are there any differences in the biological predisposition of Mediterranean communities to the health effects of seafood intake?

## 2. Aim

The aim of the present thesis is fourfold, as it consists in (1) identifying Italian communities that may be more exposed to seafood contaminants, (2) validating a food frequency questionnaire to estimate the intake of mercury through seafood consumption, (3) creating a biobank for future studies on the correlation between seafood consumption and DNA methylation, (4) selecting genes, molecular pathways and biological mechanisms that could be impacted by seafood contaminants and that will be included in future studies on DNA methylation carried out on the above mentioned biobank.

In order to fulfil our objectives, the work has been divided into four main parts: (1) the development and meta-analysis of a georeferenced database on polycyclic aromatic hydrocarbons in Western and Central Mediterranean seafood aimed at identifying geographical patterns of contamination and trends that could be related to the biology of the marine organisms, as well as to the physico-chemical properties of each hydrocarbon and to the oceanographic characteristic of this part of the Mediterranean; (2) the development and validation of a food frequency questionnaire to estimate the intake of mercury through seafood consumption among a population living in a geographic area that is usually considered a contamination hotspot—the Trieste gulf; (3) creation of a biobank made of biological samples from members of several Italian communities together with information on their dietary habits, lifestyle and general health; (4) a review of the literature pertaining to the genetic component of individual susceptibility to methylmercury and polycyclic aromatic hydrocarbons exposure in humans, to the effects that these pollutants have on human DNA methylation, and to the evidence that Mediterranean coastal communities represent an informative case study to investigate the potential impact of methylmercury and polycyclic aromatic hydrocarbons on the human genome and epigenome.

## 3. Meta-Analysis of a New Georeferenced Database on Polycyclic Aromatic Hydrocarbons in Western and Central Mediterranean Seafood (chapter published as: De Giovanni, A.; Abondio, P.; Frapiccini, E.; Luiselli, D.; Marini, M. Meta-Analysis of a New Georeferenced Database on Polycyclic Aromatic Hydrocarbons in Western and Central Mediterranean Seafood. Appl. Sci. 2022, 12, 2776. https://doi.org/10.3390/app12062776)

Abstract

The aim of this work was to collect and harmonize the results of several studies achieved over the years, in order to obtain a database of georeferenced observations on polycyclic aromatic hydrocarbons (PAHs) in Western and Central Mediterranean seafood. For each observation, some information on the taxonomy and the ecology of the sampled species are reported, as well as details on the investigated hydrocarbon, and spatial and temporal information on sampling. Moreover, two health risk indexes were calculated for each record and included in the database. Through several statistical methods, we conducted a meta-analysis of the data on some of the species in this database, identifying trends that could be related to the biology of the investigated organisms, as well as to the physico-chemical properties of each hydrocarbon and to the oceanographic characteristic of this part of the Mediterranean. The analysis of the data showed that, at a consumption rate like the one typical of the Italian population, seafood caught from the area considered in the present work seems to pose a minimal risk to health. However, we also found evidence of an in-creasing trend of PAH concentrations in Mediterranean mussels, pointing to the need for constant monitoring.

### 3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of toxic [19,20] organic compounds, made up of a variable number of fused aromatic rings of carbon and hydrogen [21,22].

Depending on their origin, PAHs can be classified in petrogenic and pyrolytic (or pyrogenic). Pyrolytic PAHs result from the incomplete combustion of organic matter, such as combustion of wood, oil, vehicular and industrial emission and forest fires; petrogenic PAHs, instead, derive from fossil fuels, and can occur as a result of oil spills and petroleum production [23,24].

As regards chemical structure, low molecular weight (LMW) PAHs comprise 2-3 aromatic rings, while PAHs comprising four aromatic rings are defined as middle molecular weight (MMW) PAHs, and those made of five or more rings are referred to as high molecular weight (HMW) PAHs

[21]. In the marine environment, MMW and HMW PAHs are mostly pyrolytic in their origin, while LMW PAHs are from petrogenic sources [25,26].

From a chemical point of view, LMW and HMW PAHs behave differently, with MMW PAHs showing intermediate behavior. In particular, HMW PAHs are way less insoluble in water, and so they tend to bind organic particulate matter, being less bioavailable for their uptake from the water. Despite this, they can still be absorbed by aquatic organisms from sediments and particulate matter present in the water column [21,27].

For non-smokers, the major source of exposure to PAHs is diet, and seafood, along with cereals, is a major contributor to the dietary intake of these compounds in Europe [28]. In 2011, the EU set maximum allowable levels of benzo(a)pyrene (BaP) and of the sum of four HMW PAHs (PAH4; i.e., benzo(a)anthracene BaA, chrysene Chr, benzo(b)fluoranthene BbF, and BaP) in several fishery products (Table 1) [29].

**Table 1.** Maximum levels for PAHs in seafood, set by EU in 2011 [29]. Concentrations are expressed in mg/kg.

| Foodstuffs | Maximum levels for BaP (mg/kg) | Maximum levels for PAH4 [1] (mg/kg) |
|---|---|---|
| Muscle meat of smoked fish and smoked fishery products | 0.002 | 0.012 |
| Bivalve mollusks (fresh, chilled or frozen) | 0.005 | 0.030 |
| Bivalve mollusks (smoked) | 0.006 | 0.035 |

[1] *PAH4* sum of BaA, Chr, BbF and BaP.

Several factors could affect PAHs concentrations in seafood. As already mentioned, physico-chemical properties of PAHs influence their bioavailability, HMW PAHs being less available for uptake from the water compared to LMW and MMW PAHs. Moreover, in fish, HMW PAHs are readily excreted thanks to their fast metabolism, whose rate is higher compared to that of lighter PAHs [27,30]. PAHs levels in seafood also depends on nutritional condition of the organism [31], and on season [32], likely as an effect of changes in pollutants environmental inputs [30], seasonal variation of pollutants elimination rate [33], hydrodynamic processes [34], and/or because of factors pertaining to the reproductive cycle of the species [35]. Even the age of the fish is related to PAHs accumulation, with earlier stages of life more prone to accumulate higher amount of contaminants because of the immaturity of detoxification pathways [33]. Finally, the level of PAHs varies with the species taken into account, and this also because of differences in PAH-metabolizing capability [36]. Most notably,

filter feeding organisms show the slowest PAHs elimination rates [32]. Accordingly, metabolic efficiency is greater in fish, intermediate in crustaceans and lowest in mollusks [37].

Over the years, several studies have investigated the levels of PAHs in seafood caught in Adriatic [30,38–53], Ionian [27,38,52,54–57], and Tyrrhenian Sea [25,32,34,35,37,48,58–68], with the aims of monitoring the environmental status of particularly impacted areas [57,66], improving the knowledge of factors influencing PAHs levels in marine species [30,53], and, ultimately, informing decision-makers when it comes to fix limits to PAHs levels in fishery products [27].

The aim of this work was to collect and harmonize the results of those studies, in order to obtain a database of georeferenced observations on PAHs in seafood caught in the Western and Central Mediterranean Sea. For each observation, information on the taxonomy and the ecology of the investigated species are reported, as well as any details on the hydrocarbons, temporal and spatial information on sampling. Additionally, two health risk indexes were calculated for each record and included in the database. Finally, we conducted a meta-analysis of the data on some Mediterranean mussel, Manila clam, red mullet and common sole, identifying trends that could be related to the biology of the investigated organisms, as well as to the physico-chemical properties of each hydrocarbon and to the water circulation in this part of the Mediterranean.

## 3.2 Materials and methods

The literature search was carried out using Scopus and PubMed, aiming for the retrieval of publications reporting PAHs levels in seafood, i.e., in marine species included in D.M. 19105, 22 September 2017, Annex 1, which details commercially relevant fish species in Italy, or having "commercial", "minor commercial", "subsistence fisheries", or "of potential interest" in the "Human uses" section on FishBase (https://www.fishbase.se/search.php), or whose specimens were recovered from the fish market. Moreover, publications had to report PAH concentrations measured in marine species caught in the Adriatic (FAO geographical subareas 17 and 18), Ionian (FAO geographical subareas 13–16, 19–21) or Tyrrhenian Seas (FAO geographical subareas 8–10, 11.2, 12).

From each publication, we extrapolated several data, including the sample location and date, species and biological tissue investigated, sample length (cm) and weight (g), sample size, and PAH concentrations detected (mg/kg) (Table S1 of [69] for the full list and description of variables included in the database). Moreover, the database comprises a column specifying whether the record comes from wild, farmed or transplanted animals.

Each record was georeferenced, providing latitude and longitude in decimal degrees of the sampling location. Following [51], a geographic precision code was assigned to each record (Table

S2 of [69]), based on whether the study provided the exact coordinates or a more or less precise description of the sample location, from which geographical coordinates were inferred.

The trophic level of each species was obtained from online resources (Table S3 of [69]) and included in the database.

Abbreviations for the biological tissue where PAHs concentration was measured, as well as for each PAH and molecular weight class (i.e., LMW, MMW or HMW PAHs), are reported in the Supplementary Materials (Table S4–S6 of [69], respectively).

A column specifying if the reported PAH concentration is expressed in fresh (FW), wet (WW) or dry weight (DW) was added to the database (Table S7 of [69]). Moreover, whenever a study provided concentration in DW, we converted that measure in WW, reporting both the original and the inferred value in separate columns. In the analyses, FW measurements were considered WW. For statistical analyses, when the measured concentration was below the limit of quantification (LOQ) or limit of detection (LOD), and the authors specified those limits, we assigned to that record a value equal to half the LOQ or LOD.

For conversion from DW to WW, we used the following formula:

$$\text{C (mg/kg w.w.)} = ((100 - \%\text{ of water}) \div 100) \times \text{C (mg/kg d.w.)} \quad (1)$$

where C is the PAH concentration, and % of water is the percentage of water in the analyzed tissue. The percentage of water in Mediterranean mussel (*Mytilus galloprovincialis*) and Manila clam (*Ruditapes philippinarum*) was assumed to be equal to 85%, based on personal data not shown. The percentage of water in common sole (*Solea solea*) liver, gills and muscle was assumed to be equal to 72.75%, 70% and 74.4%, respectively, based on [51]. Finally, the percentage of water in red mullet (*Mullus barbatus*) fillet was assumed to be equal to 80%, following [70].

Two health risk indexes (Table S8 of [69]), excess lifetime cancer risk (ELCR) and target hazard quotient (THQ), were calculated for each record and included in the database. Specifically, ELCR was calculated according to the following equation:

$$\text{ELCR} = \text{EF (day/yr)} \times \text{ED (yr)} \times \text{IR (kg/day)} \times \text{CSF (mg kg}{-1}\text{ day)} \div \text{BW (kg)} \times \text{AT} \quad (2)$$

where EF is the exposure frequency (365 days/year); ED is the exposure duration, which was assumed to be equal to the Italian mean life expectancy (83,226 yr) (ISTAT, 2019); IR is the ingestion rate, which is equal to the PAH concentration times the mean ingestion rate of the species (FAOSTAT, 2018 - https://www.fao.org/faostat/en/#data/FBS); CSF is the cancer slope factor for

each of the analyzed PAH (OEHHA, 2009 - https://oehha.ca.gov/chemicals); BW is the body weight, which was assumed to be equal to the mean body weight of the Italian population (67 Kg) (ANSA, 2013); and AT is the averaging time, which is equal to EF×ED. An ELCR above $10^{-5}$, which is the acceptable lifetime risk (ALR) [71], indicates a probability greater than 1 chance over 100,000 of developing cancer [64].

THQ, which indicates the ratio between exposure and the reference dose, was calculated according to the following equation:

$$\text{THQ} = \text{EF (day/yr)} \times \text{ED (yr)} \times \text{IR (kg/day)} \times \text{C (mg/kg)} \div \text{RFD (mg kg}-1\text{ day)} \times \text{BW (kg)} \times \text{AT} \quad (3)$$

where C is the PAH concentration, and RFD the oral reference dose for PAH. When THQ risk is above 1, it means that THQ is higher than the reference dose, and systemic effects may occur [64].

Graphs and statistics were produced using RStudio version3.6.1. All the analyses were carried out after grouping records according to PAHs molecular weight, i.e., analyses were conducted separately on LMW, MMW and HMW PAHs in each selected species.

After checking the assumption of normality of residual distributions through the Shapiro–Wilk test (R function: shapiro_test), we used the Wilcoxon rank sum test (R function: wilcox.test) with data on Mediterranean mussel, Manila clam, red mullet and common sole to calculate the statistical significance of differences between the mean concentrations of LMW, MMW and HMW PAHs in those species.

To look for seasonal trends, we used the Wilcoxon rank sum test to calculate the statistical significance of differences between the mean concentrations of each class of PAHs in cold (October–March) and warm (April–September) months. Additionally, to control for potential confounding factors, we repeated the above analysis using non-parametric ANCOVA (R function: ancova.np), with a sampling depth and sampling year as covariates. In the present work, we used the same month clustering as in [72] and [30], which is based on sea water temperature: January–March (winter), April–June (spring), July–September (summer) and October–December (autumn).

Finally, to see if there is a relationship between the latitude, sampling depth and sampling year and the PAH concentrations in Mediterranean mussel caught along the Italian coast of the Adriatic and the Tyrrhenian Seas, we used Kendall's rank correlation (R function: pcor; method = "kendall").

## 3.3 Results

### 3.3.1 Database description

Of the 10,704 records included in the database, 5790 were extracted from a database on contaminants in Mediterranean biota available at https://www.emodnet-chemistry.eu/data (accessed on 01/12/2021), while the other 4914 are from 38 scientific publications in peer-reviewed journals. The database was compiled along the lines of the work of Cinnirella and colleagues (https://doi.org/10.1594/PANGAEA.899723) on mercury concentration in Mediterranean biota [70].

The geographical distribution of sampling sites is shown in Figure 1.

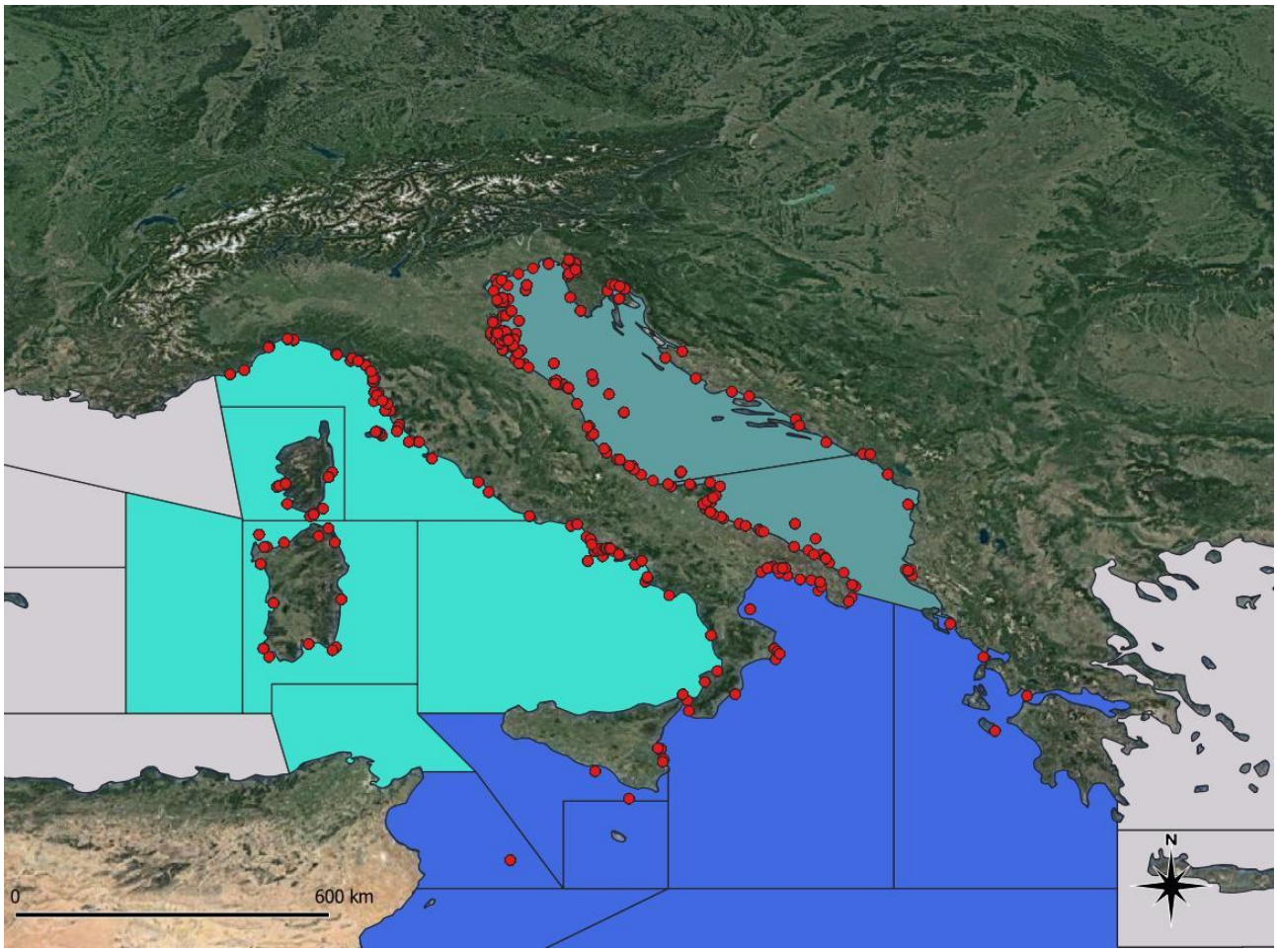



**Figure 1.** Geographical distribution of sampling sites (red dots). FAO geographical subareas are reported and colored based whether they belong to Adriatic (cadet blue), Ionian (royal blue) or Tyrrhenian Seas (turquoise).

In total, 1025 records are from the Ionian Sea, 4469 from the Adriatic Sea, and 5210 from the Tyrrhenian Sea (Figure 2).

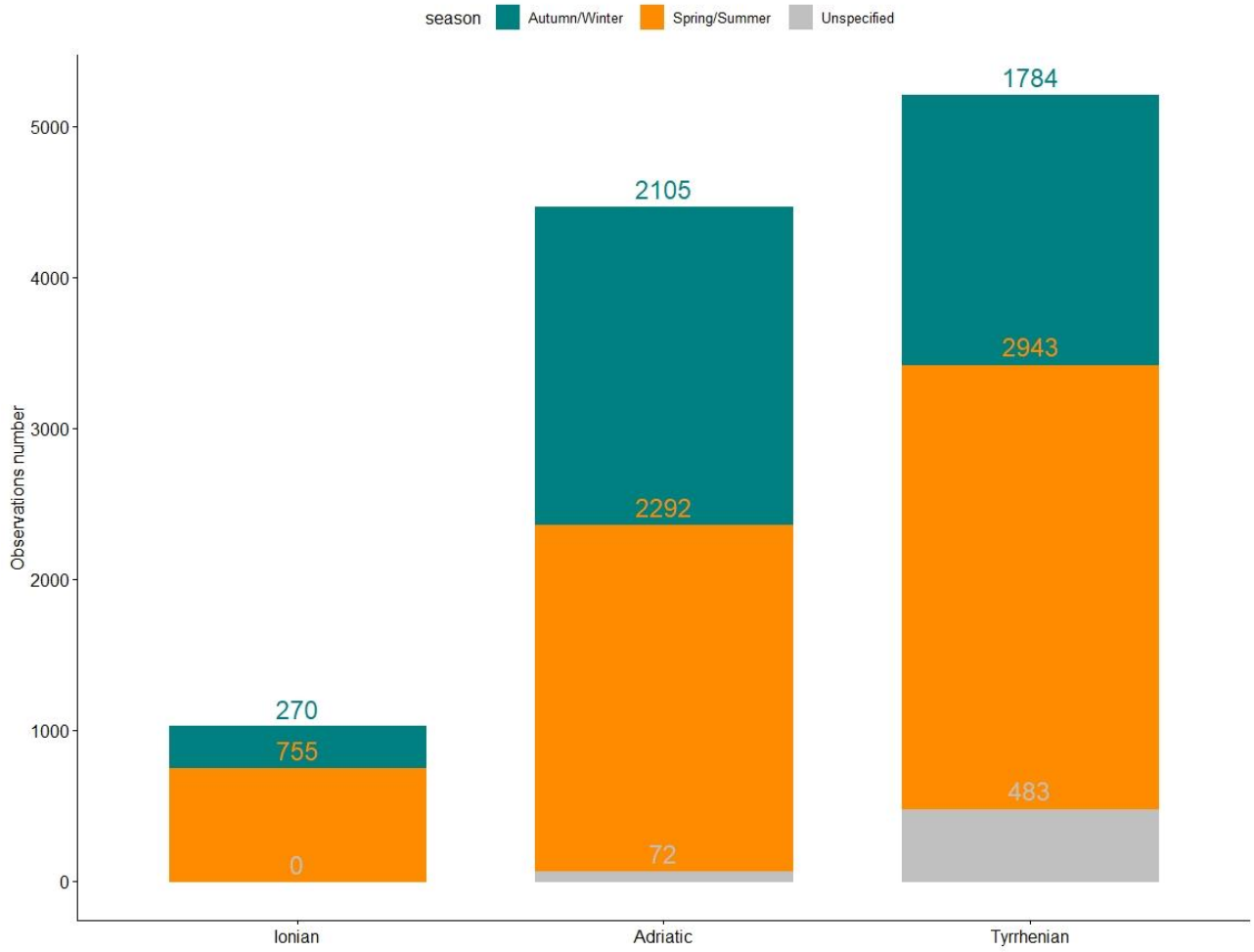



**Figure 2.** Bar plot showing the number of observations (x axis) by sea (i.e., Ionian, Adriatic, and Tyrrhenian Seas) in this database (y axis), with colors indicating the season in which sampling took place.

Mediterranean mussel (*Mytilus galloprovincialis*, code: Mytgal) is the most studied species, with 7710 records from 22 sources, followed by Manila clam (*Ruditapes philippinarum*, code: Rudphi), with 982 records from two sources, and common sole (*Solea solea*, code: Solsol), with 344 records from four sources. All the other species are present in this database with fewer than 250 observations (Figure 3).

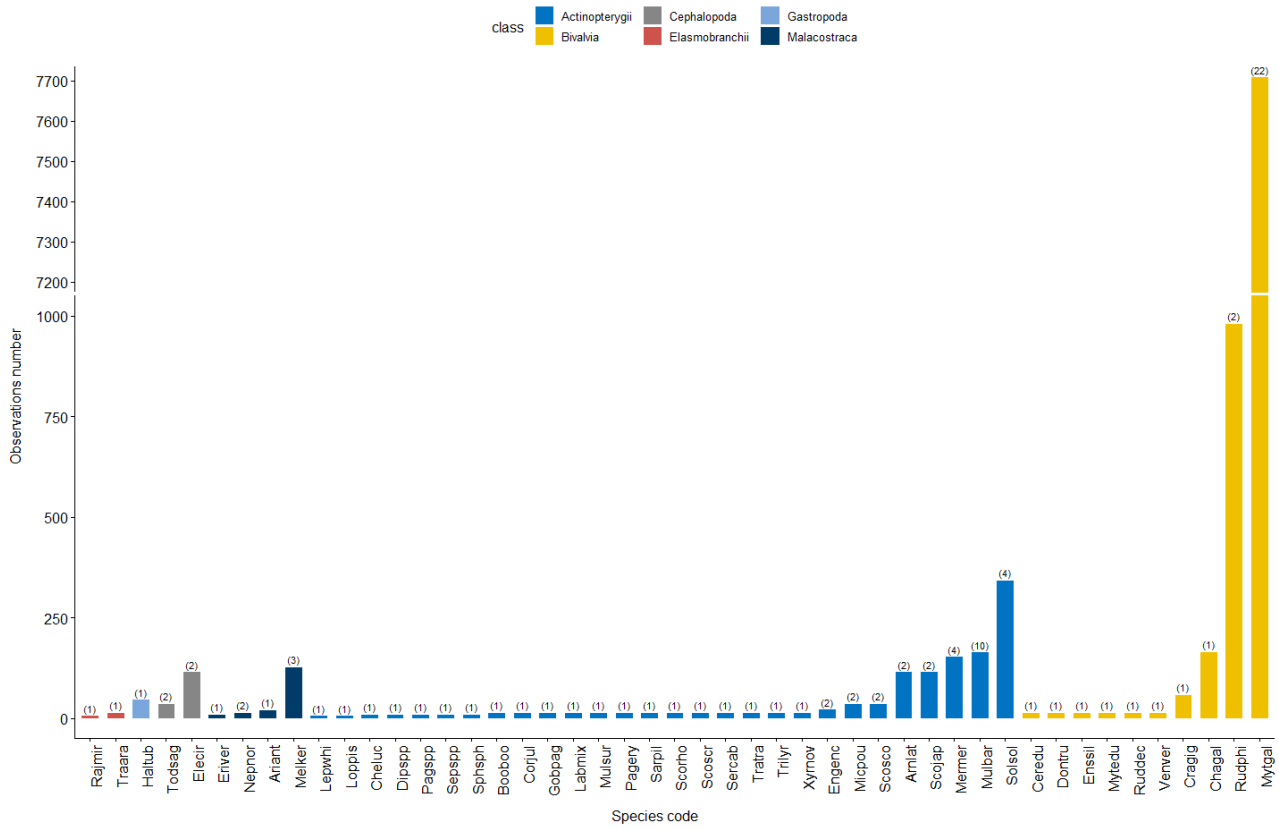



**Figure 3.** Bar plot showing the number of observations (x axis) for each marine species present in this database (y axis). The bars are arranged in ascending order of number of observations, by taxonomic class. On top of each bar, in brackets, is the number of sources from which the above observations were obtained. Each color corresponds to the taxonomic class of the species. Species codes are on the X-axis and are made of the first three letter of genus and species. The graph was realized using ggbreak library (version 0.0.7) in R [73].

