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**TESI**

# *"Detection of environmental pollutants in food and the role of bioindicator organisms in environmental monitoring"*

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*I would like to express my sincere gratitude to the people who have been close to me during the PhD course.*
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# Abstract

The research activities carried out during my three-years of doctoral studies in Veterinary Sciences are part of a study to monitor particularly widespread and potentially toxic environmental pollutants, using of marine bioindicators, specifically *Eriphia verrucosa*, *Mugil cephalus*, *Diplodus annularis* and *Mullus barbatus*; the freshwater macroinvertebrate *Procambarus clarkii*; *Apis mellifera* and the products of the hive (honey, wax, pollen).

Five biomonitoring studies in which I partecipated are presented in this doctoral thesis.

The biological indicator of the first work was the crab species *Eriphia verrucosa*. The aim was to determine the concentrations of polycyclic aromatic hydrocarbons (PAHs) and heavy metals in the muscle of specimens of the warty crab (*Eriphia verrucosa*), caught at two sites on the northern coast of the Campania region (Italy), and to assess the risks to consumers.

The qualitative and quantitative analysis of PAHs was carried out using a high-performance liquid chromatograph (HPLC). The concentrations of Pb, Cr and As in the digested samples were determined with an atomic absorption spectrometer (GF-AAS, analyzt 600, Perkin-Elmer, Bonenseewerk, Germany).

The results indicate low Pb, Cr and PAHs contamination in the study areas, pointing ut a low risk for human consumption. The total As content was high, with an average value of 5.021 mg $kg^{-1}$ w.w.

This study showed that *Eriphia verrucosa* can accumulate environmental pollutants in its muscle tissue and highlighted the importance of monitoring the presence of these pollutants in crabs, and more generally in all fish products, to ensure food safety for consumers.

The invasive species *Procambarus clarkii*, also known as Louisiana red swamp crayfish, was the biological indicator used in the second study, reported in Chapter 2.

The aim of the research was to determine the concentrations of xenobiotic heavy metals (Hg, Cd, Pb), the metalloid As and trace elements (Cu, Cr, Zn) in the abdominal muscle and hepatopancreas of sixty specimens of *Procambarus clarkii* collected from two areas with different anthropic impact in the Campania region (Italy).

The results showed a low level of contamination in the selected areas and a different distribution of contaminants both in the analysed matrices and in the capture areas, confirming the capacity of *P. clarkii* tissues to bioaccumulate heavy metals and the direct correlation with the level of environmental contamination and the duration of exposure to these contaminants.

The study presented in the third chapter uses *Apis mellifera* and the products of the hive as bioindicators.

The study started during the Covid-19 pandemic and aimed to assess the impact of the lockdown, imposed by the government, on the presence of eleven heavy metals in the environment.

Samples were collected at three different times during the pandemic lockdown: end of May (T1), after a few months of total shutdown of all human activities; end of July (T2), partial resumption of activities; end of October (T3), total resumption of activities and international mobility. Approximately 100 foraging bees, fresh wax (20 g) and pollen (20 g) were collected at each site. Detection and quantification of Cd, V, Cr, Mn, Ni, Cu, As,

Sb, Ba, Pb and Hg were performed using a Thermo Scientific™ ICAP™ RQ inductively coupled plasma mass spectrometer (Q-ICP-MS).

The results showed that the heavy metal levels bioaccumulated by our bioindicators during the lockdown (T1) were statistically lower than the levels detected during partial and total resumption of activities (T2 and T3).

In the study reported in the chapter four, the same trace elements were quantified in honey samples collected from the same sites as in the above study. The carcinogenic and non-carcinogenic risks of honey consumption in toddlers, adolescents and adults were estimated using the Target Risk Quotient (THQ) and Lifetime Cancer Risk (LTCR). The analysis performed showed that: in no sample did Pb levels exceed the residual maximum level of 100 $\mu$g / kg established in Regulation (EU) 2015/1005; for the three consumer groups considered, there is a potential carcinogenic risk due to the presence of concentrations of Ni, Cr and As; toddlers reported higher exposure levels to the contaminants.

The biological indicators selected in the last study were adult specimens of *Mugil cephalus*, *Diplodus annularis* and *Mullus barbatus*, caught in an area between Baia Domizia and the mouth of the river Sarno (Campania region, Italy).

The levels of phthalates (PAEs) and microplastics (MPs) in muscles and gills of the three fish species were determined to assess the presence of these pollutants in the environment and the toxicological risk to consumers from eating fish contaminated with PAHs and MPs.

For the analysis of PAEs, solid-phase extraction and GC-MS were used for all samples, while an infrared microscope was used for the detection of MPs after pre-digestion of the muscle and gill samples of *Mugil cephalus*. The results showed a higher bioaccumulation of PAEs in *Mullus barbatus* than

in the other two species and a higher concentration in the gills than in the muscles.

MPs (polyamide, polypropylene, and high-density polyethylene) were detected in 50% of the gill samples, but no particle was detected in the muscle samples of *Mugil cephalus*.

Although the levels of contaminants sought were not particularly high, there is a potential carcinogenic risk for younger consumers of these fish species.

Le attività di ricerca svolte nel triennio di Dottorato di Ricerca in Scienze Veterinarie si inseriscono nell'ambito di uno studio atto a monitorare contaminanti ambientali particolarmente diffusi e potenzialmente tossici, mediante l'impiego di indicatori biologici quali: organismi marini, nello specifico le specie *Eriphia verrucosa*, *Mugil cephalus*, *Diplodus annularis* e *Mullus barbatus*; il macroinvertebrato di acqua dolce *Procambarus clarkii*; e *Apis mellifera* ed i prodotti dell'alveare (miele, cera, polline).

In questa tesi di dottorato vengono presentati cinque studi di biomonitoraggio a cui ho partecipato.

L'indicatore biologico del primo lavoro è stato la specie di granchio *Eriphia verrucosa*. Lo studio riguardava la ricerca di idrocarburi policiclici aromatici (IPA) e metalli pesanti nel muscolo di esemplari di granchio favollo (*Eriphia verrucosa*) catturati in due località situate lungo la costa settentrionale della regione Campania (Italia), e la valutazione del rischio per il consumatore. La determinazione quali-quantitativa degli idrocarburi policiclici aromatici è stata effettuata mediante cromatografo liquido ad alte prestazioni (HPLC). Le concentrazioni di Pb, Cr e As nei campioni mineralizzati sono state determinate con uno spettrometro ad assorbimento atomico (GF-AAS, analyzt 600, Perkin-Elmer, Bonenseewerk, Germania). I risultati di tale lavoro hanno mostrato una bassa contaminazione da IPA, da Pb e Cr, mentre sono stati rilevati livelli di As totale elevati, con un valore medio di 5,021 mg $kg^{-1}$ w.w.

Questo studio ha mostrato che la specie *Eriphia verrucosa* può accumulare contaminanti ambientali nel tessuto muscolare e mette in evidenza l'importanza di monitorare la presenza di questi inquinanti nei granchi, e in generale in tutti i prodotti ittici, al fine di garantire la sicurezza alimentare per i consumatori.

Nello studio esposto nel secondo capitolo è stato utilizzato come organismo

bioindicatore il *Procambarus clarkii*, una specie invasiva conosciuta anche come gambero rosso della Louisiana. Lo studio mirava a determinare le concentrazioni di metalli pesanti xenobiotici (Hg, Cd, Pb), il metalloide As ed oligoelementi (Cu, Cr, Zn), in muscolo addominale ed epatopancreas di sessanta esemplari di *Procambarus clarkii* prelevati in due aree a differente impatto antropico nella regione Campania (Italia).

I risultati di tale studio, pur rilevando un basso grado di contaminazione nei siti prescelti, hanno evidenziato una differente distribuzione dei contaminanti esaminati, sia tra le differenti matrici analizzate, sia tra i siti di cattura, confermando la capacità di bioaccumulo di metalli pesanti nei tessuti di *P. clarkii* e la diretta correlazione con il grado di contaminazione ambientale e con il tempo di esposizione a tali contaminanti.

Lo studio descritto nel terzo capitolo ha utilizzato come bioindicatore *Apis mellifera* ed i prodotti dell'alveare. Il lavoro ha preso inizio nel periodo di lockdown imposto dal governo durante la pandemia da Covid-19. I campioni sono stati prelevati in tre momenti diversi rispetto al lockdown pandemico: fine maggio (T1), dopo alcuni mesi di chiusura totale di tutte le attività antropiche, fine luglio (T2), ripresa parziale delle attività, e fine ottobre (T3), ripresa totale delle attività e mobilità internazionale.

Da ciascun sito di campionamento sono stati raccolti circa 100 api bottinatrici, cera fresca (20 g) e polline (20 g). Il rilevamento e la quantificazione di Cd, V, Cr, Mn, Ni, Cu, As, Sb, Ba, Pb, Hg è stata eseguita utilizzando uno spettrometro di massa al plasma ad accoppiamento induttivo Thermo Scientific™ ICAP™ RQ (Q-ICP-MS).

I risultati hanno mostrato che i livelli di metalli pesanti bioaccumulati dai nostri bioindicatori durante il lockdown (T1) erano statisticamente inferiori rispetto ai livelli riscontrati in tempi successivi alle restrizioni e alla ripresa delle attività (T2 e T3).

Nel successivo studio, riportato nel quarto capitolo, sono stati quantificati gli stessi elementi in traccia in campioni di miele prelevati nei siti dello studio sopradescritto. Sono stati stimati i rischi cancerogeni e non cancerogeni dovuti all'ingestione di miele nei bambini piccoli, negli adolescenti e negli adulti, utilizzando il quoziente di rischio target (THQ) e rischio di cancro a vita (LTCR).

Dall'analisi eseguita è emerso che: in nessun campione i livelli di Pb hanno superato il limite massimo residuale di 100 $\mu$g/kg fissato dal Regolamento (UE) 2015/1005; per i tre gruppi di consumatori presi in considerazione esiste un potenziale rischio cancerogeno dovuto alla presenza di concentrazioni di Ni, Cr e As; i bambini piccoli hanno riportato valori di esposizione più elevati.

Nell'ultimo studio descritto i bioidicatori selezionati sono stati esemplari adulti di *Mugil cephalus*, *Diplodus annularis* e *Mullus barbatus*, catturati in un'area compresa tra Baia Domizia e la foce del fiume Sarno (regione Campania, Italia). Sono state determinate le concentrazioni di ftalati (PAE) e microplastiche (MP) in muscolo e branchie dei pesci al fine di valutare la presenza nell'ambiente di questi contaminanti e il rischio tossicologico per i consumatori esposti.

Per l'analisi degli ftalati è stata utilizzata l'estrazione in fase solida e GC-MS, effettuata su tutti i campioni, mentre la rilevazione delle microplastiche è stata effettuata su muscolo e branchie di *Mugil cephalus*, utilizzando un microscopio ad infrarossi dopo una predigestione dei campioni.

I risultati di questo studio hanno mostrato un bioaccumulo maggiore di PAE in *Mullus barbatus* rispetto alle altre due specie e una maggiore concentrazione nelle branchie rispetto al muscolo. Le MP (poliammide, polipropilene e polietilene) sono state rilevate nel 50% dei campioni branchiali, e nessuna particella è stata rilevata nel muscolo.

Nonostante i livelli dei contaminanti ricercati non siano risultati particolarmente elevati, esiste un potenziale rischio cancerogeno per i consumatori più giovani di queste specie di pesci.

# Introduction

Unlike chemical-physical monitoring, which analyses only the abiotic factors of the ecosystem, biomonitoring aims to assess and protect the health of the environment by analysing the animal or plant species that inhabit the ecosystem. This systematic and regular process of recording and collecting data allows researchers not only to create a database of detailed information about the environment and its biota, and to define the characteristics of their interrelationship, but also to detect environmental stressors. The bond between organisms and habitat is so close that it is possible to assess the conditions of the former by observing the latter and vice versa.

Some organisms, sometimes entire communities of species, are able to indicate negative changes in environmental condition, by showing responses to pollutants that are both visible to the naked eye and detected by laboratory methods. These species are referred to as bioindicators: organisms that, because of their natural characteristics, are able to detect the presence of a source of stress to the environment and, consequently, to themselves. The response to such stressors can also occur in whole animal populations ("animal sentinel system" or SSA) and be manifested in their presence/absence or in the reduction of the number of individuals of a species in a given area. It is important to point out the difference between “bioindicators proper” and "bioaccumulators". Bioindicators proper detect environmental changes at physiological (biomarkers), morphological, and spatial distribution lev-

els; bioaccumulators can accumulate concentrations of one or more pollutants in their bodies ([1]). Some contaminants are accumulated by these organisms at higher concentrations than those derived from the environment and those excreted by themselves through detoxification and metabolic processes, and may reach increasingly higher concentrations as they move up the trophic chain ("biomagnification") or be accumulated directly through the environmental matrix ("bioconcentration").

In biomonitoring studies, the choice of biological indicators is crucial: a bioindicator is valid depending on the type of research to be conducted.

The possible presence of anthropogenic pollutants in different parts of an ecosystem is an important issue for researchers to address. Some of these pollutants are harmful even at low concentrations, and their persistence in one specimen can cause harm along the entire food chain, and become a hazard to living things involved in it, including humans.

The chemicals analysed by the research teams I was part of during my PhD were: Heavy metals, the metalloid arsenic, polycyclic aromatic hydrocarbons (PAHs), esters of phthalic acid or phthalates (PAEs) and microplastics (MPs). Heavy metals are one of the most persistent and harmful sources of pollution. Their presence in the environment is mainly due to anthropogenic activities related to industrial processes, and, to a lesser extent, to natural phenomena. Although some of these substances (e.g., Cu, Cr, Se, Mn, Zi) are essential for human metabolism and have harmful effects only in high doses ([2]), others, such as lead, cadmium, mercury, and the metalloid arsenic, can accumulate slowly and gradually in the body, damaging the kidneys, bones, lungs, liver, and the neurological system, and causing cancer, diabetes and various diseases ([3]; [4]; [5]; [6]).

PAEs and MPs are chemical and physical pollutants that are widely distributed in the marine environment. They can accumulate in biota, and

thus become a threat to the marine ecosystem and humans.

Marine pollution is a growing problem worldwide due to the continuous accumulation of pollutants from anthropogenic sources. The pollutants can be difficult to degrade, persistent, and widespread, so they can accumulate in the environment over a long period of time and become a health risk to living creatures. Due to the large amount of plastics entering the marine environment (12 million tons/year) ([7]), phthalates or phthalic acid esters (PAEs) and microplastics (MPs) are among the most abundant chemical and physical contaminants in the marine environment.

Phthalates (PAEs), originally introduced in the processing of plastics to give them more flexibility (later they were also used in cosmetics, drugs, paints, medical devices, and in the production of pesticides etc.), belong to the class of endocrine disruptors due to their effects on reproduction, metabolism and growth ([8]). Some of them may also have neurotoxic, genotoxic and carcinogenic effects ([8]; [9]; [10]; [11]).

The consequences of ingestion of MPs by both marine organisms, such as fish, and living organisms associated with their food chain, such as humans, can affect various tissues, and lead to hepatotoxicity, alterations in the immune and endocrine systems, disruption of the intestinal barrier, metabolism, and intestinal dysbiosis ([12]). They can also be toxic through the release of PAEs and transport of other pollutants such as heavy metals, persistent organic pollutants (POPs) and pathogens ([13]; [14]; [12]; [15]).

PAHs are chemicals characterized by strong lipophilicity, solubility in organic solvents, and high boiling and melting points. Living organisms can also be exposed to PAHs through inhalation or skin contact as well, but ingestion is the route that can lead to more adverse health effects in animals and humans ([16]; [17]). Due to their persistence, long-range transport, and ability to bioaccumulate in the food web, PAH contamination has become

a global concern. The toxicity potential of PAHs is so high that European institutions have established concentration limits for foods intended for human consumption. The International Agency for Research on Cancer (IARC) has classified sixteen different PAHs as hazardous compounds for human health because they are potentially carcinogenic and mutagenic.

In order to protect the health of the biota, the environment and especially humans (as consumers at the last trophic level), some of my works aim to verify the possible presence of contaminants not only in animal species, but also in food derived directly or indirectly from them, assuming a value for food safety.

In this context, products that are frequently consumed by humans as foods were studied: honey, a product derived from beekeeping that is also consumed by children, and the marine species *Eriphia verrucosa*, *Mugil cephalus*, *Diplodus annularis* and *Mullus barbatus*.

The detection of contaminants in these bioindicators was aimed at both monitoring the presence of pollutants in the selected areas and determining the toxicological risk to which consumers of these products are exposed.

The research I have done in the field of environmental biomonitoring has led me to detect contaminants in different ecosystems and to use different bioindicators. For the marine environment I analysed *Eriphia verrucosa*, *Mugil cephalus*, *Diplodus annularis* and *Mullus barbatus*, for freshwater streams, *Procambarus clarkii*, for the atmosphere, soil and vegetation, *Apis mellifera* and the products of the hive (pollen, wax, honey).

# Chapter 1

# Polycyclic aromatic hydrocarbons (PAHs), arsenic, chromium and lead in warty crab (*Eriphia verrucosa*): occurrence and risk assessment

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## 1.1 Abstract

This study assesses the PAH and heavy metal levels in muscle of warty crabs (*Eriphia verrucosa*), from the northern coast of the Campania region improving the data on toxic contaminants in this crustacean.

The results showed a minimal PAH contamination; the mean concentrations were as follows: 0.2, 1.6 and 1.7 µg $kg^{-1}$ wet weight (w.w.) for BaP, PAH4 and PAH6, respectively.

Regarding the levels of the two PAHs not included in the European regulations, the BkF mean concentration was 0.1 µg $kg^{-1}$ w.w., while DahA was detected only in 10.7% of samples. Pb and Cr were also detected at low levels with mean values of 0.068 and 0.468 mg $kg^{-1}$ w.w., respectively; instead, high As levels, with a mean value of 5.021 mg $kg^{-1}$ w.w., were found.

Considering the EWIs and the ILCRs calculated in this study, the PAH, Pb and Cr contamination levels found in the edible part of the crabs resulted safe for human consumption. Contrariwise, the ILCR calculated for the As exceeded the acceptable level of cancer risk, although the calculation did not refer to the inorganic form which is the only one recognized as carcinogenic. Hence, this study shows that warty crabs can accumulate environmental contaminants in their muscle tissue representing an important route of exposure to these toxics for the local population that regularly consumes them. This finding highlights the importance of monitoring the presence of these pollutants in crabs and in general in all fish and seafood in order to ensure food safety for consumers.

## 1.2 Introduction

Over the last decades, interest and awareness of institutional bodies, researchers and consumers in seafood safety have increased significantly. Fishes, mussels and crustaceans are part of the culinary traditions of several countries worldwide and represent an essential source of nutrients being rich in proteins, fatty acids, essential amino acids and vitamins ([18]). Despite this, seafood can represent also a route of human exposure to dangerous chemical substances.

Seafood safety is strictly linked to marine environment quality because many pollutants present in the aquatic environment can be bioaccumulated and biomagnified by marine organisms; therefore, concerns have been raised about the potential risks for human health derived by the consumption of contaminated fisheries products ([19]).

The Mediterranean Sea, as a semi-enclosed basin characterized by an intense naval traffic and industrial coastal activity, represents a geographic area highly sensitive to environmental pollution ([16]). Therefore, seafood from Mediterranean basin deserves to be carefully analysed to guarantee the safety of consumers and to provide reliable scientific data that can be exploited by the institutions to implement the panel of necessary analyses to maintain high standards of food safety and quality. Moreover, the monitoring of some aquatic species, because of their natural habitat, diet and position in the food chain, represents a useful bioindicator to collect data on the current health status of the marine ecosystem.

*Eriphia verrucosa* (Fig. 1.1) is a benthonic species of crustacean, also called the warty crab, that lives in shallow waters up to the rocky coastlines. It is a common species in the Mediterranean Sea, regularly found along the Italian Tyrrhenian coasts, feeding primarily on bivalves, gastropods and

polychaetes. Moreover, the warty crab is part of the traditional cuisine of southern Italy, especially of Campania region, and is widely consumed by the local population ([20]).

*Eriphia verrucosa* fishing takes place throughout the year and without limitations for both professional and not professional fishing. There are no minimum sizes to be respected for their fishing. The warty crab, because of its geographic distribution, position in the food web and consumption by humans, represents an optimal marine species for quali-quantitative toxicological investigations.

Among numerous contaminants present in the marine environment, polycyclic aromatic hydrocarbons (PAHs) are persistent pollutants widely diffused, in particular in harbours, estuaries and coastal waters. They originate from incomplete combustion and pyrolysis of organic material, in processes as fossil fuel combustion, waste incineration and accidental oil spills ([21]; [22]). PAHs are chemicals characterized by strong lipophilicity, solubility in organic solvents and high boiling and melting points. Living organisms can be exposed to PAHs through different routes, as inhalation or dermal contact, but primarily through ingestion that is considered the mainly way of exposure causing detrimental effects on animals and human health ([16]; [17]). Based on the evidence of their toxic potential, European institutions have issued two regulations regarding presence of PAHs in food for human consumption: the Commission Regulation (EC) 1881/2006 and its amendment (Commission Regulation (EU) 835/2011 2011) that establishes the maximum levels (MLs) in molluscs and some smoked fish products of four PAH compounds (benzo[a]pyrene (BaP), benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbF) and chrysene (Cry)) and the Commission Regulation (EC) 333/2007 (2007) and its amendment (Commission Regulation (EU) 835/2011, 2011) that defines the sampling and analytical

methods approved for PAH detection in food products.

The need to officially assess the presence of PAHs in food items and to set MLs that safeguard the public health is linked to the high toxicity of these chemicals. The International Agency for Research on Cancer (IARC) listed sixteen different PAHs as dangerous compounds for human health due to their ability to be potentially carcinogens and mutagens ([23]). Despite this, the EC regulation considers just four PAH compounds for which research in products intended for human consumption is mandatory. Moreover, EC regulation limits the research of PAHs only to two categories of fishery products: bivalve molluscs and muscle meat of smoked fish and smoked fishery products.

