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TESI

"Monitoring of environmental pollutants in aquatic organisms: toxicological risk assessment"

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La Terra è un bel posto
e vale la pena lottare per lei

Ernest Hemingway

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List of abbreviations

AAS	Spettrofotometria in assorbimento atomico
IPA	Idrocarburi policiclici aromatici
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
BkF	Benzo[k]fluorantene
DahA	Dibenzo[a,h]anthracene
PCBs	Polychlorinated biphenyls
IARC	International Agency of Research on Cancer
PAHs	Polycyclic aromatic hydrocarbons
LOD	Limit of detection
LOQ	Limit of quantification
dl	Detection limit
EWI	Estimate weekly intakes
IARC	International Agency of Research on Cancer
PTWI	Provisional tolerable weekly intake
BaP	benzo[a]pyrene
BaA	benzo[a]antharacene
BbF	benzo[b]fluoranthene
Cry	chrysene
GF-AAS	Graphite furnace atomic absorption spectroscopy
HPLC	High Performance Liquid Chromatography
FAPAS	Food Analysis Performance Assessment Scheme
SEM	Standard error of mean
SD	Standard Deviation
ANOVA	Factorial analysis of variance

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Abstract

The research activity of the PhD project in Veterinary Science aimed at determining the presence of environmental pollutants in aquatic organisms to improve the current knowledge on animal biomonitoring and provide the toxicological risk assessment connected, concerning the link between pollution and human and animal health. Many industrial pollutants, especially micropollutants, have adverse effects at very low concentrations. It is therefore important to early detect the presence of these compounds directly or through particular molecular biomarkers found in living organism. As a matter of fact, the search of these substances in the natural environment, is not always easy as they are often present below the detection threshold. In aquatic ecosystems, the use of invertebrate species seems to be an appropriate way of monitoring the environmental quality, due to their general capacity to accumulate pollutants, either from sediments and surrounding water or from food sources.

The research activity wes carried out on the following aquatic organisms:

- The common octopus *(Octopus vulgaris)* is a mollusc belonging to the class Cephalopoda. It is considered a cosmopolitan species as its range extends from the eastern Atlantic to the Mediterranean Sea and at least to Senegal in Africa. *Octopus vulgaris* constitutes an important marine resource suitable for human consumption; however, they can represent a source of chemical contaminants intake particularly of heavy metals.

The aims of this study were to determine (i) the levels of heavy metals (Pb, Cd and Hg) in the muscle and digestive gland of octopus caught from two different locations along Campania coast, as well as (ii) to estimate their weekly human intake derived from the ingestion of octopus.
The analysis of 38 samples showed a higher concentration of Pb in the muscle of octopus in one of the sampling sites. Differences were observed between the two tissue types, with a higher level of Cd and Pb observed in the digestive gland compared to the muscle. Noteworthy, the consumption of *O. vulgaris* captured in some areas could increase Pb intake in heavy consumers of local fish products.

Abstract

The red swamp crayfish *Procambarus clarkii* (Girard, 1852), is an invasive freshwater species that originated in the north eastern Mexico and south-central United States. This species has been imported into Italy in the seventies, mainly for aquaculture purpose. Nowadays it is one of the most diffuse crayfish species in several European countries and is able to tolerate extreme and polluted environmentrs, accumulating heavy metals and toxins in its tissue, mainly in the hepatopancreas. The objectives of the present study were to evaluate the levels of Cd, Pb, Hg, As, Cr, Cu and Zn in muscle and in hepatopancreas of red swamp crayfish (*Procambarus clarkii,* Girard, 1852) collected from two different sites in Campania (Italy), Villa Literno and Sessa Aurunca, to provide data for both human health and the ecological risk assessment. The general evidence was that crayfishes from accumulated higher levels of metals (As, Cu, Zn and Cr) in the hepatopancreas than in the muscle. The results obtained in the current study showed low levels of Cd, Pb and Hg and largely below the MLR established by the European Commission for muscle from crustacean appendages. These results suggested a limited Cd, Pb and Hg contamination in the sampling sites and were indicative of low risk for human consumption. Our data showed that *P. clarkii* can be considered a good bioindicator for metal pollution in the study areas.

- The warty crab (*Eriphia verrucosa)* is a bentonic species found in the Mediterranean Sea, the Black Sea, and the eastern Atlantic Ocean, from Brittany to Mauritania and the Azores. It is highly fecund and is reported to feed on bivalves, gastropods, and hermit crabs, or on mollusks and polychaetes. *Eriphia verrucose* shows a preferential uptake of pollutants from sediments. The aim of this study was to evaluate the levels of As, Cr and Pb and the content of six PAHs in edible muscle of warty crab from various coastal areas of Campania region. Results showed that PAHs and metals concentration in warty crab were always lower than the legal limits established by the European Commission.

- The red seaweed, *Kappaphycus alvarezii* and *Kappaphycus striatum* are cultivated extensively as a source of carrageenan which have been used

for decades in food applications and are generally regarded as safe. There are different studies in literature on the chemical-nutritional characteristics and the benefits of their use, but further studies are needed to identify any toxicological risks related to their use.
Aims of this study were to evaluate the presence of chemical constituents and to quantify toxic and essential elements in two species of red seaweed *Kappaphycus alvarezii* and *Kappaphycus striatum* collected in Palau Bidong (Malaysia), in order to assess their potential use as additive in animal nutrition and the possible health risks related to animal consumption. The total amount of trace elements in *K. alvarezii* was almost double than in *K. striatum*, in agreement to the differences in ash percentages. On the whole, due to the low amount of protein on dry matter, red seaweeds *K. alvarezii* should be used in animals nutrtion under intensive production as mineral additive.

- The edible mussel *Mytilus galloprovincialis* is among the most commonly used sentinel organisms for the monitoring of biological effects of various contaminants in the marine. Polychlorinated biphenyls (PCBs) are environmental pollutants of industrial origin that can contaminate food, mainly food of animal origin. Although production of PCBs has been banned in many countries since the 1980s, they are still present in the environment and are considered dangerous pollutants for human health. New analytical approaches are useful to monitor the presence of such contaminants in seafood products and in the environment. In this work, we evaluate changes in protein expression of *Mytilus galloprovincialis* (Lam.) experimentally exposed to a PCB mixture and identify chemically specific protein expression signatures by using a proteomic approach. In particular, we identify 21 proteins whose levels of expression are sensibly modified after 3 weeks of exposure. The present work shows that a proteomic approach can be a useful tool to study alterations of protein expression in mussels exposed to PCBs and represents a first step toward the development of screening protocols to be used for biomonitoring surveys of fishery products.

Abstract

Le attività di ricerca svolte nel triennio di Dottorato di Ricerca in Scienze Veterinarie si inseriscono nell'ambito di uno studio di biomonitoraggio, rivolto all'individuazione di organismi acquatici utilizzabili per raccogliere informazioni circa la contaminazione ambientale da microinquinanti, organici e inorganici, potenzialmente pericolosi per gli organismi viventi.

A tale scopo sono stati selezionati i seguenti organismi acquatici:

- Il polpo comune (*Octopus vulgaris*) è un mollusco appartenente alla classe Cefalopodi, presente in tutti i mari e gli oceani, è molto diffuso anche nel Mar Mediterraneo. *Octopus vulgaris* è una specie ittica molto apprezzata dal consumatore, tuttavia può rappresentare una fonte di assunzione di contaminanti chimici come i metalli pesanti. Gli obiettivi di questo studio sono stati: (i) determinare i livelli di Pb, Cd e Hg nel muscolo e nell'epatopancreas di trentotto esemplari di *Octopus vulgaris* catturati in due diverse aree della costa campana, nonché (ii) di stimare i livelli di assunzione settimanalie di tali metalli per l'uomo consumatore di polpo. I risultati hanno evidenziato una maggiore concentrazione di Pb nella parte muscolare di *Octopus vulgaris* in uno dei siti di campionamento. Sono state osservate differenze significative anche tra i due tessuti esaminati, riscontrando livelli di Cd e Pb più elevati nell'epatopancreas che nella parte muscolare. I dati ottenuti indicano che il consumo di *O. vulgaris* catturato in alcune aree potrebbe aumentare l'assunzione di Pb nei consumatori abituali di prodotti ittici locali.

- *Procambarus clarkii* (Girard, 1852), noto anche come gambero rosso della Louisiana, è una specie invasiva di acqua dolce originaria del Messico nord-orientale e degli Stati Uniti centro-meridionali. Questa specie è stata introdotta in Italia nel corso degli anni settanta del secolo scorso, principalmente per incrementeare il settore dell'acquacoltura. Oggi è una delle specie di gambero d'acqua dolce più diffusa in Europa ed è in grado di tollerare ambienti estremi e inquinati, accumulando metalli pesanti e tossine nei suoi tessuti, principalmente nell'epatopancreas.

Abstract

Lo scopo di questo studio è stato quello di determinare le concentrazioni di metalli pesanti (Hg, Cd, Pb e As) e di altri elementi in traccia (Cu, Cr, Zn) nella parte muscolare e nell'epatopancreas di sessanta esemplari di *Procambarus clarkii* catturati durante l'estate 2017. Sono state prese in esame due aree di studio della Campania, la prima nei pressi del fiume Garigliano, l'altra del fiume Volturno.
I risultati ottenuti in questo studio mostrano livelli molto bassi di Cd, Pb e Hg in tutti i campioni di *P. clarkii* analizzati ed ampiamente inferiori ai limiti massimi stabiliti dalla Commissione Europea (Reg. CE 1881/2006).

- Il granchio favollo (*Eriphia verrucosa*) è una specie bentonica che si trova nel Mar Mediterraneo, nel Mar Nero e nell'Oceano Atlantico orientale, dalla Bretagna alla Mauritania e alle Azzorre. Divesi studi hanno dimostrato la capacità di *Eriphia verrucosa*, di accumulare xenobiotici nei sui organi e tessuti. L'obiettivo di questo studio è stato quello di valutare i livelli di As, Cr e Pb e il contenuto di sei IPA nella parte muscolare di 32 esemplari di granchio favollo provenienti da varie aree costiere della regione Campania. I risultati ottenuti in questo studio hanno evidenziato livelli bassi di Pb, As, Cr ed IPA in tutti i campioni analizzati e per i contaminanti normati, ampiamente inferiori ai limiti massimi stabiliti dalla Commissione Europea (Reg. CE 1881/2006).