Year of publication goes from 1990 to 2021, while sampling year goes from 1981 to 2019.

### 3.3.2 PAHs concentration by molecular weight and by season

We compared the concentrations of LMW, MMW and HMW PAHs in Mediterranean mussel, Manila clam, common sole and red mullet, being that these species are the ones on which more records are available in this database. Summary statistics for each class of PAHs in each species are reported in Table 2. Statistics and p-values for each test are reported in Supplementary Materials (Tables S9-S12 of [69]).

**Table 2.** Summary statistics on LMW (low molecular weight), MMW (middle molecular weight) and HMW (high molecular weight) PAHs in Mediterranean mussel, Manila clam, common sole and red mullet caught in the Adriatic, Ionian and Tyrrhenian Seas. Concentrations are in mg/kg wet weight.

| Species | PAHs class [1] | $C_{mean}$ [2] | $C_{min}$ [3] | $C_{max}$ [4] |
|---|---|---|---|---|
| *Mytilus galloprovincialis* | LMW | 0.00942 | 0.00000 | 3.96000 |
| | MMW | 0.01068 | 0.00000 | 1.05960 |
| | HMW | 0.00570 | 0.00000 | 0.34500 |
| *Ruditapes philippinarum* | LMW | 0.00137 | 0.00000 | 0.03770 |
| | MMW | 0.00197 | 0.00041 | 0.00810 |
| | HMW | 0.00058 | 0.00000 | 0.00690 |
| *Solea solea* | LMW | 0.00079 | 0.00006 | 0.01000 |
| | MMW | 0.00189 | 0.00009 | 0.01037 |
| | HMW | 0.00914 | 0.00000 | 0.73500 |
| *Mullus barbatus* | LMW | 0.01030 | 0.00011 | 0.09385 |
| | MMW | 0.00299 | 0.00042 | 0.01050 |
| | HMW | 0.00090 | 0.00000 | 0.00539 |

[1] *PAHs class*—class of PAHs based on molecular weight, [2] $C_{mean}$—mean concentration, [3] $C_{min}$—minimum concentration, [4] $C_{max}$—maximum concentration.

In all four species, MMW PAHs show the highest mean concentration, followed by LMW PAHs and, finally, HMW PAHs, which show the lowest mean concentration (Figure 4). In all cases, the difference between the mean concentration of each PAHs class was statistically significant ($p < 0.05$), except for the difference between MMW and LMW PAHs in red mullet.

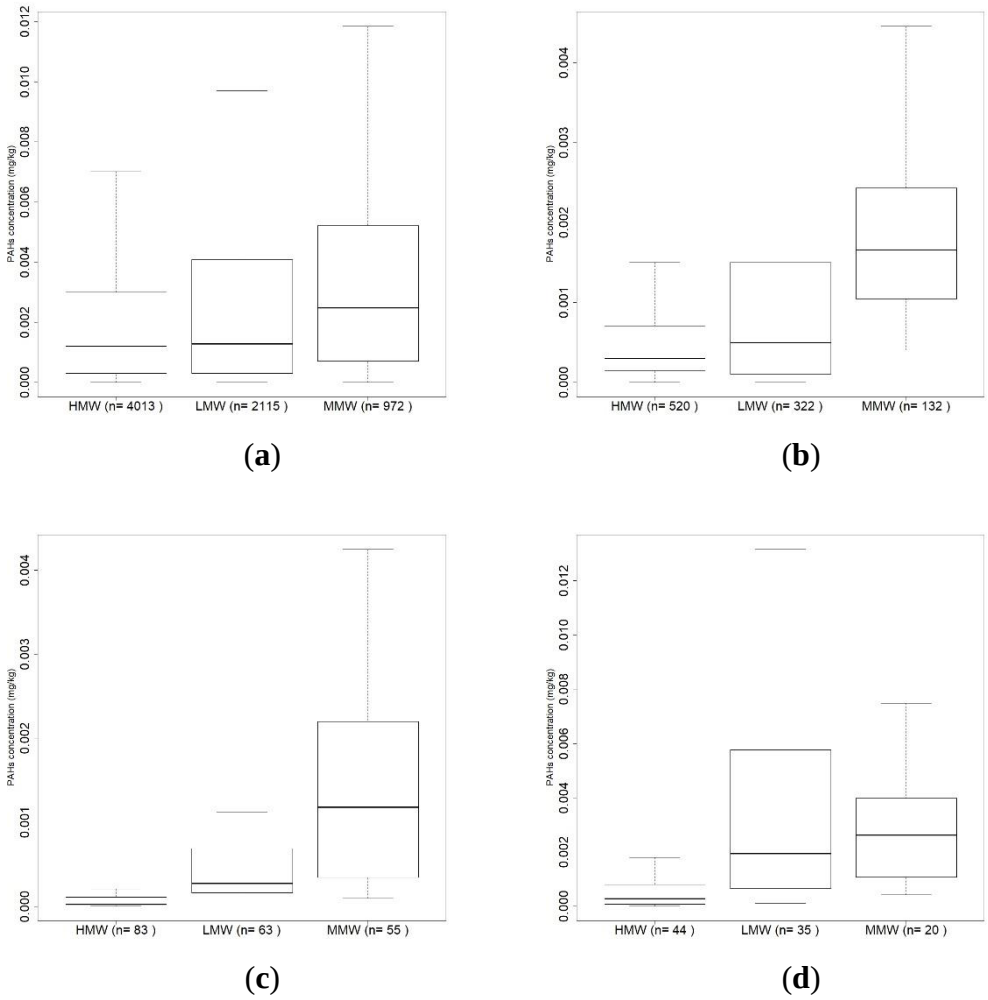


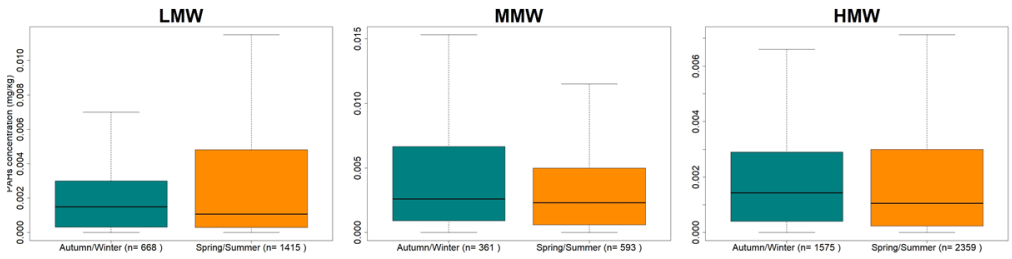

**Figure 4.** PAHs concentration (mg/kg wet weight) by molecular weight in (**a**) Mediterranean mussel, (**b**) Manila clam, (**c**) common sole, and (**d**) red mullet. Sample sizes are in brackets; outliers not shown.

For each class of PAHs, we compared mean concentrations measured in cold and warm months in Mediterranean mussel, Manila clam, and red mullet, controlling for the effect of sampling depth and sampling year (Figure 5). We excluded common sole from the analysis because all samples for which sampling date was specified in the original sources were collected in autumn (October–December). Summary statistics on each variable included in the present analysis are reported in Table 3.

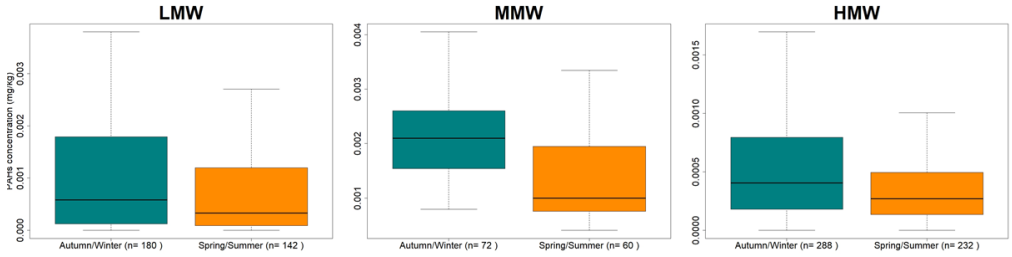
**Table 3.** Summary statistics on LMW (low molecular weight), MMW (middle molecular weight) and HMW (high molecular weight) PAHs in Mediterranean mussel, Manila clam and red mullet caught in different seasons in Adriatic, Ionian and Tyrrhenian Seas. Concentrations are in mg/kg wet weight; sampling depth is in meters.

| Species | Period | PAHs class [1] | $C_{mean}$ [2] | $C_{min}$ [3] | $C_{max}$ [4] | $Depth_{min}$ [5] | $Dept_{max}$ [6] | $Year_{min}$ [7] | $Year_{max}$ [8] |
|---|---|---|---|---|---|---|---|---|---|
| *Mytilus galloprovincialis* | Autumn/Winter | LMW | 0.00434 | 0.00000 | 0.18090 | 0.00 | 77.60 | 1995 | 2018 |
| | | MMW | 0.01292 | 0.00000 | 0.96000 | 0.00 | 118.80 | 1995 | 2018 |
| | | HMW | 0.00512 | 0.00000 | 0.34500 | 0.00 | 118.80 | 1995 | 2018 |
| | Spring/Summer | LMW | 0.01155 | 0.00000 | 3.96000 | 0.00 | 108.00 | 1981 | 2017 |
| | | MMW | 0.00908 | 0.00000 | 1.05960 | 0.00 | 108.00 | 1981 | 2017 |
| | | HMW | 0.00601 | 0.00000 | 0.18040 | 0.00 | 108.00 | 1981 | 2017 |
| *Ruditapes philippinarum* | Autumn/Winter | LMW | 0.00177 | 0.00000 | 0.03770 | - | - | 2005 | 2013 |
| | | MMW | 0.00228 | 0.00080 | 0.00770 | - | - | 2005 | 2013 |
| | | HMW | 0.00066 | 0.00000 | 0.00690 | - | - | 2005 | 2013 |
| | Spring/Summer | LMW | 0.00087 | 0.00000 | 0.00890 | - | - | 2001 | 2014 |
| | | MMW | 0.00160 | 0.00041 | 0.00810 | - | - | 2001 | 2014 |
| | | HMW | 0.00047 | 0.00000 | 0.00600 | - | - | 2001 | 2014 |
| *Mullus barbatus* | Autumn/Winter | LMW | 0.00649 | 0.00016 | 0.09265 | 70 | 70 | 2004 | 2019 |
| | | MMW | 0.00315 | 0.00042 | 0.01050 | 70 | 70 | 2004 | 2019 |
| | | HMW | 0.00088 | 0.00000 | 0.00539 | 70 | 70 | 2004 | 2019 |
| | Spring/Summer | LMW | 0.01477 | 0.00011 | 0.09385 | 70 | 70 | 2004 | 2019 |
| | | MMW | 0.00334 | 0.00060 | 0.00749 | 70 | 70 | 2004 | 2019 |
| | | HMW | 0.00118 | 0.00000 | 0.00536 | 70 | 70 | 2004 | 2019 |

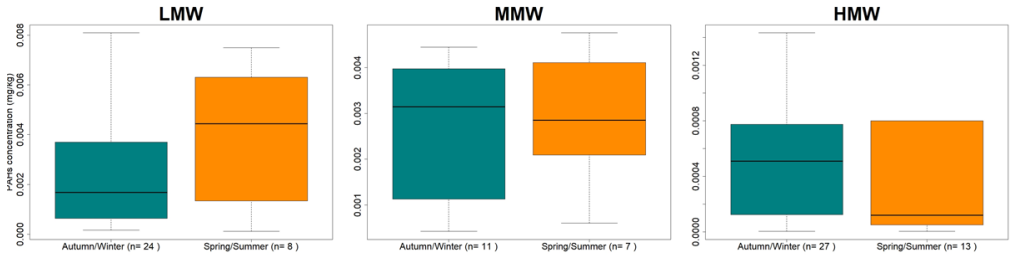
[1] *PAHs class*—class of PAHs based on molecular weight, [2] $C_{mean}$—mean concentration, [3] $C_{min}$—minimum concentration, [4] $C_{max}$—maximum concentration, [5] $Depth_{min}$—minimum sampling depth, [6] $Depth_{max}$—maximum sampling depth, [7] $Year_{min}$—year of the first sampling campaign, [8] $Year_{max}$—year of the last sampling campaign.




**(a)**




**(b)**




**(c)**

**Figure 5.** Comparison of PAHs concentrations (mg/kg wet weight) in cold and warm months in (**a**) Mediterranean mussel, (**b**) Manila clam, and (**c**) red mullet; sample sizes are in brackets; outliers not shown.

In Mediterranean mussel, the Wilcoxon rank sum test reveals that both MMW- and HMW PAHs are present at significantly ($p < 0.05$) higher concentrations in cold months (Table S13 of [69]). Moreover, ANCOVA shows that the same trend is statistically significant, even after controlling for the sampling depth and sampling year (Table S14 of [69]).

In Manila clam, the Wilcoxon rank sum test reveals that all PAHs classes (i.e., LMW-, MMW- and HMW PAHs) are present at significantly higher concentrations in cold months (Table S15 of [69]). However, as shown by ANCOVA, the above trend remains statistically significant after controlling for sampling year only in LMW- and MMW PAHs (Table S16 of [69]). Data on sampling depth for Manila clam were not sufficient to use this variable as covariate in ANCOVA.

Finally, in red mullet, both the Wilcoxon rank sum test and ANCOVA show that for any of the PAH classes, concentrations in warm months are not significantly different from those in cold months (Tables S17 and S18 of [69]). Additionally, in the case of red mullet, ANCOVA was carried out with only sampling year as a covariate, as data on sampling depth were not sufficient.

3.3.3 Latitude, depth and sampling year effect in Mediterranean mussel

We tested the presence of a relationship between the latitude, sampling depth and sampling year and the PAH concentrations in Mediterranean mussel caught along the Italian coast of the Adriatic and the Tyrrhenian Seas. Kendall's rank correlation coefficients (τb) and p-values are reported in the Supplementary Materials (Tables S19–S30 of [69]), along with maps showing the geographical distributions of sampling sites from where data used in each analysis derive (Figures S1–S4 of [69]).

3.3.3.1 Adriatic

Summary statistics on each variable included in the correlation analysis on data from the Adriatic Sea are reported in Table 4.

**Table 4.** Summary statistics on LMW (low molecular weight), MMW (middle molecular weight) and HMW (high molecular weight) PAHs in Mediterranean mussels caught in different seasons along the Italian coast of the Adriatic Sea. Concentrations are in mg/kg wet weight; sampling depth is in meters; latitude are in decimal degrees. Reported statistics were calculated after missing-data removal from concentration, sampling depth, sampling year and latitude columns.

| Period | PAHs class [1] | $C_{mean}$ [2] | $C_{min}$ [3] | $C_{max}$ [4] | $Depth_{min}$ [5] | $Depth_{max}$ [6] | $Year_{min}$ [7] | $Year_{max}$ [8] | $Lat_{min}$ [9] | $Lat_{max}$ [10] |
|---|---|---|---|---|---|---|---|---|---|---|
| Autumn/Winter | LMW | 0.00463 | 0.00002 | 0.18090 | 1.20 | 24.80 | 2006 | 2009 | 41.60 | 45.76 |
| | MMW | 0.00591 | 0.00015 | 0.11010 | 1.20 | 24.80 | 2005 | 2009 | 41.60 | 45.76 |
| | HMW | 0.00382 | 0.00002 | 0.34500 | 1.20 | 24.80 | 2005 | 2009 | 41.60 | 45.76 |
| Spring/Summer | LMW | 0.01093 | 0.00000 | 1.15500 | 0.01 | 35.00 | 2006 | 2011 | 40.20 | 45.77 |
| | MMW | 0.00396 | 0.00003 | 0.03780 | 0.01 | 30.60 | 2005 | 2017 | 40.20 | 45.77 |
| | HMW | 0.00209 | 0.00000 | 0.03150 | 0.01 | 30.60 | 2005 | 2017 | 40.20 | 45.77 |

[1] *PAHs class*—class of PAHs based on molecular weight, [2] $C_{mean}$—mean concentration, [3] $C_{min}$—minimum concentration, [4] $C_{max}$—maximum concentration, [5] $Depth_{min}$—minimum sampling depth, [6] $Depth_{max}$—maximum sampling depth, [7] $Year_{min}$—year of the first sampling campaign, [8] $Year_{max}$—year of the last sampling campaign, [9] $Lat_{min}$—minimum latitude of sampling sites, [10] $Lat_{max}$—maximum of sampling sites.

As Kendall's rank correlation revealed, after removing the effect of the sampling depth and sampling year, concentrations of LMW-, MMW- and HMW PAHs in Mediterranean mussel caught along the Italian coast of the Adriatic Sea turned out to be negatively correlated with latitude in warm

months, while the correlation becomes positive in cold months. Results are all statistically significant ($p < 0.05$), except for MMW PAHs in both periods of the year.

In Mediterranean mussel caught along the Italian coast of the Adriatic Sea, after removing the effect of the latitude and sampling year, concentrations of LMW-, MMW- and HMW PAHs are always negatively correlated with the sampling depth. Results are all statistically significant ($p < 0.05$), except in the case of MMW PAHs in warm months.

Finally, concentrations of all three classes of PAHs in warm months increase over the years, while concentrations of all three classes of PAHs in cold months decrease over the years, after removing the effect of latitude and sampling depth. Results are all statistically significant ($p < 0.05$), except for those on MMW PAHs in cold months.

3.3.3.2 Tyrrhenian

Summary statistics on each variable included in the correlation analysis on data from the Tyrrhenian Sea are reported in Table 5.

**Table 5.** Summary statistics on LMW (low molecular weight), MMW (middle molecular weight) and HMW (high molecular weight) PAHs in Mediterranean mussels caught in different seasons in the Tyrrhenian Sea. Concentrations are in mg/kg wet weight; sampling depth is in meters; latitude are in decimal degrees. Reported statistics were calculated after missing data removal from concentration, sampling depth, sampling year and latitude columns.

| Period | PAHs class [1] | $C_{mean}$ [2] | $C_{min}$ [3] | $C_{max}$ [4] | $Depth_{min}$ [5] | $Depth_{max}$ [6] | $Year_{min}$ [7] | $Year_{max}$ [8] | $Lat_{min}$ [9] | $Lat_{max}$ [10] |
|---|---|---|---|---|---|---|---|---|---|---|
| Autumn/Winter | LMW | 0.00434 | 0.00000 | 0.12000 | 0.10 | 49.02 | 1999 | 2017 | 38.54 | 43.50 |
| | MMW | 0.02649 | 0.00000 | 0.96000 | 0.10 | 49.02 | 1999 | 2017 | 38.54 | 43.50 |
| | HMW | 0.00953 | 0.00000 | 0.20237 | 0.10 | 49.02 | 1999 | 2017 | 38.54 | 43.50 |
| Spring/Summer | LMW | 0.00685 | 0.00000 | 0.20000 | 0.19 | 108.00 | 1981 | 2017 | 38.54 | 44.42 |
| | MMW | 0.01234 | 0.00000 | 1.05960 | 0.19 | 108.00 | 1981 | 2017 | 38.97 | 44.42 |
| | HMW | 0.00766 | 0.00000 | 0.18040 | 0.19 | 108.00 | 1981 | 2017 | 38.54 | 44.42 |

[1] *PAHs class*—class of PAHs based on molecular weight, [2] $C_{mean}$—mean concentration, [3] $C_{min}$—minimum concentration, [4] $C_{max}$—maximum concentration, [5] $Depth_{min}$—minimum sampling depth, [6] $Depth_{max}$—maximum sampling depth, [7] $Year_{min}$—year of the first sampling campaign, [8] $Year_{max}$—year of the last sampling campaign, [9] $Lat_{min}$—minimum latitude of sampling sites, [10] $Lat_{max}$—maximum of sampling sites.

As Kendall's rank correlation revealed, after removing the effect of the sampling depth and sampling year, concentrations of LMW-, MMW- and HMW PAHs in Mediterranean mussel caught

in the Tyrrhenian Sea increase with latitude in both cold and warm periods, and this increase is statistically significant ($p < 0.05$) for every PAH class and period of the year, excluding MMW PAHs.

In Mediterranean mussel caught in the Tyrrhenian Sea, after removing the effect of the latitude and sampling year, concentrations of LMW in warm months and of MMW in both periods of the year are negatively correlated with the sampling depth. These results are always statistically significant ($p < 0.05$), apart from those on MMW in cold months. On the contrary, in all other cases (i.e., LMW PAHs in cold months, and HMW PAHs in both periods of the year), the PAH concentrations and sampling depth turned out to be positively correlated, always reaching statistical significance, except for HMW PAHs in warm months.

Finally, concentrations of LMW-, MMW- and HMW PAHs in Mediterranean mus-sels caught in the Tyrrhenian Sea, in both cold and warm months, increase over the years, and this trend is statistically significant ($p < 0.05$) for LMW- and MMW PAHs in warm months, and for HMW PAHs in both periods of the year.

### 3.3.4 Human health risks assessment

In total, 92 out of 651 (~14%) records of BaP in bivalves exceed the limit of 0.005 mg/kg set by the EU [29] (Table 1), with most of these records (57) being on Mediterranean mussels caught in the Tyrrhenian Sea. Conversely, none of the 26 records of PAH4 in bivalves exceed the limit of 0.03 mg/kg set by the EU [29] (Table 1).

In this database, assuming a consumption rate equal to the average per capita consumption in Italy (FAOSTAT, 2018), ELCR values range from a minimum of $1.36\times10^{-10}$ to a maximum of $4.52\times10^{-4}$, which is reached by a record of dibenzo(a,i)pyrene (DaiP) in Mediterranean mussel. A total of 324 out of 5275 records (~6%) exceeds the threshold value of $10^{-5}$ for ELCR. Records exceeding ELCR threshold value are mostly on Mediterranean mussel (225), while dibenzo(a,h)anthracene (DahA) is the compound most frequently associated with such high ELCR values.

THQ values range from a minimum of $2.2\times10^{-9}$ to a maximum of ~0.13, which is reached by a record of BaP in Mediterranean mussel. Therefore, none of the records in this database exceeds the threshold value of 1 for THQ. THQ distributions for some of the most consumed species in Italy [74] are reported in Figure 6. As can be seen, among those included in the graph, European anchovy (*Engraulis encrasicolus*), blue mussel (*Mytilus edulis*) and European hake (*Merluccius merluccius*) are the first three species showing the highest median THQ.