Among dangerous pollutants which can induce detrimental effects on human health, interfering with immune and reproductive systems, also trace elements can represent a risk for usual consumer of warty crabs. Although data regarding trace elements concentrations in warty crab are poor ([24]; [25]), Ariano et al. (2015) ([20]) reported high concentrations of cadmium (Cd) in this crustacean (whole animal) that could lead to health risks for the population that usually consume this seafood. For this finding and considering that marine environment is affected by other toxic metals, this study also intends to assess the contamination levels of arsenic (As), chromium (Cr) and lead (Pb) which are elements very relevant for food safety. These metals are widespread and persistent pollutants which can be found at high concentrations in marine environment close to greatly urbanized and industrialized areas such as coastal areas, estuaries and river mouths ([26]). These elements are well known for their ability to induce harmful effects both in acute and chronic exposures; moreover, it has been reported that toxic metals as Pb can provoke severe health disease even at sub-lethal concentrations ([24]).

Human exposure occurs mainly through food consumption; in particular, fish and seafood are recognised as the mainly contributors to human As intake ([27]).

The aim of the present study is to evaluate PAHs, As, Cr and Pb concentrations in muscle of the warty crabs. In addition to the assessment of the four PAH compounds included in the European regulations for food safety and quality, we investigated also the presence of benzo[k]fluoranthene (BkF) and dibenzo[a,h]anthracene (DahA) which show a similar toxicity ([28];[29]).

Figure 1.1: *Eriphia verrucosa* (Forskål, 1775)
https://www.naturamediterraneo.com/forum/topic.asp?TOPIC_ID=18218

## 1.3 Materials and Methods

### 1.3.1 Biological Materials

Twenty-eight samples of warty crab (*Eriphia verrucosa*) were caught from two different locations, Castel Volturno (site A) and Naples (site B), located along the northern coast of the Campania region (Italy) (Fig. 1.2). All samples were collected between May and July 2016.

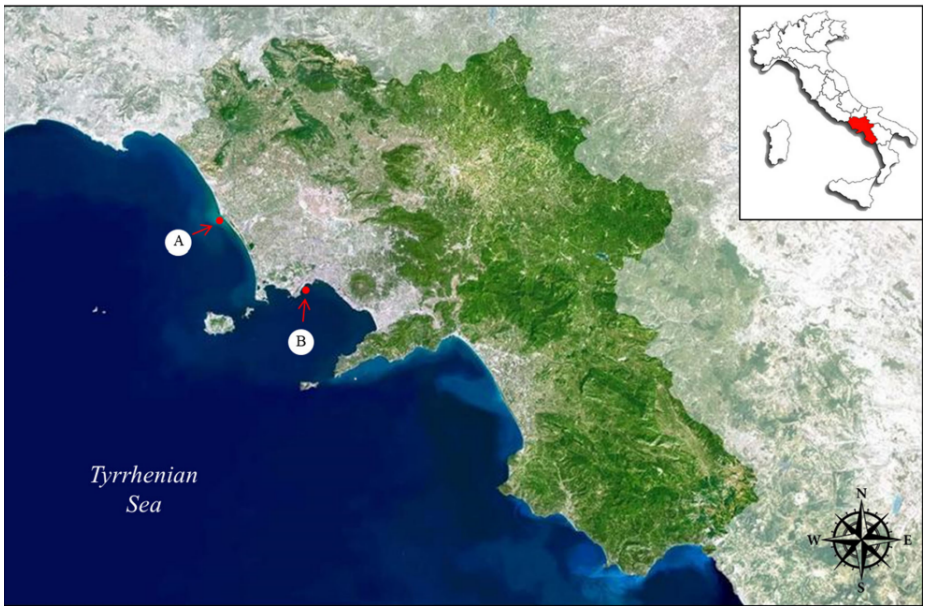


Figure 1.2: Map showing locations of the sampling sites: Castel Volturno (site A) and Naples (site B) in Campania region, Italy

Once captured, the crabs were euthanized at - 80 °C for 30 min. Then, the animals were weighed and measured using an Absolute Digimatic Caliper (Mitutoyo, Japan).

The length (cl) and width (cw) of their carapace ranged between 3.8 and 6.0 cm (mean value: 4.9 cm) and 5.0 and 8.0 cm (mean value: 6.2 cm), respectively. Then the crabs were immediately sealed in decontaminated polyethylene bags, frozen at -20 °C and kept at the same temperature un-

til delivery to the laboratory where they were dissected using steel tools including forceps, scissors straight and scalpels and analysed.

### 1.3.2 Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

Analysis of polycyclic aromatic hydrocarbons was performed according to the procedure described by Serpe et al. (2010) ([30]). In brief, the crab muscle from claws and appendages was individually separated, homogenized and weighed (2.0 ± 0.5 g). Each sample was saponified with 10 mL of a solution of potassium hydroxide (2 N in ethanol) and liquid/liquid extracted for three times with 20 mL of cyclohexane. The extract was filtered, reduced to small volume and purified using a silica Sep-Pak cartridge and eluted with acetonitrile (ACN).

The instrumental analysis was carried out by a high performance liquid chromatograph (HPLC) equipped with a fluorescence detector (Waters Alliance). Chromatographic separation was performed by an EnviroSep PP (125 × 3.2 mm, particle size 5 um, Phenomenex) LC column using the gradient elution with acetonitrile and ultrapure water as solvents at 0.5 mL $min^{-1}$. The fluorescence detection was performed at the excitation and emission wavelengths of 294 and 404 nm, respectively. External standard method was used to determine PAH concentration in the samples. Linearity of method was checked by triple injection of standard solution at concentrations between 0.4 and 20.0 ng $mL^{-1}$ obtaining a correlation coefficient ($r^2$) at least 0.999. The calibration curve was made for every sequence of analysis.

The limit of quantification (LOQ) was 0.2 µg $kg^{-1}$ for each PAH.

### 1.3.3 Analysis of Heavy Metals

Glassware and laboratory equipment were decontaminated before use with diluted ultrapure 65% $HNO_3$ (ROMIL-UpA, Cambridge, UK) and were rinsed with Milli-Q water (Millipore Corp., Bedford, MA).

For the analysis, the crab samples (0.50 ± 0.02 g) were weighed in Teflon vessels with 5.0 mL of 69% $HNO_3$ and 2.0 mL of 30% $H_2O_2$ (ROMIL-UpA) and placed in a microwave digestion system (Milestone, Bergamo, Italy). Microwave assisted digestion was performed with a mineralization program for 15 min at 190 °C. Then, the vessels were cooled at room temperature, and the digestion mixtures were diluted at the final volume of 50.0 mL by adding ultrapure water (resistivity 18.2 MΩ cm) that was produced in-house using a purification system arium® pro (Sartorius, Germany)([31]). Pb, Cr and As concentrations in the digested samples were determined with an atomic absorption spectrometer equipped with a graphite furnace and a L'vov platform (GF-AAS, Analyst 600, Perkin-Elmer, Bonenseewerk, Germany). The LOQs were 0.020, 0.050 and 0.165 mg $kg^{-1}$ for Pb, Cr and As, respectively.

### 1.3.4 Quality Assurance

In the laboratory, appropriate quality assurance procedures were implemented in order to ensure the reliability of the results in accordance with the UNI/EN/ISO/IEC 17025 Standard (2005). Quality assurance and quality control (QA/ QC) of the methods were monitored through analysis of procedural blanks, duplicate samples and standard solutions.

Standard solutions of analytes were prepared from certified stock solutions containing Pb, Cr, As (atomic spectroscopy standard, Perkin Elmer) and the PAH of interest.

Concentrations for each set of samples were determined in the medium range of the calibration curve. The performance of the method was assessed through participation in inter-laboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK).

### 1.3.5 Statistical Analysis

PAH concentrations were expressed in µg $kg^{-1}$ wet weight (w.w) as sum of BaA, Cry, BaP and BbF (PAH4) and sum of BaA, Cry, BaP, BbF, BkF and DahA (PAH6) using mean ± SEM (standard error of the mean). All metal concentrations were expressed in mg $kg^{-1}$ w.w. as mean ± SEM. Statistical significance of the influence of sampling sites (Castel Volturno Vs Napoli) has been tested using factorial analysis of variance. Furthermore, we apply ANOVA test to highlight differences between metals and PAH accumulation in the muscle of warty crabs and between the sampling areas. Multiple regressions have been used to discover statistical significance between metals and PAHs concentration and intrinsic variables (as length and width of specimens). One-sample Kolmogorov-Smirnov test confirmed normal distribution of our data. All our statistical analyses have been performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). Significant value has been established at $p < 0.05$. For statistical calculations, the contribution of the undetected PAHs was considered equal to zero; for the undetected metals, it was considered a contribution equal to 0.5 LOQ ([32]).

### 1.3.6 Estimation of Dietary Intake and the Carcinogenic Risk

In order to assess the exposition to PAHs, Cr, As and Pb of the population that regularly consumes warty crabs coming from the coasts of the Campania region and thus to evaluate the potential health risk resulting from it, the estimation of the weekly intakes were calculated using the levels of contaminants determined in the crab muscles. The calculations were carried out only for adults as they are considered the main consumers of these crustaceans. The estimated weekly intakes (EWIs) are calculated using the equation described by Lambiase et al. (2017)([33]) and reported below:

$$EWI = \frac{(C \cdot WI)}{BW}$$

where C is the mean concentrations of PAHs, Cr, As and Pb determined in crab samples; WI is the human weekly intake of crabs; and BW is the body weight (70 kg). The EWIs were calculated using both the WI of 37.7 g week$^{-1}$ obtained by the Food and Agriculture Organization (FAO) ([34])and an estimated WI of one 100-g crustacean edible portion ([35]).
In addition, to assess the carcinogenic risk associated with the intake of PAHs, Cr, As and Pb through the consumption of local crabs, the incremental lifetime cancer risk (ILCR) is also calculated using the equation described by Tiwari et al. (2017)([36]):

$$ILCR = \frac{ED \cdot EF \cdot EDI \cdot SF \cdot CF}{AT}$$

where ED is the exposure duration (83 year, Italian average life expectancy, ([37]); EF is the exposure frequency (365 day yr$^{-1}$); EDI is the estimated

daily intake (ng $kg^{-1}$ body weight (b.w.) $day^{-1}$); SF is the oral cancer slope factor in kg day $mg^{-1}$: 7.3 for BaP ([36]), 5 x $10^{-1}$ for Cr ([38]), 1.50 for As ([39]) and 8.5 x $10^{-3}$ for Pb ([38]); CF is the conversion factor (1.0 $\times$ $10^{-6}$ mg $ng^{-1}$ ); and AT is the average lifespan (30,295 days).

The ILCR for PAH4 was calculated using the PAH concentration expressed in BaP equivalent obtained employing the toxic equivalent factors (TEFs) ([36]).

## 1.4 Results

### 1.4.1 Polycyclic Aromatic Hydrocarbons (PAHs) concentration in crab

PAH levels in crab muscle samples are reported in Table 1.1. The range concentrations of PAH4 and PAH6 were between < LOQ and 4.9 µg $kg^{-1}$ w.w. for both the sums. Overall, the PAHs were detected in all samples except for seven crab muscles (25.0% of the total) that showed BaA, Cry, BaP and BbF concentrations below the LOQs. BaP was detected only in 39.3% of the total warty crabs, and its concentration ranged between < LOQ and 0.5 µg $kg^{-1}$ w.w.; BkF and DahA were detected in 25.0% and 10.7% of the total samples, respectively, and their range concentrations were < LOQ and 0.4 µg $kg^{-1}$ w.w. for both substances. The most abundant PAHs were BbF and Cry that contributed to PAH4 with 35.1% (mean: 0.9 µg $kg^{-1}$ w.w.) and 21.6% (mean: 0.4 µg $kg^{-1}$ w.w.), respectively.

The levels of PAHs assessed in muscle of *Eriphia verrucosa* varied between sampling sites (Fig. 1.3). Cry concentration was significantly higher in the crabs from Castel Volturno than those from Naples ($p < 0.01$); significant differences between site A and site B were also detected for BaP, PAH4 and PAH6 concentrations ($p < 0.05$).

The analysed individuals varied in length and width. The multiple regression analyses indicate that there was no correlation between size and concentration of all analysed PAHs ($p > 0.05$).

| | SITE A (n=13) | SITE B (n=15) |
|---|---|---|
| BaA | <LOQ-0.4 (0.0± 0.03) | <LOQ-0.5 (0.1± 0.05) |
| Cry | <LOQ-1.8 (0.7 ± 0.15) | <LOQ-1.2 (0.2 ± 0.11) |
| Bbf | <LOQ-3.4 (1.2 ± 0.27) | <LOQ-2.8 (0.6 ± 0.24) |
| Bkf | <LOQ-0.4 (0.1 ± 0.04) | <LOQ-0.4 (0.1 ± 0.03) |
| BaP | <LOQ-0.5 (0.3 ± 0.07) | <LOQ-0.5 (0.1 ± 0.05) |
| DahA | <LOQ - | <LOQ-0.4 (0.1 ± 0.04) |
| PAH4 | 0.9-4.9 (2.3 ± 0.33) | <LOQ-4.1 (1.0 ± 0.40) |
| PAH6 | 0.9-4.9 (2.4 ± 0.36) | <LOQ-4.5 (1.1 ± 0.45) |

Table 1.1: PAH concentrations (range and mean ± SEM) in *Eriphia verrucosa* expressed in µg $kg^{-1}$ w.w.

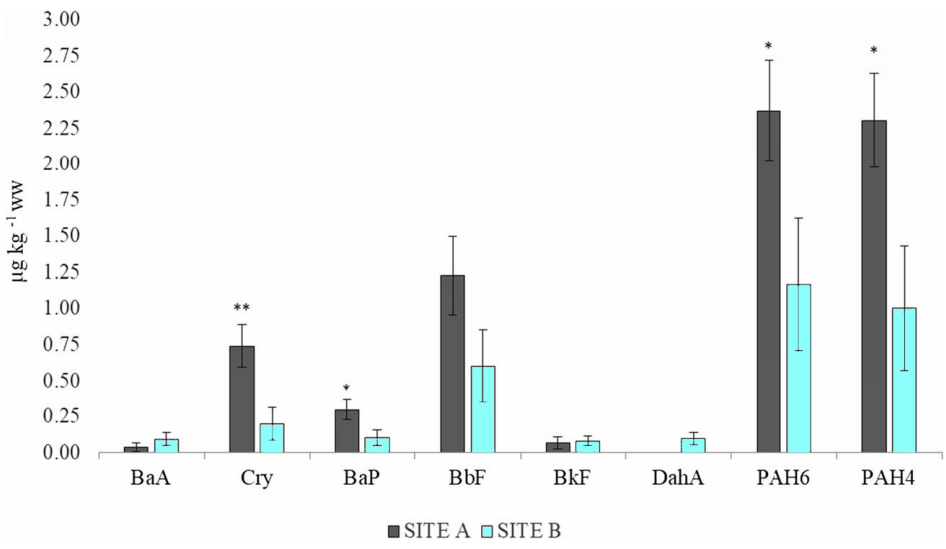


Figure 1.3: PAH concentrations in *Eriphia verrucosa* depending on sampling sites: A Castel Volturno (n = 13) vs B Naples (n = 15). Vertical bars represent average concentration (µg $kg^{-1}$ w.w.) ± SEM. Probability levels for significant differences: $p<0.01$ (**); $p<0.05$ (*)

### 1.4.2 Heavy Metals

Regarding the occurrence of metals in crab muscles, the results are reported in Table 1.2. Arsenic was the most abundant element detected in all samples; its concentration ranged from 0.985 to 14.555 mg $kg^{-1}$ w.w.

As was followed by Cr (range: <LOQ–3.216 mg $kg^{-1}$ w.w.) and Pb (range: < LOQ–0.242 mg $kg^{-1}$ w.w.).

Contrary to PAHs, there were no statistical differences between metals concentration and sampling sites ($p > 0.05$) (Fig. 1.4). The multiple regression analyses indicate that there was no correlation between size and concentration of all analysed metals ($p > 0.05$).

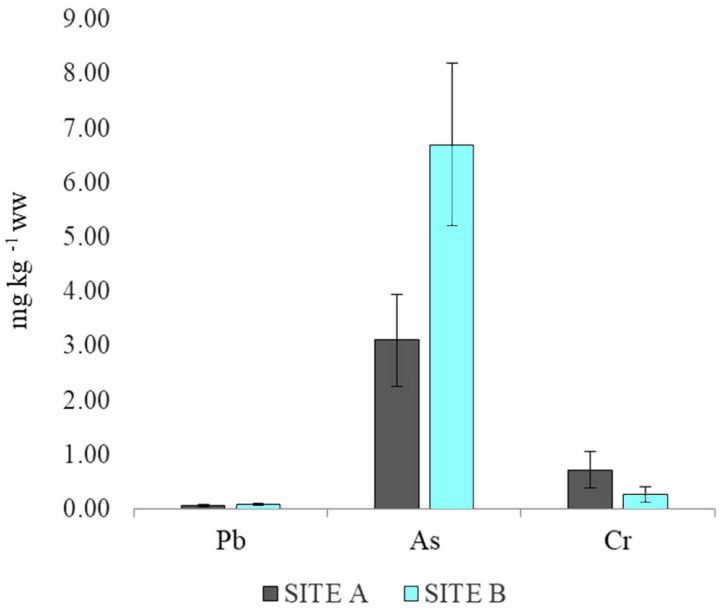


Figure 1.4: Heavy metal concentrations in *Eriphia verrucosa* depending on sampling sites: A Castel Volturno (n = 13) vs B Naples (n = 15). Vertical bars represent average concentration (mg $kg^{-1}$ w.w.) ± SEM. Probability levels for significant differences: $p<0.01$ (**); $p<0.05$ (*)

| | SITE A (n=13) | SITE B (n=15) |
|---|---|---|
| Pb | <LOQ–0.240 (0.056 ± 0.018) | < LOQ–0.242 (0.078 ± 0.016) |
| As | 1.093–10.243 (3.098 ± 0.850) | 0.985–14.555 (6.688 ± 1.491) |
| Cr | <LOQ–3.216 (1.2 ± 0.27) | < LOQ–2.318 (0.257 ± 0.150) |

Table 1.2: Metal concentrations (range and mean ± SEM) in *E. verrucosa* expressed in mg $kg^{-1}$ w.w.

## 1.5 Discussion

### 1.5.1 Polycyclic Aromatic Hydrocarbons

The crab muscle samples analysed in the current study showed low concentrations of each PAH investigated. The Commission Regulation (EU) 835/2011 2011 does not fix BaP and PAH4 MLs for crabs and in general for all crustaceans; therefore, it was not possible to assess whether the contamination levels determined in the samples were compliant with the EU Regulation. Nevertheless, the BaP and PAH4 concentrations reported herein resulted lower than the MLs set for smoked crabs and for other types of foodstuffs ([40]).

In addition to lacking of European and national regulations as regard PAH contamination in these marine organisms, there are also very few data available on this issue in literature which are also often reported in different way (dry or wet weight) making the comparisons difficult. Nevertheless, in order to assess the PAH contamination level of the crabs coming from the northern coast of Campania region in relation to crustaceans coming from other marine areas and hence the potential health risk of the consumption of this seafood, the results reported in this study were compared to the data described by few authors on crabs coming from other coastal areas.

Abdolahpur Monikh et al. (2014) ([41]) described BaP concentrations in muscles of *Portunus pelagicus*, sampled in the Persian Gulf that ranged from 170 to 956 ng $g^{-1}$ on dry weight (dw) (mean: 200 ng $g^{-1}$ dw). Considering that crustaceans have an average water content of about 80% ([42]), the mean BaP concentration expressed on wet weight became 40 ng $g^{-1}$ w.w., and therefore it was much higher than the mean values of BaP found in the present study. Zhang et al.(2020) ([43]), in a study on PAH bioaccu-

mulation in marine organisms from South Yellow Sea in China, determined in crab samples concentrations (sum of 16 PAHs) that ranged from 119.11 to 223.34 ng $g^{-1}$ dw resulting higher than our results. Moreover, they described that among benthic and benthivorous organisms, crabs showed lower PAH concentrations than shrimps and demersal fish. The mean PAH concentrations found in this study resulted instead higher than those found in crabs (*Callinectes amnicola*) from Atlas Cove (Nigeria) analysed by Olayinka et al.(2019) ([44]) who determined concentrations of the PAH6 below the detection limits in all samples.

Perugini et al. (2007) ([45]), in Norway lobster from Central Adriatic Sea, also showed Cry and BbF concentrations below the detection limit, while the BaA and BkF were slightly higher than the concentrations detected in this study. Therefore, the overall PAH level reported herein resulted comparable or lower than those described in other marine areas indicating a low risk for human health. In order to obtain more data on the bioaccumulation of PAHs in crustaceans from the coasts of the Campania region and the health risks for the population that consumes this food, the concentrations found in this study were compared with those reported by other authors that investigated this marine area. From comparing, the PAH levels detected in warty crabs resulted lower than the levels found in other fish and seafood species ([46];[47]).

The low levels of PAHs detected in the present study suggest that the exposure to these contaminants of the population of this area that consumes crabs and in general crustaceans and the consequent health risk are also low. All these findings led to suppose that the PAH concentrations found in the edible parts of the crabs can be considered at baseline levels.

It was interesting to find that the PAH concentrations determined in the crab muscles from Castel Volturno were significantly higher than those

found in the animals from Naples; this difference in concentrations could be attributed to the presence of the Volturno river that flows into the Tyrrhenian Sea at Castel Volturno. The Volturno is the longest Southern Italian river which crosses densely populated areas, such as the province of Caserta, and that collects pollutants mainly from zootechnical and agricultural activities, handcrafts and industries ([48]). Otherwise, the source of contamination of the Gulf of Naples is mainly represented by maritime transports, fishing and coastal tourism ([49]). Regarding the difference in PAH concentrations in relation to the size of crabs, it was not statistically significant in agreement to the results showed by other authors ([45]). As a concern, BkF and DahA were detected in crab muscles at levels comparable to PAHs included in Commission Regulation (EU) No 835/2011. BkF and DahA are classified by the IARC as possible and probable carcinogenic to humans, respectively.