- Le alghe rosse, *Kappaphycus alvarezii* e *Kappaphycus striatum* sono ampiamente coltivate come fonte di carragenina. Esistono diversi studi in letteratura riguardo le caratteristiche chimico-nutrizionali, ma sono necessari ulteriori studi per identificare eventuali rischi tossicologici legati al loro impiego. Gli obiettivi dello studio presentato sono stati: (i) la valutazione delle caratteristiche nutrizionali e (ii) la determinazione dei livelli di elementi in traccia essenziali e non essenziali in due specie di alghe rosse *Kappaphycus alvarezii* e *Kappaphycus striatum* raccolte a Palau Bidong (Malaysia) e (iii) il loro potenziale utilizzo come additivo nell'alimentazione animale. I dati ottenuti hanno evidenziato la possibilità di impiego delle alghe rosse *K. alvarezii* nell'alimentazione animale, anche se

a causa della bassa quantità di proteine sulla sostanza secca, potrebbero essere utilizzate solo come integratori.

- I mitili (*Mytilus galloprovincialis*) sono tra gli organismi sentinella più comunemente utilizzati per gli studi di biomonitoraggio di vari contaminanti.

I bifenili policlorurati (PCB) sono inquinanti ambientali di origine industriale che possono contaminare gli alimenti, principalmente quelli di origine animale. Sebbene la produzione di PCB sia stata vietata in molti paesi dagli anni '80, essi sono ancora presenti nell'ambiente e sono considerati inquinanti pericolosi per la salute umana. Nuovi approcci analitici possono essere utili per monitorare la presenza di tali contaminanti nei prodotti ittici e nell'ambiente. Lo scopo di questo studio è stato quello di valutare i cambiamenti nell'espressione proteica di esemplari di *Mytilus galloprovincialis* (Lam.) esposti sperimentalmente a una miscela PCB. I risultati hanno permesso di identificare 21 proteine i cui livelli di espressione vengono sensibilmente modificati dopo 3 settimane di esposizione. Il presente lavoro mostra che un approccio proteomico può essere uno strumento utile per studiare le alterazioni dell'espressione proteica nei mitili esposti ai PCB e rappresenta un primo passo verso lo sviluppo di protocolli di screening da utilizzare per le indagini di biomonitoraggio dei prodotti della pesca.

The aquatic environment appears as the final destination for most of anthropogenic contaminants released from industry, agriculture, transport and urbanization. The conservation of ecosystems and human health is based on a sound assessment of the risks associated with the presence of contaminants in the aquatic environment. As consequnce, there is the need of early detection of industrial pollutants, especially micropollutants that have adverse effects in very low concentrations; it is important to disclose the presence of these compounds directly or through certain molecular biomarkers in living organism rather than in the natural environment, where they are often below the detection threshold (Rombola et al, 2012).

Heavy metals are amongst the most frequent pollutants found in the aquatic ecosystem and their presence derives from sources both natural (volcanic activity and weathering of rocks) and anthropogenic (mining, metal production, combustion of fossil fuels, sewage sludge and waste incineration) (Markert et al, 2011; Langner et al, 2011; Abbas et al, 2008; Klavins et al, 2000). As consequence, heavy metals are present in the aquatic environment where they bioaccumulate along the food chain (Olmedo et al, 2013; Smith et al, 1998).
Some heavy metals such as cadmium (Cd), lead (Pb) and mercury (Hg) and metalloids such as arsenic (As) are of great toxicological concern and have a wide range of toxic effects in both humans and animals. Many of them are biologically essential (Cu, Se, Cr, Fe, Zn, Mn) but all have the potential to be toxic to biota above a threshold concentration.
Cadmium is a non essential heavy metal; its role in promoting significant adverse health effects in humans and animals has been widely studied, especially in relation to high-level metal exposure (EFSA, 2009; Agency for Toxic Substances and Disease Registry, 2008; IARC, 1993). In recent years studies have focused on the possible implication of low-level, long-term Cd exposure in developmental diseases, highlighting the existence of vulnerable groups in the general population (Järup et al, 1998). The adverse effects of long-term Cd exposure mainly target the kidneys (Järup et al, 2009).

Between heavy metals, Pb is well known for its ability to induce harmful effects both in acute and chronic conditions. Lead acts on central nervous system, kidneys, liver, skeletal and immune system affecting mostly children, due to their higher metabolic rate and higher ability to absorb a greater amount of Pb (Molina-Villalba et al, 2015; Luckey and Venugopal, 1977; Emmerson, 1973;).
Mercury is one of the most toxic heavy metals in the environment (Castro-González and Méndez-Armenta, 2008). Exposure to high levels of metallic inorganic or organic mercury can permanently damage the brain, kidney and developing foetus (Rice et al, 2014).
Arsenic is a metalloid and one of the most hazardous elements in the environment (Smith el al., 1998; Mandal and Suzuki, 2002). The main way of As exposure for human population is via contaminated drinking water and ingestion of contaminated food (IARC, 2012). Arsenic has been classified by the International Agency of Research on Cancer (IARC) as a group I elemenet which means it is potentially carcinogenic to humans (IARC, 2012).

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, accidental oil spills (Habibullah-Al-Mamun, et al., 2018; Tornero and Hanke 2016;). 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 ingestion is the first way of exposure that can lead to detrimental effects on animals and human health (Ferrante et al., 2018; Zaccaroni et al.,2018).
Due to their persistence, long range transport and capacity to bioaccumulate in the trophic chain, PAHs contamination has become a global issue. Based on the evidence of their toxic potential, European institutions have issued two Community regulations regarding PAHs presence in food for human consumption. The UE Reg. 1881/2006 establishes the legal and safety limits

of four PAHs compounds (benzo[a]pyrene, benzo[a]antharacene, benzo[b]fluoranthene and chrysene) taking in account, among seafood, only molluscs and smoked products. The need to officially assess the presence of PAHs in food items and to set a maximum concentration limit that prevents the product from being harmful for public health, is linked to the high toxicity of these chemicals which can induce carcinogenesis and mutagenic effects as reported by IARC. IARC listed sixteen different PAHs as dangerous compounds for human health due to their ability to be potentially carcinogens and mutagens.

The consumption of fish, shellfish, and fishmeal feeding contaminated with polychlorinated biphenyls (PCBs) represents an important source of accumulation in both humans and food-producing animals (European Food Safety Authority, 2005, 2010; Malisch et al, 2004).
PCBs include a group of 209 different congeners that differ in the number and the position of chlorine atom substituents (Thomas et al, 2008), and they are classified as (i) dioxin-like PCB (12 DL-PCB) and (ii) non–dioxin-like PCB (197 NDL-PCB) congeners. Because of their physicochemical properties, such as chemical stability, low heat conductivity, and high dielectric constants, PCBs were widely used in many industrial applications. Recently, the production and the use of PCBs have been banned in the majority of industrialized countries (Council Directive 85/647/EEC; Council Directive 96/59/EC; Stockholm Convention on Persistent Organic Pollutants, 2009). Nevertheless, they are still present in the environment because of their high stability. Furthermore, because of both their lipid solubility and the absence of adequate metabolic pathways in the organisms, PCBs tend to accumulate in fatty tissues, and they biomagnify along the trophic chain (Thomas et al, 2008; WHO 2001).

Over the last decades, interest and awareness of institutional bodies, researchers and consumers in seafood safety has increased significantly.
Regular dietary fishery products intake is recommended by nutritionists since they contain high concentrations of functional nutrients, including essential amino-acids, vitamins and omega-3 fatty acids, useful in

decreasing the risk of cardiovascular diseases (Cirillo et al, 2010; (Cederholm, 2017). Despite this, fishery products consumption can represent also a route of exposure to dangerous chemical substances. Seafood safety is strictly linked to marine environment quality, because many pollutants present in the aquatic environment can be bio accumulated and biomagnified by marine organisms; concerns have been raised about the potential risks for human health derived by the consumption of contaminated fisheries products (Cappello et al., 2018).

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 (Ferrante et al., 2018). Therefore, seafood from Mediterranean basin deserves to be carefully analysed to guarantee the consumers' safety 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 biomarker to collect data on the current health status of the marine ecosystem.

Common octopus (*Octopus vulgaris*) is mainly consumed in Southern European countries such as Italy and Spain (ISMEA, 2016). However, this species can represent a source for chemical contaminants intake. The levels of heavy metals in tissues of marine organisms is mainly influenced by biotic and abiotic factors (Has-Schön et al, 2006). *O. vulgaris* is a benthic species, living in direct contact with the seabed, which constitutes a possible pathway for trace element accumulation, and can therefore represents a source of human exposure to toxic elements (Mustafa Canli et al, 2003; Bustamante et al, 2002; Storelli et al, 2012). *Eriphia verrucosa*, is a benthonic species of crustacean also called the warty crab. The warty crab lives in shallow waters up to the rocky coastlines. It's a common species in the Mediterranean Sea, regularly found along the Italian Tyrrhenian coasts, feeding primarily on bivalves, gastropods and polychaetes (Ozogul etl al., 2013). 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. The warty crab, because of its geographic distribution, its position in the food web and its consumption by humans, represents an optimal marine species for quali-quantitative toxicological investigations (Ariano et al, 2015).

In freshwater ecosystems, the use of invertebrate species seems to be an appropriate way of monitoring the environmental quality, due to their general capacity to accumulate pollutants, either from sediments and surrounding water or from food sources (Colin et al., 2016; Devi et al, 1996). Among invertebrate species, crayfish have long been acknowledged as good bioindicator of metals contamination (Reynolds and Souty-Grosset, 2011; Caro, 2010)

The research activity has aimed at the determination of environmental pollutants in aquatic organisms to improve the current knowledge on animal biomonitoring and to provide the toxicological risk assessment, concerning the link between pollution and human and animal health.