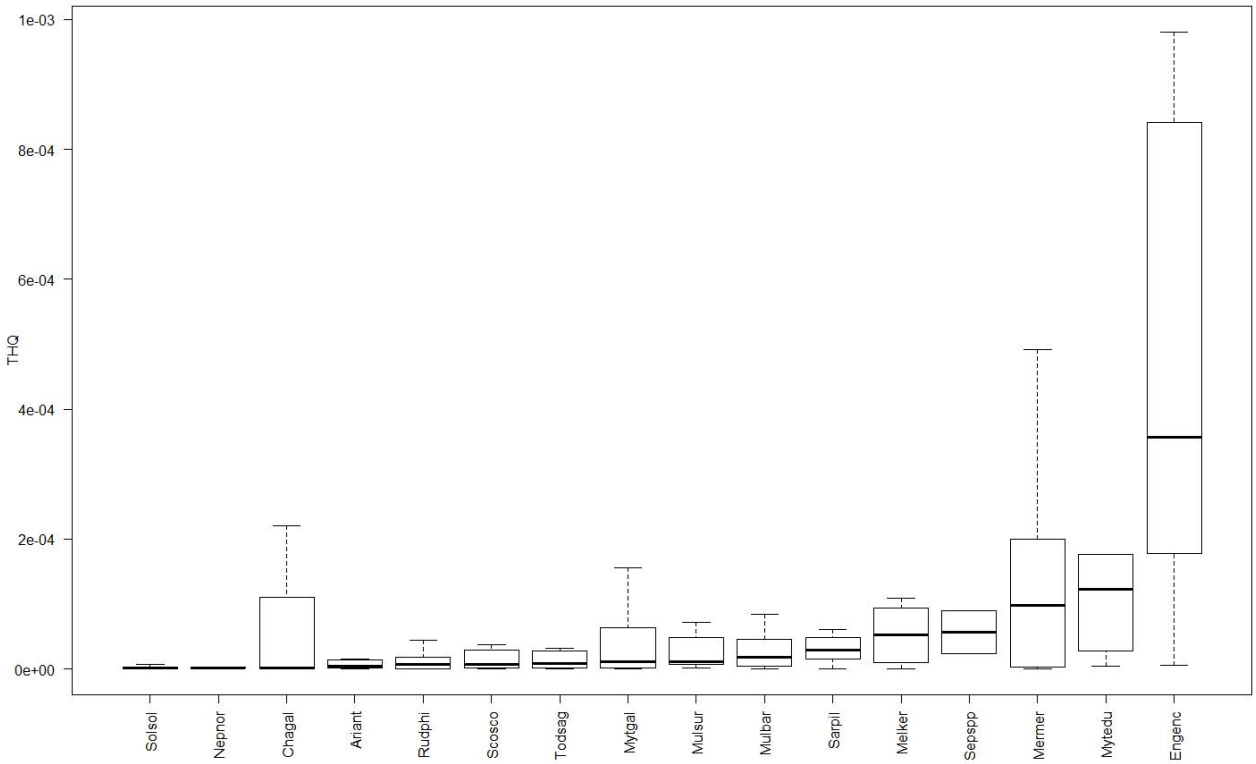



**Figure 6.** Boxplots showing the THQs distribution in several marine species included in this database. Species codes are on the X-axis and are made of the first three letter of genus and species. Boxplots are in ascending order from left to right, based on median values; outliers not shown.

## 3.4 Discussion

The development of this database on PAHs in seafood caught in the Western and Central Mediterranean Sea showed that most of the studies carried out from 1990 to 2021 focused on the Tyrrhenian and Adriatic Seas, with fewer records coming from the Ionian Sea. Moreover, Mediterranean mussel was by far the most studied species, accounting for more than half of records in the database. This is not surprising, given that, along with red mullet, Mediterranean mussel is considered one of the most suitable organisms to be used in biomonitoring studies, because of the widespread distribution, the ability to accumulate contaminants to a degree proportional to their bioavailability, as well as the ease of sampling [59,75].

In this database, MMW PAHs and LMW PAHs showed higher concentrations than HMW PAHs in Mediterranean mussel, Manila clam, common sole and red mullet. This is in line with the expectations, given the greater solubility and bioavailability of lighter PAHs and the faster metabolism of the heavier ones [30].

In a recent investigation carried out using large-scale monitoring data on PAHs in sediments of the Mediterranean Sea [76], the authors found that the two most prevalent PAHs in the Western Mediterranean basin were fluoranthene (Flu) and phenanthrene (Phe), with the former being the most abundant also in the Adriatic Sea and in the Central Mediterranean basin. In view of this, it is interesting to note that, in our meta-analysis, Flu and Phe are also the most abundant PAHs in both Mediterranean mussel and Manila clam (Figures S5 and S6 of [69]), which are benthic filter-feeding bivalves, and so may be particularly prone to absorbing PAHs accumulated in bottom sediments, after their remobilization.

The analysis on seasonality shows that PAH concentrations tend to be higher in specimens sampled in cold months (October–March), and this trend is statistically significant in both Mediterranean mussel and Manila clam, confirming what several studies, including those in this database, found independently [26,34,37,47,50,59].

As suggested by previous investigators, the reason for such a seasonal pattern may lie both in the biology of these species and in changes in the emission and mobilization of PAHs in the environment. PAHs are lipophilic compounds [67], and as such, they accumulate preferentially in lipid-rich tissues [68]. Given that the lipid content of tissues of marine species can vary under the effect of, for example, nutritional [31] and reproductive status [53,77], one might speculate that seasonal fluctuations in such parameters may be reflected in PAH concentration changes. However, this should not be the case with Mediterranean mussel, since several investigations carried out in the Mediterranean Sea found higher lipid content in mussel sampled during summer [78,79], and this is likely due to the depletion of lipids that takes place after spawning [77,79]. Another possibility is that higher PAHs concentrations in winter are the result of increased PAH emission through, for example, domestic heat [30,35,80,81] and industrial activities [82]. Moreover, in summer, the degradation of PAHs, especially of the lighter ones (i.e., LMW PAHs) [82,83], is enhanced by UV radiation, high temperatures, and ozone [84]. On the other hand, low seawater temperature in winter can inhibit the microbial degradation of PAHs [85,86]. Additionally, the resuspension of particulate matter that takes place during winter via sea storms [37] and water mixing [30] could foster PAHs availability and accumulation in filter feeding organisms, such as mussels, and this is particularly true for heavier and more recalcitrant PAHs [30,87].

In red mullet, lighter (i.e., LMW) and heavier (i.e., MMW and HMW) PAHs show opposite trends, with the former being present at greater concentrations during warm months, and the latter during cold months. Frapiccini and colleagues [53] suggested that higher LMW PAHs in summer months might be related to an increase in maritime traffic during this period of the year, while the increase in heavier PAHs concentrations observed in winter might be caused by a reduced expression

in detoxification enzymes in red mullet [88], along with the environmental factors that are hypothesized to determine the same pattern in Mediterranean mussel. Nevertheless, in our meta-analysis, the difference between PAHs concentrations in warm and cold months was not statistically significant. Moreover, it is important to note that, analyzing the liver of red mullets caught in the Northern Adriatic Sea, Guerranti and colleagues [48] found higher levels of PAHs in autumn than in spring, but they found the opposite trend in the liver of red mullets caught in the Southern Adriatic and Tyrrhenian Seas.

In this database, concentrations of PAHs in Mediterranean mussel caught along the Italian coast of the Adriatic Sea are negatively correlated with latitude in warm months, (average $\tau b \cong -0.2$). On the contrary, concentrations of PAHs and latitude show a positive correlation (average $\tau b \cong 0.2$) in cold months. We tentatively attribute the above results to the water masses circulation that characterizes the Adriatic Sea, and/or to the seasonal changes in the river discharge from the Italian coast. The major riverine freshwater input in the Adriatic Sea [89], the Po River, flows into the northern part of the basin, with greater discharge in spring and autumn, and lower discharge in summer [72]. In winter, the Po plume flows mostly southward [72] along the Italian coast, forming the Western Adriatic Current, a buoyant fresher water layer more than 50 m deep [90]. Moreover, in this period of the year, the cold Bora wind that affects the region causes the cooling of the sea surface layer, resulting in the complete vertical mixing of the sea water. This in turn leads to the resuspension of the bottom sediments and, therefore, to the remobilization of contaminants accumulated therein [72]. On the other hand, during spring and summer, the strong thermal stratification of the water results in the offshore propagation of the Po plume, which reaches the center of the basin [72].

Considering all the above, we hypothesize that the positive correlation between the PAH concentration and latitude observed in cold months may be due to, or at least favored by, the increased input of pollutants from the Po River, coupled with the resuspension of contaminants determined by the cooling action of the Bora wind in the northern Adriatic Sea. This latitudinal trend may be disrupted in warm months because of a greater contribution of pollutants from local rivers in the middle Adriatic, given the weaker influence of the Po plume on the regions south of the Po delta.

Our results seem to agree with a previous study analyzing nutrients transport along the Western Adriatic coast [90]. Indeed, what that study found is that, in winter, nutrient concentrations in water decreased southward from the Po River, while in spring, concentrations off Pescara were higher than concentrations in the Po area.

Although above explanation might sound plausible, our results should be interpreted with caution, considering the high data heterogeneity and the different geographical distribution of observations during warm and cold months, the latter being more discontinuous (Figure S2 of [69]).

The negative correlation (average $\tau b \cong -0.2$) observed between the sampling depth and PAH concentrations in Mediterranean mussel caught along the Italian coast of the Adriatic Sea is in accordance with several past investigations. A study carried out in the Western Mediterranean Sea revealed a higher PAH content in suspended particulate matter collected near the sea surface compared to that sampled from deep water [91], while another study found similar results in water samples collected in the Baltic Sea [85]. Moreover, in [25], concentrations of PAHs in Mediterranean mussels collected in the Gulf of Naples were negatively correlated with sampling depth. We hypothesize that the observed bathymetric gradient may be due, at least in the Adriatic Sea, to a greater impact of river discharge at a low depth.

Concerning the discrepancy between results of the correlation analyses on the PAH concentration and sampling year, with concentrations increasing over the years in warm months and decreasing over the years in cold months, we were not able to find a likely explanation based on information retrieved from the literature. Overall, we cannot exclude that our results are an artefact stemming from the high degree of heterogeneity of the data collected in the database. Moreover, it is notable that data on PAH concentrations in Mediterranean mussel caught along the Western Adriatic coast in cold months are from the periods 2006–2009 and 2005–2009, for LMW PAHs and for MMW- and HMW PAHs, respectively, while data on mussels caught in warm months cover a longer period of time (2006–2011 and 2005–2017 for LMW PAHs and for MMW- and HMW PAHs, respectively) (Table 4); therefore, we cannot rule out the possibility of an actual declining trend in PAH concentrations in the period covered by data on cold months, whose signal is lost in the broader period that the data on warm months span. In this regard, it is interesting to note that an oscillating temporal trend of PAHs concentrations was observed in Adriatic sediments by Rizzi and colleagues [76], with several PAHs showing a decrease in concentrations until the years between 2005 and 2010, when a new increase took place (Figure S2 of [76]).

Turning to mussels from the Tyrrhenian Sea, we observed a latitudinal trend, consistent across the three PAHs classes, in both periods of the year, that is, an increase in PAH concentrations from the southern to the northern part of the basin (average $\tau b \cong 0.1$). Interpreting the above results is challenging, given the low number of sampling sites, especially in cold months, and their uneven geographical distribution along the Italian coast. Therefore, we limit ourselves to observe that, in the Tyrrhenian Sea, the salinity of the shallower water mass (0–150 m) follows the same latitudinal trend that we observed for the PAH concentration in mussels, passing from 36.2 psu in the southern region to 38.4 in the northern [92]; salinity superior or equal to 37 psu has been shown to slow down the degradation of PAH molecules [83]. Moreover, water circulation in the Tyrrhenian Sea is dominated by a wide cyclonic path that enters the basin through the Sardinia Channel and flows along the Sicilian

and Italian coasts [92]. Thereby, it could be that mussels at more northerly latitudes are affected not only by local input of contaminants, but also by the substances that currents catch along their path. Finally, it is worth noting that, as can be seen from the marine traffic density map [93] of the European Atlas of the Seas [94], the Tyrrhenian coast of Calabria, in the southern part of the basin, is the least affected by vessel traffic.

The inconsistency between the results of the correlation analysis on PAH concentrations and sampling depth in the Tyrrhenian Sea is not easy to interpret, and again, it may represent a simple artefact of data heterogeneity. The statistically significant negative correlation between sampling depth and LMW- and MMW PAHs concentrations in warm months is in line with the results on LMW- and HMW PAHs in the Adriatic Sea and may be attributable to a greater impact of river discharge at low depth. On the contrary, we found that LMW- and HMW PAHs measured in the Tyrrhenian Sea in cold months significantly increase with sampling depth. Although we cannot find a convincing explanation to such a pattern, it is noteworthy that the Tyrrhenian Sea is rich in volcanic submarine structures [95,96], which can be important contributors of pyrolytic PAHs in the environment [97,98]. As such, we hypothesize that Tyrrhenian mussels that inhabit greater depths may be more susceptible to contamination driven by submarine volcanism, being at the same time less affected by river discharge.

The increasing trend of PAH concentrations in Mediterranean mussels from Tyrrhenian Sea is in line with recent findings suggesting that, in recent years, concentrations of these compounds in Mediterranean Sea sediments have increased, especially in the western part of the basin, probably as an effect of a parallel rise in PAH emissions from forest fires [76].

Both human and animal studies point to PAH exposure as being detrimental for the health, due to, for example, their carcinogenicity, teratogenicity and endocrine-disrupting effects [20]. Accordingly, the EU set a maximum level of several PAHs in fresh and smoked seafood to be sold [29] (Table 1). None of the records included in this database exceed those limits. Moreover, based on FAOSTAT data on per capita seafood consumption in Italy (FAOSTAT, 2018), none of the records in this database exceed the threshold value of 1 for THQ, pointing to a minimum risk of running into systemic effects at a consumption rate like the one typical of the Italian population. Conversely, in around 6% of cases, samples exceed the threshold value of $10^{-5}$ for ELCR, pointing to a probability of greater than 1 chance over 100,000 of developing cancer [64]. Ultimately, at a consumption rate like the one typical of the Italian population, seafood caught from the area considered in the present work seems to pose a minimal risk to health. However, it should be considered that the seafood ingestion rate is variable among the population [99], and that an individual is simultaneously exposed

to several PAH sources, from both ingestion and other routes. Furthermore, it is vital to note the emerging role of genetics in shaping individual susceptibility to PAHs [100].

## 3.5 Conclusions

Gathering the results of several investigations, we produced a database on PAHs in seafood from the Western and Central Mediterranean Sea. A clear imbalance in favor of studies addressing PAHs in bivalve mollusks emerged.

The meta-analysis carried out on the database led us to obtain potential hints on factors (e.g., reproductive status, water masses circulation, and river discharge season-al variability) that could determine differences in the PAH contamination of marine species.

The assessment of human health risks posed by PAH seafood contamination showed that, at a consumption rate like the one typical of the Italian population, seafood caught from the study areas seems to pose a minimal risk to health. Despite this, concerns may arise considering the individual susceptibility to PAHs exposure as well as the apparent increasing trend of PAHs levels observed in both environmental matrices and sea animals.

## 4. Mercury intake estimation in adult individuals from Trieste: hair mercury assessment and validation of a newly developed food frequency questionnaire (under review at the MPDI journal *Pollutants*)

Abstract

Seafood constitutes the primary source of exposure to the organic form of mercury in the general population, and Trieste gulf is considered a hotspot of mercury contamination, mostly due to the input from the polluted Isonzo River. In the present study, we used a newly developed quantitative food frequency questionnaire, coupled with a database on mercury in Mediterranean seafood, to obtain an estimation of the intake of mercury through seafood consumption in a sample of 32 individuals from Trieste. Then we validated the results obtained from the questionnaire against those of the analysis of total mercury measured in the hair of the same individuals, through Spearman rank correlation coefficients, Cohen's weighted Kappa statistic and Bland-Altman plot. This preliminary study shows a high accuracy of the reported questionnaire in the estimation of habitual mercury intake, similar to the one measured through the analysis of the hair.

### 4.1 Introduction

Among the general population, seafood is the main source of exposure to the organic form of mercury, i.e., methylmercury (MeHg) [101,102], with large, long-lived, predatory fishes (e.g., Atlantic bluefin tuna, swordfish, sharks) showing higher concentrations of this contaminant, as an effect of biomagnification along the food chain [103], and MeHg uptake being a lifelong process of bioaccumulation [104].

MeHg is a well-established neurotoxicant, and exposure to MeHg is associated with nervous system damage in adults and impaired neurological development in infants and children [105], as well as with an increased risk of cardiovascular disease in adults [106].

Based on epidemiological evidence accumulated over the years, national and international agencies set limits and gave recommendations in order to protect citizens' health. In 2001, the U.S. Environmental Protection Agency (US EPA) derived an oral reference dose (RfD) for MeHg—that is, the maximum oral dose that is likely to be without appreciable risk of deleterious effects during a lifetime—of 0.1 μg $kg^{-1}$ $day^{-1}$ [107,108]. Then, in 2012, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM) established a tolerable intake of 1.3 μg $kg^{-1}$ bodyweight (b.w.) per week [9,109], corresponding to an apparent no-observed-effect level (NOEL)

of ~11.5 mg $kg^{-1}$ and ~46 μg $L^{-1}$ in hair and blood, respectively. This threshold value has been adopted for all classes of consumers, even though adults may be less sensitive to the adverse effects of MeHg [17].

Hair mercury level is frequently used as a biomarker of endogenous exposure to MeHg. Indeed, MeHg is permanently incorporated into the growing hair through diffusion from the blood, and the ratio between total mercury (THg) in the hair and that in the blood is around 250:1 [110,111].

As several investigations have shown, hair THg level often depends on the habitual seafood consumption [112–124]. In the study of Elhamri and colleagues on a Moroccan coastal community [113], the authors found a linear relationship between the log-transformed data on hair THg levels and the fish consumption frequency (FCF) in times per week. Moreover, they found that subjects with a FCF of three to five times per week showed greater hair THg levels (Geometric Mean = 5.30 μg $g^{-1}$) compared to those of subjects with a FCF of one to two times per week (Geometric Mean = 1.04 μg $g^{-1}$). Accordingly, analysing 237 adults from Naples (Italy), Díez and colleagues [114] found a strong positive correlation ($r_s$=0.536; $p$<0.05) between THg in hair and fish consumption rate.

The Mediterranean Sea is generally considered a geological hot spot for Hg [17], as it is characterized by large deposits of HgS that account for about 65% of the global mercury reserves [125], and the highest concentrations of Hg in Europe tend to be found in fish caught in the Mediterranean Sea [126]. Moreover, data showed a more marked Hg bioavailability in the Tyrrhenian and the Adriatic coastal waters compared to the rest of the Mediterranean [127], and Hg levels higher than the legal limit have been discovered in seafood caught in both areas [104,128–133].

Considering all the above, and also the great amount of local seafood consumed by Mediterranean communities [17,100,118], it is not surprising that several studies revealed high hair THg levels in people from Mediterranean regions [121,134,135].

Among the methods aimed at estimating the habitual seafood consumption [136,137] or the intake of specific nutrients [138,139] and/or contaminants [140], food frequency questionnaire (FFQ) is one of the most used. FFQ is a retrospective direct method for dietary assessment, in that it collects information on foods and beverages already consumed, assessing the frequency with which foods and/or food groups are eaten over a certain time period [141]. Whenever a new FFQ is developed, whether *de novo* or from pre-existing questionnaires, it should be validated in the investigated population on which the study is going to be conducted, which means that the results should be compared with those obtained applying at least another method [142], such as a dietary record [136,143], a 24-hour recall [144,145] or the use of biomarkers [146,147].

Here, we used a newly developed quantitative FFQ (see *Appendix*)—i.e., a FFQ that asks respondents' usual portion size based on a specified measure—, coupled with a database on Hg in

Mediterranean seafood [70], to obtain an estimation of the intake of Hg through seafood consumption in a sample of 32 individuals from a coastal city of Northern Italy, i.e., Trieste, which is considered a Hg contamination hotspot [52]. Then we compared the results obtained from the questionnaire with those of the analysis of THg measured in the hair of the same individuals, with the aim of (1) validating the questionnaire, and (2) collecting preliminary data on Hg exposure among the population of Trieste.

## 4.2 Materials and methods

### 4.2.1 Sampling location

The Italian city of Trieste is located in the region of Friuli-Venezia Giulia, facing the north-western part of the Adriatic Sea (Figure 7a). Trieste gulf is considered a hotspot of Hg contamination, mostly due to the Hg input from the polluted Isonzo River (Figure 7b), which cross the cinnabar-rich deposits of the Idrija mine [52].

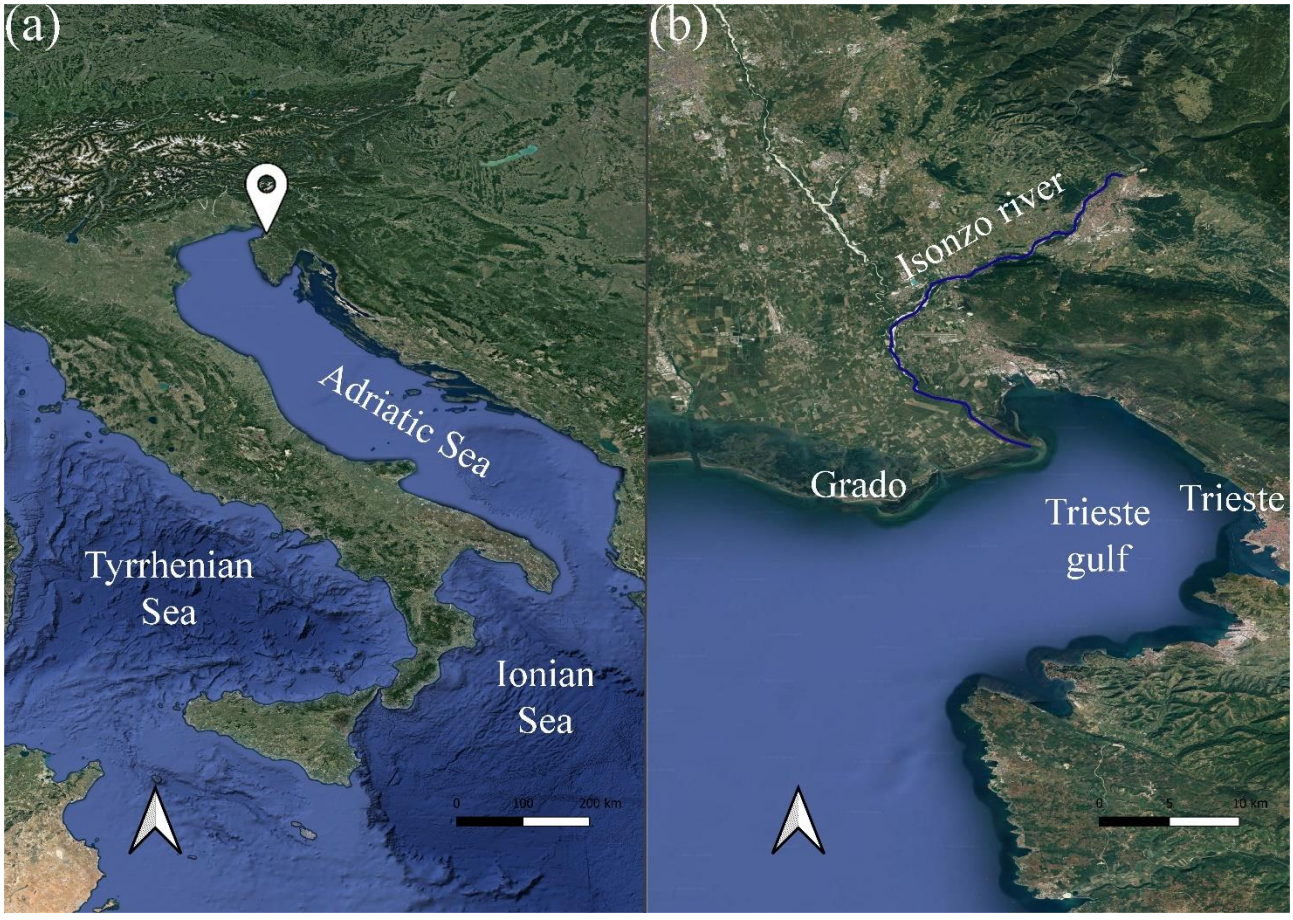



**Figure 7.** (**a**) Italian peninsula. White waypoint indicates Trieste location. (**b**) Trieste gulf with Isonzo river highlighted in blue.