On the basis of this classification, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) included also these two substances in the group of eight PAHs that are considered the only indicators of the carcinogenic potency of these contaminants in food (EFSA (European Food Safety Authority) 2008). Therefore, considering the BkF and DahA levels described herein in crabs, it may be recommended to develop new food safety plans to monitor also these two substances.

### 1.5.2 Heavy Metals

The heavy metal analysis carried out in this study showed low levels of Pb and Cr but a high presence of As in crab muscles samples. Interestingly and contrary to what was observed for PAHs, the heavy metal levels found in the crabs were higher than those found in fish and mussels coming from the

same marine area ([46]) showing that these organisms, as also described by other authors, can bioaccumulate toxic elements in their tissues when they live in polluted environment ([50]; [51]; [52]). Pb was detected at low levels in the crabs from both sampling sites resulting below MLs established in muscle meat of crustaceans (0.5 µg $g^{-1}$ w.w.) by the Commission Regulation (EC) N° 1881/2006 and its amendment ([40]). Concerning Cr and As, although some forms of these elements are recognized as carcinogenic to humans (Group 1) by the IARC ([53];[54];[55]), there are no MLs laid down for food by the European Commission.

Overall, As was the most abundant element detected in warty crab muscles followed by Cr and Pb. Comparison of the studies carried out by other authors showed that the As concentrations assessed in warty crabs from northern coast of Campania region resulted higher than those measured in the muscle of *Eriphia verrucosa* and *Rapana venosa* from Turkey ([56]), in the muscle of fiddler crab of *Uca tangeri* species (mean: 1.76 µg $g^{-1}$) collected from Spain ([57]) and also in the edible muscle of warty crab from the Black Sea that had As concentrations ranging from 1.34 µg $g^{-1}$ to 2.43 µg $g^{-1}$ w.w. ([24]). It has been reported that benthonic species that feed close to the coasts may bioaccumulate higher As levels than pelagic ones. In fact, being As naturally presents in rocks, marine environment near the coasts has an abundant amount of this element ([58]). In particular, Campania region is a territory characterised by high background levels of As of volcanic origin; in fact, high concentrations of this metal were found in the pyroclastic deposits in the NW and SE sectors of the region, including the coastal areas, as also in the Volturno River plain ([59];[60]). On the basis of this information, it was possible to hypothesize that the As levels found in the crabs analysed herein derived from natural sources due to the large volcanic area present in the region. Moreover, it is important considered

that of the total As (tAs) amount only the inorganic As (iAs) rate is harmful to human health. According to the data available in literature and the EFSA opinion, in fish and in general all seafood, the tAs include mainly arsenobetaine, and the iAs rate varies depending on the species of fish or seafood (EFSA, 2014).

However, Cubadda et al. (2016)([61]), in a study on the dietary exposure of the Italian population to iAs, found that crustaceans and molluscs are one of the food group with the highest iAs concentration (28.3 ng $g^{-1}$ w.w.).

Concerning the other two heavy metals measured, the Pb concentrations found in the muscle of *Eriphia verrucosa* in Naples and Castel Volturno sites were approximatively comparable than those measured in the muscle of warty crab from Turkey ([56]) and from Adriatic Sea ([25]). Instead, the levels of Pb resulted lower than the concentrations detected in muscles of *Rapana venosa* (0.1 to 0.7 µg $g^{-1}$) analysed by Mülayim and Balkıs (2015)([62]) and in the edible muscle of warty crab (0.13 µg $g^{-1}$ to 0.36 µg $g^{-1}$ w.w.) analysed by Durmus et al. (2018)([24]) both collected from the Black Sea. The Pb levels assessed in this study resulted also lower than the levels found in the edible muscle (0.10 µg $g^{-1}$) of Chinese mitten crabs (*Eriocheir sinensis*) from rivers and lakes of Netherlands ([63]), in muscles of the blue crab (1.08 ± 0.56 mg $kg^{-1}$) collected from the northern Bay of Bengal ([51]) and in muscles of the red crab from the Gulf of Mexico ([50]).

For Cr, the levels detected in the present study were comparable to those found in muscle of *Rapana venosa* (0.47 ± 0.01 µg $g^{-1}$) from the Black Sea ([64]) and in muscles of the blue crabs (0.68 ± 0.50 mg $kg^{-1}$ collected from the northern Bay of Bengal ([51]), while the Cr levels resulted higher than those found in muscle of *Rapana venosa* (0.1 to 0.2 µg $g^{-1}$) from the Black Sea analysed by Mülayim and Balkıs (2015)([62]) and in muscle of warty crab from Adriatic Sea ([25]). Moreover, it has been reported by many au-

thors that the bioaccumulation of toxic metals in crabs, and in general in all marine animals, depends on several physiological and biometric factors among which the body size is recognized as an important parameter ([65]; [66];[67]).

In the current study, the statistical analysis showed that the heavy metal levels were not statistically correlated to the size of the crabs ($p > 0.05$), suggesting that these parameters have a minor effect on metals accumulation in subjects inside the size range considered in this study. In fact, the length and width of warty crabs carapace in the present study ranged between 3.8 and 6.0 cm (mean value: 4.9 cm) and 5.0 and 8.0 cm (mean value: 6.2 cm), respectively. It has been described that the metal bioaccumulation is strongly influenced by metabolism in fish ([68]). Hence, same authors have been suggested that the negative correlation is probably due to a faster metabolism rate of the smaller animals, which correspond tothe younger specimens, than the older ones ([69]). Therefore, these processes could lead to a dilution of the contaminant concentration with growth. However, it has been observed that the negative correlation between metal concentrations and body size occurs when the marine pollution is at low levels; for high levels of pollution, instead, a positive correlation has been described ([69]).

### 1.5.3 Health Risk Assessment

The EWI calculated for BaP, PAH4, Cr, As and Pb that occurs through the consumption of crabs from the coasts of the Campania region are showed in Table 1.3. As regard PAHs, a human tolerable weekly intake has not been fixed. The EFSA Panel on Contaminants in the Food Chain (CONTAM) in its Scientific Opinion regarding polycyclic aromatic hydrocarbons

in food European Food Safety Authority 2008 ([70]) reported median values of consumer exposure to BaP and PAH4 for the food category fish and fishery products of 21 and 170 ng day$^{-1}$, respectively. The EWIs calculated using the values reported by EFSA 2008 ([70]) assuming a body weight of 70 kg (2.1 ng kg$^{-1}$ b.w. per week for BaP and 17 ng kg$^{-1}$ b.w. per week for PAH4) were higher than the EWIs calculated with the concentrations found in the present study.

| | BaP | PAH4 | Cr | As | Pb |
|---|---|---|---|---|---|
| EWI (WI 37.7 g week$^{-1}$)[a] | | | | | |
| min | nc | nc | 0.0135 | 0.5305 | 0.0054 |
| max | 0.0003 | 0.0026 | 1.7320 | 7.8389 | 0.1303 |
| mean | 0.0001 | 0.0009 | 0.2523 | 2.7041 | 0.0366 |
| median | nc | 0.0007 | 0.0439 | 0.9193 | 0.0304 |
| EWI EWI (WI 100 g week$^{-1}$)[b] | | | | | |
| min | nc | nc | 0.0357 | 1.4071 | 0.0143 |
| max | 0.0008 | 0.0070 | 4.5943 | 20.7929 | 0.3457 |
| mean | 0.0003 | 0.0023 | 0.6691 | 7.1728 | 0.0970 |
| median | nc | 0.0019 | 0.1164 | 2.4386 | 0.0807 |

[a] WI obtained from FAO, 2013[34]
[b] WI obtained from Di Lena et al. 2018[35]
*nc* Not calculable

Table 1.3: Estimated weekly intake (EWI) of BaP, PAH4, Cr, As and Pb calculated using both WIs (37.7 and 100 g week$^{-1}$) and expressed in µg kg$^{-1}$ b.w. week$^{-1}$

This finding showed that the exposure of the population to these contaminants through the consumption of local crabs involves a low health risk. Moreover, for human exposure risk characterization, in its opinion, EFSA used the bench mark dose lower confidence limit ($BMDL_{10}$) for a 10% increase in the number of tumour in animals ([70]).The $BMDL_{10}$ derived by EFSA (European Food Safety Authority) (2008)([70]) were 0.07 and 0.34 mg kg$^{-1}$ b.w. day$^{-1}$ for BaP and PAH4, respectively. Also considering these values, the consumption of local crabs resulting safe for human health. Regarding Cr, As and Pb, the CONTAM Panel established a TDI only for

Cr (III), supposing that all chromium in food is in this chemical form, that is 0.3 mg $kg^{-1}$ b.w. per day (2.1 mg $kg^{-1}$ b.w. per week), resulting higher than the value calculated herein. For As and Pb, the EFSA Panel has not been set any TDI or TWI values. Precisely, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) fixed a provisional tolerable weekly intake (PTWI) of 15 µg $kg^{-1}$ b.w. per week for iAs and 25 µg $kg^{-1}$ b.w. per week for Pb. These values were considered no longer suitable by the EFSA Panel that established for iAs a BMDL01 between 0.3 and 8 µg $kg^{-1}$ b.w. per day for an increased risk of cancer of the lung, skin and bladder, as well as skin lesions (EFSA, 2014) and for Pb a BMDL01 of 1.50 µg $kg^{-1}$ b.w. per day for an increased risk of cardiovascular effects and nephrotoxicity in adults (EFSA, 2012). Hence, as for the PAHs, the EWI values calculated for the metals showed a low exposure to these toxic pollutants for the population that consumes crustaceans.

Regarding the carcinogenic risk associated with the BaP, PAH4, Cr, As and Pb intakes through the consumption of crabs, the ILCRs calculated are reported in Table 1.4. For the carcinogenic risk assessment, it was set a threshold of 1.0 x $10^{-6}$ which means there is one in a million chances for an individual to develop cancer over a lifetime as a result of exposure to a carcinogen ([36];[38]); at this level, the cancer risk is considered negligible. The risk becomes serious when the ILCR exceeds the threshold of 1.0 x $10^{-4}$ ([38]). The ILCRs calculated in this study were in the range between 4.46 x $10^{-3}$ and 6.53 x $10^{-9}$. The highest ILCR values, which exceed the threshold of 1.0 x $10^{-4}$, were obtained for As and for Cr, when the ILCR was calculated using the maximum Cr concentration found in the crabs, indicating that for these elements, there is a potentially risk for human health. However, these results were obtained using the total concentration of As and Cr and not the rates recognised as carcinogenic, which are iAs

and Cr (VI); hence, due to the lack of information, it was impossible to make toxicological discussions.

| | BaP | PAH4 | Cr | As | Pb |
|---|---|---|---|---|---|
| ILCR (DI 5.38 g day$^{-1}$)[a] | | | | | |
| Min | nc | nc | 9.61 x $10^{-7}$ | 1.14 x $10^{-4}$ | 6.53 x $10^{-9}$ |
| Max | 3.03 x $10^{-7}$ | 4.91 x $10^{-7}$ | 1.24 x $10^{-4}$ | 1.68 x $10^{-3}$ | 1.58 x $10^{-7}$ |
| Mean | 1.06 x $10^{-7}$ | 1.62 x $10^{-7}$ | 1.80 x $10^{-5}$ | 5.79 x $10^{-4}$ | $4.44x10^{-8}$ |
| Median | nc | 5.88 x $10^{-8}$ | 3.13 x $10^{-6}$ | 1.97 x $10^{-4}$ | 3.69 x $10^{-8}$ |
| ILRC (DI 14.28 g day$^{-1}$)[b] | | | | | |
| Min | nc | nc | 2.55 x $10^{-6}$ | 3.02 x $10^{-4}$ | 1.74 x $10^{-8}$ |
| Max | 8.05 x $10^{-7}$ | 1.30 x $10^{-6}$ | 3.28 x $10^{-4}$ | 4.46 x $10^{-3}$ | 4.20 x $10^{-7}$ |
| Mean | 2.82 x $10^{-7}$ | 4.30 x $10^{-7}$ | 4.78 x $10^{-5}$ | 1.54 x $10^{-3}$ | 1.18 x $10^{-7}$ |
| Median | nc | 1.56 x $10^{-7}$ | 8.32 x $10^{-6}$ | 5.23 x $10^{-4}$ | 9.81 x $10^{-8}$ |

[a] DI obtained from FAO, 2013 [34]
[b] DI obtained from Di Lena et al., 2018 [35]
[c]The PAH concentrations used for the ILCR calculations were expressed in BaP equivalent
*nc* Not calculable

Table 1.4: Incremental lifetime cancer risk (ILCR) of BaP, PAH4, Cr, As and Pb calculated using both daily intakes (DIs, 5.38 and 14.28 g day$^{-1}$)

## 1.6 Conclusions

This study assesses the PAH and heavy metal levels in *Eriphia verrucosa* from the northern coast of the Campania region improving the data regarding dangerous chemical compounds in this traditional Mediterranean crustacean. The results suggested a limited contamination of Pb, Cr and PAHs in the study areas indicating a low risk for human consumption. In fact, the EWIs and ILCRs calculated for these carcinogens were compliant with the thresholds considered safe for human health. Moreover, in addition to the four PAHs included in the Commission Regulation (EU) N° 835/2011, the analysis showed the occurrence also of BkF and DahA in crab muscles.

Considering that these two substances are recognized as possible and probable carcinogenic to humans respectively, it would be necessary to put more careful attention to official controls and monitoring on toxicological investigation including also these two PAHs to assure public health. Contrariwise, higher As concentrations that had probably a natural origin were found in muscle of warty crabs. The ILCRs calculated for As exceeded the acceptable level of cancer risk indicating a potentially threat for human health. These findings should be thoroughly studied in order to understand the bioaccumulation mechanisms and to identify anthropogenic sources of As pollution in the these marine areas in addition to natural ones.

# Chapter 2

# Heavy metals in muscle and hepatopancreas of red swamp crayfish (*Procambarus clarkii*) in Campania (Italy)

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## 2.1 Abstract

The aim of this study was to carry out a quali-quantitative analysis of the presence of non-essential and essential trace elements in freshwater crayfish (*Procambarus clarkii*) edible tissues to establish the healthiness of this product and to evaluate the pollution status of the sampling sites included in the present study.

*P. clarkii* is one of the most common species of freshwater crustaceans in Italy, regularly consumed by local people. Moreover, the crayfish, due to its trophic position and diet, can be considered as an excellent bioindicator of the health status of the ecosystem. We collected sixty crayfish samples from two different sites in Campania (Italy): Villa Literno and Sessa Aurunca.

Concentrations of trace elements were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Our data showed low concentrations of Cd, Hg and Pb, with values below the European Commission MRL ([71]). We suggest that data obtained from this study showed that crayfish collected from Villa Literno and Sessa Aurunca were safe for human consumption. Furthermore, the results of this research indicated mild contamination of heavy metals of the sampling sites, indicating a good health status of the area's aquatic ecosystem.

## 2.2 Introduction

Trace elements are classified by the scientific community as non-essential and essential. Non-essential trace elements have no biological role in animal organisms and represent a serious threat to aquatic fauna. Heavy metals and metalloids such as arsenic, lead, cadmium and mercury originate from natural sources and human activities (mining, metal production, combustion of fossil fuels, sewage sludge and waste incineration) ([72], [73]) and are spread worldwide in fresh and salty waters, becoming one of the major causes of persistent aquatic pollution. Trace elements enter the food chain through bioaccumulation and biomagnification processes, contributing to compromising the balance of the food chain for a long time ([74]). Adverse effects linked to acute or chronic exposure to metals include damages to the immune system, helping the onset of infectious diseases, and interference with the endocrine system, leading to reproductive alterations.

Among the freshwater fauna, crustaceans are one of the most sensitive macroinvertebrate species to suffer negative effects of exposure to metals due to their diet, way of feeding with direct contact with sediments, and life span ([75]-[76]), and they easily accumulate trace elements in the hepatopancreas, the target organ for metals investigation ([77]–[78]).

Since crustaceans are extremely sensitive to metal effects, are widely spread in aquatic ecosystems and are regularly consumed by humans, they represent an optimal bioindicator to gain information about the health status of the ecosystem and to determine safety and quality of food intended for human consumption. In our study, we focused on the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Fig. 2.1), which is common in the sampling areas we included in the study and usually consumed by local people. Moreover, *P. clarkii* is considered by the scientific community as an

optimal bioindicator for trace elements contamination ([79],[80]). Indeed, the red swamp crayfish has been used as an indicator species to monitor the environmental quality and the contamination of biological habitats in previous studies ([77],[81]–[82]).

Nowadays, the red swamp crayfish is listed in Italy as an invasive species. It originates from the United States and Mexico and arrived in Europe during the last century, for aquaculture purposes ([83]). Unfortunately, most of the Italian farmers failed to take adequate precautions in their cultivation methods to prevent the crayfish escape from farm enclosers. Soon after, the red swamp crayfish established wild stable populations in many lakes and ponds across Italy and rapidly became the dominant freshwater crayfish ([84], [85]). Regarding the sampling areas, we focused our attention on geographic areas of the Campania region (Italy) which are well known to be characterized by high pollution of soil, fresh, salty water and groundwater. These sites represent ex-industrial areas and are located nearby illegal waste dumps ([86]). Specifically, since the 1980s Naples and Caserta have been exploited as illegal landfills of toxic waste. Such operations and the accumulation of toxic products have had a serious impact on the ecosystem of the coast and the hinterland, influencing health and future development of the local fauna and human population ([87]).

In the present study, we performed a quali-quantitative analysis of trace elements in samples of hepatopancreas and abdominal muscle of *P. clarkii* collected in two different Italian sampling sites, selected for their potential high level of metal contamination. We sought to identify sources of pollution in the study area, to assess public health risk linked to consumption of crayfishes and to improve the current knowledge about the use of *P. clarkii* as a bioindicator of heavy metal pollution in freshwater ecosystems.

Figure 2.1: *Procambarus clarkii* (Girard, 1852)

## 2.3 Materials and Methods

### 2.3.1 Sampling

Sixty samples of red swamp crayfish were collected during summer 2017. Crayfishes were captured using baited traps placed at Villa Literno (ViL), near the Volturno River, and at Sessa Aurunca (SeA), near the Garigliano River (Fig. 2.2) in the Campania region.

No data is at present available concerning pollution of the two areas, apart from one study reporting trace elements concentration in the blood of dogs from Sessa Aurunca ([88]). Specimens were then transferred alive in refrigerated boxes (4–8 °C) to the laboratory. In our facility, crayfishes were weighed and sexed. Furthermore, we measured each carapace length using a caliper (Absolute Digimatic caliper, Mitutoyo, Japan) (Tab. 2.1), from the tip of the rostrum to the edge of the carapace. Crayfishes were euthanized by thermal shock (−80 °C for 30 min).

Subsequently, the abdominal muscle and the hepatopancreas were removed under partially defrosting conditions and stored in Falcon tubes at −20 °C until further analyses.

| Sites | n | Mean Weight (g) ± SD | Mean Total Lenght (cm) ± SD | Sex |
|---|---|---|---|---|
| Villa Literno (ViL) | 30 | 28.19 ± 4.43 | 9.58 ± 0.67 | 17♀ 13 ♂ |
| Sessa Aurunca (SeA) | 30 | 27.81 ± 3.51 | 9.32 ± 0.59 | 16 ♀ 14 ♂ |

Table 2.1: Number of individuals (n), weight (g), size (mm) and sex of *Procambarus clarkii* captured at Villa Literno and Sessa Aurunca

### 2.3.2 Chemical and Instrumental Analysis

Each sample was homogenized and 0.5 ± 0.2 g of tissue was added to 5 mL of 65'$HNO_3$ and 2.0 mL of 30 $H_2O_2$. Microwave-assisted digestion was


Figure 2.2: Map showing locations of the sampling sites: Villa Literno (ViL) and Sessa Aurunca (SeA)

performed with a specific mineralization program for 25 min at 190 °C. Samples were cooled at 32 °C and the digested mixture was transferred into a 50.0 mL flask and the final volume was obtained by adding Milli-Q water ([31]). Trace elements detection and quantification were determined by ICP-OES technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. Subsequently, both metals quantification and quality assurance procedure were performed as described by Zaccaroni et al. ([88]). LODs values (limit of detection values) as wet weight were: 0.024 $\mu$g g$^{-1}$ for As; 0.0002 $\mu$g g$^{-1}$ for Cu; 0.006 $\mu$g g$^{-1}$ for Zn; 0.001 $\mu$g g$^{-1}$ for Cr; 0.0018 $\mu$g g$^{-1}$ for Cd; 0.011 $\mu$g g$^{-1}$ for Pb; 0.001 $\mu$g g$^{-1}$ for Hg. The performance of the method has been defined by interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, York, UK).

### 2.3.3 Statistical Analysis

Results are reported in wet weight as mean ± SEM (standard error) ([89]). Statistical significance of the influence of sampling sites (ViL Vs. SeA)

and statistical significance in concentrations of trace elements in target organs (muscle vs. hepatopancreas) were tested using factorial analysis of variance. Furthermore, we apply the ANOVA test to highlight differences between trace element accumulation in the hepatopancreas and the muscle and between the sampling areas.

Multiple regression was used to discover statistical significance between trace element concentration and intrinsic variables (as total weight and gender of specimens). One-Sample Kolmogorov–Smirnov Test confirmed the normal distribution of our data.

All our statistical analyses have been performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). Significant value has been established at $p < 0.05$.

## 2.4 Results

Mean concentrations of As, Cu, Zn and Cr in abdominal muscle (AbM) and hepatopancreas (Hep) of *P. clarkii* are summarized in Fig. 2.3.