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Chapter 1

Metal Concentration in Muscle and Digestive Gland of Common Octopus (*Octopus vulgaris*) from Two Coastal Site in Southern Tyrrhenian Sea (Italy)

Heavy metals in *Octopus vulgaris* captured in Campania region

Ariano A, Marrone R, Andreini R, Smaldone G, Velotto S, Montagnaro S, Anastasio A and Severino L, 2019. Metal Concentration in Muscle and Digestive Gland of Common Octopus (*Octopus vulgaris*) from Two Coastal Site in Southern Tyrrhenian Sea (Italy). *Molecules*, 24, 2401 (doi: 10.3390/molecules24132401). Received: 31 May 2019; Accepted: 28 June 2019; Published: 29 June 2019

Ariano A., Smaldone G., Zaccaroni A., Marrone R., Campani E., Anastasio A., Severino L. Levels of heavy metals in muscle and digestive gland of *Octopus vulgaris* from the southern Tyrrhenian sea.
XXVII SILAE Congress - September 9-13 2018 - Milazzo, Italy.

1.1 Abstract

Octopus vulgaris constitute an important part of most suitable marine resources for human consumption, however, they can represent a source in chemical contaminants intake such as heavy metals. In this scenario, the aim of the study was the evaluation of the concentration of Pb, Cd and Hg in the muscle and digestive gland of octopus caught from two different locations along Campania coast (Castellammare di Stabia and Napoli) and the estimation of their weekly human intake derived from the ingestion of octopus. Analysing 38 samples showed a higher concentration of Pb in the muscle of octopus in Castellammare di Stabia than in Napoli. No statistical differences were reported for Cd, Pb and Hg concentrations in the digestive gland of octopus between two sampling sites. Differences were observed between the two tissue types, with a higher level of Cd and Pb observed in the digestive gland compared with the muscle. Noteworthy, the consumption of muscle from Castellammare di Stabia could increase Pb intake in heavy consumers of local octopus. In conclusion, the present work determines that it is important to improve strategies to minimize environmental pollution sources in these areas.

1.2 Introduction

Regular dietary fishery products intake is recommended by nutritionists since they contain high concentrations of functional nutrients, including omega-3 fatty acids, useful in decreasing the risk of cardiovascular diseases (Cirillo et al, 2010). Fishery products consumption in Italy has increased from 16 kg/year per person in 2016, to 25 kg/year in 2018, with good prospects of further growth (FAO, 2018). Cephalopods constitute an important part of the marine resources most suitable for human consumption. Common octopus (*Octopus vulgaris*) is mainly consumed in Southern European countries such as Italy and Spain; consumption in Italy has a range from 1.5 to 5.1 kg per capita/year (ISMEA, 2016). However, this species can represent a source for chemical contaminants intake. The levels of heavy metals in tissues of marine organisms is mainly influenced by biotic and abiotic factors (Has-Schön et al, 2006).

O. vulgaris is a benthic species, living in direct contact with the seabed, which constitutes a possible pathway for trace element accumulation, and can therefore represents a source of human exposure to toxic elements (Mustafa Canli et al, 2003; Bustamante et al, 2002).

In the Campania region (Italy) several areas exist where the pollution of soil, marine water, and groundwater is extremely severe and represents a serious hazard to public health. Moreover, these sites are located near highly urbanized and populated areas and usually represented by ex-industrial areas or lands nearby illegal waste dumps (Maselli et al, 2010).

European Regulation 1881/06 established maximum levels of contaminants in foodstuffs, fixing specific limit for heavy metals in Cephalopods (without viscera). The Joint FAO/WHO Expert Committee on Food Additives revised its risk assessment on heavy metals in fish and adopted a PTWI of 4µg/kg b.w. week for mercury, 7 µg/kg b.w. for cadmium and 25 µg/kg b.w. for lead. Therefore, the objectives of the present study were: firstly to evaluate lead, cadmium and mercury levels in the muscle of common octopus (*Octopus vulgaris*) collected at two different sites along the Southern Tyrrhenian Sea coast (Italy) and estimate the weekly human intake (WHI) of heavy metals deriving from the ingestion of octopus compared

with the PTWI; secondly to investigate the geographical variation of the same metals in muscle and digestive gland.

Fig. 1.1 Octopus vulgaris.

1.3 Results and Discussion

Mean concentrations of heavy metals in samples of the muscle and digestive gland of *Octopus vulgaris* are summarized in Figure 1.2. Results showed significantly higher concentration of Pb ($p < 0.001$) in the muscle of *O. vulgaris* in Castellammare di Stabia (mean value 0.537 µg g^{-1}) versus Napoli (mean value 0.046 µg g^{-1}). Levels of Cd and Hg were very low in all samples of octopus muscle. No statistical differences were reported for Cd and Hg concentrations in the muscle of octopus between both sampling sites. In the digestive gland Cd was found at a mean concentration of 2.643 and 2.969 µg g^{-1}, Pb was found at a mean concentration of 1.511 and 0.763 µg g^{-1} and Hg of 0.040 and 0.101 µg g^{-1}, in Castellammare di Stabia and Napoli, respectively. No statistical differences were reported for Cd, Pb and Hg concentrations in digestive gland of octopus between Castellammare di Stabia and Napoli (Figure 1.2).

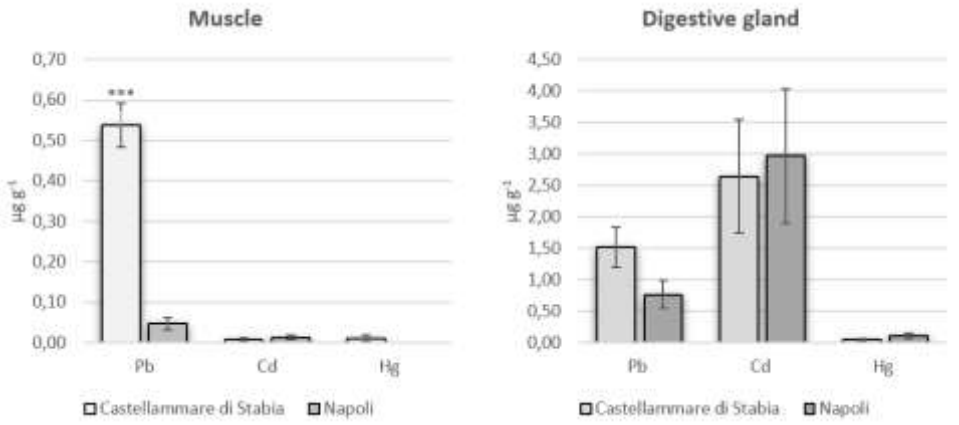

Fig. 1.2 Mean concentration (µg g^{-1} wet weigth) ± SEM of Pb, Cd and Hg in O. vulgaris muscle and digestive gland from Napoli and Castellammare di Stabia. Probability levels for significant differences from sampling sites: $p < 0.001$(***).

Metal concentrations in tissues of *O. vulgaris* captured along the Campania coast were compared to the values reported for *O. vulgaris* in other coastal waters.

In the present study, Hg concentrations in muscle of *O. vulgaris* were lower than levels found in samples from the Portuguese coast (0.49 μg g^{-1}) (Carvalho et al, 2005). Also, other works reported higher levels of Hg in the muscle (0.13–0.76 μg g^{-1}) and digestive gland (0.36–7.4 μg g^{-1}) (Raimundo et al, 2010; Seixas et al, 2005).

Lead concentration in the in muscle was higher than levels reported in samples from the Portuguese coast, ranging from 0.04 to 0.09 μg g^{-1} (Carvalho et al, 2005). However, other studies showed higher levels of Pb (range value 1.5–7.2 μg g^{-1}) in the digestive gland of *O. vulgaris* than those reported in the current study (Raimundo et al, 2004; Napoleão et al, 2001].

1.3.1 Metal Concentration versus Sampling Sites and Tissue Type

Data analysis using multivariate tests allowed an estimation of how tissue type and sampling sites influence heavy metals concentration (Table 1.1).

Tab. 1.1 Factorial analysis of variance (ANOVA) testing the effect from the collection site (Napoli versus Castellammare di Stabia), the accumulation organ type (muscle versus digestive gland) on the concentration of heavy metals (Pb, Cd and Hg) in Octopus vulgaris. df = degree of freedom. Probability levels for significant effects: p < 0.001 (***); p < 0.01 (**). MS = mean squares; F = F-ratio.

Source of variation	Dependent Variable	df	Mean square	F
Site	Pb	1	7.292	10.08**
	Cd	1	0,521	0,06
	Hg	1	0,012	1,15
Organ	Pb	1	13,583	18,77***
	Cd	1	148,532	16,08***
	Hg	1	0,081	7,68

The tissue type had a highly significant ($p < 0.001$) influence on the accumulation of Cd and Pb, leading to higher accumulation in the digestive gland (mean values of 2.81 and 1.137 µg g^{-1}, respectively) than in muscle (Figure 1.3 A). Mercury was accumulated at same concentration in both organs. Significant site-specific differences were detected for Pb ($p < 0.01$) only. Concentration of lead were significatively higher in octopus collected in Castellammare di Stabia (1.024 µg g^{-1}) versus that in Napoli (0.405 µg g^{-1}) (Figure 1.3 B).