### 4.2.2 FFQ development and administration

Following recommendations made in [142] and [141], we developed a quantitative FFQ consisting of close-ended questions on the habitual consumption of 52 aquatic species. These species were selected through the following procedure: using Hg concentrations data retrieved from literature [46,52,55,66,104,128,129,131–133,148–167], coupled with a large database on Hg in Mediterranean biota [70], we calculated the average concentrations of Hg in edible tissues of more than 350 Mediterranean aquatic species; among these, we selected those species that are included in the ministerial decree of 22 September 2017 (n. 19105), listing species that are commercially relevant in Italy, and that show an average Hg concentration in edible tissue exceeding the maximum allowable level of Hg in seafood of 0.5 mg/kg, set by EU [168], thus obtaining a list of 41 commercially relevant aquatic species, particularly contaminated by Hg in the Mediterranean Sea; finally, we further expanded the above list adding several species that are most consumed by the Italian population (ISMEA, 2011).

For each of the 52 species, the questionnaire assesses respondents' habitual consumption frequency and portion size over a period spanning the last six months before the interview.

Apart from the questions on seafood consumption, the questionnaire includes questions on other routes of exposition to Hg—i.e., breakage of Hg thermometer, occupation, dental amalgam fillings—, smoking and general state of health.

The questionnaire was interviewer-administered.

### 4.2.3 Hg intake estimation from FFQ

Once collected, the questionnaires were used to calculate Hg weekly intake of the participants, via the following formula:

$$\Sigma \text{ (F (times/week)} \times \text{C (mg/kg))} \div \text{BW (kg)} \quad (4)$$

where F is the consumption frequency of each species in times per week, C is the Hg concentration in edible tissues of each species in mg/kg, and BW is the bodyweight of the participant in kg.

After discovering, through comparison with hair THg levels, that the questionnaire tends to overestimate Hg intake in individuals consuming higher amount of seafood, we applied a standardized filtering process to all 32 questionnaires; namely, for each participant, the estimation of Hg weekly intake was achieved using only those species that had been consumed at the two highest frequencies. For example, if a participant had consumed a given species twice a week, another species twice a month, and another one once a month, we excluded the last species from the calculation of the Hg weekly intake of that participant.

### 4.2.4 Hair sampling and THg measurement

Hairs of participants were collected following the procedure recommended by the World Health Organization [110]. After a cleaning step involving rinsing the hair with 70 % isopropyl alcohol, a bundle of hair approximately 0.75-1.0 cm in diameter was cut from the occipital region of each participant, using blunt-tipped, clean stainless steel. To make sure that an adequate amount (50 mg) of hairs had been collected, samples were also weighed with a precision scale. Once cut, the hair bundle was wrapped closed to the scalp end with a small Post-it note and held together with a plastic clip. Finally, the hair sample was placed in a marked paper envelope, which was in turn stored in a sealed plastic bag.

The measurement of THg concentration in hair samples was carried out using a Mercury Analyzer DMA-80 Milestone, which is a spectrophotometer based on atomic absorption determination after thermal decomposition of the samples.

The analysis process consists of introducing the sample into a small oven that can reach 650 ºC. After drying and incineration of the sample, the volatile compounds are carried by a flow of $O_2$ to a catalyst, where the released mercury is reduced to $Hg^0$, which is later retained in a small gold amalgam. After the other volatile compounds are purged into an activated carbon trap, the amalgam is heated to 900 ºC and the volatilized mercury passes through two measurement cells where it is quantified by detection using atomic absorption spectroscopy (AAS).

The detection system contains a mercury lamp that emits light at a wavelength of 253.65 nm and a silicon diode UV detector to quantify mercury. For the evaluation of the signal, the area under the peak generated in the measurement is used. From this area, the Hg concentration is calculated, interpolating in one of the two calibration curves assigned to the two measurement cells, the first measuring up to 20 ng and the second measuring from 20 ng to 1000 ng.

Through the equations of these two calibrations, the amount of mercury expressed in ng is reached, which is then divided by the mass in grams of the sample and gives us the result in

concentration (μg $kg^{-1}$). A solvent-free, easy, fast and waste-free method was employed [169]. Each hair bundle was cut in pieces of ~1.5 cm in order to assess the monthly Hg intake, as each 1-1.5 cm segment of hair incorporates the Hg assimilated during the preceding month [170]. For each segment obtained, a quantity of ~10 mg of sample was weighted into a quartz cuvette. After that, 50 μL of ultrapure water were added to the sample in the cuvette, which was then introduced into the DMA-80. Samples were analysed using the same temperature and time parameters as in [169], and for each 1.5 cm of sample we made two replicates, using their mean for statistical analyses.
As can be seen from figures 10 and 11, not all participants had long enough hairs to assess the Hg intake during the preceding six months, and for six male participants only the first 1.5 cm of hair was available.

### 4.2.5 Hg intake estimation from hair THg

For each participant, to estimate Hg intake from hair THg level, we used the average of the THg concentrations measured in each ~1.5 cm segment of the hair sample. First, we derived the blood THg level using the above-mentioned ratio of 250:1 between the THg levels in the two matrices; hence, we obtained the estimate of the Hg dietary intake with the formula reported in [109]:

$$C\ (\mu g/L)\ ) \times b \times V\ (L) \div A \times f \times BW\ (kg) \qquad (5)$$

where C is Hg concentration in blood in μg/L, b is the elimination constant, which is equal to the ration between ln(2) and Hg half-life in blood, which was assumed to be equal to 50 days as in [109], V is the blood volume, which was assumed to be equal to 5 L as has been assumed by WHO and US EPA [109], A is the gastrointestinal absorption factor (0.95), f is the fraction of absorbed dose distributed to blood, which was assumed to be equal to 0.05 as in [109], and BW is the bodyweight of the participant in kg.

### 4.2.6 Statistical analysis

Statistical analyses were carried out using RStudio version 3.6.1.

After assessing the normality of data via Shapiro-Wilk normality test (R function: shapiro.test) and QQ-plots (R function: qqPlot), we used Kruskal-Wallis rank sum test (R function: kruskal.test) to assess the statistical significance of differences between Hg intakes or hair THg concentrations in the two sexes, and we calculated Spearman rank correlation coefficients (R function: cor.test; method

= “spearman”) to evaluate the association between Hg intakes or hair THg concentrations and age of participants.

To assess the validity of the FFQ, we compared the Hg intakes obtained with the two methods—i.e., the FFQ and the hair THg measurement—by calculating Spearman rank correlation coefficients between the two, and using Cohen’s weighted Kappa statistic (R function: cohen.kappa), which measures the extent to which the two methods assign the same participant to the same quintile. Finally, for the FFQ validation, we also used the Bland-Altman plot (R function: blandr.draw) [171,172], which is a graphical method that is recommended in conjunction with correlation coefficients when agreement between two quantitative methods has to be evaluated [142]. In fact, correlation between results obtained with two different methods measuring the same quantity does not necessarily imply agreement between the two methods [173]. Briefly, the Bland-Altman plot consists in a scatter plot, in which on the Y-axis is the difference between each paired measurements (A and B)—i.e., the difference between the Hg intake estimated from the FFQ and that derived from the hair THg measurement—, while on the X-axis is the average of these measurements ((A+B)/2) [173]. When the difference between the two paired measurements is plotted against their mean, the two methods can be deemed in agreement if at least 95% of the data points lie within ±1.96*s* of the mean difference or within the non-parametric lower and upper limits of agreement (LOA) [174].

## 4.3 Results

### 4.3.1 Sample characteristics

Information on participants are reported in Table 6. The 32 participants are all adult and resident in Trieste and include 13 males and 19 females. Mean age is 35 among males (median=32, SD=10.90, range: 21-55), and 48 among females (median=51, SD=11.63, range:20-65). Nine individuals, seven males and two females, are current smokers, and six participants, one male and five females, have dental amalgam fillings, while two female individuals were not able to provide this latter information.

**Table 6.** Information on participants from Trieste, including results of non-filtered and filtered FFQs and hair THg analysis.

| Variables | Male participants (n=13) | Female participants (n=19) | Overall (n=32) |
|---|---|---|---|
| Mean age ± SD (median, range) | 35±10.90 (32, 21-55) | 48±11.63 (51, 20-65) | 42.81±12.82 (45.50, 20-65) |
| Current smokers | 7 | 2 | 9 |
| Dental amalgam fillings | 1 | 5 | 6 |
| Mean Hg w.i.[a] from n.f.[b] FFQ (μg $kg^{-1}$ b.w.) ± SD (median, range) | 4.03±3.13 (3.46, 0.52-9.55) | 4.11±5.28 (1.99, 0.00-17.64) | 4.08±4.47 (2.62, 0.00-17.21) |
| Mean Hg w.i.[a] from f.[c] FFQ (μg $kg^{-1}$ b.w.) ± SD (median, range) | 1.98±1.08 (2.12, 0.36-3.53) | 2.57±2.63 (1.23, 0.00-8.01) | 2.33±2.13 (1.70, 0.00-8.00) |
| Mean hair THg (mg $kg^{-1}$) ± SD (median, range) | 2.91±2.16 (2.07, 0.72-7.43) | 1.42±0.48 (0.48, 0.02-9.63) | 2.02±2.42 (1.08, 0.01-9.63) |
| Mean Hg w.i.[a] from hair THg (μg $kg^{-1}$ b.w.) ± SD (median, range) | 2.16±1.61 (1.54, 0.53-5.52) | 1.05±1.82 (0.36, 0.01-7.15) | 1.50±1.80 (0.80, 0.01-7.15) |

[a] w.i., weekly intake
[b] n.f., non-filtered
[c] f., filtered

4.3.2 Exposure assessment

4.3.2.1 FFQ

Including all the species consumed by participants, without any filtering procedure, we obtained a median Hg weekly intake of 2.62 μg/kg b.w. (mean=4.08 μg/kg b.w., SD=4.47, range: 0.00-17.21 μg/kg b.w.), which is more than twice the EFSA maximum recommended intake. Among males, the median weekly intake is equal to 3.46 μg/kg b.w. (mean=4.03 μg/kg b.w., SD=3.13, range: 0.52-9.55 μg/kg b.w.), while among females it is equal to 1.99 μg/kg b.w. (mean=4.11 μg/kg b.w., SD=5.28, range: 0.00-17.64 μg/kg b.w.). The difference between Hg weekly intake in females and in males is not statistically significant, moreover we found no statistically significant correlation between the inferred weekly intake and age, nor in the overall sample, neither in the two sexes separately.

Gilthead seabream (*Sparus aurata*) is the most consumed species in our sample, with an average consumption rate of 161.82 g/week, followed by common sole (*Solea solea*), with an average consumption rate of 79.62 g/week, and squid (*Loligo vulgaris*), with an average consumption rate of 67.19 g/week. Whereas the first three species which contribute most to the habitual Hg intake in our sample are European seabass (*Dicentrarchus labrax*), with an average intake of 31.92 μg/week,

Gilthead seabream, with an average intake of 30.85 μg/week, and Norway lobster (*Nephrops norvegicus*), with an average intake of 21.64 μg/week.

After applying the above-described filtering procedure, i.e., including only those species that had been consumed at the two highest frequencies in the estimation of Hg intake of each participant, and assuming a negligible intake for the others, we obtained a median Hg weekly intake of 1.70 μg/kg b.w. (mean=2.33 μg/kg b.w., SD=2.13, range: 0.00-8.00 μg/kg b.w.), which is still slightly higher than EFSA maximum recommended intake. Among males, the median weekly intake after filtering of the questionnaires is equal to 2.12 μg/kg b.w. (mean=1.98 μg/kg b.w., SD=1.08, range: 0.36-3.53 μg/kg b.w.), while among females it is equal to 1.23 μg/kg b.w. (mean=2.57 μg/kg b.w., SD=2.63, range: 0.00-8.01 μg/kg b.w.). Also in this case we found no statistically significant difference between Hg weekly intake in males and in females. Moreover, we found no statistically significant correlation between the inferred weekly intake and age, nor in the overall sample, neither among female participants, while inferred weekly intake and age show a statistically significant negative correlation ($\tau=-0.5$, $p < 0.05$) among male subjects. After the filtering procedure, we found that Gilthead seabream is the species which contributes most to the habitual Hg intake in our sample, with an average intake of 26.03 μg/week, followed by European seabass, with an average intake of 23.13 μg/week, and tuna (*Thunnus* spp.), with an average intake of 18.73 μg/week.

4.3.2.2 Hair analysis

After averaging the THg concentrations measured in every ~1.5 cm segment of each participant's hair (Figures 10 and 11), we found a median hair THg level in the analysed sample of 1.08 mg $kg^{-1}$ (mean=2.02 mg/kg, SD=2.42, range: 0.01-9.63 mg/kg), which is well below the NOEL of ~11.5 mg/kg set by EFSA. Concerning the difference between the two sexes, results of the analysis of the hair are consistent with those of FFQs, with male individuals being more exposed to Hg compared to female participants, and this difference is statistically significant ($p < 0.05$). Indeed, among males, the median hair THg level is equal to 2.07 mg/kg (mean=2.91 mg/kg, SD=2.16, range: 0.72-7.43 mg/kg), while among females it is equal to 0.48 mg/kg (mean=1.42 mg/kg, SD=0.48, range: 0.02-9.63 mg/kg). Finally, consistently with results obtained from FFQs analysis, we found no statistically significant correlation between the hair THg level and age, nor in the overall sample, neither in the two sexes separately.

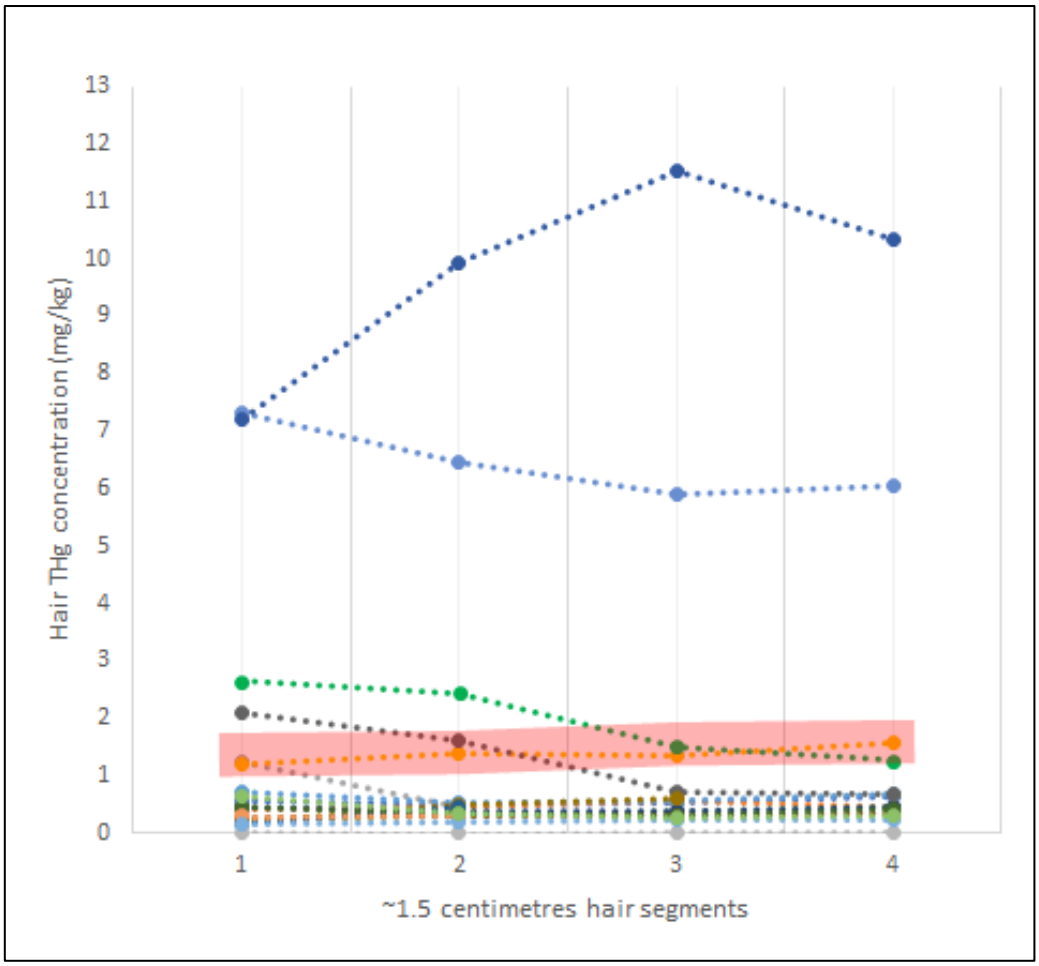



**Figure 8.** Hair concentrations (Y-axis) in the first four ~1.5 centimetres segment of hair (X-axis) in female participants, starting from the nearest (1) to the farthest (4) from the scalp. Each line colour corresponds to a participant, with bigger coloured dots representing measurements. On top of the graph are shown the periods around which the detected Hg was ingested. The red transparent bar represents the evolution of the mean hair THg level.

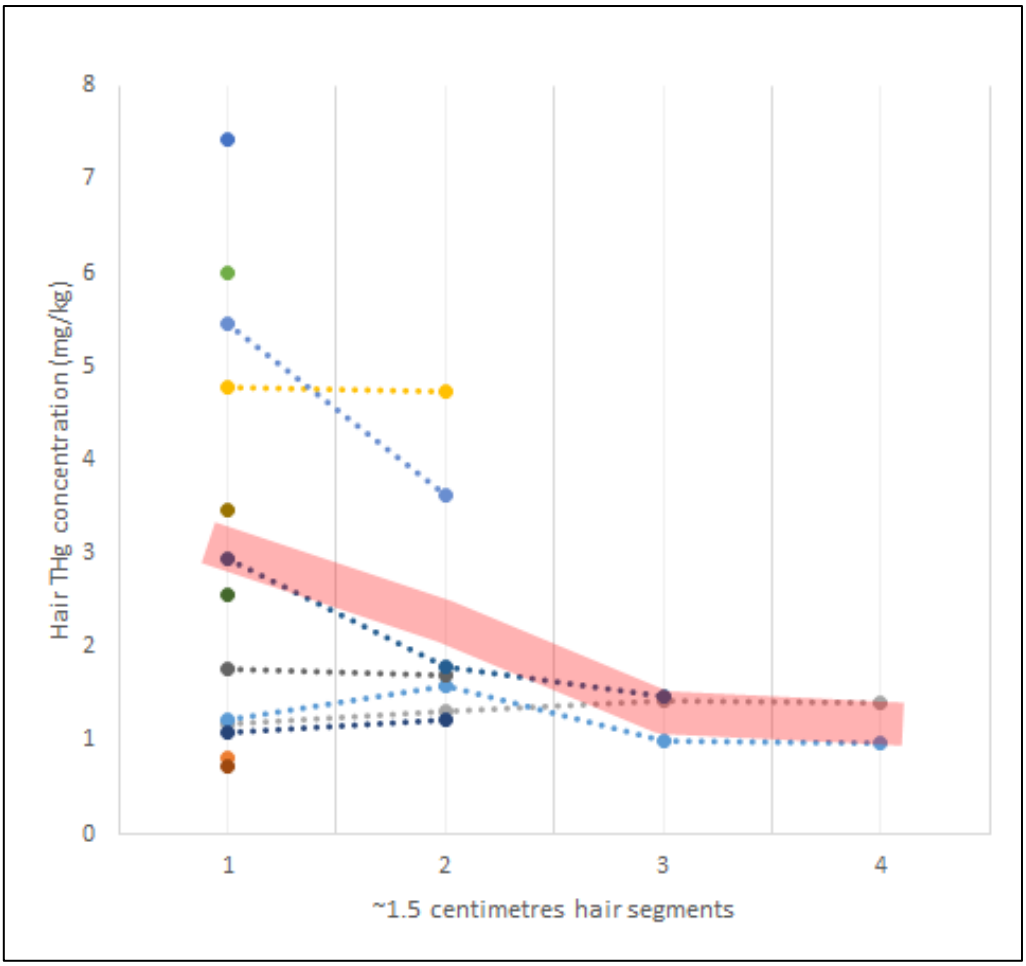



**Figure 9.** Hair concentrations (Y-axis) in the first four ~1.5 centimetres segment of hair (X-axis) in male participants, starting from the nearest (1) to the farthest (4) from the scalp. Each line colour corresponds to a participant, with bigger coloured dots representing measurements. On top of the graph are shown the periods around which the detected Hg was ingested. The red transparent bar represents the evolution of the mean hair THg level.

The hair THg level was used to derive the Hg dietary intake of each participant using (2). The median Hg weekly intake derived from hair THg level is equal to 0.80 µg/kg b.w. (mean=1.50 µg/kg b.w., SD=1.80, range: 0.01-7.15 µg/kg b.w.), which is lower than EFSA maximum recommended intake and the values obtained from FFQs, both before and after filtering. Among males, the median weekly intake is equal to 1.54 µg/kg b.w. (mean=2.16 µg/kg b.w., SD= 1.61, range: 0.53-5.52 µg/kg b.w.), while among females it is equal to 0.36 µg/kg b.w. (mean=1.05 µg/kg b.w., SD=1.82, range: 0.01-7.15 µg/kg b.w.).

4.3.3 FFQ validation

Spearman rank correlation coefficient shows that the Hg weekly intakes derived from non-filtered FFQs are positively correlated with those derived from hair THg concentrations (Table 7), and that this positive correlation is strong and statistically significant (R=0.76, $p < 0.05$).

**Table 7.** Spearman rank correlation coefficient (ρ) and Cohen's weighted Kappa values calculated by comparing Hg weekly intake from non-filtered FFQ and from filtered FFQ with Hg weekly intake from hair THg.

| | **ρ (p-value)** | **Cohen's weighted Kappa** |
|---|---|---|
| Hg w.i.[a] from n.f.[b] FFQ— Hg w.i.[a] from hair THg | 0.76 (5.54e−07) | 0.69 |
| Hg w.i.[a] from f.[c] FFQ— Hg w.i.[a] from hair THg | 0.61 (1.90e−04) | 0.57 |

[a] w.i., weekly intake
[b] n.f., non-filtered
[c] f., filtered

Weighted Kappa statistic is equal to 0.69, pointing to a moderate agreement between the two methods [175]. Then, we built the Bland-Altman plot upon the two series of data. In the case of few participants, FFQ and hair THg measurement returned intake values that are markedly more discordant than the other paired values. Accordingly, the Shapiro-Wilk normality test showed that the differences between each paired measurements are not normally distributed. As stated by Bland and Altman, when there are one or more extreme discrepancies between the method—i.e., the difference between one or more pair of measurements differs considerably from the others—a nonparametric approach may be preferable. Therefore, as suggested by Frey and colleagues [176], we used quantile estimation based on one and two order statistics—i.e., Harrell-Davis quantiles, sample quantiles, and Sfakianakis-Verginis quantiles estimator—to derive the non-parametric limits of agreement. The mean difference between the two methods is equal to 2.57. The resulting plot (Figure 10a) shows that 93.75% of the data points—i.e., 30 out of 32—lie within the acceptability range determined by sample quantiles (lower LOA=−1.13, upper LOA=12.51), by Harrell-Davis quantiles (lower LOA=−0.99, upper LOA=11.55), and by Sfakianakis-Verginis quantiles estimator (lower LOA=−1.02, upper LOA=11.76).