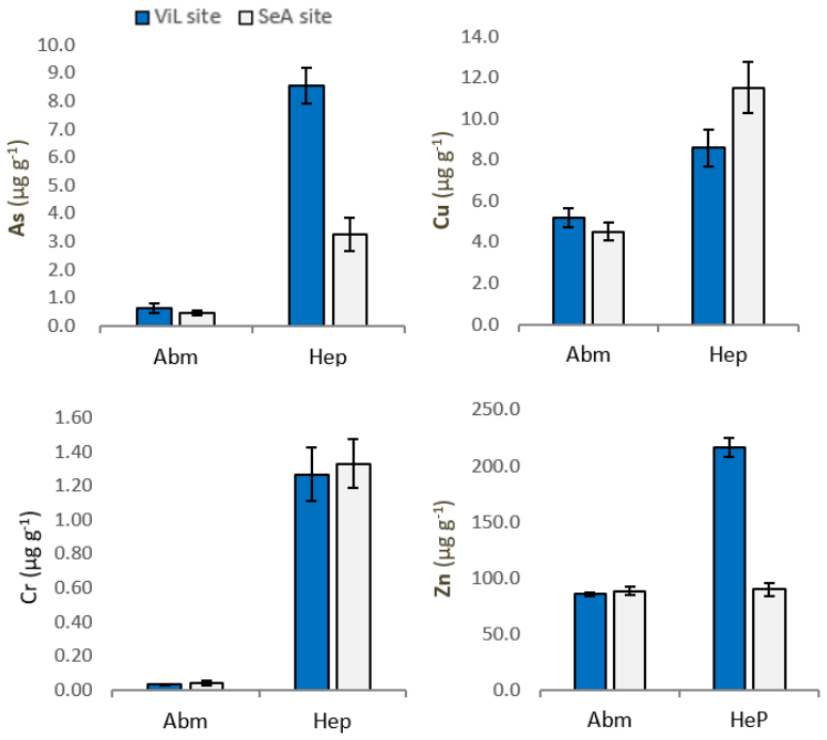


Figure 2.3: Concentrations of As, Cu, Zn and Cr in *Procambarus clarkii* abdominal muscle (AbM) and hepatopancreas (Hep) from Villa Literno (ViL) and Sessa Aurunca (SeA). Vertical bars represent average concentration ($\mu$g g$^{-1}$ wet weigth) $\pm$ SEM

Our results show a variability in the concentration of two trace elements in *P. clarkii*, depending on sampling sites. Specifically, the levels of As and Zn were significantly higher ($p < 0.01$) in *P. clarkii* tissue from ViL site. Significant differences in organ accumulation of As, Cr, Cu and Zn have been highlighted. Indeed, trace elements concentration was significantly higher in hepatopancreas than in muscle (Table 2.2).

In Hep, Arsenic was found at a mean concentration of 8.534 and 3.248 $\mu$g

$g^{-1}$ , while in AbM mean values were 0.627 and 0.456 $\mu$g $g^{-1}$ , in ViL site ($p < 0.01$) and SeA site ($p < 0.01$), respectively. Our data show, both in samples from SeA site and Vil site, significant differences ($p < 0.01$) between Cu concentration in Hep and AbM. In addition, significant differences ($p < 0.01$) were found for Zn between Hep and AbM at ViL site. Finally, higher concentrations of Cr were found in the crayfish Hep compared AbM at both sampling sites ($p < 0.01$).

| Trace Elements ($\mu$g $g^{-1}$ Wet Weight) | AbM ViL Site | Hep ViL Site | AbM SeA Site | Hep SeA Site |
|---|---|---|---|---|
| As | $0.627^{A} \pm 0.173$ | $8.534^{B} \pm 0.628$ | $0.456^{A} \pm 0.092$ | $3.248^{B} \pm 0.605$ |
| Cu | $5.172^{a} \pm 0.450$ | $8.577^{b} \pm 0.896$ | $4.518^{A} \pm 0.461$ | $11.512^{A} \pm 1.239$ |
| Zn | $85.553^{A} \pm 1.788$ | $216.643^{B} \pm 8.225$ | 87.961 ±3.753 | 89.617 ±6.091 |
| Cr | $0.031^{A} \pm 0.002$ | $1.265^{B} \pm 0.157$ | $0.042^{A} \pm 0.016$ | $1.328^{B} \pm 0.144$ |
| Cr | 0.031 A ±0.002 | $1.265^{B} \pm 0.157$ | $0.042^{A} \pm 0.016$ | $1.328^{B} \pm 0.144$ |
| Cd | <dl | 0.020 ±0.002 | <dl | 0.018 ±0.002 |
| Pb | <dl | 0.015 ±0.002 | <dl | 0.012 ±0.001 |
| Hg | <dl | <dl | <dl | <dl height |

Probability levels for significant differences depending on organ type: AbM versus Hep: A, B: $p < 0.01$, a, b: $p < 0.05$.

Table 2.2: Concentrations of As, Cu, Zn and Cr in *Procambarus clarkii* abdominal muscle (AbM) and hepatopancreas (Hep) from Villa Literno (ViL) and Sessa Aurunca (SeA). Vertical bars represent average concentration ($\mu$g $g^{-1}$ wet weigth) ± SEM

Results showed negligible levels of Cd and Pb in all samples of the crayfish AbM. In the Hep, Cd was found at a mean concentration of 0.020 and 0.018 $\mu$g $g^{-1}$ ; Pb was found at a mean concentration of 0.015 and 0.012 $\mu$g $g^{-1}$ in Villa Literno (ViL site) and Sessa Aurunca (SeA site), respectively. Mercury was found under the detection limit (dl) in all analysed samples (Table 2.2). The analysed individuals varied in size and weight ranges, including males and females.

The multiple regression analyses indicate that there were no correlations between weight, gender and concentration of all analysed trace elements ($p > 0.05$).

## 2.5 Discussion

The absence of a relation between trace elements and gender agrees with other published studies on *P. clarkii* ([81],[90], [91]). Moreover, we did not appreciate a significant link between trace element concentration in the analysed tissues and the weight of specimens, suggesting that these parameters have a minor effect on metal accumulation in subjects inside the weight range considered in this study ([31]).

Arsenic concentrations found in the crayfish muscle are comparable to results obtained by Bellante et al. (0.537 $\mu$g g$^{-1}$ w.w.). In the same study the concentration of As in hepatopancreas was lower than those found in the present study (1.128 $\mu$g g$^{-1}$ w.w.) ([90]).

Comparable levels of As in muscle were found by Gedik et al. in crayfish from Lousiana ([92]). Devesa et al. ([93]) report arsenic concentration ranging from 9.2 to 12 $\mu$g g$^{-1}$ in muscle and from 2.5 to 2.6 $\mu$g g$^{-1}$ in hepatopancreas of crayfish from Southern Spain, higher than those found in the present study. On the contrary, Mistri et al. ([94]) and Tan et al. ([95]) report mean As concentration in both Hep and AbM lower than those detected in present study.

Regarding essential trace element concentrations, previous studies reported variable values of copper and zinc levels in the crayfish tissues. Among them, Bellante et al. ([90]) reported Cu levels in crayfish hepatopancreas and muscle ranging from 1.149 to 48.3 $\mu$g g$^{-1}$ (mean value 12.3 $\mu$g g$^{-1}$ ) and from 1.34 to 12.72 $\mu$g g$^{-1}$ (mean value 5.19 $\mu$g g$^{-1}$ ) w.w., respectively. These data agree with the results of the present study. Similarly, Kuklina et al. ([96]) and Mistri et al. ([94]) report comparable Cu concentrations in both tissues. Despite this, another study conducted in Lousiana established a range for Cu and Zn concentrations in the crayfish muscle ranging

from 23.8 to 44.2 $\mu$g g$^{-1}$ and from 41.3 to 55.8 $\mu$g g$^{-1}$, respectively ([97]). Moreover, a recent study conducted in Central Italy showed Cu levels that varied from 23 to 1031 $\mu$g g$^{-1}$ in Hep and from 27 to 187 $\mu$g g$^{-1}$ in AbM ([98]). Cu levels in the hepatopancreas and muscle reported by those authors were higher than those detected in the present study, while Zn levels in Hep and AbM were lower than those found in ViL and SeA sites. Regarding Cr concentrations, Bellante et al. ([90]) reported levels in crayfish hepatopancreas and muscle of 0.915 $\mu$g g$^{-1}$ and 0.24 $\mu$g g$^{-1}$ w.w., respectively. Mancinelli et al. ([81]), reported Cr in muscle tissue of *P. clarkii* (0.20–0.29 $\mu$g g$^{-1}$ ) at higher concentrations than those found in AbM in ViL and SeA. Kuklina et al. ([96]) and Tan et al. ([95]) report similar Cr concentrations in the Hep to those detected in present research, while levels detected in AbM are higher in Campania samples with respect to these two studies. Detection of Cd and Pb has been widely explored in crayfish. The levels of Cd in AbM of ViL site and SeA site, respectively, are generally comparable with those found in the muscle of *P. clarkii* from Preola Lake (<dl–0.01 $\mu$g g$^{-1}$ d.w.) and Gorgo Medio Lake (<dl–0.03 $\mu$g g$^{-1}$ d.w.) in Sicily, Italy ([90]), and lower than those reported in crayfish muscle from Trasimeno Lake (0.05 $\mu$g g$^{-1}$ and 2.2 $\mu$g g$^{-1}$) and Bolsena Lake (0.03 $\mu$g g$^{-1}$) in Central Italy ([81], [98]).

The levels of Pb accumulated in AbM and Hep determined in our research are also lower than concentrations measured in other areas ([98] - [82]). Cadmium concentrations found in Hep of ViL site and SeA site are comparable to those measured in hepatopancreas of *P. clarkii* from Preola Lake and Gorgo Medio Lake in Sicily, Italy ([90]), but lower than the ones reported by other authors ([77]). In 2016, Goretti et al. ([98]), detected Cd (mean value 8.2 $\mu$g g$^{-1}$ unpolluted area; 28.2 $\mu$g g$^{-1}$ polluted area) and Pb (mean value 8.5 $\mu$g g$^{-1}$ unpolluted area; 3.2 $\mu$g g$^{-1}$ polluted area)

in the hepatopancres of *P. clarkii* from Trasimeno Lake (Cental Italy) at higher levels than those found in ViL and SeA sites. Same results were reported for both Cd and Pb by Tan et al. ([95]), Mistri et al. ([94]) and Kuklina et al. ([96]). The general evidence was that crayfishes from ViL and SeA accumulated higher levels of metals (As, Cu, Zn and Cr) in Hep than in AbM, in accordance with those reported in literature ([77], [92] – [90]). Almost all studies on the distribution of trace elements in crayfish tissues showed that the hepatopancreas is the target organ of storage and detoxification of heavy metals ([77] - [78]). However, in the present study, no statistical differences were reported for Cd and Pb concentrations in AbM and Hep of *P. clarkii*, probably due to the negligible concentrations of these non-essential trace elements in the aquatic environment of both sampling sites.

### 2.5.1 Concern for Public Health

Even though no European or Italian regulation for As, Cu, Zn and Cr concentration in crustaceans and food products exists (because they are considered as essential trace elements, necessary for specific physiological functions), some tolerable upper intake levels have been proposed by both American and EU governmental and research entities (National Institutes of Health, U.S., Department of Health and Human Services; German Federal Institute for Risk Assessment, BfR; Scientific Committee on Food, European Commission, SCF; EFSA).

Copper is easily found in the environment and is essential for normal growth and metabolism ([99]). Additionally, it is a component of the respiratory metalloprotein hemocyanin in crustaceans ([100]). Therefore, relatively high copper amounts may be found in crayfish tissues, mainly in the hep-

atopancreas ([77], [101]). The role of Cu in crayfish metabolism and its great variability in data reported by other studies make comparison difficult, but the concentrations of Cu found in the present study are generally similar or higher than those reported in nine crayfishes captured both in polluted and unpolluted study areas ([90]). Detected levels of copper are well above the recommended dietary allowances for toddlers and for adults (0.14–0.15 $\mu$g g$^{-1}$ respectively) set by NIH ([102]) and of 0.08 $\mu$g g$^{-1}$ for adults defined by BfR, SCF and EFSA ([103] - [104]).

The concentrations of Zn were higher than concentrations found by other authors in polluted and unpolluted areas ([90], [97], [105]). Our results are indicative of high Zn levels, especially in the ViL site. These levels exceed the tolerable upper intake level (UL) defined by the SCF of 0.41 $\mu$g g$^{-1}$ for adults ([106]). Anyway, it should be noted that crayfish consumption is not that common among the Italian population, and the quantity of flesh usually consumed is generally reduced, so for both Cu and Zn a reduced exposure, and consequent health risk, is expected.

Chromium levels detected in Hep and AbM are comparable or lower than those reported by other authors ([81], [90]). Furthermore, Cr concentrations in AbM are below the threshold concentration suggested by FDA ([107]) of 1.089 $\mu$g g$^{-1}$ w.w. for human consumption. Anyway, it is important to remember that also an excess of these metals can potentially cause harmful effects in organisms ([78], [108]). No UL has been defined for Chromium, but the WHO suggested to not exceed a 250 $\mu$g/day supplementation, equivalent to a daily dose of 4.16 $\mu$g g$^{-1}$, if using a standard weight of 60 kg ([109], [103]).

Although our results are suggestive of higher levels of As in Hep, especially in the ViL site, concentrations of As in AbM are comparable to those reported in the literature and considered concentration responsible for low

risk for human consumption ([92]). No UL has been set for As at present by any governmental institution, but a maximum concentration of 50 $\mu$g $L^{-1}$ has been defined ([102]), well below the mean concentrations detected in present study. Anyway, it should be remembered that the substantial portion of arsenic present in fish and mollusks is in the organic form and, as stated by Trumbo et al. ([102]) as well, these forms are less toxic than inorganic form (for whom the assessment is done). Consequently, any increased health risk from food products such as fish and mollusks is unlikely.

Regarding non-essential trace elements, The European Union legislation (Commission Regulation (EC) 1881/2006 and its amendment ([71]) on food safety clearly establish the MRLs for total Cd, Pb and Hg which can be detected in the muscle of crustaceans (0.5 $\mu$g $g^{-1}$ w.w. for Cd; 0.5 $\mu$g $g^{-1}$ w.w. for Pb and 0.5 $\mu$g $g^{-1}$ w.w. for Hg) intended for human consumption ([110],[71]). The results obtained in the current study show lower levels of Cd, Pb and Hg in AbM and Hep from ViL and SeA sites than the MRLs reported by EU regulations. Furthermore, our data are largely below the established MRLs, suggesting a limited Cd, Pb and Hg contamination of the aquatic environment of the study areas, and good food safety of aquatic products derived from these geographic areas.

## 2.6 Conclusions

The accumulation of trace elements in *P. clarkii* tissues reflects the concentrations of metals in the surrounding environment ([111]) and our data suggest that *P. clarkii* could be considered a good bioindicator for metal pollution. The higher Cu and Zn concentrations found in *P. clarkii* tissues, especially for Zn from ViL site, could be related to higher anthropic activity in these areas, as already proved by a paper by Zaccaroni et al. ([88]). However, these results must be evaluated with caution because of the small number of samples collected and the lack of legal limits for the detection of some trace elements concentration in crustaceans and other fish products. The higher As concentrations in crayfish Hep, especially from ViL site, must be further clarified in order to identify possible sources of contamination in these areas. Further studies are also needed in determining the percentage of organic and inorganic arsenic in crayfish tissues. Ongoing studies on metals in a greater number of *P. clarkii*, in other biological and environmental samples and in other geographical areas, will provide more useful information to confirm this species as indicator of environmental contamination.

# Chapter 3

# Effects of Covid-19 pandemic lockdown and environmental pollution assessment in Campania region (Italy) through the analysis of heavy metals in honeybees

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## 3.1 Abstract

The Covid-19 outbreak had a critical impact on a massive amount of human activities as well as the global health system. On the other hand, the lockdown and related suspension of working activities reduced pollution emissions. The use of biomonitoring is an efficient and quite recent tool to assess environmental pollution through the analysis of a proper bioindicator, such as bees.

This study set out to ascertain the impact of the Covid-19 pandemic lockdown on the environmental occurrence of eleven heavy metals in the Campania region (Italy) by analysing bees and bee products. A further aim of this study was the assessment of the Honeybee Contamination Index (HCI) in three different areas of the Campania region and its comparison with other Italian areas to depict the current environmental pollutants levels of heavy metals. The results showed that the levels of heavy metals bioaccumulated by bees during the pandemic lockdown (T1) were statistically lower than the sampling times after Covid-19 restrictions and the resumption of some or all activities (T2 and T3). A comparable trend was observed in wax and pollen. However, bee, pollen, and wax showed higher levels of Cd and Hg in T1 than T2 and T3. The analysis of the HCI showed a low contamination level of the sampling sites for Cd and Pb, and an intermediate-high level as regards Ni and Cr.

The biomonitoring study highlighted a decrease of heavy metals in the environmental compartments due to the intense pandemic restrictions. Therefore, *Apis mellifera* and other bee products remain a reliable and alternative tool for environmental pollution assessment.

## 3.2 Introduction

Environmental pollution due to anthropogenic activities has led to direct and indirect contamination of all natural ecosystems. In particular, heavy metal contamination of soil, air, and water is alarming due to its adverse impacts on living organisms ([112]). In the latter period, a decrease in concentrations of environmental pollutants was recorded during the Covid-19 pandemic ([113]). Indeed, the Covid-19 pandemic had a critical impact on health, society and economy, but, at the same time, it helped to reduce environmental pollution ([114]). This effect could be related to the highly restrictive government measures imposed to contain the spread of the pandemic. The restrictions affected the mobility of people and vehicles and suspended some industrial activities ([115]).

In Italy, one of the countries significantly hit by this pandemic, the lockdown was set between March 9 and May 3, 2020. The restrictions imposed the closing of factories, schools, shopping malls, blocking public transportation and sporting events, and tourism. Subsequently, with the reduction of the state of emergency, represented by fewer people hospitalized in intensive care, restrictions were gradually reduced until the complete reopening of working activities and mobility.

The sudden block of all global anthropogenic activities significantly affected the environmental quality ([116]). Numerous studies underline the positive effects on water and air quality during the Covid-19 lockdown, comparing the percentage of contaminants found before and during the lockdown ([117]; [115]). In terms of environmental pollution, greenhouse gas emissions, nitrogen dioxide, black carbon, and water contamination have decreased significantly ([114]). In India, several studies conducted on different rivers have found a reduction in industrial waste contamination

and an improvement in water quality of about 40–50%, also leading to a reduction in heavy metal concentrations ([118]; [119]; [120]). Therefore, continuous monitoring of ecosystems is necessary and valuable to obtain real-time information on environmental quality. Although different methods are available to assess the environmental quality, an original and efficent approach could be the use of bioindicator species. Among these, bees proved to be a valid bioindicator due to their foraging activities ([121]); hence, their application in environmental monitoring could be also reliable during Covid-19 emergency. Indeed, bees travel over large areas of approximately 7 $km^2$ from the colony and encounter various differently contaminated environmental substrates ([122]; [123]; [124]). They can be a carrier for heavy metals from the environment to hives in a variety of ways, such as by accumulating airborne particulate matter on their furry bodies during flight, through the water or by picking up heavy metals from pollen and nectar accumulated in the plants due to contaminated soil ([125]; [126]; [127]).

Studies conducted on the concentration of heavy metals suggest that the accumulation on the body of bees is site-specific, and once transported to the hive, these heavy metals can occur in various bee products such as honey, wax, and propolis ([128]; [129]; [130]), leading to a potential risk to human health as well.

The following study is placed in a context of environmental biomonitoring during the Covid-19 pandemic in Italy. The occurrence of heavy metals in bees and bee products in different areas of the Campania region (Italy) was evaluated just after lockdown, after partial restriction, and resumption of activities. Hence, this study set out to ascertain the impact of the Covid-19 pandemic on environmental pollution by heavy metals using *Apis mellifera* (Fig. 3.1) and other bee products as potential monitoring tool

and the assessment of the contamination level in three different areas of Campania region.

Figure 3.1: *Apis mellifera* (Linnaeus, 1758)
https://it.wikipedia.org/wiki/Apis_mellifera#/media/File:
European_honey_bee_extracts_nectar.jpg

## 3.3 Materials and Methods

### 3.3.1 Sampling

The biomonitoring study was conducted in the Campania region during the 2020 Covid-19 global pandemic. Bees, pollen, and wax were sampled at three different times during the national lockdown: late May (T1), after few months of total shutdown of all anthropogenic activities, late July (T2), partial resumption of activities, and the end of October (T3), total resumption of activities and international mobility.

The sampling sites (n = 8) were chosen in three different suburban areas of the region. They were located as follows: one in the municipality of Vico Equense, in the Sorrento peninsula area; four in the Vesuvius area, in the municipalities of Torre del Greco, Terzigno, Trecase, and Ottaviano; three in the Caserta province, in the municipalities of Cancello Arnone, Castel Volturno, and Mondragone (Fig. 3.2).

The sampling was conducted after receiving consent from beekeepers to their anonymous participation in the study. Specimen collection was done respecting the colonies and trying not to interfere with bees' activities. For each apiary, the samples were: about 100 foraging bees; fresh wax (20 g); pollen (20 g) collected from each site using stainless steel pollen-collecting traps positioned at least 24 h before the collection operation. Some precautions were taken during the sampling phase in order to avoid accidental contamination of the samples: the beekeeping equipment was made of stainless steel and was cleaned from all residues before and after use; the sampling operator made use of new, intact, clean and specific personal protective equipment (PPE); the samples were taken with disposable gloves and scalpels, placed in sterile containers and stored at a temperature

of − 20 °C.


Figure 3.2: Area of study and sampling sites (Google Maps, 2022)

### 3.3.2 Chemical and Instrumental Analysis

The samples were homogenized using a mixer, and 0.5 ± 0.2 g aliquots of each sample were spiked with 5 mL $HNO_3$ (65% w/w) and 2 mL of $H_2O_2$ (30% w/w). Then, the samples underwent wet mineralization via a Milestone microwave for 30 min at 190 °C. At the end of the digestion, the samples were cooled and transferred to flasks. The final volume of 25.0 mL was obtained by adding MilliQ water.

Trace elements detection and quantification were performed using a Thermo Scientific™ ICAP™ RQ inductively coupled plasma mass spectrometer (Q-ICP-MS) with a Burgener Mira-Mist nebulizer, a Quartz cyclonic spray chamber, cooled to 2.7 °C, and skimmer cones. The instrument was op-

erated using the Thermo Scientific™ Qtegra™ Intelligent Scientific Data Solution™ (ISDS) Software. The operating conditions of the Q-ICP-MS equipment were optimized using a tuning solution (Ba, Bi, Ce, Co, In, Li, U 1.00 µg/L, Thermo Scientific) on masses $^{115}$In, $^{7}$Li, $^{59}$Co, $^{238}$U, $^{209}$Bi and $^{104}$Ce was used for oxide and doubly charged interference checks. The analysis was performed in KED (Kinetic Energy Discrimination) mode using Helium as collision gas, and the parameters were: plasma gas flow (Ar): 14,8 mL/min; nebulizer gas flow: 0.98 L/min; auxiliary gas flow: 0.85 L/min; ICP RF Power: 1550 W; CeO/Ce = 0.0057. Cell gas flow was 4.8 mL/min for He.