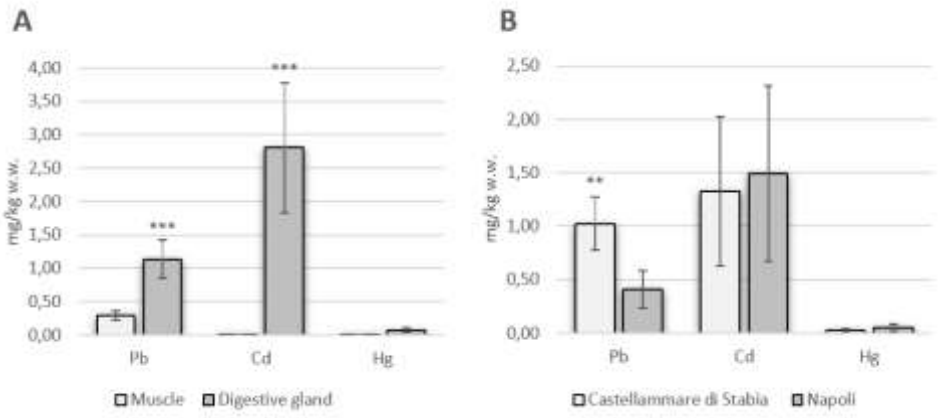

Fig. 1.3 Concentration of Pb, Cd and Hg in O. vulgaris depending on (A) organ type: muscle versus digestive gland; (B) sampling sites: Napoli versus Castellammare di Stabia. Vertical bars represent average concentration (µg g^{-1} wet weigth) ± SEM. Probability levels for significant differences: $p < 0.001$*(***);* $p < 0.01$ *(**).*

All digestive gland samples of octopus showed the highest Cd and Pb concentration, confirming the primary role of this district in the bioaccumulation and detoxification processes of Cd and Pb and confirming

the presence of these metals at both sampling areas (Bustamante et al, 2002; Storelli et al, 2005). Also, a similar distribution of Hg in the two different tissue has already been described in other studies (Storelli et al, 2005; Pereira et al, 2009).
Cadmium concentrations in muscle and digestive gland in the present study were comparable with those previously reported by other authors (Carvalho et al, 2005; Piras et al, 2013).

1.3.2 Metal Concentration versus Biological Parameters

The analyzed individuals varied in size and weight ranges, including males and females. The multiple regression analyses indicate that there was no correlation between weight, gender and concentration of Pb, Cd and Hg ($p>0.05$). The lack of relations between heavy metals concentration in muscle and digestive gland of *O. vulgaris* and weight suggest that, within the range of weight of the studied specimens these parameters had minor effect on metal accumulation. These observations were already described in other studies on *O. vulgaris* captured along Portuguese coast (Raimundo et al, 2004). The absence of relation between Pb, Cd and Hg levels and gender agrees with other studies on cephalopods (Raimundo et al, 2004; Bustamante et al, 1998).

1.3.3 Concern for Public Health

Levels of Cd and Hg in present study were low in all samples of muscle and were below the legal limit for human consumption. Anyway, the average concentrations of Pb in samples of muscle from Castellammare di Stabia were above the maximum concentration level of 0.3 µg g^{-1}, leading to the exclusion of this product for human consumption (Commission regulation (EC) No 1881/2006).
To establish possible human health implications related to consumption of octopus, the Pb estimate weekly intakes (EWI) were subsequently compared with the provisional tolerable weekly intake (PTWI) of 25 µg/kg of body weight (JECFA, 2011). Estimating a weekly consumption of 100 g of *O.*

vulgaris muscle, EWI values were found to be 53.7 µg/week. This value accounted for 3.06% of the tolerable weekly intake set by EFSA. Considering the level of Pb, the consumption of octopus muscle from Castellammare di Stabia may increase Pb intake, but it would not contribute significantly to the PTWI. In contrast, it may contribute greatly to high EWI values in heavy consumer of octopus, when all other main contributors to dietary Pb intake and professional exposure were included in the exposure assessment.

1.4 Materials and Methods

1.4.1 Sampling

Thirty-eight samples of common octopus (*Octopus vulgaris*) were fished directly from two different locations along Campania coast (Italy) in autumn of 2016 (Figure 1.4).

Fig. 1.4 Map showing locations of the sampling sites: Napoli (site A) and Castellammare di Stabia (site B).

Once captured, the octopus were weighed, their total length were measured and then they were immediately sealed in individual polyethylene bags, frozen at −20 °C and kept at the same temperature until dissection. Sex was also determined for each individual (Table 1.2). In the laboratory, the digestive gland of each organism was totally removed under partially defrost conditions without rupture of the outer membrane. Subsequently, the

digestive gland was treated separately from the remaining tissues and an interior portion was sampled for metal analysis.

Tab. 1.2 Number of individuals (n), weight (g), size (mm) and sex of O. vulgaris captured along the Campania coast.

Sites	*n*	Weight range (g)	Total lenght range (cm)	Sex
Napoli	19	845 ± 143	71.7 ± 18.3	15 ♀ 4 ♂
Castellammare di Stabia	19	740 ± 229	67.6 ± 21.5	8 ♀ 11 ♂

Arms and mantle were dissected including the skin. Each tissue was subsequently homogenized by means of a laboratory mixer and stored at −20 °C until further analyses

1.4.2 Chemical and Instrumental Analysis

Glassware and laboratory equipments were decontaminated before use with diluted ultrapure 65% HNO_3 (Romil UpA, Cambridge, UK) and rinsed with Milli Q water (Millipore, Bedford, MA, USA). Aliquots of each sample (0.50 ± 0.02 g) were digested in 5 mL of ultrapure 65% HNO_3 and 2 mL of 30% H_2O_2 (Romil UpA, Cambridge, UK) in a microwave digestion system (Milestone, Bergamo, Italy). The final volume was obtained by adding Milli-Q water. Metal concentrations in the digested samples were determined with an atomic absorption spectrometer (Aanalyst 600, Perkin-Elmer, Madrid, Spain) equipped with a graphite furnace and a L'vov platform for Pb and Cd. A flow injection analysis hydride system (FIAS 100, Perkin-Elmer) was used for the determination of the total Hg. The equipment was calibrated with standard solutions (Perkin-Elmer), resulting in a calibration curve with three concentrations for Pb and Hg and four concentrations for Cd.

Recovery of the metals was determined by adding known amounts to metal-free samples, which were then subjected to the same digestion procedure. The resulting solutions were analyzed for metal concentrations. Recovery of metals from spiked samples ranged from 85 to 120%. Concentrations of each heavy metal were expressed as milligrams per kilogram wet weight (Ariano et al. 2015).

1.4.3 Quality Assurance

Quality was monitored through analysis of procedural blanks, duplicate samples, and standard solutions. Standard solutions of analytes were prepared from certified stock solutions of Cd, Pb, and Hg with a relative matrix modifier (atomic spectroscopy standard, Perkin Elmer). 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 interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK). The FAPAS studies were conducted with fish tissue. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by determining the standard deviation of 10 independent blanks spiked at 1, 2, 4 and 8 µg g^{-1} for Cd and 25, 50 and 100 µg g^{-1} for both Pb and Hg, with an external standardization curve (Nthunya et al, 2017; Nthunya et al, 2018; Russo et al, 2013).

1.4.4 Statistical Analysis

All metal concentrations were expressed in wet weight as mean ± SEM (standard error from mean) (Pereira et al, 2009). Factorial analysis of variance was used to test statistical significance of the influence of the sampling site (Napoli versus Castellammare di Stabia), the target tissue for metal accumulation (muscle versus digestive gland) on the concentration of heavy metals. Moreover, ANOVA and Mann-Whitney test was used to detect differences between metal concentration in muscle and digestive gland and sampling area. Statistical significance between concentration of

metals and the variables (total weight and gender) were analysed using multiple regression.

The normal distribution of data was confirmed by the One-Sample Kolmogorov-Smirnov Test. Statistical analyses were performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). The result, of $p < 0.05$, was considered significant.

1.5 Conclusions

The present study provided data on heavy metals concentrations in *Octopus vulgaris* from the Southern Tyrrhenian Sea (Italy). The concentration of Cd and Pb found in tissues of octopus sampled at Castellammare di Stabia and Napoli witness for their presence in the environment. Mercury occurred only at trace concentrations at both sampling area. The results also indicate that there was no correlation between weight, gender and concentration of heavy metals. Instead, large variations in Cd and Pb concentration existed across muscle and digestive gland of this species.

Considering Hg and Cd intake, the consumption of octopus muscle from both sampling sites does not contribute significantly to the PTWI of 4 µg/kg b.w. week and 7 µg/kg b.w. week, respectively. In contrast, the consumption of muscle from Castellammare di Stabia could increase Pb intake in the heavy consumer of local octopus, but it would not contribute significantly to the PTWI of 25 µg/kg b.w. week.

The capability of digestive gland to accumulate higher levels of Cd than muscle, as reported by Roldán-Wong et al could provide a new tool for the monitoring of the geographical distribution of this metal, even when present at negligible levels in the edible part. The presence of Pb at a higher concentration at Castellammare di Stabia in both muscle and digestive gland was probably due to a major anthropogenic pressure in this site (Roldán-Wong et al, 2018).

Monitoring studies on heavy metals in a greater number of samples of *Octopus vulgaris* and other species of the marine food chain, as suggested by Sangiuliano et al, will provide more detailed information of the human exposure to metals in these areas (Sangiuliano et al, 2017). Although monitoring of levels of chemical contaminants in marine food resources is fundamental for human health and ecological approach, it is needful to improve strategies to minimize environmental pollution sources in these areas.

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Chapter 2

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

Ariano A., Scivicco M., Campani E., Damiano S., Genovese A., Severino L. Trace elements in muscle and digestive gland of red swamp crayfish (*Procambarus clarkii*) in Campania Region (Italy): preliminary observations.
XXVIII SILAE Congress - September 16-20 2019 – La Habana, Cuba.

2.1 Abstract

Heavy metals represent one of the major classes of contaminants in aquatic environments and are today a worldwide threat to aquatic species. They are widespread environmental contaminants, potentially hazardous to wildlife even at very low concentrations and freshwater crustaceans are amongst the most sensitive of macroinvertebrate species. The objectives of the present study were to evaluate the levels of Cd, Pb, Hg, As, Cr, Cu and Zn in muscle and in hepatopancreas of red swamp crayfish (*Procambarus clarkii,* Girard, 1852) collected from two different sites in Campania (Italy), Villa Literno and Sessa Aurunca, to provide data for both human health and the ecological risk assessment.

The general evidence was that crayfishes from accumulated higher levels of metals (As, Cu, Zn and Cr) in the hepatopancreas than in the muscle. The results obtained in the current study showed low levels of Cd, Pb and Hg and largely below the MLR established by the European Commission for muscle from crustacean appendages. These results suggested a limited Cd, Pb and Hg contamination in the sampling sites and were indicative of low risk for human consumption. Our data showed that *P. clarkii* can be considered a good bioindicator for metal pollution in the study areas.

Further studies will provide more detailed information on the role of this species as an indicator of environmental contamination and a clearer scenario of the distribution of heavy metals in their habitat.