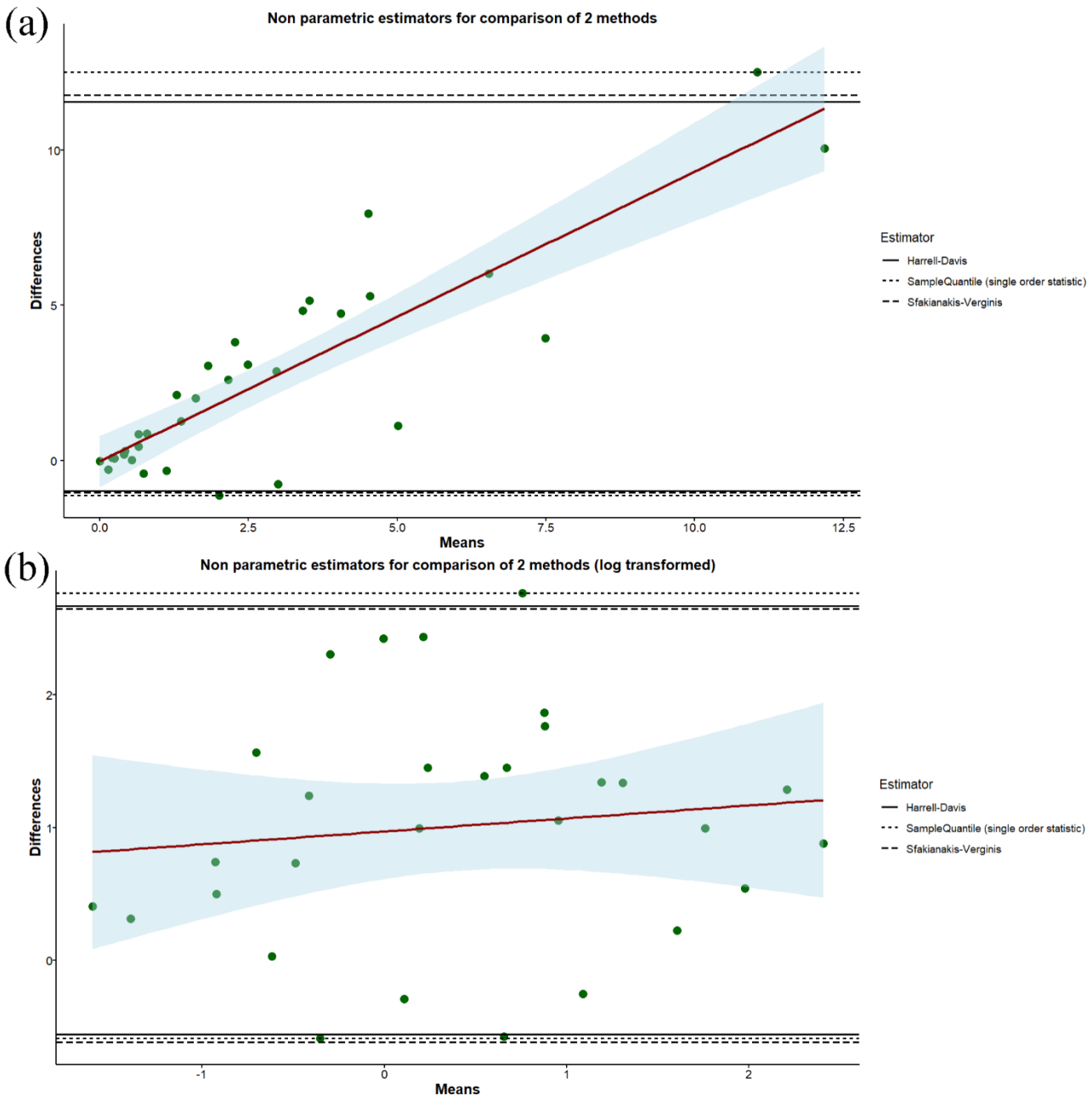



**Figure 10.** (**a**) Bland-Altman plot built on weekly Hg intakes from non-filtered FFQs and hair THg concentration. On the Y-axis is the difference between each paired measurements—i.e., the difference between the Hg intake estimated from the non-filtered FFQ and that derived from the hair THg measurement—, while on the X-axis is the average of these measurements. (**b**) Bland-Altman plot built on log-transformed weekly Hg intakes from non-filtered FFQs and hair THg concentration.

The Bland-Altman plot in Figure 6a also reveals an ever-increasing overestimation of Hg intakes by FFQs as hair THg level increases. In other words, the mean difference between the two methods is approximately proportional to the magnitude of the measurement, and this is made apparent by the substantial slope of the red line shown in the plot. Therefore, we tried to build the plot using the logarithms of the two data series, as suggested by Bland and Altman [171], after

excluding two female participants for whom weekly Hg intake from FFQ was equal to zero. In this new plot (Figure 10b), 93.75% of the data points—i.e., 30 out of 32—lie within the acceptability range determined by sample quantiles (lower LOA=−0.59, upper LOA=2.77), while 90% of the data points—i.e., 27 out of 30—lie within the acceptability range determined by Harrell-Davis quantiles (lower LOA=−0.56, upper LOA=2.67), and 96.67% of the data points—i.e., 29 out of 30—lie within the acceptability range determined by Sfakianakis-Verginis quantiles estimator (lower LOA=−0.62, upper LOA=2.65). Also in this case, the proportionality between the mean difference and the magnitude of the measurement persists, even if at a lower level.

Given the bias that non-filtered FFQ shows in the assessment of the Hg weekly intake, we filtered participants' answers through several procedures, each time assessing the resulting improvement through the Bland-Altman plot. In this way, we found that the best results are obtained when only those species that had been consumed at the two highest frequencies are included in the calculation of the Hg weekly intake of each participant. Also in this case, Spearman rank correlation coefficient shows that the Hg weekly intakes derived from filtered FFQs are positively correlated with those derived from hair THg concentrations (Table 2), and that this positive correlation is strong and statistically significant (R=0.61, $p < 0.05$). Weighted Kappa statistic is equal to 0.57, pointing to a weak agreement between the two methods [175]. The Bland-Altman plot built upon the same filtered data (Figure 11a) shows that 93.75% of the data points—i.e., 30 out of 32—lie within the acceptability range determined by sample quantiles (lower LOA=−4.06, upper LOA=6.79), by Harrell-Davis quantiles (lower LOA=−3.35, upper LOA=6.18) and by Sfakianakis-Verginis quantiles estimator (lower LOA=−3.16, upper LOA=6.31).

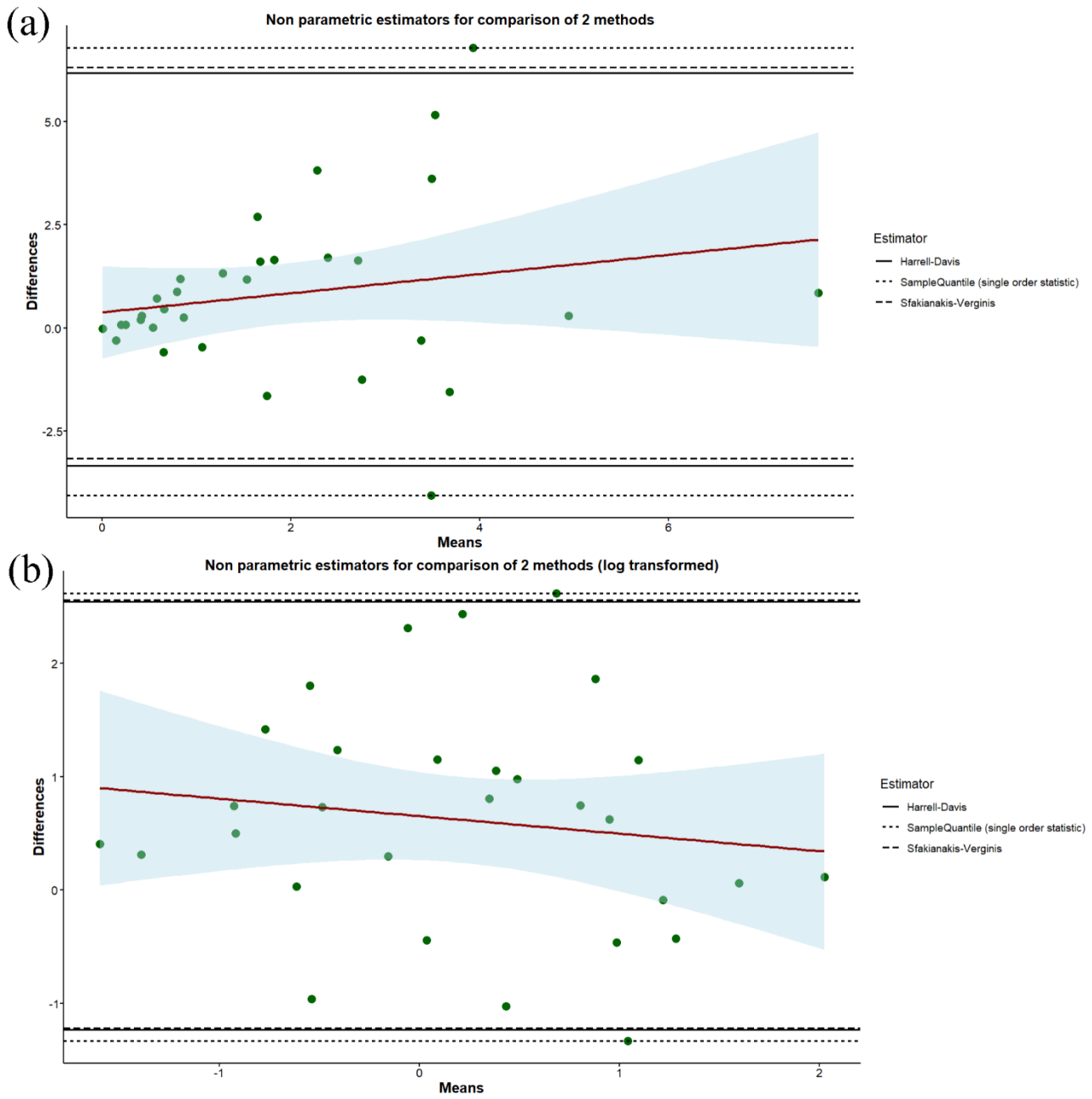



**Figure 11.** (**a**) Bland-Altman plot built on weekly Hg intakes from filtered FFQs and hair THg concentration. On the Y-axis is the difference between each paired measurements—i.e., the difference between the Hg intake estimated from the filtered FFQ and that derived from the hair THg measurement—, while on the X-axis is the average of these measurements. (**b**) Bland-Altman plot built on log-transformed weekly Hg intakes from filtered FFQs and hair THg concentration.

The proportionality between the mean difference and the magnitude of the measurement is more subtle compared to that observed in the plot built upon non-filtered FFQs. Then we built the Bland-Altman plot using the log-transformed hair THg concentrations and Hg intakes from filtered FFQs. Also in this case (Figure 11b), 93.75% of the data points—i.e., 30 out of 32—lie within the acceptability range determined by sample quantiles (lower LOA=−1.33, upper LOA=2.62), by

Harrell-Davis quantiles (lower LOA=−1.23, upper LOA=2.55) and by Sfakianakis-Verginis quantiles estimator (lower LOA=−1.22, upper LOA=2.56). In this latter plot, the mean difference between the two methods slightly decreases as the magnitude of the measurement increases.

## 4.4 Discussion

### 4.4.1 FFQ reliability

It has been suggested that the correlation coefficients in validation studies should not be below 0.3–0.4 [136,142], and results derived from both the non-filtered and the filtered questionnaire developed in the present study meet this requirement.

As regards weighted Kappa statistic, in his original paper, Cohen suggested that values of Kappa ≤ 0 indicate no agreement, and interpreted values between 0.01 and 0.20 as none to slight, values between 0.21 and 0.40 as fair, values between 0.41 and 0.60 as moderate, values between 0.61 and 0.80 as substantial, and values between 0.81 and 1.00 as almost perfect agreement [177]. However, other authors suggest that any kappa below 0.60 should be interpreted as an indication of inadequate agreement among the two methods, especially in a clinical context [175]. On the basis of such premises, we conclude that the FFQ developed in the present study, both before and after filtering, shows a moderate to substantial agreement with the hair THg measurement.

A strong correlation does not imply agreement between the two methods [172], and that is why correlation should be used alongside the Bland-Altman plot [142]. Results of the Bland-Altman plots with non-parametric LOAs show that the filtered FFQ is more reliable in assessing the Hg weekly intake compared to the non-filtered version, as the latter tends to overestimate Hg intake in individuals consuming higher amount of seafood. However, it is to note that the requirement for the validation—i.e., at least 95% of the data points lying within ±1.96*s* of the mean difference or within the non-parametric lower and upper LOA—is met only in the case of log-transformed data series from non-filtered FFQs and hair THg concentration, using Sfakianakis-Verginis quantiles as LOA. In most other cases, only 93.75% of the data points lie within the non-parametric range of acceptability, with the above percentage dropping to 90% in the case of log-transformed data series from non-filtered FFQs and hair THg concentration, using Harrell-Davis quantiles as LOA.

Overall, the results of the present study are promising and point to a fair efficacy of the newly developed FFQ in assessing habitual Hg intake.

As stated by Cade and colleagues in their review [142], for the Bland-Altman method, a sample size of at least 50 is desirable, while, for validation studies using correlation coefficients, this

number rises to 100. Therefore, administering the FFQ to a larger sample may strengthen the validity assessment.

Another potential source of error in the present study stems from the fact that we don't know the exact geographic origin of the seafood consumed by the participants. This may lead to misleading results of the FFQ, as seafood contamination also depends on local oceanographic factors, and on the vicinity to emission sources.

### 4.4.2 Hg exposure

The FFQ, both before and after filtering, returns a median Hg weekly intake that is higher than the value derived from hair THg measurement. In particular, non-filtered FFQ returns a value (2.62 μg/kg b.w.) that is more than twice the EFSA maximum recommended intake of 1.3 μg/kg b.w. per week [9,109], and filtered FFQ returns a value (1.70 μg/kg b.w.) that is only slightly higher than the same limit. On the other hand, the median Hg weekly intake derived from hair THg measurement is equal to 0.80 μg/kg b.w., which is lower than EFSA maximum recommended intake.

The median hair THg concentration in the analysed sample from Trieste is equal to 1.08 mg/kg, which is well below the NOEL of ~11.5 mg/kg set by EFSA. However, it is to note that there is no consensus on the actual hair THg level above which health risk may occur, as several investigations led to the establishment of threshold values ranging from 1, set by US EPA [178], to 14 mg/kg, set by Joint FAO/WHO Expert Committee on Food Additives (JECFA) [179]. 16 out of the 32 participants in this study exhibit hair THg concentration higher than 1 mg $kg^{-1}$, 6 participants exhibit concentrations higher than 3.75 mg/kg, which is the concentration at which adverse health effects are possible according to German Human Biomonitoring Commission [180], and only a female participant exhibits a THg concentration slightly greater than EFSA NOEL of 11.5 mg/kg in one single ~1.5 cm segment of her hair, roughly corresponding to the period from February to March 2021.

The median hair THg concentration obtained in the present study is greater than that found by Basu and colleagues [121] for the populations of Africa (0.69 mg/kg) and Europe (0.30 mg/kg), for the populations living on the coast of Atlantic (0.62 mg/kg) and Arctic (0.74 mg/kg) oceans, and on the coast of the Mediterranean Sea (0.88 mg/kg), while it is lower than that found in the same study for the populations of Americas (2.02 mg/kg), Eastern Mediterranean (1.68 mg/kg), South-East Asia (3.10 mg/kg) and Western Pacific (1.40 mg/kg), for the coastal populations of the Pacific Ocean (1.75 mg/kg), and for fish consumers—i.e., non-Indigenous or non-Arctic groups who consume relatively high amounts of seafood—(3.04 mg/kg).

The coastal town of Grado, in Friuli-Venezia Giulia, is less than 30 km from Trieste (Figure 1b) and is strongly impacted by Hg pollution due to maritime traffic, a local chlor-alkali plant, and the former Idrija mercury mine in Slovenia. An investigation carried out on 19 inhabitants of this town found a hair THg concentration ranging from 1.13 to 20.16 mg/kg, the highest values being measured among fishermen, with a median value (3.90 mg/kg) well above that found in the present study. On the other hand, in another study on the same geographic area [181], the authors found a mean hair THg concentration of 0.83 mg/kg among mothers of children with advanced fine motor skills, and of 1.24 mg/kg among mothers of children who showed normal or delayed skills. Both of these values are lower than the mean hair THg concentration exhibited by the sample analysed in our investigation (2.02 mg/kg), and, accordingly, the two groups of mothers in that study showed a fresh fish intake during pregnancy of 0.47 and 0.66 servings per week, respectively, while the 32 participants from Trieste show a mean seafood intake of 1.28 servings per week.

Compared to our results, lower hair THg concentrations are also shown by the general population of several towns on the coast of Sicily (mean concentration=0.23 mg/kg, SD=0.4) [119], by people living in Priolo, near the chlor-alkali plant of Augusta (Sicily), one of the largest in Europe (mean concentration=1.37 mg/kg, median concentration=1.00 mg/kg), and by the general population of Naples (mean concentration=0.638 mg/kg). On the contrary, higher THg concentration were found in fishermen of several coastal towns of Sicily (mean concentration=6.45 mg/kg, SD=7.03), in people from Augusta (mean concentration=2.61 mg/kg, median concentration=1.90 mg/kg), and in tuna consumers from Carloforte (Sardinia) (median concentration=9.6 mg/kg, range: 1.4-34.5 mg/kg).

It is important to note that, due to its small size, the sample analysed in the present study may not be representative of the entire population of Trieste.

Finally, it comes as no surprise the fact that, as revealed by questionnaires, Gilthead seabream, European seabass, squid and tuna are the species that contribute the most to the habitual Hg intake of the participants, since the first three species are among those that are most consumed in Italy, and tuna is one of those species that are most contaminated.

## 5. Sampling of Italian communities

During the project, we took biological samples from people belonging to several Italian communities. These samples will be analysed for DNA methylation, in order to look for potential correlations between this epigenetic modification and environmental factors such as diet. The study that lies ahead can help to test the hypotheses that have emerged in the present thesis.

After participants signed the informed consent, oral mucosa cells were collected through buccal swabs, rubbed against oral mucosa following a protocol approved by the Prevention and Protection Service of the University of Bologna. Upon sampling, each participant replied to a written questionnaire on dietary habits, smoking status, employment, and general health. On sampling, also personal data on grandparents and parents were collected. In particular, surnames of participants ancestors are useful because they allow to estimate the antiquity of the presence of their lineage in the region, while knowing where they were born and lived is essential for meet the grandparents criterion, i.e. the sampling criterion that states that to get a sample representative of the genetic variability of a population, it's necessary to sample individuals whose grandparents are born in the same geographic area in which that population lives.

We took oral mucosa samples from 200 individuals coming from 22 Italian towns (Figure 12). Information on participants, divided by place of living, are reported in Table 8.

The sample includes 10 sets of brothers and 8 father-son pairs.

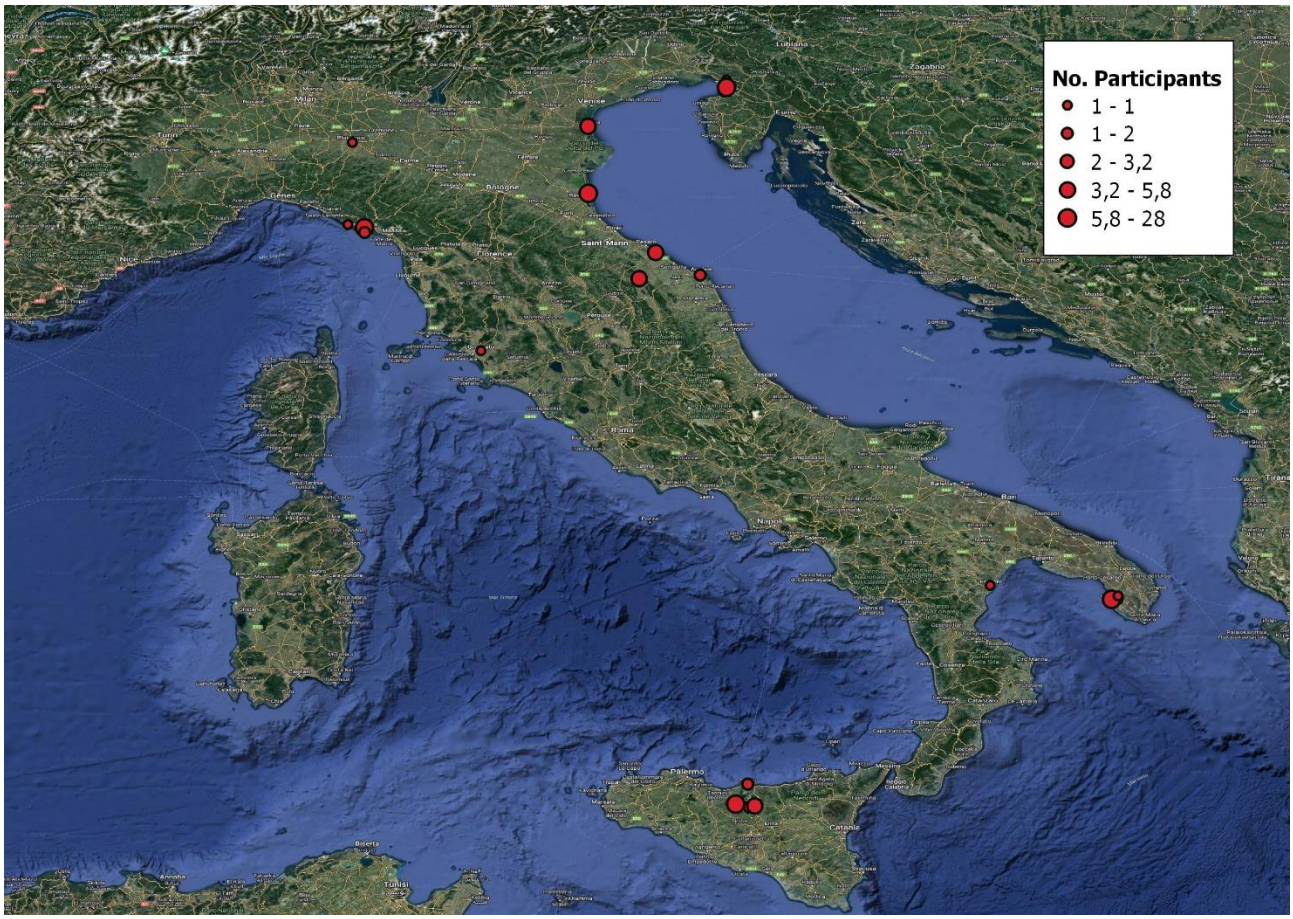



**Figure 12.** Map showing towns (red circles) from which participants come from. The diameters of the circles are proportional to the sample size.