The Q-ICP-MS was used to determine Cd, V, Cr, Mn, Ni, Cu, As, Sb, Ba and Pb in bees, pollen, and wax samples. All samples were analysed in duplicate, and each sample was measured in triplicate by Q-ICP-MS detection ([131]; [132]; [133]).

The solutions were prepared using water (18.2 MΩ cm resistivity) purified with Millipore Mill-Q® purification system, concentrated nitric acid ($HNO_3$ 65% m/m, Suprapur®, Merck, Germany) and hydrogen peroxide ($H_2O_2$ (30% w/w), Suprapur®, Merck). An $HNO_3$ 1% v/v (Suprapur®, Merck, Ultrapure) solution was used to clean the Q-ICP-MS apparatus between quantifications. A volume of 5 mL of $HNO_3$ (65% w/w) and 2 mL $H_2O_2$ (30% w/w) were added to digest both samples and standard solutions. The calibration standards were prepared with multi-element standard solution CertiPUR® (Merck, Darmstadt, Germany) 1000 mg $L^{-1}$ at concentrations: 0.5, 1.0, 2.5, 5.0, 10.0 µg $L^{-1}$ . An internal standard mix comprising 50 µg $L^{-1}$ Ge, 5 µg $L^{-1}$ Ir, 10 µg $L^{-1}$ In and 25 µg $L^{-1}$ Y was introduced online with an internal standard mixing kit. The internal standard elements were appropriately matched to analyte elements ([134]; [135]).

The Limit of Detection (LOD) was 0.00015 ppm for each metal but Ni, Ba and Cu, where it was 0.00600 ppm and Hg (0.00003 ppm).

### 3.3.3 Honeybee Contamination Index

For the assessment of environmental pollution based on heavy metal concentrations in bees was used the Honeybee Contamination Index (HCI) proposed by Goretti et al. (2020) ([122]):

$$HCI_i = \log \frac{C_{bees}}{C_{bees\_i}}$$

where ($C_{bees}$) is the element concentration in bees and ($C_{bees\_i}$) is the reference threshold limit reported by DiSTAL –UniBo (2010) and Gutierrez et al. (2015). $C_{bees\_i}$ varied from high ($C_{bees1}$) and low ($C_{bees2}$) reference thresholds of contamination, respectively (Table 3.1). The lack of data allows assessing the HCI only for Cd, Pb, Cr, and Ni.

### 3.3.4 Statistical Analysis

Data analysis and graph processing were performed using R Software version 3.6.0 and the following packages: ggplot2, ggsci, FactoMinerR, FactoInvestigate and factoextra ([136]; [137]; [138]; [139]; [140]; [141]).

## 3.4 Results and Discussion

### 3.4.1 Heavy Metal Concentrations and Honeybees Contamination Index

Heavy metal concentrations (expressed as wet weight) found in honeybees in the three different times are displayed in Table 3.1. The amount of bee's body water was 68% based on average value reported by Goretti et al. (2020) ([122]). The samples collected at T3 (the most representative condition of the current environmental situation) showed that Trecase was the city with higher levels of Cd (39.50 µg/kg), V (40.60 µg/kg), Sb (5.25 µg/kg), Ba (1189.10 µg/kg), and Pb (95.55 µg/kg); Torre del Greco showed higher values for Ni (233.00 µg/kg), and As (50.30 µg/kg); Ottaviano reported higher concentration for Hg (42.15 µg/kg), whereas Vico Equense showed higher levels for Cr (147.55 µg/kg), Cu (10,437.95 µg/kg), and Mn (10,531.75 µg/kg). However, statistically significant differences among the sampling areas were observed only for Cu, Cd, and Ba as discussed below. The heavy metals concentrations assessed in bees in Campania were in line with Giglio et al. (2017), which reported similar mean data in the suburban area of Trieste in June of 2013. They reported values (expressed as µg/kg dry weight) of Cd: 52 ± 6, V: 60 ± 16, Cr: 261 ± 16, Ni: 358 ± 37, Cu: 12, 820 ± 920, As: 50 ± 18, and Pb: 127 ± 17. In contrast, a recent study by Goretti et al., (2020) ([122]) stated higher concentration levels (µg/kg dry weight) for Cd: 300 ± 500, Pb: 340 ± 310, Ni: 1340 ± 1840, Mn: 98, 980 ± 79, 590, whereas similar or lower values were observed for Cr: 270 ± 140 and Cu: 14, 390 ± 2900 from analyses conducted in Umbria region between 2014 and 2015. Instead, Ruschioni et al. (2013) ([142])reported mean concentrations (µg/kg) in the range of 20–100 for Cd, 30–150 for Cr,

40–162 for Ni, 50–370 for Pb in ten natural reserves in the Marche Region monitored from 2008 to 2010.

The HCI used to assess the environmental pollution level was calculated following Goretti et al. (2020) ([122]) methods, based on a reference threshold limit of four heavy metals deriving by DiSTAL-UniBo and Gutierrez et al. (2015) ([143], [144]). This index was calculated according to the concentrations at T3 for the reason mentioned above. The HCI showed a low contamination level for Cd and Pb, whereas, Ni and Cr levels led to an intermediate-high HCI values (Fig. 3.3). Hence, the detected concentrations of Ni and Cr pointed out a relevant environmental pollution in Campania region.

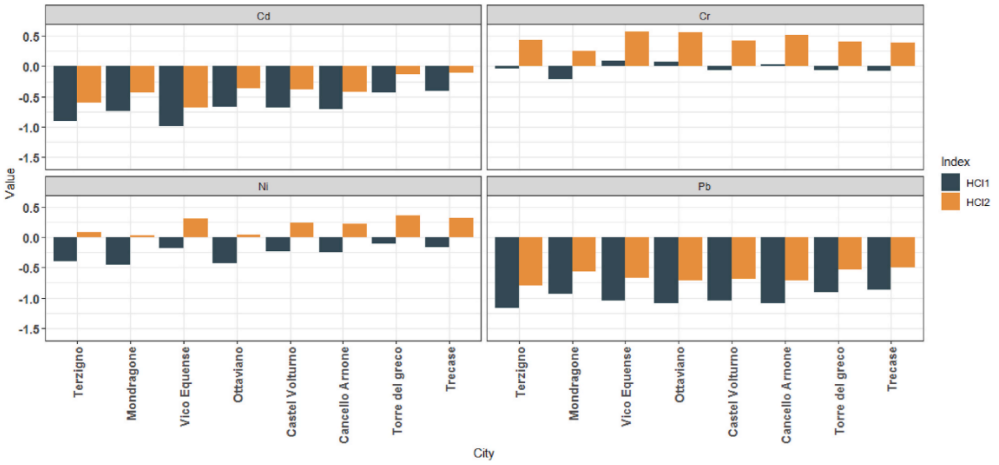


Figure 3.3: Level of environmental pollution for Cd, Cr, Ni, and Pb in eight sites of Campania region based on the Honeybee Contamination Index (HCI), calculated through minimum (HCI1) and maximum (HCI2) reference threshold limit

| Element | $C_{bees1}$ (mg $kg^{-1}$ w.w.) | $C_{bees2}$ (mg $kg^{-1}$ w.w.) |
|---|---|---|
| Cd | 0.10 | 0.05 |
| Pb | 0.70 | 0.30 |
| Cr | 0.12 | 0.04 |
| Ni | 0.30 | 0.10 |

Table 3.1: Maximum and minimum reference threshold limit for Cd, Pb, Cr, and Ni (mg $kg^{-1}$ w.w.) in contaminated honeybees

### 3.4.2 Impact of Covid-19 Pandemic on Environmental Pollution

The lockdown due to the Covid-19 pandemic has changed people habits and work activity with effects on air and soil quality. Several measures to contain the Sars-Cov 2 infection were taken such as travel ban (except for health or work reason), school and retail shop closures (except for basic needs shop), suspension of sport, cultural, religious events and sport activities ([145]). For this reason, bees contamination during and after different types of social restriction was assessed. The data were arranged according to the three different sampling times (T1, T2, and T3) and were tested for the homogeneity of the variances (Bartlett's test) and normality (Shapiro-Wilk's test). Finally, a one-way analysis of variance with post-hoc Tukey's test was performed to highlight any likely statistically significant difference. Comparing the three sampling times, the levels of the analysed elements showed significantly different means at the 95% confidence level between the first sampling time (T1) and the other two (T2 and T3) (Fig. 3.4). The most striking result to emerge from the data analysis is the lowest environmental concentration for most elements in the samples collected immediately after the pandemic Covid-19 lockdown (T1 time) (Fig. 3.5). However, it is also worth noting that, unlike the other elements, Cd and

Hg showed the highest levels at the T1 period throughout the three sampling times and for most of the sampling sites. This result might be related to the different persistence of these two elements in the environment. Cd concentration was higher in lockdown, as also reported by ([116]). A similar trend in water samples was also described by Tokatlı and Varol (2021) ([146]) in three sites in Northwest Turkey. Besides, Hg showed a different trend with the highest levels just after lockdown. Hg concentrations were lower in post-lockdown or partial lockdown. These two metals probably have a more remarkable environmental persistence, and more time could be needed for their decrease to appreciate the effects in the long term (T2 and T3) (Fig. 3.5). In addition, despite the reduction of industrial activities and urban traffic, the increase of atmospheric Hg emissions, as a consequence of intensive use of household appliances (e.g., boilers and heaters) throughout the lockdown, likely led to a different trend in the environmental occurrence of this metal ([147]; [148]) (see Fig. 3.6).

Regarding Cd, which is naturally present in soil and sediment, almost all of its air emissions come from anthropogenic sources, mainly from non-ferrous metal smelting and refining, fossil fuel combustion and municipal waste incineration ([149]). Cd air emissions can also come from intensive use of phosphate fertilizers ([150]) that may have been used even during the lockdown period. Another source of Cd contamination of bees is possible through plant uptake and transfer to flowers. The extreme mobility of Cd in the air and in the plant-soil system ([151]) makes it one of the most toxic metals ([142]). As early as 1991, a study by Yaaqub et al. (1991) ([152]) stated that much of the Cd occurrence in bees (33% – 72%) is due to its widespread occurrence in the air. This theory is consistent with Harrison and Williams (1982) ([153]), who state that highly mobile Cd is transferred primarily by large-scale atmospheric transport.

No statistically significant difference emerged among the sampling sites, except for Cu and Cd ($p < 0.001$) and Ba ($p < 0.05$) that showed higher levels in the Vesuvius area as regards Cd and in the Sorrento peninsula, for Cu and Ba: these differences could depend upon either anthropogenic activities (agricultural runoff) or the site-specific characteristics of the volcanic area ([154]).

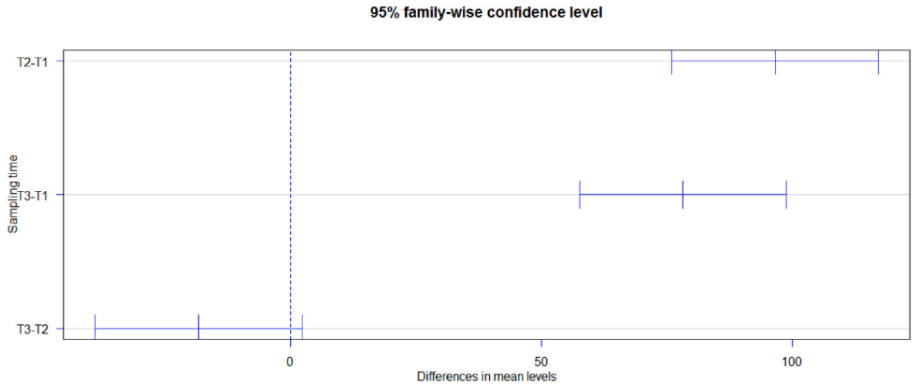


Figure 3.4: Differences in mean levels of concentration of heavy metals according to the different sampling times (T1, T2, and T3)

### 3.4.3 Effects of Covid-19 Restrictions on Elements Concentration in Bee Matrices

The effect of pandemic restrictions was also evaluated in bee product matrices such as pollen and wax. Hence, the concentrations of elements were measured at three times (Fig. 3.4; Fig. 3.5). Levels in pollen were the highest at T2 and the lowest at T1 for most of the elements, whereas Cd and Hg still reported a different behavior (T1>T3>T2) (Fig. 3.5).

The concentration detected in pollen fully confirms the same trend observed in bees for all elements. Likewise, Cd in wax was higher in the samples collected just after the Covid-19 pandemic lockdown and, along with Hg (T3>T1>T2) reported the lowest levels during partial-lockdown. The rest

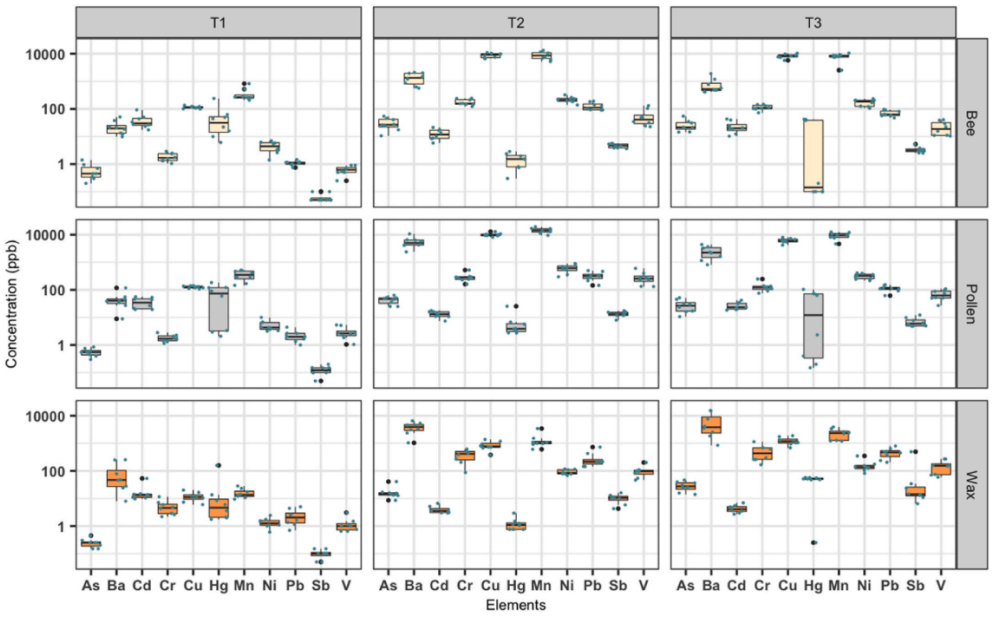


Figure 3.5: Heavy metals concentrations (on exponential scale) in three bee product matrices from Campania region according to three different sampling times: just after lockdown (T1), partial restriction (T2), resumption of any activity (T3)

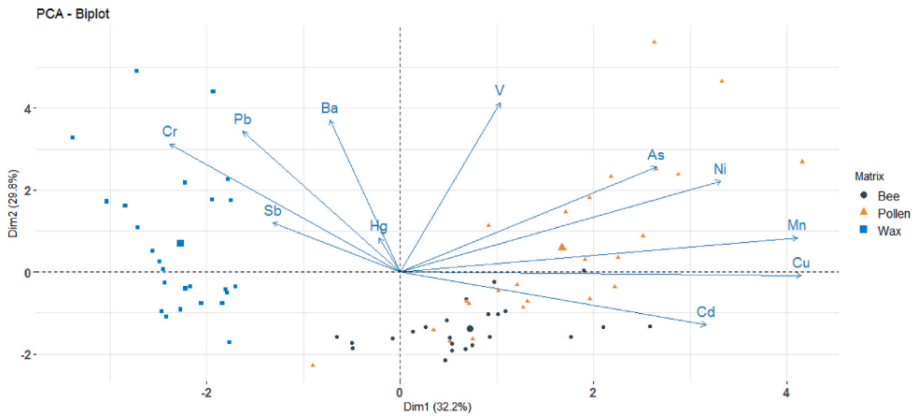


Figure 3.6: Principal component analysis (PCA) biplot showing the differentiation of the three bee product matrices by the first two principal axes

of the elements reported the highest concentrations in samples collected after the resumption of activities (Fig. 3.5). The levels of most contaminants in pollen and wax confirm a decrease in pollution after substantial pandemic restrictions, as emerged from the analysis of bees. In pollen and

partially in wax, Cd and Hg levels increased just after the lockdown, confirming the trend highlighted in bees. In addition, the lowest levels of Cd and Hg occurred in partial lockdown both in bees and bee product matrices, suggest that the decrease of these two metals could be appreciated in the long term. Bees and bee product matrices contamination may be affected by seasonality due to weather conditions: the rainfall that usually occurs in winter-autumn lead to higher elements levels of contaminants than in spring-summer ([155]; [156]). As a matter of fact, in Campania region the rainfall are more abundant and frequent in September–October rather than July and May ([157]). However, the results of this study showed an opposite trend highlighting that the reduction in environmental pollution might have been influenced by pandemic restrictions rather than seasonality.

### 3.4.4 Principal Components Analysis (PCA)

In order to highlight any matrix contribution in the accumulation of heavy metals, a Principal Component Analysis (PCA) was performed. The plane described by the first two components (Dim 1 and Dim 2) accounts for more than 60% of the total variance, whereas the individuals are clearly separated according to the qualitative variable "Matrix", which better illustrates their distance on the plane. On this basis, a hierarchical clusterization of the individuals revealed two clusters: the first is characterized by high values of Cr, Pb, Ba, Sb and low values of Cu, Mn, Cd, Ni, and As and was identified by the wax. The second cluster is characterized by high values of Cu, Mn, Cd, Ni, and As and low values of Cr, Pb, Ba, and Sb: both bees and pollen belong to this cluster, revealing a causal relationship between contamination of pollen and bioaccumulation in the tissues of bees. A possible explanation might be that the bees accumulate environmental

pollutants during their foraging activity, mainly through pollen collection. These results can be partly motivated by the study of Zhelyazkova et al. (2004) ([158]), which evaluated how the concentration of some contaminants changes in the hemolymph of bees. Among these, Mn and Cd were predominant, respectively 21.7 and 17.7 times higher than in bees not subjected to contaminated feeding. The co-occurrence and the proximity of Cu and Cd in the PCA plane may suggest their agricultural origin, whereas Ni and As are likely to have a volcanic origin.

## 3.5 Conclusions

The study mainly focused on assessing the contaminant levels just after strict lockdown, partial-lockdown, and post-lockdown. The analysis of bees, pollen, and wax suggests that levels of most contaminants decreased during the intense restriction, likely due to the reduction of industrial and urban activity. However, Cd and Hg showed an opposite trend. Overall, the limitation set during the Covid-19 pandemic lockdown had a counter-effect that reduced anthropogenic activities, resulting in a lower occurrence of heavy metals in the environment. In contrast, an immediate increase in the levels of these chemicals emerged along with the resumption of these activities.

As regards biomonitoring, *Apis mellifera* resulted in an efficient bioindicator for monitoring heavy metals and toxic elements; however, the analysis of bee products may also support this assessment. The HCI allowed estimating the environmental pollution in the examined areas for some elements by comparing with reference threshold limits (Cd, Cr, Ni, and Pb). All sites showed low contamination levels for Cd and Pb and an intermediate-high level of Ni and Cr in one city in each area. However, the concentrations of Cd related to the Vesuvius area were statistically higher than in the other two areas, whereas higher levels of Cu and Ba occurred in the Sorrento peninsula.

In conclusion, whilst the restrictions due to the Covid-19 pandemic impacted the socio-economic sphere, an improvement of environmental quality was confirmed, likely through the reduction of some sources of anthropogenic pollution, generally related to work activities and vehicle emissions. Overall, although the primary purpose of this study was to gain an insight into the impact of the Covid-19 pandemic on environmental pollution, these

findings also strengthen the idea that the use of bees as a biomonitoring tool may well have a bearing in urban-scale environmental investigations, with more manageable and less costly analyses.

# Chapter 4

# Dietary exposure to heavy metals through polyfloral honey from Campania region (Italy)

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## 4.1 Abstract

Honey may have potential benefits due to its nutrient and bioactive molecules. On the other hand, it is a food that could be affected by environmental pollution; therefore, honey may contain contaminants such as heavy metals.

The present study aimed to quantify eleven heavy metals and essential elements (Hg, Cd, V, Cr, Ni, Cu, As, Sb, Pb, Ba, Mn) in honey collected in the Campania region (Italy) and analysed through Q-ICP-MS. Secondly, carcinogenic and non-carcinogenic risks due to ingestion of honey in toddlers, adolescents, and adults were estimated based on the Target Hazard Quotient (THQ) and Lifetime Cancer Risk (LTCR). No statistically significant difference emerged among the different areas. The risk assessment did not report concerns for non-carcinogenic risk. However, the three groups showed a potential carcinogenic risk for Ni, Cr, and As, even though toddlers reported higher exposure values.

The finding of this study provides pieces of knowledge on levels of contaminants in honey in Campania. Furthermore, it can aid in understanding the resulting risk due to honey ingestion.

## 4.2 Introduction

Honey (Fig. 4.1) is a well-known sweet and viscous natural substance used as food since prehistoric times ([159]). Indeed, it is an excellent source of nutrients such as sugars (mainly glucose and fructose), proteins, vitamins, minerals, phenols, and others. According to Codex Alimentarius (2019) ([160]) definition, honey is produced by honeybees from the nectar of flowers, from secretions of plants, as well as excretions of plant-sucking insects on the living parts of plants (in the case of honeydew honey), which the bees collect, transform through the addition of specific compounds, deposit, dehydrate, store and leave in the honeycomb to ripen and mature. The type of plant characterizes the honey variety and its chemical-physical composition ([161]).