2.2 Introduction

The aquatic environment appears as the final destination for most of anthropogenic contaminants released from industry, agriculture, transport and urbanization. The conservation of ecosystems and human health is based on a sound assessment of the risks associated with the presence of contaminants in the aquatic environment. As consequence, there are the need of early detection of industrial pollutants, especially micropollutants that have adverse effects in very low concentrations; it is important to disclose the presence of these compounds in living organisms rather than in the natural environment, where they are often below the detection threshold. Heavy metals are amongst the most frequent pollutants found in the aquatic ecosystem and their presence derives from sources both natural (volcanic activity and weathering of rocks) and anthropogenic (mining, metal production, combustion of fossil fuels, sewage sludge and waste incineration) (Klavins et al, 2000). As consequence heavy metals are present in the aquatic environment where they bioaccumulate along the food chain (Smith et al, 1998).

Some heavy metals such as cadmium (Cd), lead (Pb) and mercury (Hg) and metalloids such as arsenic (As) are of great toxicological concern and have a wide range of toxic effects in both humans and animals. Among metals, copper (Cu), zinc (Zn) and biologically essential but all have the potential to be toxic to biota above a threshold concentration (Anderson et al, 1997; Schmitt et al, 2006).

In Campania region (Italy) exist several areas where the pollution of soil, marine water, freshwater and groundwater is extremely severe and represent a serious hazard to public health. Moreover, the sites in question in the Campania region are located near highly urbanized and populated areas and usually represented by ex-industrial areas, intensive agricultural areas or lands nearby illegal waste dumps (Maselli et al., 2010). Some areas, particularly Naples and Caserta, were subjected to extensive illegal dumping operations of toxic wastes since the 1980s. The highly toxic wastes dumping operations that have taken place both along the coast and the hinterland, have extremely adverse effects on health, livelihoods and the future prospect

of sustainable development of the local population (Marfe and Di Stefano, 2016).

In aquatic ecosystems, the use of invertebrate species seems to be an appropriate way of monitoring the environmental quality, due to their general capacity to accumulate pollutants, either from sediments and surrounding water or from food sources (Colin et al., 2016; Devi et al, 1996). Among invertebrate species, crayfish have long been acknowledged as good bioindicator of metals contamination (Reynolds and Souty-Grosset, 2011; Caro, 2010).

Fig. 2.1 Particular of Procambarus clarkii (Girard, 1852).

The red swamp crayfish *Procambarus clarkii* (Girard, 1852*)* is an invasive freshwater species that originated in the north eastern Mexico and south-central United States and has been imported into Europe in the seventies of last century, mainly for aquaculture purpose (Gherardi, 2006). In Italy, *P.*

clarkii was introduced since 1977, where most farmer have failed to take adequate precautions in their cultivation methods to prevent the crayfish from escaping from farm enclosers. Afterwards the red swamp crayfish has established wild stable populations in many lakes and ponds across Italy and rapidly has become the dominant freshwater crayfish in all habitats it colonized (Gherardi et al., 1999; Delmastro, 1992;). Nowadays is one of the most diffuse crayfish species in several European countries, also because it is invasive and tolerant to the adverse environmental conditions, due to flexibility of its biological cycle (Goretti et al., 2016; Scalici and Gherardi, 2007). In addiction *P. clarkii* live in direct contact with sediments and with its relatively long life span tend to accumulate heavy metals in some tissues, mainly in the hepatopancreas, well known to ho have a primary role in metals bioaccumulation and detoxification processes (Bianchi et al., 2011; Alcoro et al., 2006; Martín-Díaz et al., 2006; Anderson et al, 1997). Furthermore, the accumulation of metals in their tissues is strictly connected with dose and time of exposure (Kouba et al., 2010). For these reasons *P. clarkii* shows the features of a potential bioindicator for metals pollution in freshwater ecosystems. In fact, the red swamp crayfish has been used as indicator species to monitor the environmental quality and the contamination of the biological habitat in previous bioaccumulation studies (Mancinelli et al, 2018; Gedik et al., 2017; Goretti et al., 2016; Bellante et al, 2015; Suárez-Serrano et al., 2010; Alcoro et al., 2006;).

The aims of the present study were (i) to evaluate heavy metals concentrations (Cd, Pb, Hg, As, Cu, Zn and Cr) in abdominal muscle and hepatopancreas of *Procambarus clarkii* collected from different locations of Campania (Italy), (ii) in order to identify possible sources of contamination in the study areas and to assess the health risk connected to human consumption of crayfishes.

Moreover, (iii) to improve the current knowledge about the use of *Procambarus clarkii* as bioindicator of heavy metals pollution in freshwater ecosystems.

2.3 Materials and Methods

2.3.1 Sampling

Sixty samples of red swamp crayfish (*P. clarkii*) were caught using baited traps during summer 2017 in two sites of Campania region (Italy), at Villa Literno, near the Volturno River and Sessa Aurunca, near the Garigliano River (Fig. 2.2). After capture, the specimens were transferred alive in refrigerated box (4 - 8°C) to the laboratory, where they were weighed, sexed, and the carapace lengths were measured from the tip of the rostrum to the edge of the carapace using a caliper (Absolute Digimatic caliper, Mitutoyo, Japan) (Tab. 2.1). After measurements, the specimens were euthanized by thermal shock (-80 °C for 30 min). Subsequently, the abdominal muscle and the digestive gland of each organism were totally removed under partially defrost conditions and stored in falcon tubes at -20 °C until further analyses.

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

Tab. 2.1 Number of individuals (n), weight (g), size (mm) and sex of P. clarkii captured at Villa Literno and Sessa Aurunca.

Sites	*n*	Weight range (g)	Total lenght range (cm)	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 ♂

2.3.2 Chemical and instrumental analysis

Before Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) analysis, each sample was homogenized by means of a laboratory mixer and 0.5 ± 0.02g was placed in a Teflon vessel with 5.0 ml of 65% HNO_3 and 2.0 ml of 30% H_2O_2 (Romil UpA). The vessel was sealed and placed in a microwave digestion system (Milestone, Bergamo, Italy). Microwave assisted digestion was performed with a mineralization program for 25 min at 190°C. The vessel was then cooled at 32°C, the digestion mixture was transferred into a 50.0 ml flask and the final volume was obtained by adding Milli-Q water (Ariano et al, 2019).
Concentrations of trace elements were determined by ICP-OES technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. The calibration curve and two blanks were run during each set of analyses to check the purity of the chemicals. Reference material (BCR-422 cod muscle, IRMM Institute for Reference Materials and Measurements, DORM -2 fish protein, National Research Council, Canada) were also included for quality control. All the values of the reference materials were within certified limits. Instrumental detection limits expressed as wet weight (wet wt.) and determined following the protocol

described by Perkin Elmer ICP application study number 57 (Barnard et al, 1993).
LODs values (limit of detection values) expressed as wet weight were: 0.024 µg g^{-1} for As; 0.011 µg g^{-1} for Pb; 0.0018 µg g^{-1} for Cd; 0.001 µg g^{-1} for Cr; 0.006 µg g^{-1} for Zn; 0.0002 µg g^{-1} for Cu; 0.001 µg g^{-1} for Hg.
The performance of the method was assessed through participation in interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK)

2.3.3 Statistical Analysis

All metals concentrations were expressed in wet weight as mean ± SEM (standard error from mean) (Pereira et al., 2009) Factorial analysis of variance was used to test statistical significance of the influence of the sampling site (ViL vs SeA), the target tissue for metal accumulation (muscle vs hepatopancreas) on the concentration of trace elements. Moreover, ANOVA and Mann-Whitney test was used to detect differences between elements concentration in muscle and epatopancreas and sampling area. Statistical significance between concentration of trace elements and the variables (total weight and gender) were analysed using multiple regression. Normal distribution of data was confirmed by the One-Sample Kolmogorov-Smirnov Test. Statistical analyses were performed using MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium). $P < 0.05$ was considered significant.

2.4 Results

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

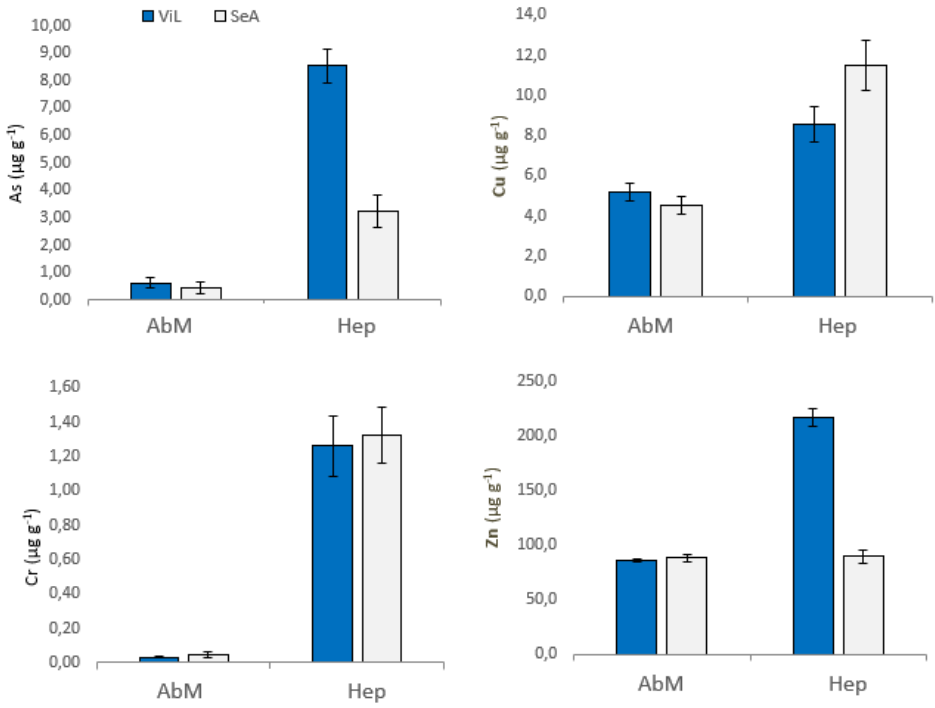

Fig. 2.3 *Mean concentration (µg g-1 wet weigth) ± SEM of trace elements (As, Cu, Zn and Cr) in P. clarkii muscle (AbM) and hepatopancreas (Hep) from Villa Literno (ViL) and Sessa Aurunca (SeA).*

Results showed negligible levels of Pb and Cd in all samples of crayfish AbM. In the Hep Cd was found at a mean concentration of 0.018 and 0.013 µg g^{-1}, Pb was found at a mean concentration of 0.015 and 0.012 µg g^{-1}, in

Villa Literno (ViL site) and Sessa Aurunca (SeA site), respectively. Mercury was found under the detection limit (dl) in all analyzed samples.