**Table 8.** Information on participants from which oral mucosa samples were gathered.

| Place of living | Region | No. participants | Male participants | Female participants | Mean age |
|---|---|---|---|---|---|
| Policoro | Basilicata | 1 | 0 | 1 | 53.00 |
| Marina di Ravenna | ER[1] | 7 | 7 | 0 | 43.00 |
| Piacenza | ER[1] | 1 | 1 | 0 | 38.00 |
| Muggia | FVG[2] | 2 | 1 | 1 | 39.00 |
| Monrupino | FVG[2] | 1 | 1 | 0 | 38.00 |
| Trieste | FVG[2] | 28 | 10 | 18 | 44.18 |
| La Spezia (Cadimare) | Liguria | 2 | 2 | 0 | 62.50 |
| La Spezia | Liguria | 6 | 6 | 0 | 44.33 |
| Monterosso al Mare | Liguria | 1 | 1 | 0 | 69.00 |
| Portovenere | Liguria | 2 | 2 | 0 | 46.50 |
| Ancona | Marche | 2 | 2 | 0 | 58.00 |
| Fano | Marche | 4 | 4 | 0 | 46.00 |
| Pergola | Marche | 4 | 4 | 0 | 50.75 |
| Alezio | Puglia | 2 | 2 | 0 | 37.50 |
| Gallipoli | Puglia | 18 | 16 | 2 | 48.61 |
| Sannicola | Puglia | 1 | 0 | 1 | 27.00 |
| Caltavuturo | Sicilia | 15 | 6 | 9 | 49.40 |
| Castellana Sicula | Sicilia | 2 | 2 | 0 | 62 |
| Cefalù | Sicilia | 2 | 2 | 0 | 61.50 |
| Petralia Soprana | Sicilia | 5 | 1 | 4 | 68.60 |
| Grosseto | Toscana | 1 | 0 | 1 | 41.00 |
| Chioggia | Veneto | 4 | 4 | 0 | 54.50 |

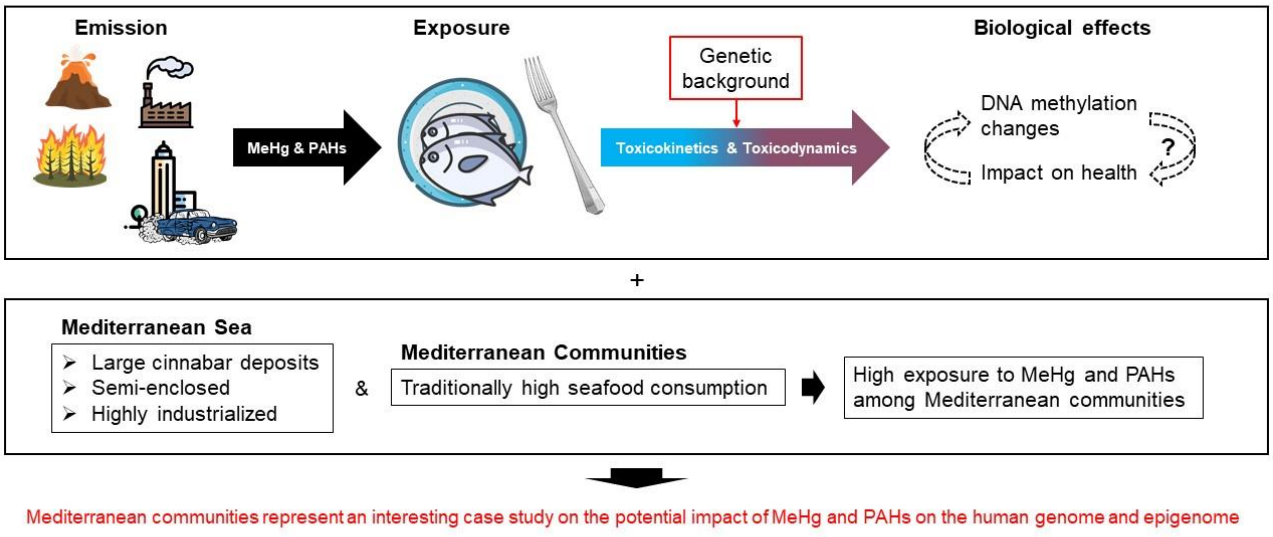
[1]ER, Emilia-Romagna

[2]FVG, Friuli-Venezia Giulia

**6. Methylmercury and Polycyclic Aromatic Hydrocarbons in Mediterranean Seafood: A Molecular Anthropological Perspective** (chapter published as: De Giovanni, A.; Giuliani, C.; Marini, M.; Luiselli, D. Methylmercury and Polycyclic Aromatic Hydrocarbons in Mediterranean Seafood: A Molecular Anthropological Perspective. Appl. Sci. 2021, 11, 11179. https://doi.org/10.3390/app112311179)

Abstract

Eating seafood has numerous health benefits; however, it constitutes one of the main sources of exposure to several harmful environmental pollutants, both of anthropogenic and natural origin. Among these, methylmercury and polycyclic aromatic hydrocarbons give rise to concerns related to their possible effects on human biology. In the present review, we summarize the results of epidemiological investigations on the genetic component of individual susceptibility to methylmercury and polycyclic aromatic hydrocarbons exposure in humans, and on the effects that these two pollutants have on human epigenetic profiles (DNA methylation). Then, we provide evidence that Mediterranean coastal communities represent an informative case study to investigate the potential impact of methylmercury and polycyclic aromatic hydrocarbons on the human genome and epigenome, since they are characterized by a traditionally high local seafood consumption, and given the characteristics that render the Mediterranean Sea particularly polluted. Finally, we discuss the challenges of a molecular anthropological approach to this topic.



## 6.1 Introduction

Despite being usually considered a healthy food [7], seafood carries several contaminants that can negatively affect human health [8]. It is recognized that the benefits of fish intake exceed the potential risks, but here we address how contaminants levels in seafood are significantly affected by biological and ecological factors [53,83,135,182–184]. Moreover, seafood habitual intake is a crucial factor in determining contaminants exposure [99,185].

In the present study, we first give a glimpse into the latest findings on the genetic diversity underlying differences in human response to mercury (Hg) and polycyclic aromatic hydrocarbons (PAHs) exposure, and on the impact of these pollutants on human DNA methylation patterns. We decided to include only epidemiological investigations assessing environmental chemical exposures using biomarkers (such as hair and blood mercury, and PAHs urine metabolites). We excluded studies addressing occupational exposure because we were interested in the potential effects on human molecular variability of Hg and PAHs from seafood. As detailed before, Hg in aquatic organisms is mostly found in the form of methylmercury (MeHg), while in occupational exposure, elemental Hg vapor is the major contributor of Hg load in the human body [118,121]. Concerning PAHs, occupational settings are associated with exposure levels much higher than those resulting from diet [186]. Then, we address the ecological evidence that makes Mediterranean coastal communities a potential informative case study to explore this topic.

We decided to focus on MeHg and PAHs because they are two of the most concerning and widespread seafood contaminants, and because of their high levels in Mediterranean seafood.

## 6.2 Seafood contaminants

### 6.2.1 MeHg

Hg is a heavy metal found naturally in the Earth's crust. From here, mercury is released into the atmosphere via natural phenomena such as volcanic activity and forest fires, and human activities, such as the burning of coal, oil and wood, and mining. In particular, artisanal and small-scale gold mining in developing countries has recently replaced coal combustion as the largest anthropogenic mercury emission source globally [187]. Once released into the environment, it starts to circulate following what is known as the global mercury cycle, which can last up to 3000 years [188]. When mercury passes into water, it is readily transformed by bacteria in its organic form, methylmercury, which can interact with biological components and eventually biomagnify along aquatic food chains

[126]. Many studies have suggested that climate change will increase mercury inputs and methylmercury production and bioaccumulation in aquatic ecosystems [187]. Seafood is recognized as the main source of mercury in the general population, and MeHg accounts for the majority (70–100%) of Hg found in muscle tissue of fishes, molluscs and crustaceans [101].

MeHg is a well-established neurotoxicant, and exposure to MeHg has been associated with nervous system damage in adults and impaired neurological development in infants and children [105]. Decrements in memory, attention, language, and visual–motor skills in childhood have been associated with MeHg biomarkers at birth in populations with moderate MeHg exposure from regular seafood consumption [189]. Even low mercury levels (i.e., levels lower than 4 μg/g in hair; 20 μg/L in cord blood, or approximately 12 μg/L in adult blood) can negatively affect fetal and infant growth and cause neurologic outcomes [190]. Urinary levels of Hg are frequently used to estimate the level of exposure to Hg vapours or inorganic Hg (IHg), whereas blood, hair and toenail [118] Hg predicts MeHg exposure.

Because of the threat that mercury poses to human health, the EU set a maximum level of mercury in seafood of 1 or 0.5 mg/kg, depending on the species, after which seafood shall not be placed on the market [191], while the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a tolerable intake of 1.6 μg/kg bodyweight per week for methylmercury in order to protect the developing fetus from neurotoxic effects [192]. On the basis of multiple epidemiological studies [193,194] that observed adverse effects in children as consequences of maternal exposures, the European Food Safety Authority (EFSA) eventually decreased this limit to 1.3 μg/kg bodyweight per week [109], corresponding to a Hg level of ~11.5 mg/kg and ~46 μg/L in hair and blood, respectively. This threshold value has been adopted for all classes of consumers, even though adults may be less sensitive to the adverse effects of MeHg [17]. Furthermore, the US-EPA established an oral reference dose (RfD) for MeHg—that is, the maximum acceptable oral dose for this contaminant—of $1 \times 10^{-4}$ mg/kg day$^{-1}$ (US-EPA, 2010).

### 6.2.2 PAHs

PAHs are a class of organic compounds consisting of two or more fused benzene rings, deriving from the incomplete combustion or pyrolysis of organic materials. Natural sources of PAHs include volcanoes, forest fires and petroleum seeps, while the combustion of fossil fuels, oil and wood are among the main anthropogenic sources [51,195]. Due to their physicochemical properties, PAHs are persistent pollutants, in that they can stay in the environment for long periods [196]. They represent the largest share among the main organic contaminants present in the marine environment, due to

marine traffic and possible accidents involving oil tankers [64]. Although most PAHs are metabolized a short time after uptake, thanks to their lipophilic nature, a fraction accumulates in lipid-containing tissues such as liver, eggs and muscle [53]. The most important non-occupational source of human exposure to PAHs is the consumption of contaminated food, including seafood [197], especially mollusks and crustaceans [198]. Sixteen PAHs are categorized as priority environmental pollutants, and some of them are deemed to be probable human carcinogens by the US Environmental Protection Agency (US-EPA), with benzo(a)pyrene (B(a)P) arousing more concern because of being the most carcinogenic, teratogenic and toxic compound [199]. The most used biomarkers of PAH exposure are metabolites of PAHs, particularly 1-hydroxypyrene (1-OHP), and PAH–DNA or protein adducts. 1-OHP is the principal product of pyrene metabolism [200], and its urinary excretion has been attributed mainly to the ingestion of PAHs through the diet [201]. Rather, PAH–DNA adducts, which are the products of the Phase I metabolism of PAHs, are deemed a biomarker that integrates multiple BB(a)P exposure routes (including inhalation, dermal absorption, and ingestion) and reflects a biologically effective dose [202]. PAH–DNA adduct formation is significantly influenced by individual susceptibility, which is linked to specific genetic polymorphisms [202,203]. Urine PAHs metabolites and, to a less extent, PAH–DNA adducts are also related to parent air PAH exposures, both at elevated exposures in occupational cohorts, and at low levels of air pollution [200,204].

The EU set a maximum level of B(a)P in seafood to be sold ranging from 2 μg/kg wet weight, for muscle meat of fish (other than smoked fish), to 10 μg/kg for bivalve mollusks [191]. Concerning human exposure, the US-EPA set an RfD for several PAH compounds, including anthracene (0.3 mg/kg $day^{-1}$), acenaphthene (0.06 mg/kg $day^{-1}$), fluorene (0.04 mg/kg $day^{-1}$), fluoranthene (0.04 mg/kg $day^{-1}$), pyrene (0.03 mg/kg $day^{-1}$), naphthalene (0.02 mg/kg $day^{-1}$) and B(a)P (0.0003 mg/kg $day^{-1}$).

Several findings point to an important role of genetic diversity in shaping individual susceptibility to Hg [205] and PAHs [206] exposure, and consequently some authors claim the urgent need to include this factor in risk assessment and decision making [18]. Moreover, Hg and PAHs, similar to several other environmental toxicants [207], can impact the human epigenome through different mechanisms, and some of the epigenetic alterations driven by these two substances were shown to be associated with adverse health effects [208,209].

Being a semi-enclosed sea, delimited by highly industrialized countries and characterized by large deposits of cinnabar (HgS), the Mediterranean Sea is at a high-risk for contamination by toxic compounds [16], and evidence exists that significant anthropogenic chemical inputs into the Mediterranean began in prehistoric times [11]. In line with this, several studies have shown higher levels of contaminants in marine organisms from the Mediterranean Sea compared to those from other

geographic areas [164]. At the same time, the European countries bordering the Mediterranean are among the world's highest seafood consumers, with Spain, Italy and France accounting for more than half of the European expenditure on fish and fishery products, despite having only around a third of the EU's population (EUROSTAT, 2014). Accordingly, high Hg concentrations in the blood and hair of several Mediterranean communities [135] have been found. Given such a traditionally high consumption of contaminated seafood, in our view, it is important to gain insights into the molecular diversity underpinning potential differences in susceptibility to MeHg and PAHs exposure in these communities. Moreover, Mediterranean populations might represent an interesting case study to investigate the potential impact of Hg and PAHs on the human genome and epigenome.

Modern technologies allow us to explore human genomic and epigenomic variability in a cost- and time-efficient way, enabling us, for example, to portray molecular diversity at the populational level [210], and to detect natural selection footprints in genomic regions [211].

### 6.3 Human genetic diversity

Epidemiological investigations are showing the role of genetics in shaping individual susceptibility to MeHg and PAHs. Through a literature search, we identified 18 (Table 9 and Table S1 of [100] for further details) and 3 (Table 10 and Table S2 of [100] for further details) epidemiological studies addressing the role of genetic polymorphisms in MeHg and PAHs toxicokinetics, respectively. Below, we describe some of the main findings of the above studies. Please refer to the tables for the full list of retrieved publications.

**Table 9.** List of epidemiological studies investigating the influence of genetic polymorphisms on MeHg toxicokinetic. Genes in which the above polymorphisms were identified, biomarkers affected, and samples studied are shown for each study.

| Study | Genes | Biomarker | Sample |
|---|---|---|---|
| [212] | GCLC; GSTP1 | Erythrocyte Hg | Swedish cases of acute myocardial infarction/stroke and controls |
| [213] | GSTM1; GSTT1 | Hair Hg | Students in Austria |
| [214] | GCLM; GSTP1 | Erythrocyte Hg | Fish-eating Swedish individuals |
| [215] | GSTP1; MT4; GSTM1; GCLC; GSTT1 | Blood and hair Hg | Students in Austria |
| [216] | GSTM1; GSTT1 | Maternal and cord blood Hg | Korean mothers and their infants |
| [217] | GSTT1; GSS; GSTP1; SEPP1 | Hair and urinary Hg | Michigan dental professionals |
| | MT1M; MT2A; MT1A | Hair and urinary Hg | Michigan dental professionals |
| [218] | APOE | Cord blood Hg | Children in Taiwan |
| [219] | GCLM; GSTM1 | Blood and hair Hg | Amazonian population in Brazil cronically exposed to MeHg from fish |
| [220] | TF | Umbilical cord Hg | Children from Bristol |
| [221] | GCLC; GCLM; GSTM1 | Plasmatic Hg and MeHg; whole blood Hg | Fish-eating communities of Brazilian Amazon |
| [222] | ABCB1; ABCC1; ABCC2 | Cord blood Hg | Pregnant women from Greece, Italy and Spain |
| [223] | APOE | Cord blood Hg | Children in Taiwan |
| [224] | ABCB1; ABCC1; ABCC2 | Hair Hg | Seychellois mother–child pairs with a diet rich in fish of mixed African, European and East Asian origin |
| [225] | GLRX2; GSTA4; GSTM3; GSTO1; SELS; MT1M; (see Table S1 of [100] for the whole list) | Blood and urinary Hg | American dental professionals |
| [226] | CBS; TXNRD2; SEPHS2; CYP1A2; CBS; MTRR; (see Table S1 of [100] for the whole list) | Blood Hg | Inuit from Canada |
| [227] | GCLC; GCLM; GSTP1 | Maternal blood and hair Hg and cord blood Hg | Seychellois mother–child pairs with a diet rich in fish of mixed African, European and East Asian origin |
| [228] | BDNF; GSTP1 | Hair Hg | Children in Valentia |

**Table 10.** List of epidemiological studies investigating the influence of genetic polymorphisms on PAHs toxicokinetics. Genes in which the above polymorphisms were identified, biomarkers affected, and samples studied are shown for each study.

| Study | Genes | Biomarker | Sample |
|---|---|---|---|
| [229] | XRCC1 | Sperm PAH–DNA adducts | Infertile adult men from Nanjing, China |
| [230] | MPO; NAT2; ERCC5 | Blood PAH–DNA adducts | Non-smoking healthy women from eastern Golestan Province, Iran |
| [202] | CYP1A1; GSTT2; CYP1B | Cord blood B(a)P–DNA adducts | Mother–infant pairs from Krakow, Poland |

B(a)P, Benzo(a)pyrene.

6.3.1 MeHg exposure and human genetic diversity

The majority of ingested MeHg passes into the bloodstream, by which route it reaches all tissues. Here, MeHg enters cells thanks to its ability to form water-soluble complexes with the amino acid cysteine. After forming a complex with reduced glutathione (GSH) (Figure 13), MeHg is excreted by the liver cells into the bile. At this point, the glutathione is hydrolyzed, leading to the release of the methylmercury–cysteine complex. The latter is mostly secreted into the intestine tract, where MeHg is demethylated by intestine microflora. The resulting inorganic Hg is then eliminated via the feces [170].

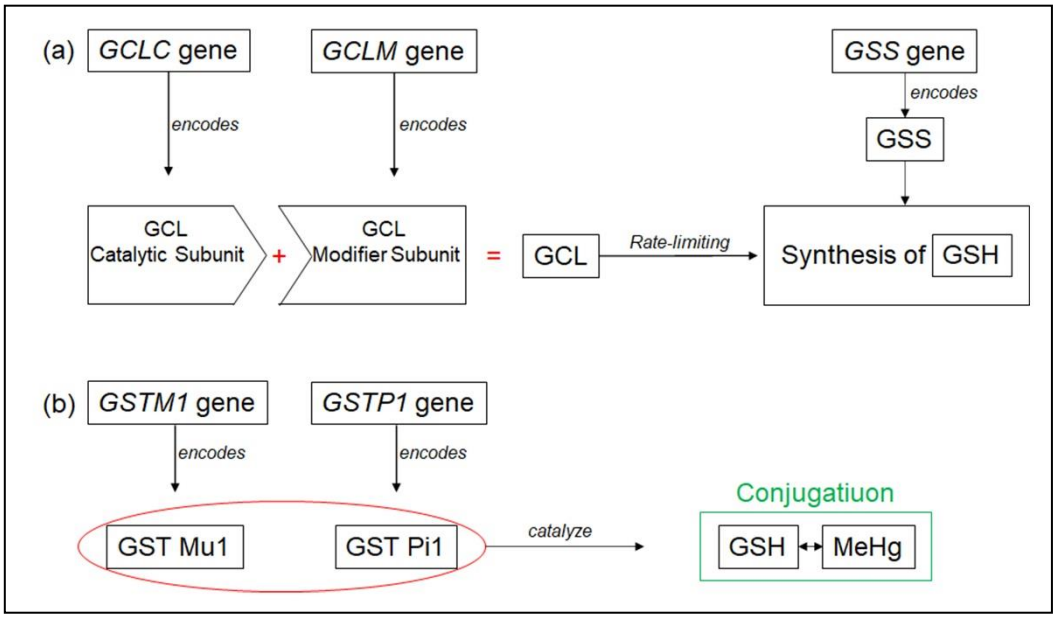



**Figure 13.** Schematic representation of the role played by several genes in MeHg elimination from the body. (**a**) The GCLC and GCLM genes encode the catalytic and modifier subunits of the glutamate–cysteine ligase (GCL) enzyme, respectively. The GCL is the first rate-limiting enzyme of glutathione (GSH) synthesis. The GSS gene encodes the glutathione synthetase (GSS), another enzyme involved in GSH synthesis. (**b**) The GSTM1 and GSTP1 genes encode the glutathione S-transferases (GST) Mu1 and Pi1, respectively, which catalyze the conjugation of GSH to MeHg.

In recent years, epidemiological studies on populations exposed to MeHg have been showing that several genes mediating the toxicokinetics of Hg are polymorphic in humans, and may influence inter-individual variability in Hg exposure biomarker values and health outcomes (Table 9 and Table S1 of [100] for further details). In line with this, as demonstrated by kinetic studies, MeHg half-life, which is a direct determinant of the Hg body burden [231], can vary widely in humans, which may be due also to a naturally occurring biological basis for the variation in MeHg toxicokinetics.

Goodrich and colleagues [217] analyzed Single-Nucleotide Polymorphisms (SNPs; genetic variants due to a base substitution or the insertion or deletion of a single base) variability in a cohort of dental professionals exposed to inorganic Hg via dental amalgams and to MeHg via seafood consumption, in order to investigate potential associations with Hg levels in hair and urine. In this study, fish consumption as estimated by a self-administered survey was the best predictor of measured hair Hg level, and two SNPs were associated with this biomarker. In particular, SEPP1 3′UTR (rs7579) T allele was associated with lower hair Hg per unit of intake from fish consumption, while the GSS 5′ (rs3761144) minor allele (i.e., the less common allele of a SNP) (G) was associated with

increasing hair Hg concentration per unit of fish Hg. SEPP1 encodes a selenoprotein, which combats the oxidative stress created by Hg by binding the toxicant directly via a selenocysteine residue. The latter is an amino acid unique to selenoproteins that can bind Hg–selenium conjugates or MeHg. Interestingly, as demonstrated by previous studies, the 3′UTR T allele is linked to greater SEPP1 expression and Hg-binding capacity. The GSS gene encodes for an enzyme, glutathione synthetase (Figure 13a), that is involved in the synthesis of GSH, to which Hg is conjugated before being eliminated (Figure 13b). The association of the minor allele with increasing hair Hg concentration may be ascribable to a decreased expression of GSS and, thus, to decreased GSH synthesis, which in turn could impact the body's ability to eliminate MeHg as a GSH conjugate, with the higher body burden reflected in hair Hg levels.

The study of de Oliveira and colleagues [221] was the first to investigate the genetic predisposition to mercury accumulation in the plasma, where this pollutant is more bioavailable and therefore potentially harmful to human health. In this study, authors focused on riverside communities of the Brazilian Amazon, for which the only source of Hg exposure was the intake of contaminated fish. They genotyped two glutathione-related genes, GSTM1 and GCLC. The first encodes a glutathione S-transferase, an enzyme that catalyzes the conjugation of GSH to MeHg (Figure 13b), while the second encodes the catalytic subunit of the glutamate-cysteine ligase (GCL), the first rate-limiting enzyme of glutathione synthesis (Figure 13a). What the study found is that null homozygotes for GSTM1, that is, individuals that possess two copies of a non-functional allele for this gene, showed higher plasmatic MeHg levels (MeHgP) compared to subjects with functional GSTM1, which may be related to their lower MeHg-conjugating activity, lower MeHg excretion, and a higher MeHg retention. Moreover, individuals carrying at least one T allele for GCLC (rs17883901) also had significantly higher MeHgP.

As recent findings suggest, apart from being associated with hair mercury level, the SNPs in glutathione-related genes can influence the impact of methylmercury exposure on early child neurodevelopment. Wahlberg and colleagues [227] analyzed GSH-related gene variability in mothers with a diet rich in fish coming from the population of Seychellois. Genotypes of these mothers were analyzed in association with maternal hair and blood Hg, cord blood Hg, and children's mental and motor development, as expressed by the Mental Developmental Index (MDI) and the Psychomotor Developmental Index (PDI), respectively. The authors genotyped SNPs within three genes: GCLC, whose function have been described above; GCLM, encoding the modifier subunit of the GCL (Figure 13a), and GSTP1, which encodes a glutathione S-transferase (Figure 13b). What they found is that individuals with GCLC rs761142 TT genotype showed higher mean maternal hair Hg than AG and GG. Moreover, individuals carrying the combination of GCLC rs761142-TT and GCLM

rs41303970-CC genotypes showed higher hair Hg than G plus T carriers. Finally, increasing Hg in maternal and cord blood was associated with lower PDI among GCLC rs761142 TT carriers, while increasing Hg in hair was associated with lower MDI among GSTP1 rs1695 GG carriers.

Another recent study carried out on children from Valencia [228] showed that hair Hg levels were associated with worse neurobehavioral development, and that several SNPs located in the GSTP1 (rs1695) and BDNF (rs1519480, rs7934165, rs7103411) genes modified the association between Hg levels in children's hair samples and two indexes of neurobehavioral function. The brain-derived neurotrophic factor (BDNF), in particular, is a protein that promotes neural survival in adult brains, and is poorly expressed in several diseases, such as Alzheimer's and Parkinson's.

Two recent reviews [205,232] focusing on this topic collectively listed thirty-two genes whose variation is related to Hg body burden and susceptibility to Hg toxicity, and, in particular, twelve of these genes are related to hair Hg level and/or to MeHg exposure outcomes.