In the last two decades, worldwide honey production has increased by about 67% (1260063 tonnes in 2000 – 1862598 tonnes in 2019) proving a most significant market interest for this food. Honey is not only appreciated for its organoleptic features but also for its health-promoting effect. The high number of bioactive compounds with antioxidant and anti-inflammatory properties significantly reduce cellular proliferation, glucose, fructosamine, and glycosylated hemoglobin serum concentration, oxidation of low-density lipoproteins, asthma and bacterial infections ([162]). Furthermore, high microbiological stability due to acidity, low water availability, and microbial activity inhibitors was described ([163]). For these reasons, honey consumption may be suggested following LARN (Levels of Absorption Reference of Nutrients and Energies for the Italian population) ([164]). However, honey may be a source of potentially toxic elements such as heavy metals and trace elements due to honeybee's bioaccumulation capability ([165];[166]). Heavy metals are persistent pollutants, naturally occurring in the envi-

ronment or widespread by anthropogenic activity. The primary emission sources of these chemicals are industrial processes, combustion of fossil fuel refining, vehicles emissions, disposal of municipal wastes, and application of pesticides and fertilizer ([167]). Toxic elements can lead to acute or chronic intoxication, although some (e.g., Cu, Cr, Se, Mn, Zi) are essential for human metabolism and have detrimental effects only at high doses ([2]). They can alter the function of the immune and nervous system and organs such as lungs, liver, and kidneys ([3];[4];[5];[6]). Each element has a specific mode of action, but underlying toxicity mechanisms include ROS generation, glutathione depletion, and bonding to sulfhydryl groups damaging cells and activating carcinogenic processes ([168];[169]). As a result, the consumption of honey, which may be affected by the surrounding environmental conditions, could be a significant source of exposure to chemical contaminants, representing a potential public health issue. Previous research assessed the honey contamination in different regions, reporting high levels of heavy metals such as Pb, Cd, As, Cr, and Ni ([170]; [166]; [171]). However, some studies described the peculiar ability of honeybees to "filter" the nectar, reporting no relevant heavy metals levels in honey ([172]; [173]).

Therefore, this study set out to evaluate the levels of eleven heavy metals and essential elements (Hg, Cd, V, Cr, Ni, Cu, As, Sb, Pb, Ba, Mn) in honey collected from apiaries located in different Campania (Italy) municipalities, with the aim to fill the gap of data related to this area. To the best of our knowledge, although some research has been carried out on heavy metals in honey, few published studies have quantified the levels of these elements in samples collected in Southern Italy. Therefore, our study may provide a significant account of honey safety in the Campania region (Italy), ascertaining human exposure to these elements.

Figure 4.1: Honey
https://pinvi.net/il-miele-millefiori-come-nasce-caratteristiche-proprieta/

## 4.3 Materials and Methods

### 4.3.1 Sampling

Thirtytwo honey samples in three suburban areas (eight sites) of the Campania region were collected: Caserta province (Cancello ed Arnone, Castel Volturno, and Mondragone), Vesuvius area (Torre del Greco, Trecase, Terzigno and Ottaviano), and Sorrento peninsula (Vico Equense). Four honey samples were collected for each site at the end of October 2020. 50 g of fresh honey belonging to polyfloral species were collected directly from beekeepers. The samples were placed individually in sterile test tubes, frozen at a temperature of − 80 °C, and then analysed.

### 4.3.2 Chemical and Instrumental Analysis

0.50 g of sample were homogenized, and 5 mL of $HNO_3$ (65% w/w) and 2 mL of 30% $H_2O_2$ (30% w/w) were added. Then the samples underwent wet mineralization through a Milestone microwave for 30 min at 190 °C, and finally, the samples were cooled and transferred to a flask, and the final volume was adjusted to 25.0 mL by adding Millipore MillQ® water (18.2 MΩ cm resistivity).

Metals analysis was performed using a Thermo Scientific™ ICAP™ RQ inductively coupled plasma mass spectrometer (Q-ICP-MS) with a Burgener Mira-Mist nebulizer, a Quartz cyclonic spray chamber, cooled to 2.7 °C, and skimmer cones. The instrument was operated using the Thermo Scientific™ Qtegra™ Intelligent Scientific Data Solution™ (ISDS) Software. The operating conditions of the Q-ICP-MS equipment were optimized using a tuning solution (Ba, Bi, Ce, Co, In, Li, U 1.00 $\mu$g/ L, Thermo Scientific) on masses $^{115}$In, $^{7}$ Li, $^{59}$Co, $^{238}$U, $^{209}$Bi, and $^{140}$Ce was used for oxide and

doubly charged interference checks. The analysis was performed in KED (Kinetic Energy Discrimination) mode, and the parameters were: collision gas: He, plasma gas flow (Ar): 14,8 mL/min; nebulizer gas flow: 0.98 L/min; auxiliary gas flow: 0.85 L/min; ICP RF Power: 1550 W; CeO/Ce = 0.0057. Cell gas flow was 4.8 mL/min for He. The Q-ICP-MS was used for the determination of As, Ba, Cd, Cr, Cu, Mn, Hg, Ni, Pb, Sb, and V in honey. All samples were analysed in duplicate, and each sample was measured in triplicate by Q-ICP-MS detection ([132]; [133];[131]). The solutions were prepared using water (18.2 MΩ cm resistivity) purified with a Millipore Mill-Q® purification system, concentrated nitric acid ($HNO_3$ 65% m/m, Suprapur®, Merck, Germany) and hydrogen peroxide ($H_2O_2$ 30% m/m, Suprapur®, Merck).

An $HNO_3$ 1% v/v (Suprapur®, Merck, Ultrapure) solution was used to clean the Q-ICP-MS apparatus between quantifications. The calibration standards were prepared with multi-element standard solution CertiPUR® (Merck, Darmstadt, Germany) 1000 mg $L^{-1}$ at concentrations: 0.5, 1.0, 2.5, 5.0, 10.0 µg $L^{-1}$. An internal standard mix comprising 50 µg $L^{-1}$ Ge, 5 µg $L^{-1}$ Ir, 10 µg $L^{-1}$ In and 25 µg $L^{-1}$ Y was introduced online with an internal standard mixing kit. The internal standard elements were appropriately matched to analyte elements ([134];[135]).

### 4.3.3 Estimated Daily Intake

The exposure population assessment to the elements through the ingestion of honey was calculated by estimated daily intake (EDI) according to the following formula suggested by the United States Environmental Protection Agency (USEPA) ([174]):

$$EDI = \frac{C \cdot IR \cdot EF \cdot TE}{BW \cdot AT}$$

where C is the concentration of each PTE detected in the samples (mg/ kg); IR is the Intake Rate of honey (kg/day) for toddlers, adolescents, and adult population; BW is Body Weight (11.3, 52.6, and 69.7 $kg_{bw}$ for toddlers, adolescent, adult, respectively ([175]). EF is Exposure Frequency to the contaminant (350 day/year); TE is Total Exposure (70 years), and AT is the Average Lifetime time for non-carcinogenic risk (TE × 365 day/year). The data relating to IR were retrieved from Chronic Food Consumption per day (g/day) of EFSA ([176]):

- Toddlers = median 10.0 g/day, 95th percentile 25.3 g/day
- Adolescents = median 13.3 g/day, 95th percentile 19.0 g/day
- Adults = median 10.0 g/day, 95th percentile 35.8 g/day

### 4.3.4 Non-Carcinogenic Risk Assessment

#### Target Hazard Quotient

The Target Hazard Quotient ($THQ_m$) for non-carcinogenic effects of each PTE through dietary exposure was calculated through the following formula suggested by USEPA:

$$THQ = \frac{EDI}{RfD_m}$$

RfDm is Oral Reference Dose ($mg/kg_{bw}/day$) (Table 4.1) proposed by US EPA. Some studies used the same RfD for Hg ([177]; [178]); Cr ([179]; [180]; [181]); Ni ([180]; [182]; [181]); As ([183]; [181]); Mn ([180]). $THQ_{Pb}$ is a particular case because, according to US EPA, setting a reliable threshold for Pb is challenging. We used $THQ_{Pb}$ proposed by other studies ([182]; [178]; [184]; [181]). A $THQ_m$ (dimensionless) $> 1$ entails a high non-carcinogenic

risk.

### Multiple Exposure to Toxic Elements

In the case of multiple exposure to several contaminants, the cumulative risk arising from the dietary exposure to all elements was assessed through the Hazard Index (HI). This index stands for the sum of $THQ_m$ for each element and is calculated as follows:

$$HI = \sum_m THQ_m$$

An HI $> 1$ entails a high risk as far as non-carcinogenic risk is concerned.

| Element | RfD (mg/ $kg_{bw}$/day) | Reference | CSF (mg/ $kg_{bw}$/day)$^{-1}$ | Reference |
|---|---|---|---|---|
| HD | 0.0003* | [185] | / | |
| Cd | 0.0001 | [185] | 0.38 | [186]; [187]; [181] |
| V | 0.005** | [185] | / | |
| CR | 0.003*** | [185] | 0.50 | [188] |
| Ni | 0.02▲ | [185] | 1.70° | [188] |
| Cu | 0.04 | [185] | / | |
| As | 0.0003▲▲ | [185] | 1.50 | [185] |
| Ba | 0.20 | [185] | / | |
| Sb | 0.0004▲▲▲ | [185] | / | |
| Pb | 0.0035 | [182]; [178]; [181]; [189] | 0.0085°° | [188] |
| Mn | 0.10 | [190] | / | |

** Vanadium and Compounds
*** Chromium VI
▲ Nickel Soluble Salts
▲▲ Inorganic Arsenic
▲▲▲ Antimony (metallic) and Antimony Tetraxide
° Nickel subsulfide
°° Lead and Compounds
* Mercuric Chloride and other Mercury salts

Table 4.1: Oral reference Dose (mg/$kg_{bw}$/day) and Cancer Slope Factor (mg/$kg_{bw}$/day)$^{-1}$ for each element

### 4.3.5 Carcinogenic Risk Assessment

The carcinogenic effects related to the ingestion of food contaminated by Ni, Cr, Pb, As, and Cd were evaluated through the Lifetime Cancer Risk (CR) ([174]), that is:

$$LTCR = EDI \cdot CSF$$

CSF is the Cancer Slope Factor $(\text{mg/kg}_{bw}/\text{day})^{-1}$ that estimates the probability of developing cancer through Ni, Cr, Pb, As, and Cd ingestion. The $\text{CSF}_{Cd}$ proposed (Table 4.1) was previously used by Gebeyehu and Bayissa (2020) ([186]) and Real et al. (2017) ([181]). USEPA considers an LTCR (dimensionless) $> 1 \times 10^{-4}$ as an unacceptable risk of developing cancer over a human lifetime, whereas values between $1 \times 10^{-6}$ and $1 \times 10^{-4}$ are considered an acceptable range for carcinogenic risk ([190]). Instead, Health Canada and AEP propose the value of $1 \times 10^{-5}$ as the maximum safety threshold for the risk of developing cancer ([191]). The cumulative cancer risk is the risk estimation due to exposure to multiple carcinogenic elements and was calculated as:

$$LTCR_{tot} = \sum_{k=1}^{n} LTCR_k$$

Where LTCRk is the Life Time Cancer Risk for the cancer element k.

### 4.3.6 Statistical Analysis

Data analysis and graph processing were performed using R Software version 3.6.0 and ggplot2 package ([136];[140]).

## 4.4 Results and Discussion

### 4.4.1 Elements Concentration in Honey

The levels of each element obtained by the analysis of honey samples are listed in Table 4.2. No statistically significant difference emerged among the areas and municipalities regarding the levels of heavy metals. The concentrations ranged between 0.70 $\mu$g/kg (Hg) and 1713 $\mu$g/kg (Mn). Higher mean values were observed for Cu (1049 $\pm$ 360 $\mu$g/kg), Mn (881.1 $\pm$ 405.9 $\mu$g/kg), Ba (260 $\pm$ 145 $\mu$g/kg), and Ni (167 $\pm$ 80.4 $\mu$g/kg). Other elements reported concentration equal to 5.48 $\pm$ 3.55 $\mu$g/kg for Cd, 12.0 $\pm$ 8.30 $\mu$g/kg for V, 72.3 $\pm$ 19.2 $\mu$g/kg for Cr, 14.9 $\pm$ 8.36 $\mu$g/kg for As, 3.34 $\pm$ 3.02 $\mu$g/kg for Sb, 30.1 $\pm$ 8.91 $\mu$g/kg for Pb, and 31.81 $\pm$ 26.03 $\mu$g/kg for Hg.

Previous studies assessed the levels of heavy metals in honey in several regions of Italy (Fig. 4.2). However, these studies considered fewer elements. Perna et al., 2021 ([192]) analysed samples of honey collected from agricultural, forestry, urban, and industries areas of the Basilicata region. They reported similar values for Cd (3.31 $\pm$ 2.33 (range: 0.40–9.36) $\mu$g/kg) and Ba (187 $\pm$ 122 (range: 10.77–544 $\mu$g/kg), whereas higher levels for Mn (881 $\pm$ 406 (range: 523–1713) $\mu$g/kg). Squadrone et al., 2020 ([193]) considered monofloral and polyfloral honey samples from the pre-Alpine area. They reported similar levels for Cr (68.0 $\pm$ 63.0 $\mu$g/kg), Cu (950 $\pm$ 940 $\mu$g/kg), Ni (220 $\pm$ 190 $\mu$g/kg), and As (16.0 $\pm$ 21.0 $\mu$g/kg), whereas higher levels for V (9 $\pm$ 49 $\mu$g/kg), Pb (58 $\pm$ 160 $\mu$g/kg), and Mn (2100 $\pm$ 1700 $\mu$g/kg). Bontempo et al. (2017)([194]) collected seven different botanical samples in Northern, Central, and Southern Italy, reporting similar data for Ni (200 $\pm$ 300 $\mu$g/kg) and Ba (200 $\pm$ 200 $\mu$g/kg) but lower levels of

Cr (0 ± 0 $\mu$g/kg) and Cu (600 ± 1000 $\mu$g/kg) in polyfloral honey. Quinto et al., 2016([195]) analysed honey samples from Foggia, Naples, Caserta, Campobasso, Isernia, Matera, L'Aquila, and the province of Rome. As regards the area of the Campania region (Caserta and Naples), the levels of the elements that occurred in this area were lower than the levels reported in our study. Di Bella et al. (2015) ([196]) reported mean values of Ni (220 ± 110 (range: 110–330) $\mu$g/kg) in line with our data, although levels of Cr (140 ± 70 (range: 70–190) $\mu$g/kg) and Pb (50 ± 30 (range: 30–70) $\mu$g/kg) were higher from an analysis of polyfloral honey in Sicily and Calabria. Meli et al., 2015 ([197]) analysed twenty-one honey samples collected from Marche, chiefly belonging to the polyfloral species. The analysis showed similar levels for Mn (870 ± 560 (range: 370–2930) $\mu$g/kg) but higher values for Cd (20 ± 18 (range: 1–53) $\mu$g/kg), Cr (270 ± 230 (range: 20–670) $\mu$g/kg), and Pb (180 ± 160 (range: 10–450) $\mu$g/kg). Naccari et al., 2014 ([198]) reported higher levels of Cd (15.3 ± 4 (range: <LOQ - 24) $\mu$g/kg) and Pb (170.9 ± 68 (range: 78–390) $\mu$g/kg) in seven samples for each honey species (Carob, Chestnut and Eucalyptus). Pisani et al., 2008 ([199]) considered several botanical origin (including polyfloral) honey samples of the Siena area. Their data-related to polyfloral honey showed similar values for Cd (4.25 ± 3.58 (range: 1.00–15.3) $\mu$g/kg) and Sb (3.40 ± 2.19 (range: 1.20–13.3) $\mu$g/kg), whereas levels of Ni (273 ± 413 (range: 77–2760) $\mu$g/kg), Ba (915 ± 424 (range: 408–2634) $\mu$g/kg), Pb (76.4 ± 55.9 (range: 28.2–304) $\mu$g/kg), and Mn (1680 ± 3610 (range: 130–1690) $\mu$g/kg) were higher than our data. According to the literature, as mentioned earlier, almost all elements reported a fair variability likely due to the area characteristics (urban, industrial, agricultural, volcanic), botanical origin, and collection period. On the other hand, Ni levels are often in agreement with those of other studies in different sites of Italy, namely

Basilicata, Calabria, Sicily, Piedmont, Marche and Tuscany regions ([192]; [193];[195] ; [196]; [197]; [199]).

| Concentration ($\mu$g/kg) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cd | V | Cr | Ni | Cu | As | Sb | Ba | Pb | Hg | Mn |
| Mean | 5.48 | 12.03 | 72.34 | 167.0 | 1049 | 15.0 | 3.34 | 259 | 30.09 | 31.81 | 881 |
| Sd | 3.55 | 8.29 | 19.18 | 80.35 | 360 | 6.36 | 3.02 | 145 | 8.91 | 26.0 | 06 |
| Median | 5.08 | 8.53 | 67.4 | 137 | 1107 | 12.2 | 2.40 | 245 | 28.7 | 44.6 | 721 |
| Min | 1.15 | 5.35 | 48.1 | 64.3 | 510 | 5.35 | 1.50 | 103 | 16.5 | 0.70 | 522 |
| Max | 10.80 | 29.7 | 101 | 321 | 1465 | 26.1 | 10.60 | 502 | 42.8 | 55.9 | 1713 |

Table 4.2: Elements detected in honey in eight sites of the Campania

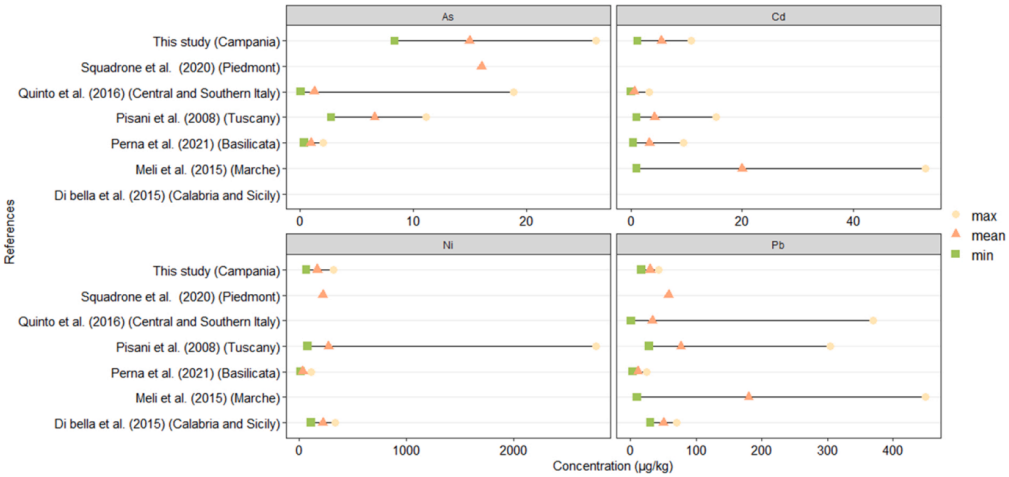


Figure 4.2: As, Cd, Ni and Pb levels in honey collected in Italy according to different studies in Italian regions

Where available, data on polyfloral honey were used for the comparison (Fig. 4.2). Pb is the only element in honey that present legal limits in Europe.

According to Commission Regulation (EU) 2015/1005 ([200]) related to maximum levels of Pb in foodstuffs, the threshold value in honey is 100 $\mu$g/kg wet weight (EU, 2015). The higher level of Pb was 55.95 $\mu$g/kg: therefore, all samples showed concentrations essentially lower than the EU Regulation threshold limit.

### 4.4.2 Bioaccumulation Comparison among Honey, Bee and other Beekeeping Matrices

This study was part of a broader investigation that evaluated the heavy metals in honeybees and beekeeping matrices (wax and pollen) and the impact of the Covid-19 pandemic on environmental pollution ([201]). Unlike the other beekeeping matrices, honey showed the lowest concentration for most elements. This result corroborates the hypothesis that the activity of the worker bees during the honey elaboration process may have a bearing on the levels of metals in the finished product, and this evidence matches that observed in earlier studies ([202]; [203]; [172]; [173]). It could conceivably be hypothesized that the levels of heavy metals in the honey is affected by enzymes and molecules (gluconic and ascorbic acid) responsible for chelation of elements and complex formation, leading to the absorption and accumulation of metals in specific body anatomic sections or excretion with feces, rather than their accumulation in honey ([173]). Furthermore, the different contamination levels may be due to the chemical-physical features of the matrices: lipophilic elements may preferentially accumulate in the lipids, whose concentrations are higher in honeybees, wax, and pollen than in honey. In addition, pollen and honeybee are more exposed to airborne particulate matter, accumulating elements on their outer surface.

### 4.4.3 Risk Assessment through Honey Consumption

#### Non-Carcinogenic Risk

To assess non-carcinogenic risk, THQ was calculated based on intake of honey for toddlers, adolescents, and adults (in a median and 95th percentile scenario) and RfD proposed by EFSA and US EPA (Tab. 4.1). The values

ranged from 7.09E-05 (median exposure) in adults for Ba to 0.40 (95th percentile exposure) in toddlers for Hg (Fig. 4.3). THQ did not exceed the threshold value of 1 for each element for toddlers, adolescents, and adults in both scenarios indicating that non-carcinogenic health effects were not significant. Likewise, HI reported values below the safety threshold (namely $< 1$). The highest value was 0.69 and occurred in toddlers (95th percentile). Hence, the exposure to all elements showed a low probability to cause adverse non-carcinogenic health effects over a lifetime.

**Carcinogenic Risk**

LTCR was used to assess carcinogenic risk based on CSF proposed by US EPA and US DOE (Table 4.1). Not all elements are carcinogenic; therefore, CSF is only available for Cd, Cr, Ni, As, and Pb (probably carcinogenic). According to USEPA, LTCR values above $1 \times 10^{-4}$ are considered unacceptable regarding the risk of developing cancer ([190]). For Ni exposure, LTCR $> 1 \times 10^{-4}$ was observed in toddlers, whereas the threshold was reached in adolescents and adults to the third quartile (75th percentile). Considering the Health Canada criteria ([191]), the levels of Cr for all age groups and As in toddlers exceeded the threshold of $1 \times 10^{-5}$ (Fig. 4.4). Accordingly, a carcinogenic risk emerged from cumulative LTCR assessment: a higher risk was observed in toddlers (median: 2.44E-04; 95th percentile: 6.17E-04) than adolescents (median: 6.98E-05; 95th percentile: 9.95E-05) and adults (median: 3.95E-05; 95th percentile: 1.42E-04). Previous studies reported significant levels of these heavy metals. Ullah et al. (2022) ([170]) declared an LTCR $>$ 1E-04 and 1E-05 for Ni and Cd, respectively, based on an analysis of honey collected in Pakistan.