Trace elements concentrations in tissue of *P. clarkii* varied between sampling sites. In fact, levels of As and Zn were significatively higher ($p < 0.001$) in *P. clarkii* from ViL site. No significant differences were found for the other trace elements analyzed.

The tissue type had a highly significant influence on the accumulation of As, Cr, Cu and Zn leading to higher accumulation in the Hep than in AbM (Fig. 2.3). In the Hep As was found at a mean concentration of 8.534 and 3.248 µg g^{-1}, in the AbM was found at a mean concentration of 0.627 and 0.456 µg g^{-1}, in ViL site ($p < 0.001$) and SeA site ($p < 0.001$), respectively. The results indicated that at the SeA site, significant differences ($p < 0.001$) were found between concentration in Hep and AbM for Cu. Similar significant differences were also detected for Cu concentrations ($p < 0.05$) at ViL site. In addition, significant differences ($p < 0.001$) were found for Zn between Hep and AbM at ViL site. Finally, higher concentrations of Cr were found in crayfish Hep than in the AbM in both sampling sites ($p < 0.001$).

The analyzed individuals varied in size and weight ranges, including males and females. The multiple regression analyses indicate that there was no correlation between weight, gender and concentration of all analyzed trace elements ($P > 0.05$).

2.5 Discussion

The lack of relations between elements concentration in muscle and hepatopancreas of *P. clarkii* and weight suggest that, within the range of weight of the studied specimens these parameters had minor effect on trace elements accumulation (Ariano et al, 2015). The absence of relation between trace elements and gender agrees with other studies on *P. clarkii* (Mancinelli et al, 2018; Bellante et al 2015; Elia et al, 2006)

Occurrence of Cd and Pb has been widely explored in crayfish. The levels of Cd in AbM of ViL site and SeA site, respectively, were generally comparable those found in the muscle tissue of *P. clarkii* from Preola Lake (<dl – 0.01 µg g^{-1} d.w.) and Gorgo Medio Lake (<dl – 0.03 µg g^{-1} d.w.) in Sicily, Italy (Bellante et al, 2015) and lower than those reported in crayfish muscle from Trasimeno Lake (0.05 µg g^{-1} and 2.2 µg g^{-1}) and Bolsena Lake (0.03 µg g^{-1}) in Central Italy (Mancinelli et al, 2018; Goretti et al, 2016). The levels of Pb accumulated in the AbM and Hep determined in the present study were lower than concentrations measured in other areas (Suarez-Serrano et al, 2010; Goretti et al, 2016; Bellante et al, 2015).

The Cd concentrations found in the Hep of ViL site and SeA site were approximatively comparable than those measured in the hepatopancreas of *P. clarkii* from Preola Lake and Gorgo Medio Lake in Sicily, Italy (Bellante et al, 2015) and lower than those reported by other Authors (Alcoro et al, 2006). In 2016, Goretti et al, detected Cd (mean value 8.2 µg g^{-1} unpolluted area; 28.2 µg g^{-1} polluted area) and Pb (mean value 8.5 µg g^{-1} unpolluted area; 3.2 µg 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 (Goretti et al, 2016).

The hepatopancreas and muscle tissues of crayfish collected from Southern Spain had As concentrations ranging from 9.2 to 12 µg g^{-1} and from 2.5 to 2.6 µg g^{-1}, respectively (Devesa et al, 2002).

Bellante et al reported that As levels in hepatopancreas and muscle were 1.128 and 0.537 µg g^{-1} wet weight (wet wt), respectively. The As levels in hepatopancreas and muscle reported by Devesa et al, were higher than those detected in the present study (Devesa et al, 2002). Instead, Bellante et al,

found comparable levels of As in crayfish muscle and lower levels of As in the hepatopancreas than in the present study (Bellante et al, 2015). Comparable levels of As in muscle, were also found by Gedik et al from crayfish from Lousiana (Gedik el al, 2017).

Other studies have reported metals (Cu, Zn) levels in crayfish tissues with varying results. For example, Bellante et al, reported that Cu levels in crayfish hepatopancreas and muscle ranged from 1.149 to 48.3 µg g^{-1} (mean value 12.3 µg g^{-1}) and from 1.34 to 12.72 µg g^{-1} (mean value 5.19 µg g^{-1}) wet wt, respectively. In our study, the Cu levels found in AbM and Hep were approximatively comparable with levels reported by above researchers (Bellante et al, 2015). In another study Cu and Zn levels in crayfish muscle collected from Lousiana ranged from 23.8 to 44.2 µg g^{-1} and from 41.3 to 55.8 µg g^{-1}, respectively (Moss et al, 2010). In another study conducted in Center Italy, Cu levels varied from 23 to 1031 µg g^{-1} in Hep and from 27 to 187 µg g^{-1} in AbM, respectively (Goretti el al, 2016). The Cu levels in hepatopancreas and muscle reported by above Authors were higher than those detected in the present study. The Zn levels in Hep and AbM reported by above Authors were lower levels than those found in ViL and SeA sites

Bellante et al, reported that Cr levels in crayfish hepatopancreas and muscle were 0.915 µg g^{-1} and 0.24 µg g^{-1} wet wt., respectively (Bellante et al, 2015). Mancinelli et al, reported Cr in muscle tissue of *P. clarkii* (0.20 – 0.29 µg g^{-1}) at higher concentrations than those found in AbM in ViL and SeA (Mancinelli et al, 2018).

The general evidence was that crayfishes from ViL and SeA accumulated higher levels of metals (As, Cu, Zn and Cr) in the Hep than in the AbM, according with the literature (Gedik et al, 2017; Goretti et al, 2016; Alcoro et al, 2006; Bellante et al, 2015). Almost all studies on the distribution of metals in crayfish tissues showed that the hepatopancreas was the main organ of storage and detoxification of heavy metals (Bianchi et al., 2011; Alcoro et al., 2006; Martín-Díaz et al., 2006; Anderson et al, 1997). 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 heavy metals in the aquatic environment of both sampling sites.

2.5.1 Concern for Public Health

The European Union legislation (Commission Regulation (EC) 1881/2006 and its amendment Commission Regulation (EU) 420/2011) on food safety established the MRLs for total Cd, Pb and Hg in the muscle meat of crustaceans (0.5 µg g^{-1} wet wt. for Cd; 0.5 µg g^{-1} wet wt. for Pb and 0.5 µg g^{-1} wet wt. for Hg). The results obtained in the current study showed lower levels of Cd, Pb and Hg in AbM and Hep from ViL and SeA sites than those reported by other Authors and were largely below the MRLs established by the European Union legislation (Commission Regulation (EC) 1881/2006). These results suggested a limited Cd, Pb and Hg contamination in the study areas and were indicative of low risk for human consumption.

There is no European or Italian regulation for Cr, Cu, Zn, and As levels in crustaceans and food products.

Cu, Zn and Cr are not regulated, because they are essential metals and considered necessary for specific physiological functions. However, an excess of these metals can cause harmful effects in organisms (Schmitt et al, 2006; Anderson et al, 1997).

Chromium levels detected in Hep and AbM were comparable or lower than those reported by other Authors (Bellante et al, 2015; Mancinelli et al, 2018). Furthermore, Cr concentrations in AbM were below the threshold concentration suggested by FDA 1993 of 1.089 µg g^{-1} wet wt. for human consumption (FDA, 1993).

Copper is very usual in the environment and essential for normal growth and metabolism (Eisler, 1998). Instead, it is a component of the respiratory metalloprotein hemocyanin in crustaceans (Rainbow, 2002), therefore, relatively high copper amounts may be found in crayfish tissues, mainly in the hepatopancreas (Alcoro et al, 2006; Bruno et al, 2006). The role of Cu in crayfish metabolism and its great variability on data reported by other studies make a tentative comparison difficult, but the concentrations of Cu found in the present study were generally comparable or higher than those reported in crayfishes captured in other polluted and unpolluted locations (Bellante et al, 2015).

The concentrations of Zn were higher than concentration found by other Authors in polluted and unpolluted areas (Bellante et al, 2015; Moss et al, 2010; Sànchez-Lòpez et al, 2004). Our results were indicative of high Zn levels, especially in the ViL site.

Although our results were indicative of higher levels of As in Hep, especially in the ViL site, the concentrations of As in AbM were comparable than those reported by other studies with low risk for human consumption (Gedik et al, 2017).

2.6 Conclusion

The accumulation of metals in *P. clarkii* tissues reflecting the concentrations of metals in the surrounding environment (Kouba et al, 2010). Our data showed that *P. clarkii* can be considered a good bioindicator for metal pollution in the study areas. Although these results must be treated with caution because of the small number of samples and the lack of legal limit for some metals in crustaceans and other fish products, we speculate the higher Cu and Zn concentrations found in *P. clarkii* tissues, especially for Zn from ViL site, could be related to a high agricultural activity in these areas. The higher As concentrations in crayfish Hep, especially from Vil site, must be well studied in order to identify possible sources of contamination in the 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 information on the role of this species as indicator of environmental contamination.

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Chapter 2 - References

Chapter 3

Determination Polycyclic aromatic hydrocarbons (PAH), arsenic, chromium and lead in warty crab (*Eriphia verrucosa*)

Heavy metals and PAHs in *Eriphia verrucosa*

Ariano A., Serpe F.P., Sanso N., Velotto S., Severino L., Esposito M. PAHs concentrations in wart crab (*Eriphia verrucosa*) from the coastal areas of Campania region, Italy. XXVII SILAE Congress - September 9-13 2018 - Milazzo, Italy.