### 6.3.2 PAHs exposure and human genetic diversity

PAHs metabolism is a complex process consisting of two major phases (Figure 14): In the first phase, following ingestion or inhalation, the xenobiotic compound is epoxidated by enzymes belonging to the cytochromes P450 family, with the formation of diols and dihydrodiols [197]. Dihydrodiols can thus bind to the DNA, to give rise to DNA adducts, starting the mutagenic processes and eventually leading to cancer [32]. Then, in the second phase, the intermediate diols conjugate with glutathione, thanks to glutathione s-transferase enzymes. This leads to the formation of polar compounds, which can be easily excreted by renal or biliary routes [233]. The liver is the major site of the metabolism of PAHs. However, in the case of ingestion, gut micro flora and intestinal cytochrome P450 enzymes can also contribute to the process [197].

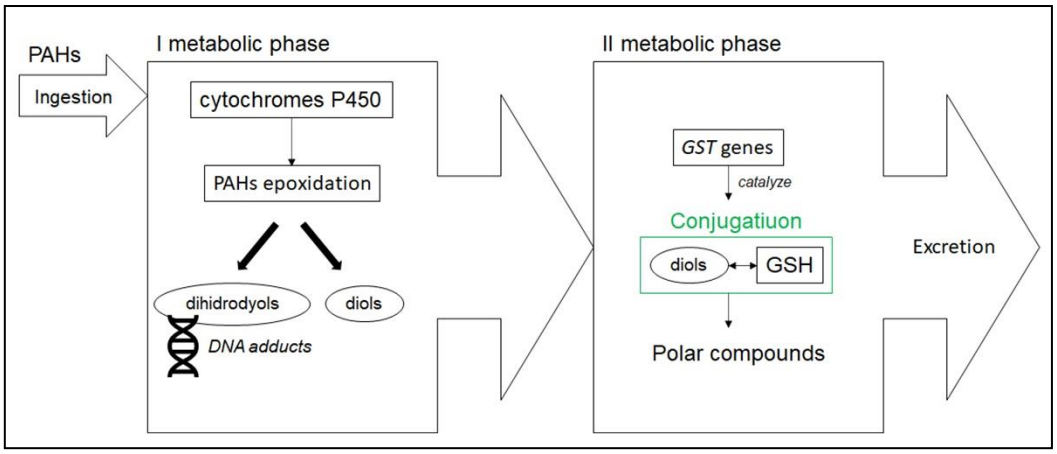



**Figure 14.** Schematic representation of the metabolic phases following PAHs ingestion. In the first phase, following ingestion or inhalation, PAHs are epoxidated by enzymes belonging to the cytochromes P450 family, with the formation of diols and dihydrodiols. Dihydrodiols can thus bind to the DNA, to give rise to DNA adducts. Then, in the second phase, the intermediate diols conjugate with glutathione, thanks to glutathione s-transferase enzymes. This leads to the formation of polar compounds, which can be easily excreted.

Epidemiological studies showed that polymorphisms at several genes influence levels of biomarkers of exposure to PAHs in several human populations (Table 10 and Table S2 of [100] for further details).

As suggested by much evidence, PAH–DNA adducts may be a potential source of heritable prezygotic DNA damage in spermatozoa. Ji and colleagues [229] detected PAH–DNA adducts in ejaculated sperm of infertile adults environmentally exposed to low levels of PAHs, showing that the consumption of PAH-rich meals at least three times a week contributed significantly to an increase in DNA adduct formation. Moreover, the authors demonstrated an association between specific XRCC1 polymorphisms and an increase in sperm adduct levels. XRCC1 encodes a protein that is essential to providing an efficient repair of the DNA, and thus polymorphisms at this gene may be useful to identify individuals susceptible to DNA damage resulting from PAHs exposure.

Myeloperoxidase (MPO), an enzyme central to the microbicidal activity of neutrophils, and *N*-acetyltransferase 2 (NAT2), which functions to both activate and deactivate drugs and carcinogens, are involved in Phase I and Phase II of PAH metabolism, respectively, while ERCC5 is a single-strand-specific DNA endonuclease that participates in DNA excision repair. The SNPs in these genes have been shown to affect the PAH-driven formation of the DNA adduct. In a study [230] of more than one hundred healthy female non-smokers from Golestan Province, a region in north-eastern Iran, characterized by very high levels of exposure to PAH probably due to diet and methods of food preparation, the DNA adduct level in blood was significantly lower in homozygotes for NAT2 slow

alleles, which are responsible for a less efficient detoxification of carcinogen-reactive metabolites, and the ERCC5 (rs1047768) non-risk-allele genotype. In contrast, DNA adduct level was higher in the MPO (rs2333227) homozygote risk-allele genotype.

In a cohort of non-smoking Polish mothers and newborns, Iyer and colleagues [202] observed a significant interaction between maternal exposure to airborne PAHs, measured by personal air monitoring, and SNPs in selected B(a)P metabolism genes on cord blood B(a)P–DNA adducts. These genes included: maternal CYP1A1 and GSTT2, and newborn CYP1A1 and CYP1B1. CYP1A1 and CYP1B1 are involved in metabolizing the parent B(a)P compound to the reactive B(a)P 7,8-diol-9,10-epoxide (BPDE) metabolite, which is involved in the formation of B(a)P–DNA adducts. In contrast, GSTT2 is involved in shifting B(a)P metabolism so as to prevent the formation of the reactive BPDE. In particular, the authors concluded that the T allele at the GSTT2 SNP position in mothers is protective with regard to cord B(a)P–DNA adduct formation, while the maternal and newborn G allele at the CYP1A1 SNP position, and the newborn G allele at the CYP1B1 SNP position, are not.

### 6.4 The epigenetic impact of seafood contaminants

Several studies have demonstrated the role of the environment in shaping human molecular variability at the epigenetic level in different populations [210,234–236]. Epigenetic changes are defined as any stable changes in the chromatin structure that are heritable from cell to cell, and can result in alteration of gene expression without altering DNA sequences [234]. The epigenome functions as an interface between the inherited genome and the dynamism imposed by the environment [237], and, as such, can be affected by the latter.

DNA methylation is among the most frequently studied epigenetic modifications. It consists in the covalent addition of a methyl group from a methyl group donor, the coenzyme S-adenosylmethionine (SAM), to the fifth carbon atom of a cytosine ring, and it is catalyzed by the DNA methyltransferase (DNMT) enzyme family. In mammals and insects, cytosine methylation is found almost exclusively in the context of CpG dinucleotides [234].

Changes in DNA methylation status influence genes' accessibility, thus altering gene expression, and aberrant DNA methylation has been discovered in a wide range of pathophysiological conditions [238].

Environmental chemicals, such as Hg and PAHs, can interfere with the one-carbon and citric acid metabolism pathways, resulting in anomalous DNA methylation status all over the genome [207]. Hg and PAHs can alter DNA methylation profiles in specific genes [239].

Three reviews address this topic [207,239,240]. Briefly, Ruiz-Hernandez and colleagues retrieve and discuss two and three epidemiologic studies investigating the association between DNA methylation and Hg [241,242] and PAHs [243–245], respectively, in adults. Considering all the strengths and weaknesses of the various studies, e.g., the lack of adjustments for potential confounding, such as sex, age, smoking status and tissue cell heterogeneity, the authors' conclusion is that the evidence they accrued supports the importance of environmental exposures in modulating the epigenome, but is insufficient to support causality because of the heterogeneity among epidemiologic studies in addressing the residual confounding of the associations, differences in DNA methylation assessment methods, and random error. The review of Culbreth and Aschner concludes that, despite some inconsistencies across different studies, dependent on the tissue or species examined, MeHg undoubtedly induces epigenetic modifications, and these modifications can potentially mediate its toxicity. In particular, regarding DNA methylation changes, controlled exposure studies on human and animal in vitro and animal in vivo models reveal that MeHg can lead to hypomethylation of the DNA in brain-derived tissue, but not in the liver, while selected individual genes show exposure-driven DNA hypermethylation.

Through a literature search, we identified seven (Table 11 and Table S3 of [100] for further details) and eight (Table 12 and Table S4 [100] for further details) epidemiological studies assessing the impact of MeHg and PAHs on DNA methylation. Below, we describe some of the main findings of the above studies. Please refer to the tables for the full list of retrieved publications.

**Table 11.** List of epidemiological studies investigating the impact of MeHg exposure on DNA methylation. Genes in which the differentially methylated CpG dinucleotides were identified, biomarkers measured, tissues from which DNA was extracted, and samples studied are shown for each study.

| Study | Genes | Biomarker | Tissue (DNA) | Sample |
|---|---|---|---|---|
| [241] | GSTM1 | Whole blood Hg | Whole blood | Women from San Francisco |
| [242] | SEPP1 | Hair Hg | Buccal mucosa | Michigan dental professionals |
| [246] | TCEANC2; ANGTP2; PRPF18; FOXD2 | Cord whole blood Hg and MeHg | Cord blood | Newborns from Baltimore, USA |
| [247] | PARM1; PFKFB3; LGMN; CCDC68; LRBA; FBXO31; (see Table S2 of [100] for the whole list) | Maternal toenail Hg | Cord blood | Mother–infant pairs from USA |
| [248] | EMID2 | Infant toenail Hg | Placenta | Rhode Island infants |
| [249] | PON1 | Maternal red blood cell Hg | Cord blood and children buffy coat | Mother–children pairs from Massachusetts, USA |
| | GRIN2B; NR3C1 | Maternal hair Hg | Children saliva | Children from Europe and US populations |

**Table 12.** List of epidemiological studies investigating the impact of PAHs exposure on DNA methylation. Genes in which the differentially methylated CpG dinucleotides were identified, biomarkers measured, tissues from which DNA was extracted, and samples studied are shown for each study.

| Study | Genes | Biomarker | Tissue (DNA) | Sample |
|---|---|---|---|---|
| [250] | ACSL3 | PAM | Umbilical cord white blood cell | Nonsmoking Dominican and African American mother-infants pairs |
| | Global DNA methylation | Pyr and B(a)P in PAM | Umbilical cord blood leukocytes | Nonsmoking women from NYC |
| [251] | IRS2 | Nap, Ace, Fl, Phe and Ant in VAT | VAT | Nonsmoking women from Korea with myoma |
| [252] | LINE1 | B(a)P-DNA adducts | Buffy coat | Nonsmoking pregnant women from Tongliang County, China |
| [253] | 239 quality-controlled autosome CpGs | Urinary ΣOH-PAHs, 9-OH-Phe and 1-OH-Pyr | Whole blood | Nonsmoking healthy Chinese individuals |
| [254] | PAX3 | ΣH_PAHs in maternal serum | Fetal neural tissue | Mother–fetus pairs |
| [209] | ZIC4 | ΣH_PAHs in fetal liver tissue | Fetal neural tissue | NTD fetuses |
| | PLEC1 | Urinary ΣOH-PAHs | | Nonsmoking healthy Chinese individuals |

1-OH-Pyr, 1-hydroxypyrene urinary metabolite; 2-OH-Nap, 9-hydroxynaphthalene urinary metabolite; 9-OH-Phe, 9-hydroxyphenanthrene urinary metabolite; Ace, acenaphthene; Ant, anthracene; B(a)P, benzo(a)pyrene; Fl, fluorine; Nap, naphthalene; NTD, neural tube defects; PAM, personal air monitor; Phe, phenanthrene; PMA, phenylmercuric acetate; Pyr, pyrene; VAT, visceral adipose tissue; ΣH_PAHs, sum of high-molecular weight PAHs including pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene; ΣOH-PAHs, total urinary monohydroxy-PAH metabolites.

### 6.4.1 The impact of the exposure to MeHg on DNA methylation

Epidemiological investigations on different populations have demonstrated the ability of Hg to impact the DNA methylation pattern in several genes (Tables 3 and S3 [100] for further details).

A study [241] showed increased DNA methylation of the GSTM5 promoter in women with higher Hg levels in whole blood. This gene is a member of th eGSTM gene family, which encodes for enzymes that are involved in the metabolism of several environmental agents.

Hg can also influence the DNA methylation status of genes that are involved in the protection against chemical toxicity. As previously mentioned, the SEPP1 gene encodes a protein known to bind Hg that has antioxidant properties. Its promoter shows a trend of DNA hypomethylation with increasing hair Hg levels, which was predicted by estimated Hg from fish consumption [242].

Some of the neurologic outcomes of the exposure to Hg were associated with DNA methylation changes [208]. The suppressive effect that MeHg exposure has on the expression of the

BDNF gene, which is poorly expressed in depressed patients, seem to be mediated also by the hypermethylation of the DNA [255]. As demonstrated by Maccani and colleagues [248], after crossing the placenta, MeHg can disrupt placental DNA methylation patterns, leading to the DNA hypomethylation of the EMID2 gene, and likely to adverse neurobehavioral outcome in infants. Cardenas and collaborators [249] found that, in male children, maternal prenatal blood mercury levels were associated with DNA hypomethylation of the Paraoxonase 1 gene (PON1), a gene involved in drug and fatty acids metabolism, and the DNA methylation pattern of this gene predicted lower cognitive test scores during early childhood. It is important to note that cord blood Hg level has previously been demonstrated to be a more accurate measure of prenatal MeHg exposure than maternal hair Hg level, and so DNA methylation changes associated with this biomarker potentially reflect MeHg effects more accurately [240]. In another very recent study , carried out on 406 mother–child pairs from a population who consume large amounts of fish and who are characterized by hair Hg levels that are higher than those in European and US populations, the authors found a positive association between prenatal MeHg exposure and DNA methylation in two nervous system-related genes, GRIN2B and NR3C1, measured in children's saliva. GRIN2B encodes a subunit of receptors that are important for the regulation of neural morphology, learning and memory, while NR3C1 is a receptor that is crucial to the stress responses in the brain. As stated by the authors, the observed DNA methylation changes associated with MeHg prenatal exposure at these two genes are predicted to lead to lower gene expression, and are likely to influence neurodevelopment and mental health.

### 6.4.2 The impact of the exposure to PAHs on DNA methylation

Both in vitro and in vivo analyses have revealed the ability of these substances to disrupt human DNA methylation patterns [207] (Tables 4 and S4 of [100] for further details).

The developing fetus is particularly susceptible to PAH-induced DNA damage, and studies support the hypothesis that this may be due also to epigenetic dysregulations caused by these chemicals. In a study of non-smoking African-American and Dominican women from New York City, the authors found that prenatal exposure to PAHs measured using a personal air monitor was associated with lower global DNA methylation levels measured in umbilical cord blood DNA.

Kim and colleagues [251] analyzed samples of visceral adipose tissue of non-smoking female patients with myoma. They showed that the DNA methylation level of IRS2 gene increased as the concentrations of PAHs in adipose tissue increased. Interestingly, the IRS2 gene mediates the effects of insulin on various cellular processes, and it has been associated with several diseases, such as type 2 diabetes. Furthermore, promoter methylation of the IRS2 gene turned out to mediate the

transcriptional silencing of this gene in the same study, and this led the authors to suggest that exposure to PAHs might contribute to the pathogenesis of insulin resistance through the methylation-mediated suppression of IRS2.

PAHs can accelerate human aging through epigenetic modifications. In their study of Chinese and Caucasian populations, Li and collaborators [253] first developed a DNA methylation age predictor based on the methylation status of many CpG sites across the genome, and then defined two aging indicators: Δage, defined as methylation age minus chronological age; and aging rate, defined as the ratio between methylation age and chronological age. Evaluating the association of PAHs exposure biomarkers with the above-defined aging indicators, the authors found that the increase in several urine PAHs metabolites was associated with an increase in both Δage and aging rate.

Possible hints of PAHs-mediated DNA methylation changes that may affect neurodevelopment emerged also from a study of pregnant women living close to a coal-fired power plant in China [252]. In that study, the authors analyzed cord blood samples for PAH–DNA adducts and assessed global DNA methylation by measuring genomic long interspersed nuclear elements (LINE1) methylation. LINE1 is one of the transposable repetitive elements, repetitive DNA sequences scattered across the genome and found in most eukaryotic organisms, which can change their position. Changes in LINE1 methylation can disrupt gene expression, and have been associated with birth defects, such as NTDs. In Lee and collaborators' study, a significant inverse relationship was observed between PAH–DNA adducts and LINE1 DNA methylation. Interestingly, the latter was a positive predictor of IQ (Intelligence Quotient) scores at 5 years of age in women enrolled before the closure of the power plant.

Neural tube defects (NTDs) are common and severe congenital malformations that arise from a failed or disordered closure of the neural tube during embryogenesis. Studies have linked NTDs to abnormal genome-wide DNA methylation. Authors found that PAX3, a gene encoding a transcription factor involved in development, is hypermethylated in NTD cases, and that the mean DNA methylation level of this gene in fetal neural tissue is positively correlated with median concentrations of PAHs in maternal serum [254]. Moreover, mean DNA methylation levels in the promoter region and 5′ UTR of ZIC4 gene tended to be inversely associated with levels of HMW-PAHs in the livers of NTD fetuses in a recent survey [209]. ZIC4 encodes a zinc finger protein whose absence can hamper cerebellum development in both humans and mice.

Concerning potential mechanisms underlying DNA methylation changes driven by Hg and PAHs, several findings support different hypotheses. Evidence exists that MeHg exposure is associated with the reduced expression or biochemical activity of DNMT, but Hg may also affect the methionine cycle, thus influencing the availability of SAM for DNA methylation [18]. Moreover,

various studies support the hypothesis that oxidative stress mediates the effects of PAHs exposure on DNA methylation, via both the suppression of DNMT and excessive SAM consumption [209].

Even if not exhaustive, given that they report all the evidence on the subject beyond the scope of this paper, the above-described results demonstrate the ability of these important seafood pollutants to impact the human epigenome and, in particular, the DNA methylation profiles.

### 6.5 An anthropological perspective

Human populations that traditionally consume seafood are at an increased risk of MeHg exposure and bioaccumulation. This is supported by recent data that demonstrate that populations consuming more fish or marine mammals have greater blood MeHg values than those consuming marine foods less than once a week [101]. Moreover, evidence exists that fish-eating populations tend to show the typical symptoms associated with Hg exposure at a high rate [189]. One of the first studies addressing this topic was carried out on a cohort of 1022 consecutive singleton births from the Faroe Islands [256], where maternal exposure to MeHg is derived from the consumption of pilot whale meat. This study found a statistically significant relationship between higher prenatal Hg exposure and poorer scores on tests of neurologic function [257]. In a cross-sectional study conducted on the adults of six fishing villages of the Pantanal region of Brazil, Hg exposures associated with fish consumption, as measured by hair mercury levels, were associated with detectable alterations in performance in tests of fine motor speed, dexterity, and concentration, and the magnitude of the effects increased with hair mercury concentration, consistent with a dose-dependent effect [258].

At present, unlike the case of MeHg, there is no direct evidence that populations that consume high amounts of seafood are more exposed to PAHs. Nonetheless, evidence exists that traditional fish smoking methods can introduce potentially harmful combustion by-products into the smoked fillets, leading to concentrations of PAHs that pose a threat to human health [259,260]. Moreover, human exposure to PAHs in seafood may date back to ancient times: with fish, shellfish and sea mammals being rich in fats, and considering the high lipophilicity of PAHs, these foods may have had absorbed substantial amount of PAHs from the bitumen used for prehistoric container production [14]. Finally, as detailed below, several investigations revealed high levels of PAHs in several commercially relevant marine species, with concentrations sometimes exceeding legal limits.

Modern technologies allow us to explore human molecular variation, both at a locus-specific and at a genome-wide level, enabling us to answer several questions about population evolutionary history and the relationship between environment and human biodiversity.

The first evidence of human adaptation to a toxic chemical was reported in arsenic-exposed women from the northern Argentinean Andes [261]. The inhabitants of this region, which is characterized by elevated arsenic concentrations in available drinking water, show a uniquely efficient arsenic metabolism. Accordingly, the authors found that the AS3MT gene, which encodes the arsenite methyltransferase and functions as the major gene for arsenic metabolism in humans, strongly differentiates the Argentinean Andes population from a highly related Peruvian population much less exposed to this environmental toxicant. Then, they confirmed that SNPs mapping in that gene was positively selected.

Similar results were obtained from investigations on another Andean community. Through analyses of ancient human remains from the Camarones Valley, it has been shown that the inhabitants of the area have been exposed to arsenic-contaminated drinking water for the last 7000 years [262]. Interestingly, a decreasing trend has been detected in the average hair and bone arsenic levels, starting from Archaic hunter–gatherers and leading to the current populations, and this evidence has been interpreted as the potential result of an adaptive increasingly efficient metabolic detoxification [263]. In support of the above scenario, analyses carried out through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), targeting SNPs strongly associated with arsenic metabolization, showed that, contrary to alleles associated with increased toxicity risk, protective variants are much more frequent in exposed populations compared to a southern Chilean community [264].

Potential hints of human adaptation to PAHs exposure come from the comparison between the exome sequence (i.e., the sequence of nucleotides that make up the protein-coding portion of the genome) of the aryl hydrocarbon receptor (AHR) gene in Neanderthal, Denisovan and modern human individuals [15]. Once activated by endogenous or exogenous ligands, such as diet-derived metabolites and PAHs, respectively, the complex made up of AHR and other proteins passes from the cytoplasm to the nucleus. Here, following further biochemical mechanisms, the AHR regulates the CYP1A1/1A2/1B1 genes expression, thus initiating PAHs metabolism, with the resultant production of PAHs' reactive metabolites and DNA adducts. Hubbard and colleagues found that modern humans carry the same allele at a codon of the AHR gene that is unique to our species, and which is associated with a reduced AHR activation by PAHs, specifically 2,3,7,8-tetrachlorodibenzofuran (TCDF), B(a)P and benz(a)anthracene, compared to Neanderthal and other primates receptors. On the other hand, modern humans and Neanderthal AHR showed similar levels of activation by endogenous ligands. Based on the above results, the authors postulate that exposure to potentially toxic environmental AHR ligands, such as PAHs derived from controlled fires in caves,

may have driven the selection of genetic variants conferring a reduced sensitivity to AHR exogenous ligands, and thus a lower DNA adduct synthesis [15].

As demonstrated also by the above-mentioned studies, molecular anthropologists are only starting to depict the role that environmental toxicants could have had in human evolution, and this is thanks to modern genomic technologies. Moreover, environmental toxicants can impact human biological variability at multiple levels, as shown by above-mentioned studies on the DNA methylation.

Environmental pressures can shape DNA methylation variability across human groups, and methods have been developed to explore the epigenetic side of human diversity at different levels. In a study analyzing the DNA methylation profiles of three human populations at 450,000 CpG sites, the authors found that DNA methylation differences contribute to the phenotypic variability of these populations, and that 68% of differentially methylated CpG sites were significantly related to underlying genetic variation, while the remaining 32% was probably related to external stimuli able to induce epigenetic changes with an impact on subsequent generations (e.g., toxic xenobiotics, differences in dietary or hormone exposure, or stress response) [265].

Many toxicants are sources of differences within human populations [239]. In Giuliani and colleagues' [266] investigation, for example, the authors compared the DNA methylation of individuals living in areas that were heavily sprayed with Agent Orange during the Vietnam War with that of individuals from non-contaminated areas. What they found is that past exposure to dioxin, the main ingredient of Agent Orange, led to DNA methylation changes among Vietnamese individuals from areas heavily sprayed, and in those whose parents participated in the war in sprayed zones.

## 6.6 Mediterranean coastal communities as an informative case study

Certain Mediterranean communities may be more exposed to the negative effects of seafood contaminants, for both environmental and cultural reasons.