Likewise, Pipoyan et al. (2020) ([166]) reported a carcinogenic risk for Ni (ILCR $>$ 1E-04) as well as As (ILCR in the range of 3.36E-06 and

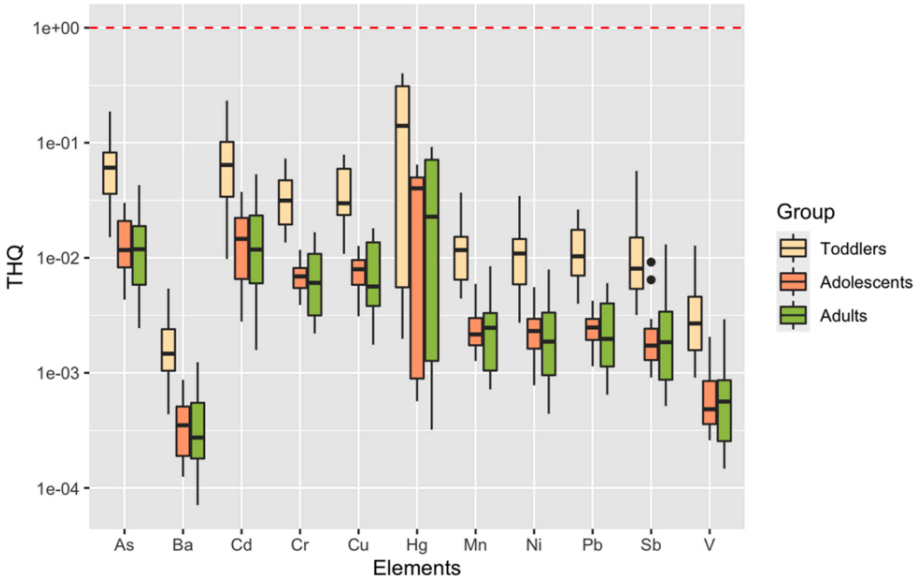


Figure 4.3: Target Hazard Quotient (THQ) values for non-carcinogenic risk based on elements exposure in toddlers, adolescents, and adults

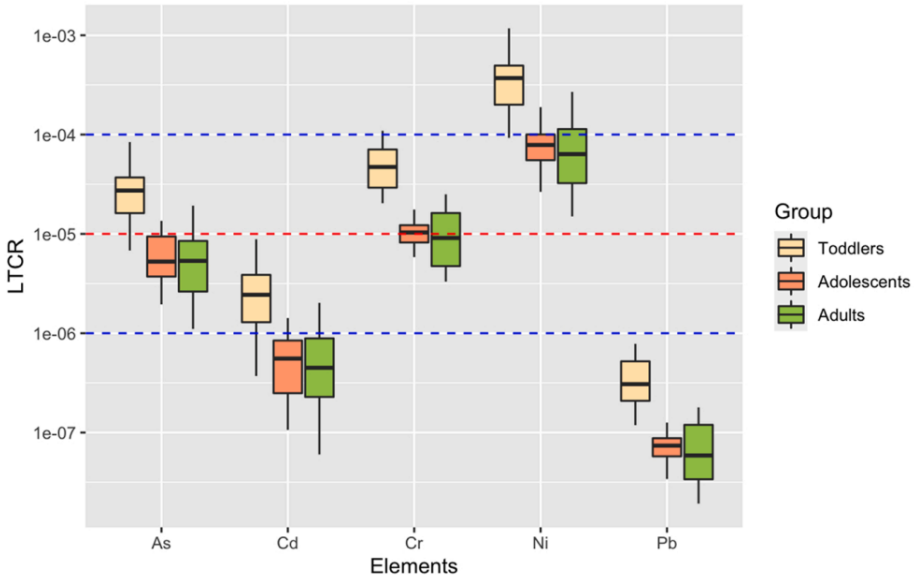


Figure 4.4: Lifetime Cancer Risk (LTCR) values based on carcinogenic elements exposure in toddlers, adolescents, and adults

1.94E-05) related to honey from Armenia. Instead, in Nigeria, Orisakwe et al. (2019)([171]) showed a higher LTCR for As, equal to 5.25E-02.

Based on the above, honey collected in some municipalities of the Campania region can accumulate high levels of Ni, Cr, and As, similarly to other areas

of the world, pointing out a potential risk for its consumption, mainly among toddlers.

## 4.5 Conclusions

Eleven heavy metals and essential elements were analysed in polyfloral honey collected in different municipalities of the Campania region. No statistically significant difference was observed among sampling sites.

The analysis revealed that no sample exceeded the threshold limit of Pb (100 $\mu$g/kg), set by Regulation (EU) 2015/1005. Risk assessment in toddlers, adolescents, and adults based on median and 95th percentile honey intake showed no concern for non-carcinogenic risk; In contrast, closer attention deserves to be paid to the exposure to Ni, Cr, and As for carcinogenic risk in the three groups, even though higher values emerged among toddlers.

In conclusion, these findings suggest that environmental pollution has a lower impact on the occurrence of heavy metals in honey, which could probably benefit from the capability of honeybees in the detoxification of this product. However, because of the possible risks from honey consumption, setting regulation threshold limits for the Ni, Cr, and As should be considered.

# Chapter 5

# Occurrence of phthalate esters and preliminary data on microplastics in fish from the Tyrrhenian sea (Italy) and impact on human health

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## 5.1 Abstract

Phthalic acid esters (PAEs) are chemical pollutants widely distributed in the marine environment. They can accumulate in biota, posing a risk to the marine ecosystem and humans. The aim of this study was to measure the content of PAEs in the gills and muscles of three fish species (*Mugil cephalus*, *Diplodus annularis*, and *Mullus barbatus*) caught along the coast of Campania (Italy), as well as to ascertain the dietary exposure to PAEs' through the consumption of fish. Secondly, a preliminary insight into MPs pollution in this area was provided through the analysis of *Mugil cephalus* organs. Solid-phase extraction and GC-MS were used for the PAEs analysis, while an infrared microscope was used to detect MPs after a pre-digestion of the samples. Risk assessment was based on estimated daily intake (EDI) and lifetime cancer risk (LTCR).

The results showed higher bioaccumulation of PAEs in *Mullus barbatus* than in the other two species and higher concentration in gills than in muscles. MPs (polyamide, polypropylene, and high-density polyethylene) were detected in 50% of the gill samples, but no particle was detected in the muscle samples of *Mugil cephalus*. A low carcinogenic and non-carcinogenic risk from the consumption of fish emerged, although a potential risk for the development of cancer was found in the worst-case, especially in toddlers. In conclusion, this study provides insight into PAEs pollution in the Tyrrhenian Sea (Italy), their distribution in fish with different behaviors, and the potential risk to the consumer. Moreover, the data on pollution by MPs in this area could form the basis for future studies.

## 5.2 Introduction

Marine pollution is a worldwide burden due to the continuous accumulation of pollutants from anthropogenic sources. Some contaminants can accumulate in the environment over time, becoming a health hazard to living beings. Among these, phthalates or phthalic acid esters (PAEs) represent a critical environmental issue due to their ubiquity. PAEs are characterized by 1,2-benzenedicarboxylic acid and alkyl chains that affect their physicochemical properties, such as water solubility and biodegradation ([204]). They are not chemically bound to the plastic matrix and tend to migrate to the air, water, soil, and food due to particular chemical-physical and biological conditions (temperature, humidity, salinity, solar radiation) ([205]; [206], [207], [208]).

PAEs are endocrine disruptors due to their effects on reproduction, metabolism, and growth in humans ([8]). Some of them can also have neurotoxic, genotoxic, and carcinogenic effects ([8]; [9]; [10]; [11]). For this reason, PAEs such as di-n-octyl phthalate (DnOP), di-7-methyloctyl phthalate (DiNP), di-2-ethylhexyl phthalate (DEHP), di-butil phthalate (DBP), di-ethyl phthalate (DEP), di-methyl phthalate (DMP) are listed in the Hazardous Substances Data Bank (HSDB). PAEs have been employed in cosmetics, slow-release capsules and dietary supplements, varnishes, medical devices, and pesticides, although their primary use is as plasticizers that add flexibility to plastics ([209]; [210]; [11]). As a result, the huge amounts of plastic waste in the marine environment, estimated to 12 million tons/year ([7]), make it likely the primary vector of PAEs in the sea ([211]). For this reason, PAEs can bioaccumulate in marine organisms such as fish due to their ingestion/contact directly or indirectly through small fragments or fibers of plastics (0.1 µm – 5 mm), namely microplastic (MPs) ([212]; [213];

[214]). Indeed, Lu et al. (2021) ([215]) demonstrated the association between the levels of PAEs (i.e., DEP) and MPs occurrence in estuarine fish. This also poses a risk to the human diet, a significant route of exposure to these chemicals ([216]; [217]). According to Wang et al. (2018) ([218]) and Domenech & Marcos (2021) ([219]), seafood contributes up to 30.01% of total PAEs and 22.04 $\times$ $10^3$ p/year of MPs to intake. Therefore, this study initially investigated the levels of six PAEs in the gills and muscles of stationary and commercial fish species (*Mugil cephalus*, *Diplodus annularis*, *Mullus barbatus*) caught along the Campania coast characterized by intense, productive activity.

The aim was to understand the pollution by PAEs and their distribution among species. In addition, the presence of MPs in the gills and muscles of *Mugil cephalus* was studied to obtain the first data on microplastic pollution in this area and its co-occurrence with PAEs.

Finally, the potential risk from the ingestion of PAEs by fish consumption was assessed through a deterministic approach.


Figure 5.1: *Mugil cephalus* (Linnaeus, 1758)

Figure 5.2: *Diplodus annularis* (Linnaeus, 1758)

Figure 5.3: *Mullus barbatus* (Linnaeus, 1758)

## 5.3 Materials and Methods

### 5.3.1 Sampling

Three different fish species, such as *Mugil cephalus*, *Diplodus annularis*, and *Mullus barbatus*, were caught by local fishermen. Fifteen adult fish for each species (tot = 45) with median weight are selected for PAEs analysis. Overall, forty-five gills (considering left and right gills as one sample) and forty-five muscles were collected. Instead, 8/15 gills and 8/15 muscle samples belonging to *Mugil cephalus* were used for MPs analysis. Due to the size of the organs, only the samples of *Mugil cephalus* allowed the research for both PAEs and MPs in gills and muscles. For the same reason, only some of *Mugil cephalus* samples were analysed. The average length was 30 - 40 cm for *Mugil cephalus*, 10 - 20 cm for *Diplodus annularis*, and 10 - 15 cm for *Mullus barbatus*. Three species were equally collected (fifteen for each species) at each sampling site from July 2020 to February 2021.

The sampling sites extended from Domizia Bay (N 41° 8' 44.081", E 13° 48' 8.643") to the Sarno River mouth (N 40° 41' 29.594" E 14° 26' 31.318") of the Campania coast (Tyrrhenian Sea). The samples were frozen within a few hours of collection and then dissected to collect gills and muscles.

### 5.3.2 Chemical and Reagents

DnOP, DiNP, DEHP, DBP, DEP, DMP and deuterated DEHP (DEHP-D4) standards were purchased from Sigma-Aldrich (Shneldorf, Germany). Florisil and Bondesil were supplied by VWR International and Agilent Technologies (Palo Alto, CA, USA), respectively.

Solvents for cleaning and other reagents (i.e. n-hexane, dichloromethane,

acetone, heptane) were purchased from Merck & Company, Inc. (Kenilwortf NJ, USA).

### 5.3.3 Phthalates Extraction and Purification

Extraction and purification for PAE analysis were performed according to the method described by Tsumura et al. (2001) ([220]), with minor modifications ([221]). No plastic equipment was used, to avoid external contamination of PAEs and glassware was preliminarily heated in a muffle and washed with acetone and n-hexane before use. For the extraction, 1 g and 0.5 g aliquot of muscle and gills, respectively, previously freeze-dried, was collected in a test tube to which 10 ml of nhexane-dichloromethane (50:50) were added. The tube was mechanically shaken and placed in an ultrasonic bath for 20 minutes. Then, the sample was centrifugated at 2500 rpm for 10 minutes to collect the supernatant in a round-bottom flask (the procedure was performed in duplicate).

The supernatant was concentrated using Rotavapor at 45°C and mixed with 5 ml of n-hexane. After, the sample was purified on a manually packed chromatographic column. The column was prepared with wadding (preconditioned with acetone and n-hexane and placed in an oven at 100°C for one hour), 2.0 grams of Florisil, and 0.5 g of Bondesil PSA, and 1 g of anhydrous sodium sulfate ($Na_2SO_4$).

Finally, the sample was eluted, mixed with 10 ml of acetone and hexane (4:1), and concentrated using Rotavapor. Finally, 2 ml of n-hexane was added to the sample collected in an amber glass vial (2 ml).

### 5.3.4 GC-MS Analisys

A GC/MS (Agilent 7890A GC system coupled to an Agilent 5975C mass selective detector (MSD) (Agilent Technologies, Santa Clara, CA, USA)) was used for the quantification of PAEs.

The parameters were set as follows: initial oven temperature: 60°C (holding time 0.5 minutes); injector temperature: 280 °C. After the injection, the ramp rate was programmed from 60°C to 240 °C at 10 °C/min and until 300 (holding for 18 min) at 15 °C/min. The transfer line of the GC-MS interface was held at 280 °C. Samples (1 $\mu$L) were injected in splitless mode into the capillary gas chromatography column. The carrier gas was high-purity helium (99.999%) at a 1.0 mL/min flow rate.

### 5.3.5 Sample Pretreatment for Microplastics Analisys

The dry sample was ground, filtered with sequentially decreasing sieves and then digested. 1g of sample was mixed with 20 ml of 30% $H_2O_2$ and placed in an oven at 30°C for 24 h. Supplementary $H_2O_2$ was added when evaporated. Then, NaCl (1.2 g/cm$^3$) was added, allowing a densiometric separation. The supernatant was collected and mixed with 100 ml Milli-Q water. The digested sample was filtered through a silicon (Si) filter (diameter: 10 mm$^2$, pore size: 5 $\mu$m) and placed in a vacuum filtration system (glass funnel with a tube on the bottom of the stem).

### 5.3.6 Microplastic Identification and Quality Control/Assurance

The identification was carried out through FTIR Nicolet™ iN10 infrared microscope (Thermo Fisher Scientific Madison, WI, USA). A transmittance

and reflection mode was set to characterize the polymers. The particle size sieve was set at 0-5000 µm, whereas the spectral range was set at 4000–675 $cm^{-1}$ with a collection time of 3 seconds and 16 co-scans. The spectral resolution was 8 $cm^{-1}$, and the aperture size was from 50 × 50 µm to 150 × 150 µm. The spectra were compared with the library, and matches ≥70% were accepted ([222]; [223]; [224]) based on the analysis performed through Omnic™ Picta™ software.

Potential environmental contamination was avoided following some precautions, according to Prata et al. (2021) ([225]): use of a laminar flow hood during each step of analysis; filtration of the liquids (water and $H_2O_2$) before use (0.22 $\mu$m filter); use of no plastic equipment and clothes; heating of glass and steel in muffle at 450°C; covering filtration equipment with Petri dish during the process; application of the blank test. No MPs were detected due to indoor airborne contamination.

### 5.3.7 Risk Assessment

The potential risk due to PAEs through fish intake was evaluated based on Tolerable Daily Intake (TDI) and Life-Time Cancer Risk (LTCR). At first, the Estimated Daily Intake (EDI, ng/$kg_{bw}$/day) was calculated (Eq. 1) and compared with the TDI of each PAEs, if any, (Table 5.1) proposed by EFSA (2019) ([226]) and WHO (2003)([227]).

Eq. 1

$$EDI_{PAE} = \frac{IR \cdot C_{PAE}}{BW}$$

- $C_{PAE}$ Concentrations of each PAE detected in fish muscles (ng/g)
- IR: Intake Rate of fish (g/day) for toddlers (median: 50.00, $95^{th}$ percentile: 94.63), adolescents (median: 56.50, $95^{th}$ percentile: 177.73), and adult population (median: 57.50, $95^{th}$ percentile: 167.2) ([228])

- BW: Body Weight ($kg_{bw}$) for toddlers (11.3), adolescents (52.6), adults (69.7) ([175])

To assess the carcinogenic risk of PAEs such as DEHP, the LTCR (dimensionless) was used. The cancer risk was evaluated based on equation (Eq. 2) proposed by USEPA.

Eq. 2

$$LTCR = \frac{EDI \cdot EF \cdot TE}{AT} \cdot SF$$

- EF: Exposure Frequency to the contaminant (350 day/year)
- TE: Total Exposure (70 year)
- AT: Average Lifetime time for non-carcinogenic risk (TE x 365 day/year)
- SF: Slope Factor $(ng/kg_{bw}/day)^{-1}$ related to each PAE (Table 5.1)

USEPA considers an LTCR $>$ 1 x $10^{-4}$ as an unacceptable risk of developing cancer over a human lifetime, whereas values between 1 x$10^{-6}$ and 1 x $10^{-4}$ are considered an acceptable range for risk ([190]). Instead, Health Canada and Alberta Environment and Parks (AEP) propose a threshold of 1 x $10^{-5}$ for the risk of developing cancer ([191]).

| PAE | TDI (µg/$kg_{bw}$/day) | SF (mg/$kg_{bw}$/day) |
|---|---|---|
| DBP | 10 ([226]) | / |
| DEHP | 50 ([226]) | 0.014 ([190]) |
| DiNP | 150 ([226]) | / |
| DEP | 500 ([227]) | / |
| DMP | / | / |
| DnOP | / | / |

Table 5.1: Tolerable daily intake (TDI) and slope factor (SF) for each phtalic acid esters (PAEs)

### 5.3.8 Statistical Analysis

Data analysis and graph processing were performed using R Software version 3.6.0 and the following packages: ggplot2, ggsci, FactoMinerR, FactoInvestigate and factoextra ([136]; [138]; [139]; [140]; [141]).

Data were tested for the homogeneity of the variances (Bartlett's test) and normality (Shapiro-Wilk's test). Finally, a one-way analysis of variance with post hoc Tukey's test was performed to ascertain any statistically significant difference.

## 5.4 Results and Discussion

### 5.4.1 Occurrence of Phthalic Acid Esters in Different Fish Species

The concentrations detected in gills and muscles from the three species are listed in Fig. 5.4. The levels of PAEs in both organs, except DEHP, showed significantly different means at the 95% confidence level between *Mullus barbatus* and the other two species ($p < 0.05$). Gills of *Mullus barbatus* reported highest median values (median, min – max) for almost all PAEs: DBP (296, 97-680 ng/g), DEP (222, 87 – 411 ng/g), DiNP (344, 76 – 6600 ng/g), DMP (625, 181 – 1273 ng/g), DnOP (198, 80 – 6523 ng/g). Differently, the concentrations of contaminants in muscles in *Mullus barbatus* and the other two species showed a low variability among species (Fig. 5.4). $\sum$ PAE in gills showed higher levels in *Mullus barbatus* (2009, 1009 – 15388 ng/g) than *Diplodus annularis* (1609, 532 – 5303 ng/g) and *Mugil cephalus* (1040, 591 – 9388 ng/g). Likewise, $\sum$ PAE in the muscle of *Mullus barbatus* (914, 497 – 7259 ng/g) was higher than *Diplodus annularis* (898, 566 – 1823 ng/g) and *Mugil cephalus* (638, 509 - 1766 ng/g).

The higher contamination of of PAEs in *Mullus barbatus* was likely due to its habitat and their speciation in seawater.

Namely, PAEs have higher concentrations near the surface and seafloor due to their release from particular matter and sediments, which resulted in higher contaminant concentrations in *Mullus barbatus* that is a benthic species, than *Diplodus annularis* and *Mugil cephalus*, which are benthopelagic and pelagic euryhaline species, respectively ([229]; Zhang et al., 2018, [230]).

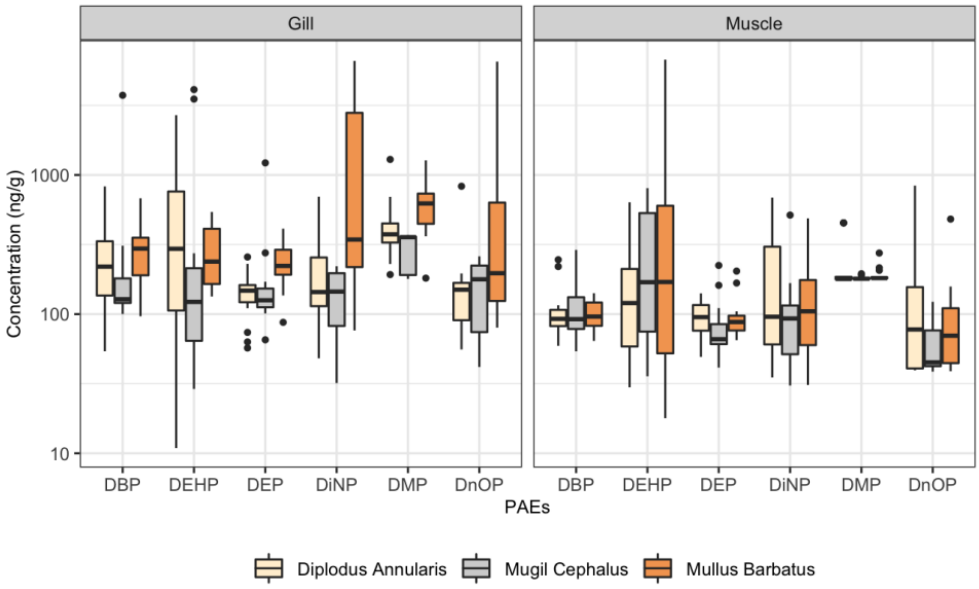


Figure 5.4: Concentration (ng/g) on the logarithmic scale of phthalic acid esters (PAEs) detected in gills and muscles belong to three species.