3.1 Abstract

The warty crab (*Eriphia verrucosa)* is a bentonic species found in the Mediterranean Sea, the Black Sea, and the eastern Atlantic Ocean, from Brittany to Mauritania and the Azores. It is highly fecund and is reported to feed on bivalves, gastropods, and hermit crabs, or on mollusks and polychaetes. *Eriphia verrucose* shows a preferential uptake of pollutants from sediments. The aim of this study was to evaluate the levels of As, Cr and Pb and the content of six PAHs in edible muscle of warty crab from various coastal areas of Campania region.

The results of the present study suggested a limited contamination of Pb, Cr and PAHs in the study areas and were indicative of low risk for human consumption. The higher As concentrations in muscle of *E. verrucosa*, especially for smaller specimens, must be well studied in order to well understand the bioaccumulation mechanisms and to identify possible sources of contamination in the these areas. Further studies are also needed in determining the percentage of organic and inorganic As in warty crab tissue.

3.2 Introduction

Over the last decades, interest and awareness of institutional bodies, researchers and consumers in seafood safety has increased significantly. Fishes, mussels and crustaceans are part of the culinary traditions of several countries worldwide and represent an essential source of nutrients. Seafood is rich in proteins, fatty acids, essential amino-acids and vitamins (Cederholm, 2017). Despite this, it can represent also a route of exposure to dangerous chemical substances. Seafood safety is strictly linked to marine environment quality, because many pollutants present in the aquatic environment can be bio accumulated 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 (Cappello et al., 2018). 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 (Ferrante et al., 2018). 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 bbioindicator to collect data on the current health status of the marine ecosystem. *Eriphia verrucosa*, is a benthonic species of crustacean also called the warty crab. The warty crab 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 (Ozogul etl al., 2013). 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. The warty crab, because of its geographic distribution, its position in the food web and its 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, accidental oil spills (Habibullah-Al-Mamun, et al., 2018; Tornero and Hanke 2016;). 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, ingestion is the first way of exposure that can lead to detrimental effects on animals and human health (Ferrante et al., 2018; Zaccaroni et al.,2018).

Fig. 3.1 Eriphia verrucosa (Forskål, 1775).

Due to their persistence, long range transport and power of bioaccumulate in the trophic chain, PAHs contamination has become a global issue. Based on the evidence of their toxic potential, European institutions have issued two regulations regarding PAHs presence in food for human consumption. The European Commission (EC) regulation N. 1881/2006 establishes the maximum residual limits (MRLs) in mollusks and some smoked fish products of four PAHs compounds: benzo[a]pyrene (BaP), benzo[a]antharacene (BaA), benzo[b]fluoranthene (BbF) and chrysene (Cry). The EC regulation N. 836/2011 defines the sampling and analytical methods approved for PAHs detection in foods products. The need to officially assess the presence of PAHs in food items and to set MRLs that prevents the product from being harmful for 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. Despite this, the EC regulation consider just four PAHs compounds for which research in products intended for human consumption is mandatory. Moreover, EC regulation limit the research of PAHs only to two categories of fisheries 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. Despite data regarding trace elements concentration in warty crab are poor (Dumus et al.,2018; Zotti et al., 2106), a previous study evaluated Cd concentration in the warty crab (Ariano et al., 2015) detecting in the whole crustacean, cadmium values high enough to rise concern for the estimated weekly intake of Italian population that usually consume warty crab as traditional food (Ariano et al., 2015). For this reason, this study analyses also arsenic, Cromium and Lead values in warty crab meat. Between trace elements evaluated, lead is a non-essential metal well known for its ability to induce harmful effects both in acute and chronic conditions. Lead acts on central nervous system, kidneys, skeletal and immune system affecting mostly children, due to their higher metabolic rate and higher ability to absorb a mayor quantity of Pb (Moline et al., 2015). Although trace elements values detected are lower than the European legal

ones, it is important to monitoring their concentration in traditional seafood, as they are consumed on regular basis by local population. Moreover, heavy metals as lead, can lead to severe health disease even if absorbed in sub-lethal doses constantly (Dumus et al., 2018).

The aim of the present study is to evaluate trace elements and PAHs concentration in muscle of the warty crabs. For this reason in addition to the investigation on the four PAHs compounds included in the European regulations for food safety and quality, we investigated also presence and levels of benzo[k]fluorantene (BkF) and dibenzo[a,h]anthracene (DahA); PAHs compounds similar in toxicity but not included in the European regulations (Spink et al., 2008; Lundstedt etal., 2007; Ronald W.P., 2000).

3.3 Materials and methods

3.3.1 Biological material

Thirtytwo samples of male warty crab (*Eriphia verrucosa*) were caught from two different locations along northern coast of Campania region (Italy).
Once captured, the crabs were weighed, the length and width of their carapace was measured using an Absolute Digimatic caliper (Mitutoyo, Japan) and then immediately sealed in decontaminated polyethylene bags, frozen at -20 °C and kept at the same temperature until delivery to the laboratory.

Fig. 3.2 Map showing locations of the sampling sites: Castelvolturno (site A) and Mergellina (site B).

3.3.2. Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

The crab muscle from claws and appendages was individually separated (2 ± 0.5 g), homogenized (Ultra-Turrax disperser T25, Bicasa, Bernareggio, Italy) and saponified with 10 ml of a solution of potassium hydroxide (2 N in ethanol) in a water bath at 80 °C for 2 h. The digest was cooled at room temperature and 10 ml of ultrapure water were added, extracted with 20 ml of cyclohexane and then centrifuged at 3000 rcf for 5 min at 4 °C (Megafuge, Heraeus). The extraction was carried out three times. The supernatants were combined (about 60 ml), filtered with a filter paper (Millipore) containing anhydrous sodium sulphate and reduced to small volume (about 0.2 ml) using a Rotovapor at 40 °C (Büchi, Flavil, Switzerland). The residue was dissolved in 3 ml Acetonitrile (ACN), applied on a Sep-Pak cartridge pre-activated with 3 ml ACN and eluted with 3 ml ACN. Eluate was dried under nitrogen flow in a thermoblock at 50 °C; the residue was dissolved in 1 ml ACN, 0.45 µm filtered (nylon syringe on-line filter, Millipore) and analysed by High Performance Liquid ChromatographY (HPLC).
External standard method was used to determine PAH concentration in the samples. Linearity of method was checked by triple injection of an 11 PAH standard solution ranging from 0.5 µg/kg to 15 µg/kg. Linear regression was applied to construct a calibration curve reporting peak area vs PAH concentration. A calibration curve was made for every sequence of analysis. Measurement uncertainty was calculated by identifying and quantifying the uncertainty components of the analytical process, in particular the relative expanded uncertainties (Uc) were calculated for each PAH (UNI CEI ENV 13005:2000) (Serpe F.P. et al, 2010).

3.3.3 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).
Before graphite furnace atomic absorption spectroscopy (GF-AAS) analysis, the sample (0.5 ± 0.02g) was placed in a teflon vessel with 5.0 ml

of 65% HNO_3 and 2.0 ml of 30% H_2O_2 (Romil UpA). The vessel was sealed 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. The vessel was then cooled at 30°C, the digestion mixture was transferred into a 50.0 ml flask and the final volume was obtained by adding Milli-Q water.
Metal concentrations in the digested samples were determined with an atomic absorption spectrometer (Analyst 600, Perkin-Elmer, Bonenseewerk, Germany) equipped with a graphite furnace and a L'vov platform for Pb, Cr and As.

3.3.4 Quality Assurance

Quality was monitored through analysis of procedural blanks, duplicate samples, and standard solutions. Standard solutions of analytes were prepared from certified stock solutions of Pb, Cr and As with a relative matrix modifier (atomic spectroscopy standard, Perkin Elmer). Concentrations for each set of samples were determined in the medium range of the calibration curve.
The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by determining the standard deviation of 10 independent blanks spiked at 25, 50 and 100 µg g^{-1} for Pb, Cr and As with an external standardization curve. The performance of the method was assessed through participation in interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK).

3.3.5 Statistical Analysis

All metal concentrations were expressed in wet weight as mean ± SEM (standard error of mean). The Statgraphic Centurion XV statistical package, version 15 (StatPoint Technologies, Inc., Warrenton, VA) was used to determine significant differences among means. The comparison was done with multiple range tests.

3.4 Results

3.4.1 Polycyclic Aromatic Hydrocarbons (PAHs) concentration in crab

PAHs concentration in crab muscle samples are summarized in Figure 3.3 and 3.4. Data are expressed as the mean concentration of each metals with associated SEM.

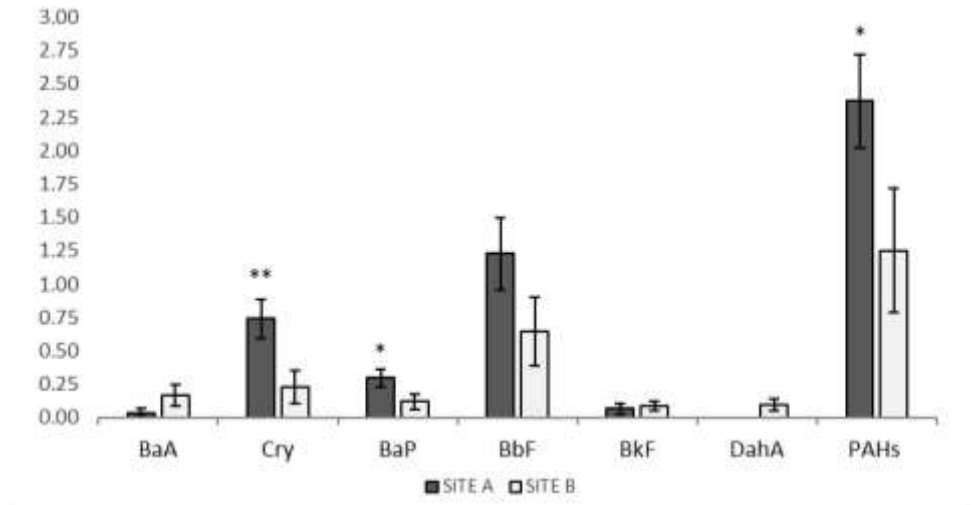

Fig. 3.3 Concentration of BaA, Cry, BaP, BbF, BkF, DahA and sum PAHs (BaA + Cry + BaP + BbF + BkF + DahA) in E. verrucosa depending on sampling sites: (A) Castelvolturno (n=16) vs (B) Mergellina (n=16). Vertical bars represent average concentration (µg g-1 wet weigth) ± SEM. Probability levels for significant differences: p<0.001(***); p<0.01 (**); p<0.05 (*).