The Mediterranean Sea is a semi-enclosed basin, surrounded by countries highly industrialized and with high agricultural development, and, as such, is of particular concern with respect to contamination by toxic compounds. The Mediterranean Sea is generally considered a geological hot spot for mercury [17], as it is characterized by large deposits of HgS that account for about 65% of the global mercury reserves [125]. Moreover, it should not be surprising that several studies have shown higher levels of contaminants in marine organisms from the Mediterranean Sea compared to those from other geographic areas [70,135,164,267,268], with levels of PAHs and especially Hg often exceeding recommended limits for human consumption [64,104,128,135]

Concerning Hg, in particular, the highest concentrations in Europe tend to be found in fish caught in the Mediterranean Sea [126]. Data showed a more marked Hg bioavailability in the Tyrrhenian and the Adriatic coastal waters compared to the rest of the Mediterranean [127], and MeHg levels higher than the legal limit have been discovered in seafood caught in both areas [104,128,130–133,269], as well as in the Ionian Sea (Sidimar 2018), and in different classes of marine organisms, including fishes, crustaceans, and mollusks. MeHg contamination hotspots are represented by the Trieste gulf [52], the coastal waters between Cattolica and Rimini, in the Central Adriatic Sea [133], and those between Anzio and Civitavecchia, in the Central Tyrrhenian Sea [159]. Turning to PAHs, potential contamination hotspots are generally deemed to be located near Taranto, Trieste [52], Naples [66,75], Genoa and Palermo [75]. It is also worth reporting that some studies revealed possible risks to human health arising from the consumption of PAHs-contaminated seafood from the Mediterranean.

In Europe, coastal populations consume greater amounts of seafood compared to inland populations [118]. Moreover, Mediterranean coastal populations are characterized by food habits based on local seafood consumption [17], and European countries bordering the Mediterranean are among the world's highest seafood consumers, with Spain, Italy and France accounting for more than half of the European expenditure on fish and fishery products, despite having only around a third of the EU's population (EUROSTAT, 2014). In Italy, apparent consumption of fish and seafood products amounted to 28.4 kg per capita, a share significantly higher than the EU average (EUROFISH, 2015). The traditionally high consumption of local seafood can lead to high MeHg and PAHs exposure levels among Mediterranean communities.

The above scenario is supported by several lines of evidence. Assuming an exposure frequency of 365 days a year, an exposure duration of 80 years (equivalent to the average lifetime in Italy in 2011), an ingestion rate of 18 g per day, and a body weight of 70 kg, after measuring Hg levels in various species of demersal fish commonly consumed in Italy, Storelli and Barone calculated a high target hazard quotient (THQ) and estimated weekly intake (EWI) for larger fish specimens caught in the Adriatic Sea [132]. In a study taking into account various commercially relevant marine species from the Ionian Sea, assuming a consumption rate greater than once per week, the authors found a possible risk for chronic systemic effects derived from Hg content [156]. Similar findings were derived from several other studies on seafood from various parts of the Mediterranean Sea, focusing on levels of Hg [133,158] and PAHs [55].

Given all the above, and considering the high average seafood consumption in the Mediterranean regions, we highlight the need for policymakers to take into account evidence of

potentially high exposure to seafood contaminants among Mediterranean communities, especially as regards MeHg, and to consider if it is reasonable to revise law limits and/or recommendations.

In line with the above-mentioned evidence, studies on newborns and preschool children from Mediterranean populations have shown high Hg concentrations in blood and hair [135]. Analyzing data collected from over 200 cross-sectional studies measuring Hg biomarkers in human populations, Basu and collaborators [121] found geographic differences in Hg exposure, with pooled central median blood mercury concentrations being higher in general background populations (i.e., those with no particular or significant exposure to mercury) living in certain geographic areas, including the Eastern Mediterranean. They also found that subpopulations that consume high amounts of seafood are approximately four times more exposed than the general background population. In particular, exposures were higher in Indigenous people in many regions of the world, in populations living in proximity to water bodies or associated with marine ecosystems, among which were populations living along the Mediterranean Sea.

Višnjevec and colleagues [118] compared the results of several investigations on Hg exposure in European countries, and found that the highest hair Hg levels were found in Madeira fishermen, habitual tuna consumers in Sardinia, and Greenlandic children and mothers.

A study on the adult population of Naples, Italy, found a strong correlation between total mercury concentrations (THg) in hair and fish consumption, while almost no association was found between THg and number, surface and area of dental amalgam fillings, another possible source of MeHg in the general population, thus confirming previous findings of the major role of seafood consumption in human exposure to this pollutant [270]. Moreover, the same study found THg levels higher than the reference dose adopted by the U.S. Environmental Protection Agency (0.1 µg per kg body weight) in 5.9% of the samples.

Finally, it is interesting to note that two of the three Mediterranean countries taken into consideration in the EU-funded human biomonitoring study named DEMOCOPHES , i.e., Spain and Cyprus, with the fourth being Slovenia, are respectively the first and the third countries as regards mercury levels in the hair of mothers compared to all the others, with Spain being also the second European country for per capita seafood consumption in 2016 [271], as well as the Mediterranean country with the second highest share of domestic wild capture consumption compared to imported seafood (FAOSTAT, 2018), after Croatia, which was not included in the DEMOCOPHES study.

Some subgroups of the Mediterranean coastal population may be more exposed than others to the harmful action of seafood pollutants. As shown by the above-described review from Basu and colleagues [121], coastal communities are more exposed to MeHg. A study [272] of mother–infant pairs from Croatia found higher levels of Hg and selenium (Se) in hair, blood, placenta and cord blood

of mothers from the coast compared to those living in continental areas, due to higher fish consumption. Interestingly, in this study, the authors also evaluated the relationship between Hg and Se levels and a polymorphism (rs28366003) in the MT2A-5 gene, which encodes a protein that plays a role in the detoxification of heavy metals, but they did not find any association.

Fishermen, in particular, have shown a tendency for a greater accumulation of Hg derived from fish, and this is related to their higher mean consumption of this food compared to the general population. Evidence in this sense comes from a fishing community on the Mediterranean coast of Morocco. In this community, researchers measured hair Hg levels, and found that these were closely related to fish intake, and that fishermen and their families were the most exposed population subgroup [113]. Additionally, in Sicily, greater Hg accumulation has been proven in fishermen, as they showed significantly higher mean hair Hg levels ($6.45 \pm 7.03$ μg $g^{-1}$ vs. $0.23 \pm 0.4$ μg $g^{-1}$ in the control group) [119]. Similar results have been derived from investigations carried out on Italian coastal communities from the northern Adriatic Sea [273].

What may emerge from all the above is that Mediterranean fishing communities could represent an informative case study to gain insight into the potential impact of Hg and PAHs on the human genome and epigenome.

Additionally, to fulfil the need for an "ecogenetic approach" to the study of the health effects of environmental chemicals stressed by Basu and colleagues [18], what we suggest is to extend the research on Mediterranean seafood contamination by Hg and PAHs by including information about the genomic and epigenomic backgrounds of the exposed communities. Such an approach would involve the following main steps: identification of communities that are particularly exposed to seafood contaminants, in terms of both cultural (i.e., traditional high consumption of local seafood) and environmental factors (i.e., subsisting on resources caught from pollution hotspots), and the selection of communities that would represent the control group, for example inland communities, characterized by a very low fish intake; simultaneous collection of biological samples (e.g., buccal mucosa cells) and information on the family history, diet and lifestyle of participants (e.g., through a Food Frequency Questionnaire); analysis of the genetic variability and DNA methylation profiles of those genes implicated, for example, in fatty acids metabolism and in susceptibility to environmental chemicals. Such an approach would enable us to answer different questions, such as, are there any biological differences between fishing and non-fishing communities that could have been caused by different seafood intakes? Are there any differences in the biological predisposition of Mediterranean communities to the health effects of seafood intake?

## 6.7 Challenges

The main challenges in such a study refer to the epigenetic investigation, and this is for several reasons. First of all, DNA methylation may be influenced by several factors [239], including many dietary components (e.g., folate, vitamin B6, vitamin B12, betaine, methionine and choline) [234], other environmental chemicals [207], pathogens load, various environmental and climatic conditions [274], sex and age [207], socioeconomic status [275], and genetic background [210,237]. Consequently, it is difficult to tell which is the actual correlative factor underlying the observed patterns, even when an association between a given factor or biomarker has been detected.

The simultaneous analysis of genetic and epigenetic data, coupled with information on eating habits and lifestyle and personal details of participants, would allow us to account for several potential confounders, possibly distorting the association between estimated exposure and epigenetic changes. To this end, it is important to gather information that is crucial to depict the whole set of environmental stimuli affecting the individual's methylome (e.g., smoking status, diet, occupation), as well as to identify, sample and to compare populations that differ markedly when it comes to seafood consumption rate and/or levels of Hg and PAHs in fish consumed.

However, it is worth noting that the potential simultaneous exposure of individuals to a plethora of chemicals constitutes one of the main challenges in the field.

Seafood, along with other food items, contains several nutrients and contaminants, which can impact human biology at a molecular level, and this may confound the association between MeHg and/or PAHs exposure and DNA methylation.

Among seafood contaminants, the heavy metals arsenic (As), cadmium (Cd) and lead (Pb) also constitute an emerging issue due to their concentrations often exceeding regulatory limits [132,276,277] and studies have demonstrated their ability to elicit DNA methylation changes [239]. This is quite expected, as comparisons of the mechanisms of action reported similar biological pathways of these metals inducing toxicity, such as ROS generation, weakening of the antioxidant defense, enzyme inactivation, and oxidative stress (for a detailed review see [278]).

Moreover, epidemiological studies showed a general hypomethylation of LINE-1 elements after PAHs, As, Cd and Pb exposure.

In vitro and animal studies performed under rigorous experimental conditions constitute a powerful method to identify the impact of single chemicals on DNA methylation. In this respect, molecular anthropological investigations could help to make a list of candidate genes to be tested through functional studies, or vice versa, could constitute a method to evaluate the real effect.

Another crucial aspect to consider is the tissue-specificity of DNA methylation [207]. Most of the retrieved epidemiological studies on MeHg and PAHs epigenetic effects measured DNA methylation in blood, with only one study using buccal mucosa [242], another study sampling saliva , one study measuring adipose tissue DNA methylation [251], and two studies using neural tissue [209,254]. Molecular anthropologists, on the other hand, often collect saliva or buccal mucosa cells as an alternative DNA source, because whole blood is difficult to collect during fieldwork [279,280]. It is also important to note that, despite the risk of discordant results due to potential tissue-specific DNA methylation changes (and especially to heterogeneity in cell composition), several studies demonstrated high correlations between the DNA methylation profiles of blood and saliva [281–283], pointing to the suitability of saliva as a source for genomic DNA in cohort studies. In the same way, recent investigations have shown that DNA methylation also correlates well between saliva and the brain [284]. Additionally, buccal cells also offer potential advantages to human epigenetic studies, as they represent a better surrogate tissue for brain tissues, with both being ectodermal tissues, and because they can be collected via a non-invasive method (buccal swabs) [285]. Finally, it should be noted that statistical methods to account for cell composition in DNA methylation assays are implemented and available [286].

Seafood is not the only source of exposure to Hg and PAHs. Working with dental amalgam fillings and working or residing among artisanal and small-scale gold mining sites result in elevated exposures to elemental and inorganic Hg [121], which may lead to DNA methylation changes [242,287]. In the same way, several occupations, such as coke oven manufacturing, chimney sweeping, paving and roofing, entail relevant exposure to PAH mixtures [288,289], with consequent impacts on the DNA methylation status [245,290]. Asking participants about their occupation is therefore of fundamental importance in order to address potential confounders of the association between exposure level to MeHg or PAHs via seafood consumption and DNA methylation changes.

As regards the influence of genetics on the individual's biological response to MeHg and PAHs exposure, it should be considered that, sometimes, the same genes are involved in the toxicokinetic of and/or susceptibility to different substances. This, obviously, complicates the detection of genes that may be subject to natural selection driven by a specific chemical. This is the case of GSTP1, MT4 and ALAD genes, whose variation can influence the toxicokinetic of Hg (Tables 1 and S1), but also of Cd [291] and Pb [292–294]. The same is true for NAT2 gene polymorphisms, which influence the toxicokinetics of both Hg [226] and PAHs [230] (Tables 3 and 4).

A further level of complexity is related to the fact that concentrations of these chemicals in edible tissues of aquatic organisms are influenced by several factors.

Several studies have highlighted the role of trophic level, habitat and size of the organism in determining the level of MeHg in sea animals. In particular, despite discordant evidence [130,155], MeHg concentration in fishes often increases with trophic level [148,159], size and age [164], with MeHg uptake being a process of bioaccumulation during the whole life [104], and other studies show that MeHg concentrations are affected also by changes in feeding habits during fish lifespan [158]. The relationship between size and MeHg concentration in marine invertebrates is rather less clear: while some results point to a positive correlation between these two variables in bivalves and crustaceans [133,295], others show a negative [269,296] or no significant correlation [133] in the same taxonomic classes, or even in the same species. As regards PAHs, only few studies have tried to assess the influence of biological factors on their accumulation in aquatic organisms [53], and these led to discordant results. Several studies found no significant correlation between PAHs concentrations and fish size or age [41,46,297,298], while Frapiccini and colleagues [51] found a negative correlation between body size and PAH concentrations in the liver and gills of common soles caught in the Po Delta and off Chioggia. Additionally, the sex [297–299] and the reproductive stage [53] of the organism seem to affect PAHs accumulation and metabolism in fish.

MeHg and PAHs concentration in fish also vary with seasons [37,41,48,53,59,162] and with the geographic origin of the fished specimen, with some areas of the Mediterranean being more polluted than others.

Addressing the above factors through a questionnaire is not easy, if not impossible, which means that a proper estimate of the habitual seafood intake or, more generally, of the eating habits of the sampled individuals, does not always correspond to a precise estimate of their habitual MeHg and/or PAHs intake [300].

To overcome these limitations, the most effective solution is the use of biomarkers of exposition. In particular, as already mentioned, hair mercury level has proven to predict exposition to MeHg [272,301], whose primary route in the general population is seafood consumption [302], while urinary excretion of the metabolite 1-OHP has been attributed mainly to the ingestion of PAHs through the diet [201,303].

However, it is important to note that, unlike MeHg, seafood is not always the main dietary contributor to PAHs intake, and that the concentrations of PAHs in food are also influenced by cooking procedures [304]; as a consequence, it would be difficult to tell whether eventual epigenetic modifications correlated with 1-OHP urinary level are actually driven by PAHs in seafood, unless a very detailed questionnaire on eating habits is collected.

As regards the uncertainties on the geographic origin of seafood consumed, sampling fishermen could help trace the origin of the fish they eat, as fishermen tend to consume their own

catch (unpublished data). Moreover, as already mentioned, fishermen represent an interesting case study, given their exposure to high levels of MeHg and, potentially, PAHs, due to their traditionally high seafood consumption. Studying fishermen, however, implies the inclusion of a potential further modifier of the effect of MeHg and PAHs on DNA methylation, that is, the typical lifestyle of fishermen. Fishing is strongly demanding, both physically and psychologically, implying, among the several challenges, working long hours, frequent night shifts, the unpredictability of the sea, and prolonged separation from the family [305]. Moreover, several investigations have linked the above factors to health conditions and harmful habits that are common among fishermen from different parts of the world, including the Mediterranean Sea [306], which comprise tobacco smoking, alcohol abuse, sleep deprivation, chronic stress, and so on . Such habits are known to impact human DNA methylation , and hence must be taken into consideration when asking sampled individuals about their daily life.

## 6.8 Conclusions

The Mediterranean Sea is considered a pollution hotspot for both natural and anthropogenic factors. As a consequence, Mediterranean communities may be particularly exposed to MeHg and PAHs through ingestion, due to their traditional high consumption rate of local seafood, and much evidence supports the above scenario. MeHg and PAHs can impact DNA methylation patterns in humans, even at low doses. Moreover, some of the epigenetic changes associated with MeHg and PAHs exposure are in turn associated with their known health outcomes. Finally, increasing evidence points to a significant contribution of human genetic variability in determining individual susceptibility to the chronic exposure to these chemicals, which, in certain cases, may be the results of population adaptation to certain ecological settings. In this framework, and also considering the growing concern about MeHg pollution due to climate change, we highlighted the benefit of an integrated approach, including molecular anthropologists and environmental and marine chemists, to the investigation of the relationship between the molecular diversity of Mediterranean communities and the exposure to MeHg and PAHs through seafood intake. Such an approach will help us to cope with uncertainties when it comes to risk assessment and decision-making about contaminant limits in seafood.

## 8. General discussion, conclusions and future prospects

When investigating the association between a given environmental stimulus and an epigenetic modification, one should consider several potential confounding factors. In particular, if the estimation of the exposure level to a given contaminant has to be done via an indirect method— e.g., via a FFQ—, it is of vital importance to take into account all the factors that could make the assessment imprecise. As emerged by the meta-analysis reported in section 3, one of these factors is the seasonal and geographic variability in seafood contamination. The meta-analysis of numerous investigations on PAHs in Western and Central Mediterranean seafood has revealed that the concentration of PAHs in edible tissues of several marine species varies with season—concentrations tend to be higher in cold months—and latitude—concentrations of PAHs in Mediterranean mussel caught along the Italian coast of the Adriatic Sea are negatively correlated with latitude in warm months, and positively correlated with latitude in cold months, whereas PAH concentrations increase from the southern to the northern part of the Tyrrhenian basin.

Then, I developed and validated a new FFQ and compared its results against those of the analysis of total mercury measured in the hair of the same individuals, as described in section 4. The reported newly developed questionnaire shows a high accuracy in the estimation of habitual mercury intake, similar to the one measured through the analysis of the hair. Moreover, the questionnaire allowed to gain insights into the Hg exposure level among a Mediterranean community living in an area which is considered a contamination hotspot—i.e., the Trieste gulf—, which is confirmed as being suitable for inclusion in a study on the molecular impact of this contaminant.

As reported in section 5, I have created a biobank made of oral mucosa samples from members of several Italian communities, together with information on their dietary habits, lifestyle and general health gathered through our newly developed FFQ. These samples will be analysed using the genome-wide methylation screening tool Infinium MethylationEPIC, Illumina. This technology allows to look into DNA methylation patterns at a genome-wide level. The aim of the study is to search for correlation between DNA methylation and environmental factors, such as diet and, in particular, seafood consumption rate as estimated by our food frequency questionnaire.

The information gathered through the questionnaires will enable us to account for several factors potentially affecting DNA methylation patterns, i.e. sex, age, smoking status and general health. Moreover, disposing of several couples of relatives allows the investigation of the role played by inheritance and common genetics background in DNA methylation changes.

Finally, the review reported in section 6 serves as a guide for the molecular analyses that will be carried out in the future, as it describes genes, molecular pathways and biological mechanisms that could be impacted by seafood contaminants and that are worth including in the investigation.

*Appendix*

## Food Frequency Questionnaire

1) How many generations is your family engaged in fishing? (for fishermen)

2) What kind of fishing do you practice now and for how long? (for fishermen)

3) How many days you go fishing now? (for fishermen)

4) How many hours per day you spend fishing now? (for fishermen)

5) What percent of the seafood you eat is part of your own catch? (for fishermen)

6) Are you following a particular diet now? If so, since when?

7) Are you taking some dietary supplement now? If so, what's its commercial name? Since when?

8) How often did you eat seafood in the last six months?

| | |
|---|---|
| ☐ Never | ☐ Twice a week |
| ☐ Less than once a month | ☐ 3-4 times a week |
| ☐ Once a month | ☐ 5-6 times a week |
| ☐ 2-3 times a month | ☐ Once a day |
| ☐ Once a week | ☐ 2 or more times a day |

9) How often did you eat the following species in the last six months?

**Species**: Kitefin shark (*Dalatias licha*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Mediterranean mussel (*Mytilus galloprovincialis*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Gulper shark (*Centrophorus granulosus*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Gilthead seabream (*Sparus aurata*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Dogfish (*Squalus blainville*, *Squalus acanthias*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: European anchovy (*Engraulis encrasicholus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Porbeagle (*Lamna nasus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: European seabass (*Dicentrarchus labrax*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Ray (*Leucoraja circularis*, *Raja clavata*, *Raja asterias*, *Raja miraletus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Clam (*Ruditapes philippinarum*, *Ruditapes decussatus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Black scorpionfish (*Scorpaena porcus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Common octopus (*Octopus vulgaris*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Mediterranean moray (*Muraena helena*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Rainbow trout (*Oncorhynchus mykiss*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Smooth-hound (*Mustelus mustelus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Salmon (*Salmo salar*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Atlantic bonito (*Sarda sarda*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: European hake (*Merluccius merluccius*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Squid (*Loligo vulgaris, Loligo forbesi*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Blackmouse catshark (*Galeus melastomus*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Shrimp (*Parapenaeus longirostris, Aristeus antennatus, Aristaeomorpha foliacea, Metapenaeus monoceros, Solenocera membranacea, Metapenaeus stebbingi*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Mediterranean spearfish (*Tetrapturus belone*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Prawn (*Penaeus kerathurus, Penaeus semisulcatus, Penaeus japonicus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Tuna (*Thunnus thynnus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Cuttlefish (*Sepia officinalis, Sepia orbignyana, Sepia elegans, Sepia spp.*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Lesser spotted dogfish (*Scyliorhinus canicula*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Atlantic mackerel (*Scomber scombrus, Scomber colias, Scomber japonicus, Scomber spp.*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Albacore (*Thunnus alalunga*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: European perch (*Perca fluviatilis*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Norway lobster (*Nephrops norvegicus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Sole (*Microchirus ocellatus, Solea solea, Buglossidium luteum*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Swordfish (*Xiphias gladius*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Sea trout (*Salmo trutta*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Little tunny (*Euthynnus alletteratus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Pelagic stingray (*Pteroplatytrygon violacea*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: European pilchard (*Sardina pilchardus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Spotted flounder (*Citharus linguatula*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Mullet (*Mullus barbatus, Mullus surmuletus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Common/rockpool prawn (*Palaemon serratus, Palaemon elegans, Palaemon spp.*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Stargazer (*Uranoscopus scaber*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Angler (*Lophius budegassa, Lophius piscatorius*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Poor cod (*Trisopterus minutus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Thickback sole (*Microchirus variegatus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: European conger (*Conger conger*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Small red scorpionfish (*Scorpaena notata*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Greater forkbeard (*Phycis blennoides*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

| | | | | |
|---|---|---|---|---|
| ☐ 50-150 grams | ☐ 250-350 grams | ☐ 450-550 grams | ☐ 650-750 grams | ☐ 850-950 grams |
| ☐ 150-250 grams | ☐ 350-450 grams | ☐ 550-650 grams | ☐ 750-850 grams | ☐ 950-1000 grams |

**Species**: Big-scale sand smelt (*Atherina boyeri*)

Frequency:

| | | |
|---|---|---|
| ☐ Less than once a month | ☐ Twice a week | ☐ 2 or more times a day |
| ☐ Once a month | ☐ 3-4 times a week | |
| ☐ 2-3 times a month | ☐ 5-6 times a week | |
| ☐ Once a week | ☐ Once a day | |

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Grouper (*Epinephelus caninus*, *Epinephelus marginatus*, *Epinephelus aeneus*, *Epinephelus fasciatus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: Silver scabbardfish (*Lepidopus caudatus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species**: European lobster (*Homarus gammarus*)

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

10) Are there any other species that you ate frequently during the last six months? List as many as possible, specifying the consumption frequency and the habitual portion size.

**Species:** _____

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species:** _____

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species:** _____

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
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Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species:** _____

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
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Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species:** _____

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

**Species:** _____

Frequency:

☐ Less than once a month ☐ Twice a week ☐ 2 or more times a day
☐ Once a month ☐ 3-4 times a week
☐ 2-3 times a month ☐ 5-6 times a week
☐ Once a week ☐ Once a day

Habitual portion size:

☐ 50-150 grams ☐ 250-350 grams ☐ 450-550 grams ☐ 650-750 grams ☐ 850-950 grams
☐ 150-250 grams ☐ 350-450 grams ☐ 550-650 grams ☐ 750-850 grams ☐ 950-1000 grams

11) Do you have any dental amalgam filling? If so, how many and since when? _____

12) Do you smoke? If so, how many cigarettes/packs a day? _____

13) Have you ever done a permanent wave treatment or other kinds of hair treatment? If so, what kind? How long ago? _____

14) Do you know if you had contact with mercury during the last six months? If so, how and how long ago?

15) Have you ever broken a mercury thermometer? If so, how long ago?

16) Body weight: ____ kg

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