Indeed, these species live on sandy and muddy bottoms, which could lead to greater exposure to these contaminants ([231]). In addition, feeding (carnivorous in *Mullus barbatus* and omnivorous-detrital in *Mugil cephalus* and *Diplodus annularis*), as well as tissue fat (higher in *Mullus barbatus*) could influence PAEs accumulation ([232]; [230]; [233]). As regards the differential bioaccumulation in the organs, a significantly higher concentration of PAEs was found in the gills than in the muscle ($p < 0.05$). Gills are in close contact with the sea and dissolved contaminants since they are involved in water filtration, gas exchange, and osmoregulation.

On the other hand, the muscle was exposed to pollutants after absorption, detoxification, and distribution processes. These anatomic and physiologic differences could explain the expected trend of bioaccumulation and distribution of PAEs, as also reported by Hu et al. (2016) ([230]).

### 5.4.2 Occurrence of Microplastic in Fish

A preliminary background about the potential contamination by MPs in addition to PAEs was provided through the analysis of the gills and muscles of *Mugil cephalus*. Some studies reported an increase in (eco)toxicological risk due to the co-occurrence of PAEs and MPs than the single pollutant, although there is still a lack of literature ([234]; [235]).

The analysis revealed the presence of MPs as well as inorganics and plastic additives (e.g., bis(2-hydroxyethyl)dimerate). MPs were detected in 4/8 gill samples ranging from 453 – 3885 µm, while no MPs were detected in muscle samples. Three different polymers were found: fibers of polyamide (nylon), fiber of polypropylene (PP), and fragments of high-density polyethylene (HDPE) (Tab. 5.2). The occurrence of nylon and PP fibers are likely linked to the use and loss of ropes, nets, and fishing gear, as well as the release of textiles from sewage ([223]; [236]; [237]). Instead, HDPE could be linked to housewares, pipes, packaging, industrial wrappings and film scattered in the sea ([238]; [239]).

These preliminary data also provide the potential inability of MPs to migrate in the muscles of *Mugil cephalus*, although further study needs to confirm this trend. However, Su et al. (2019) ([240]) and Huang et al. (2020) ([241]) did not detect MPs in the muscles of fish from the East and the South China Sea, respectively. Instead, other studies reported contrary results ([222]; [223]; [236];[242]). Since no standardized methods are available for MPs analysis, there could be inconsistencies in the results, with possible under- or over-estimation of the particle amount ([225]). Several studies have detected MPs in other internal organs, such as fish liver ([243]; [222]) or brain ([244]), whereas toxicological studies highlighted the bioavailability of MPs in the particle uptake pathway according to in vitro,

murine models ([245]), and some evidence in goldfish liver ([246]): this process seems to occur in a size-dependent manner (<10 µm).

Instead, EFSA reported that MPs <150 µm could cross the intestine of mammals, but few data are available on their behavior after absorption ([247]). However, it is reported that deep penetration of microparticles into organs is limited to smaller particles (<1.5 µm) due to filtration through the spleen and liver via bile ([247]; [248]). Hence, the absence of MPs in muscles (in the considered size range) could be attributed to the organism's efficient barrier and filtration system.

However, for the reasons mentioned above and the limitation of the analytical instrument (< about 10 µm), lower size particles may have reached the organ without detecting them. Overall, MPs in fish should be of concern beyond its presence in muscle because several culinary preparations involve cooking whole fish, potentially releasing contaminants in the cooking broths, and consuming parts other than muscle. Furthermore, as previously mentioned MPs could absorb and release PAEs as well as other pollutants such as heavy metals, persistent organic pollutants (POPs), and pathogens that may distribute throughout the body ([13]; [14]; [12]; [15]).

| | Gill | | Muscle | |
|---|---|---|---|---|
| Sample | Polymer | Amount | Polymer | Amount |
| 1 | Polyamide (Nylon) | 1 | ND | |
| 2 | Polypropylene (PP) | 1 | ND | |
| 3 | High-density polyethylene (HDPE) | 1 | ND | |
| 4 | ND | | ND | |
| 5 | Polyamide (Nylon) | 1 | ND | |
| 6 | ND | | ND | |
| 7 | ND | | ND | |
| 8 | ND | | ND | |

Table 5.2: Type and amount of microplastics (MPs) detected in gills and muscles of fish samples

### 5.4.3 Phthalates Risk Assessment

The final part of the study aimed to assess the potential risks to consumers from exposure to PAEs as a risk assessment model for exposure to MPs in humans is yet to be developed. Two different scenarios were evaluated: best and worst case.

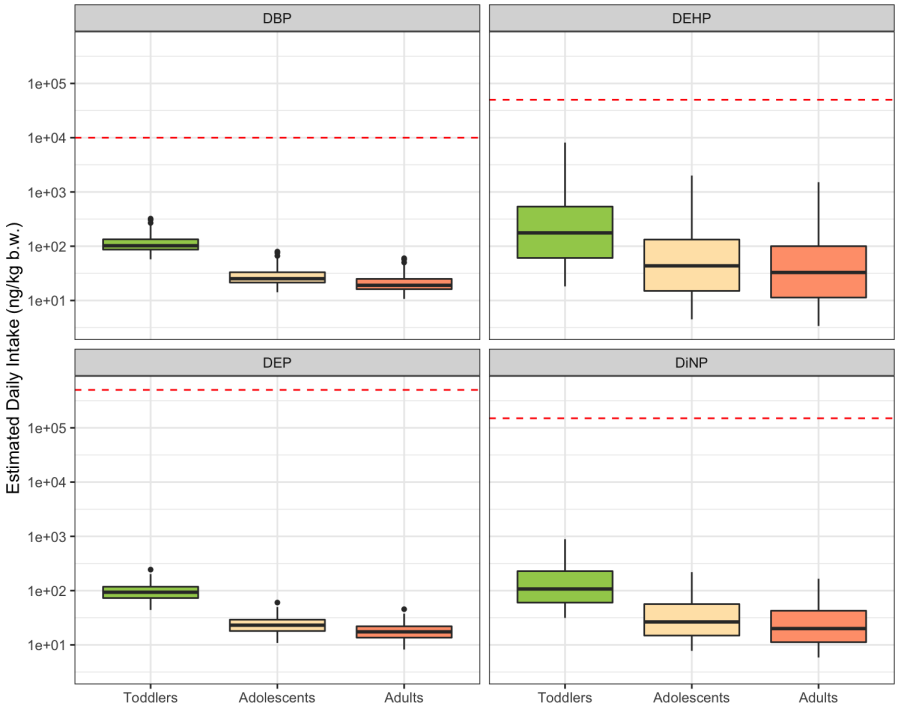


Figure 5.5: Estimated Daily Intake (EDI) (best case) on a logarithmic scale of four PAEs detected in fish muscles compared to respective Tolerable Daily Intake (TDI) (red line)

In the former, the values (median, min-max) of EDI referred to toddlers' exposure were 102.14 (57.44 – 322.48) ng/day/kg$_{bw}$ for DBP; 176.37 (18.20 – 8119.85) ng/day/kg$_{bw}$ for DEHP; 93.36 (44.04 – 243.90) ng/day/kg$_{bw}$ for DEP, and 107.18 (31.42 – 886.91) ng/day/kg$_{bw}$ for DiNP. Lower values were reported for adolescents and adults. In adolescents, the values were 25.23 (14.19 – 79.67) ng/day/kg$_{bw}$ for DBP; 43.57 (4.49 – 2006.03) ng/day/kg$_{bw}$ for DEHP; 23.06 (10.88 – 60.26) ng/day/kg$_{bw}$ for DEP; and

26.48 (7.76 – 219.11) ng/day/kg$_{bw}$ for DiNP. In adults, the values were 19.04 (10.71 – 60.12) ng/day/kg$_{bw}$ for DBP, 32.88 (3.39 – 1513.88) ng/day/kg$_{bw}$ for DEHP; 17.40 (8.21 – 45.47) ng/day/kg$_{bw}$ for DEP, and 19.98 (5.85 – 165.35) ng/day/kg$_{bw}$ for DiNP (Fig. 5.5). Instead, toddlers reported worst-case values of 193.34 (108.71 - 610.33) ng/day/kg$_{bw}$ for DBP, 333.78 (34.43 – 1649.24) ng/day/kg$_{bw}$ for DEHP, 176.68 (83.34 – 461.60) ng/day/kg$_{bw}$ for DEP, 202.85 (59.46 – 1678.56). In adolescents, the values were 77.99 (43.86 – 246.25) ng/day/kg$_{bw}$ for DBP; 134.67 (13.89 – 6200.57) ng/day/kg$_{bw}$ for DEHP; 71.28 (33.62 – 186.24) ng/day/kg$_{bw}$ for DEP; and 81.84 (23.99 – 677.27) ng/day/kg$_{bw}$ for DiNP. In adults, the values were 55.37 (31.14 – 174.83) ng/day/kg$_{bw}$ for DBP, 95.61 (9.86 – 4402.10) ng/day/kg$_{bw}$ for DEHP; 50.61 (23.87 – 132.22) ng/day/kg$_{bw}$ for DEP, and 50.10 (17.03 – 480.83) ng/day/kg$_{bw}$ for DiNP (Fig. 5.6).

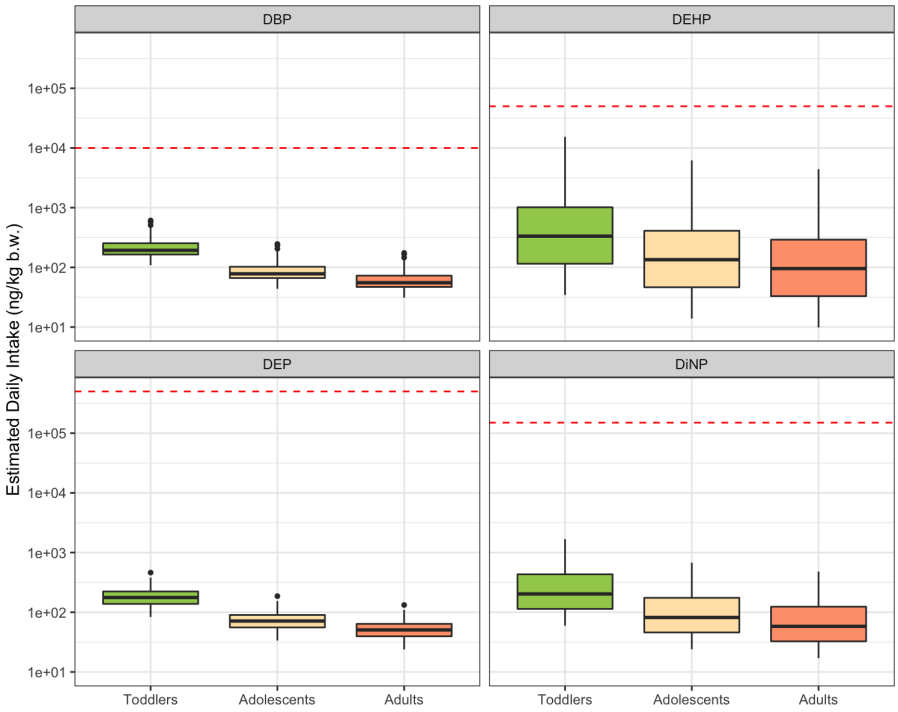


Figure 5.6: Estimated Daily Intake (EDI) (worst case) on a logarithmic scale of four PAEs detected in fish muscles compared to respective Tolerable Daily Intake (TDI) (red line)

The assessment of cumulative risk based on Group Phthalates concentration as DEHP equivalent (GPDEq) ([226]) considering DEHP (x1), DBP (x5), and DiNP (x0.3) reported in the best case values equal to 719.21 (314.82 – 9998.33) ng/day/kg$_{bw}$ in toddlers; 177.68 (77.77 – 2470.12 ng/day/kg$_{bw}$ in adolescents, and 134.09 (58.69 – 1864.10) ng/day/kg$_{bw}$ in adults, whereas in the worst case were 1361.18 (595.83 – 18922.85) ng/day/kg$_{bw}$ in toddlers; 549.21 (240.40 – 7635.03 ng/day/kg$_{bw}$ in adolescents, and 389.91 (170.67 – 5420.50) ng/day/kg$_{bw}$ in adults. Carcinogenic risk assessment based on LTCR estimation showing in the best-case values of 2.36 e06 (2.44e-07 – 1.09e-04) for toddlers, whereas adolescents and adults reported lower results: 5.74e07 (range: 5.93e-06 – 2.64e-05) and 4.41e-07 (range: 4.55e-08 – 2.03e-05), respectively (Fig. 5.7).

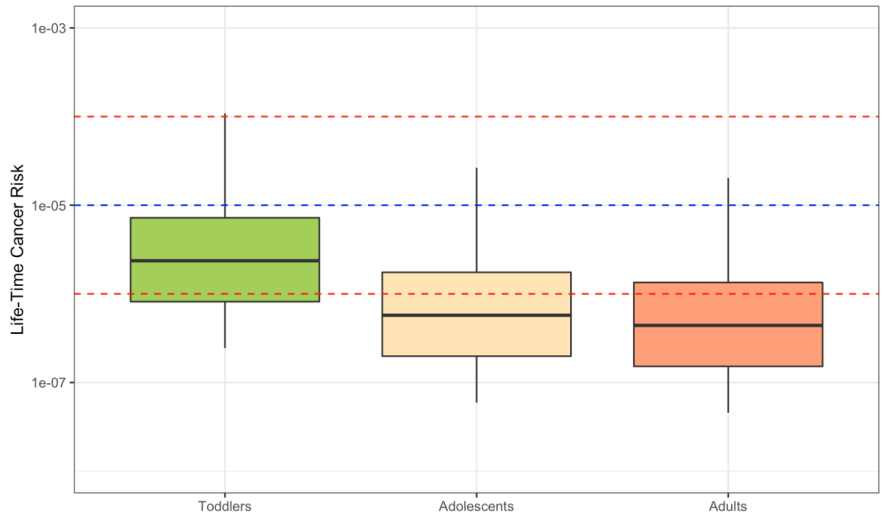


Figure 5.7: Life-Time Cancer Risk (LTCR) on a logarithmic scale due to exposure (best case) to PAEs detected in fish muscles compared to threshold values for carcinogenic risk (red and blue line)

Instead, in the worst-case, toddlers, adolescents, and adults reported values of 4.48e-06 (4.62e-07 – 2.06e-04), 1.80e-06 (1.86e-07 – 8.32e-05), and 1.28e-06 (1.32e-07 – 5.90e-04), respectively (Fig. 5.8).

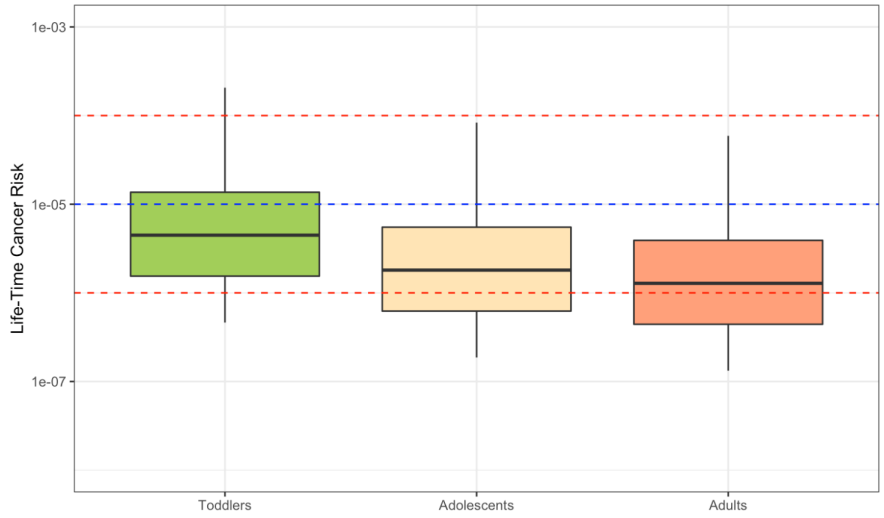


Figure 5.8: Life-Time Cancer Risk (LTCR) on a logarithmic scale due to exposure (worst case) to PAEs detected in fish muscles compared to threshold values for carcinogenic risk (red and blue line)

Overall, the median EDI values did not exceed the TDI proposed for the four PAEs, suggesting a non-carcinogenic risk. The cumulative risk as DEHP equivalent also showed a value below the TDI. The carcinogenic risk was estimated based on DEHP, considering its ability to induce hepatocellular carcinomas and adenomas.

The risk of developing cancer did not occur in any of the three groups, although thresholds (1E-$10^{-4}$ or 1E-$10^{-5}$) were exceeded for the levels above the third quartile. The estimates are based on a conservative approach that assumes 100% of the bio-accessibility and bioavailability of PAEs. However, Koch et al. (2005) ([249]) demonstrated in a human study that most orally administered DEHP (>70%) was absorbed. On the other hand, fish is only one of the several sources of exposure to PAEs that could significantly increase risk. Other commonly consumed foods such as cereals, vegetables, meat, dairy products, and beverages may contribute strongly to oral exposure ([218]).

## 5.5 Conclusions

Using fish as potential bioindicators, data on PAEs marine pollution in Campania (Italy) and bioaccumulation of these pollutants in the organs of three different species were obtained. The results show that fish behavior and characteristics probably influence the bioaccumulation of PAEs, which is higher in *Mullus barbatus* than in the other two species (*Mugil cephalus* and *Diplodus annularis*).

The gills were the organ with the highest contaminant load due to their anatomical, functional and physiological characteristics. In addition, three different plastic polymers (nylon, PP, HDPE) were detected in fish gills, whereas no MPs were found in muscles, indicating that MPs are unlikely to accumulate in these tissues.

Based on muscle contamination, the carcinogenic risk of exposure to PAEs from fish consumption was low in all age groups, although a potential carcinogenic risk was found in the worst case, especially in toddlers.

In conclusion, safer food choices could be made based on fish characteristics and behavior. The occurrence of PAEs in the Campania marine environment could be of concern and increase the dietary intake of xenobiotics related to fish consumption. Further investigations are needed to provide more in-depth data on MPs pollution and their exposure, as well as the potential correlation with PAEs. In addition, a probabilistic risk assessment for MPs needs to be developed to better characterize the risk of ingestion of these emerging pollutants related to fish consumption.

# Conclusions

In a short period of time, the increase in population and agricultural, zootechnical and industrial production has changed the state of the environment, affecting the quality of life of millions of plant and animal species, as well as that of humans.

In order to protect nature and everything related to it, it is of utmost importance to understand whether and to what extent anthropogenic activities damage the ecosystem and introduce pollutants into it.

Environmental monitoring is the systematic collection of qualitative and quantitative data, conducted using predefined methods and with the goal of assessing the state of the environment.

In the past, monitoring was based only on the analysis of the abiotic characteristics of an ecosystem (chemical-physical monitoring). However, the results obtained by this method were difficult to interpret, as pollutant concentrations often proved to be very low and too susceptible to undetectable changes. To overcome this problem, researchers began to include biotic factors in the analysis. This gave rise to biomonitoring: the regular and systematic assessment of the environment through scientific methods that use animal or plant species to measure the effect of pollutants on the environment ([1]).

The advantages of using bioindicators and bioaccumulators include: the possibility of monitoring wide areas, obtaining data that are continuous

in space and time; the possibility of evaluating synergistic effects of different contaminants on the organism; better detection of some substances: levels of contaminants in the bioaccumulating organism are often several orders of magnitude higher than those of the matrix in which it resides (water, air, sediment); economic affordability, especially over long periods and large areas, in the case of widespread pollution. However, the use of living organisms as bioindicators presents some problems. Biomonitoring based only on the analysis of bioindicators (not bioaccumulators) allows only indirect assessment of pollutant concentrations. Moreover, due to the high variability of environmental conditions and organism response, interpreting the results of a biomonitoring study can be more challenging than simply analysing instrumental data ([1]).

The studies I conducted during my PhD confirm the usefulness of bioindicators for environmental monitoring, acknowledge their limitations, and agree with the scientific community on the need for a system that utilizes of both biomonitoring and chemical-physical monitoring, as biomonitoring was never intended to replace the latter, but to support it, by allowing for more targeted and objective instrumental investigation through comprehensive, widespread surveys in the area (screening) ([250]).

The work presented in this thesis has mainly used animal species as bioindicators. However, animals are not the only organisms suitable for this purpose: microorganisms and plant organisms are used in biomonitoring studies and are often particularly suitable for assessing the presence of contaminants in the environment. Mosses and lichens are often used in air pollution studies. There are numerous biological characteristics that make these organisms excellent bioindicators. Mosses have a high ability to absorb and accumulate substances present in the atmosphere. Since these plants do not have cuticles and stomata, gas exchanges occur over the entire surface

of the plant ([250]).

In an ongoing study, we are working in collaboration with the Department of Biology of the Federico II University of Naples, to determine the concentrations of heavy metals (Cr, As, Cu, Cd, Pb, Sb, and Hg) in the moss species *Scorpiurum circinatum* and in beekeeping products, in three different areas of the Campania region (Italy). The study includes the collection and processing of data from active monitoring, carried out through the application of moss-containing retinas (moss bags), and from passive monitoring, carried out through the analysis of bees and hive products from hives usually present in the study areas.

During my PhD in Veterinary Sciences, I had the opportunity to approach different application areas in the field of environmental protection.

One of them is bioremediation, a method that involves the decontamination of environmental matrices through the use of living organisms. In this context, our research group, in collaboration with the Anton Dohrn Zoological Station in Naples (SZN), has carried out an ongoing study investigating the ability of some microalgae species (*Tetraselmis sp.*), isolated from the mouth of the Sarno River and enclosed in calcium alginate marbles, to phyto-purificate marine ecosystems from heavy metals.

Bioremediation is an approach that is increasingly being studied and used in the scientific world. While various microbes are capable of degrading toxic organic compounds commonly released into the environment, photosynthetic organisms such as microalgae have many applications in heavy metal decontamination.

If biomonitoring is an important tool in understanding the level of environmental pollution in terms of protecting our planet, it is equally important to study and implement various plans and strategies to decontaminate ecosystem.

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