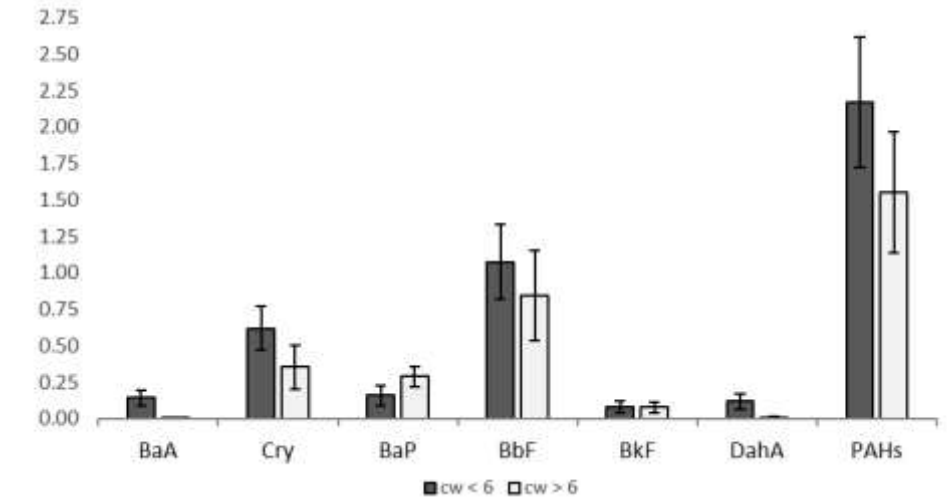

Fig. 3.4 Concentration of BaA, Cry, BaP, BbF, BkF, DahA and sum PAHs (BaA + Cry + BaP + BbF + BkF + DahA) in E. verrucosa depending on samples size: carapace width < 6 cm (n=17) vs carapace width > 6 cm (n=15) Vertical bars represent average concentration (µg g-1 wet weigth) ± SEM. Probability levels for significant differences: p<0.001(***); p<0.01 (**); p<0.05 (*).

3.4.2 Metals concentration in crab

Metals concentrations crab muscle samples are summarized in figure 3.5 and 3.6. Data are expressed as the mean concentration of each metals with associated SEM.

The levels of some PAHs in muscle of *E. verrucosa* varied between sampling sites. In fact, levels of Cry were significatively higher ($p < 0.01$) in crabs from Catelvolturo site. Similar differences were also detected for BaP and sum of PAHs (BaA + Cry + BaP + BbF + BkF + DahA)

concentrations ($p < 0.05$). No significant differences were found for As, Cr and Pb and other PAHs.

The analyzed individuals varied in size ranges. The multiple regression analyses indicate that there a positive correlation between size and concentration of As ($p < 0.05$). No significant differences were found for Cr, Pb and PAHs.

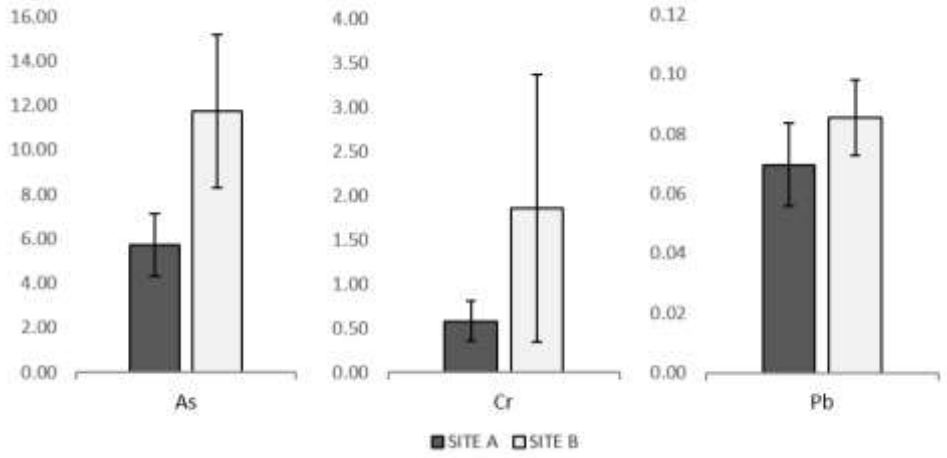

Fig. 3.5 Concentration of As, Cr and Pb in E. verrucosa depending on sampling sites: (A) Castelvolturno (n=16) vs (B) Mergellina (n=16). Vertical bars represent average concentration (µg g-1 wet weigth) ± SEM. Probability levels for significant differences: p<0.001(***); p<0.01 (**); p<0.05 (*).

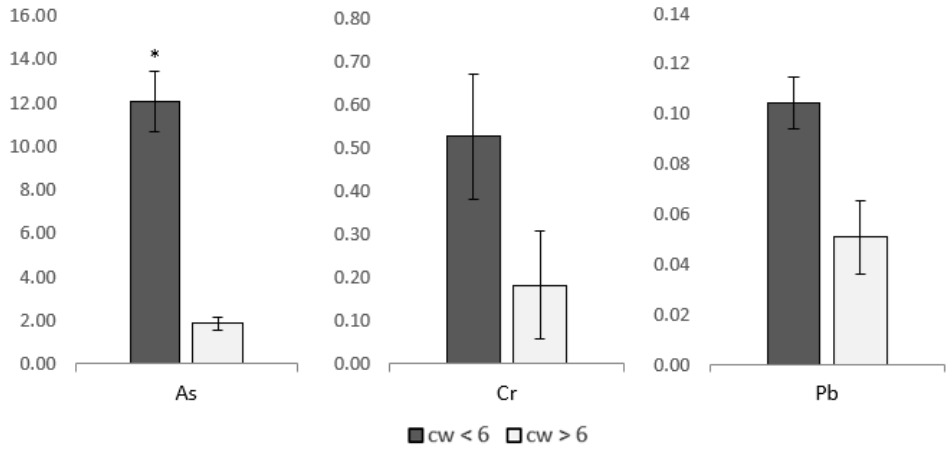

Fig. 3.6 Concentration of As, Cr and Pb in E. verrucosa depending depending on samples size: carapace width < 6 cm (n=17) vs carapace width > 6 cm (n=15) Vertical bars represent average concentration (µg g-1 wet weigth) ± SEM. Probability levels for significant differences: p<0.001(***); p<0.01 (**); p<0.05 (*).

3.5 Discussion and conclusion

Occurrence of metals concentrations have not been widely explored in crab. The Pb concentrations found in the muscle of *Eriphia verrucosa* in Mergellina and Castelvoltuno sites were approximatively comparable than those measured in the muscle of warty crab from Turkey (Bat and Öztekinin in 2016). Comparable levels of Pb in warty crab muscle, were also found by Zotti et al, in the Adriatic Sea (Zotti et al, 2016).

The edible muscle of warty crab (*E. verrucosa*) collected from the Black Sea had Pb concentrations ranging from 0.13 µg g^{-1} to 0.36 µg g^{-1} wet wt. (Durmus et al, 2018).

The levels of Pb in muscle of warty crab, were lower than those found in the edible muscle (0.10 µg g^{-1}) and in the whole body (0.12 µg g^{-1}) of chinese mitten crabs (*Eriocheir sinensis*) from Netherlands (Hoogenboom et al, 2015). Other Authors, reported that Pb levels in muscle of *Rapana venosa* from the Black Sea ranged from 0.1 to 0.7 µg g^{-1} (Mülayim et al, 2015). The Pb levels reported by above Authors were higher than those detected in the present study.

The As concentrations found in the present study for both sampling sites were higher than those measured in the muscle of *Eriphia verrucosa* and *Rapana venosa* from Turkey (Levent et al 2016). Suner et al, detected As (mean value 1.76 µg g^{-1}) in the muscle of fidder crab (*Uca tangeri*) from Spain at lower levels than those found in the present study (Suner et al, 1999). Furthermore, the edible muscle of warty crab (*E. verrucosa*) collected from the Black Sea had As concentrations ranging from 1.34 µg g^{-1} to 2.43 µg g^{-1} wet wt. (Durmus et al, 2018).

Chromium levels detected in the present study were comparable to those found in muscle (0.47 ± 0.01 µg g^{-1}) of *Rapana venosa* from The Black Sea (Topcuoğlu et al, 2002). Another Author, reported that Cr levels in muscle of *Rapana venosa* from the Black Sea ranged from 0.1 to 0.2 µg g^{-1} (Mülayim et al, 2015). Zotti et al, found neigligible levels (< detection limit) of Cr in muscle of warty crab from Adriatic Sea (Zotti et al, 2016).

Such as data on heavy metals, previous data on the presence of PAHs in the crabs are also rare. Monikh et al. described the levels of some PAHs of

environmental interest in crab *Portunus pelagicus* sampled in the Persian Gulf. The benzo[a]pyrene (BaP) levels in crab muscle reported by above Authors ranged from 170 to 956 ng g^{-1} on dry weight (mean value 200 ng g^{-1} on dry weight). Considering for crustaceans an average content of 80% of water, the concentration value BaP expressed on dry weight must be corrected in order to be able to express it on wet weight, thus obtaining a value of about 40 ng g^{-1} of BaP on wet wt., and higher than the values of BaP found in the present study (Monikh et al, 2014).

The PAHs levels in *E. verrucosa* were also approximately comparable to data reported by literature for other species of crab (Perugini et al, 2007). The results also showed that BkF and DahA (classified by the IARC as possible and probable carcinogenic to humans, respectively) were detected at levels comparable to PAHs included in Regulation 1881/2006.

Although not exist European or Italian regulation for PAHs levels in crustaceans, comparing our results to maximum levels of PAHs in smoked foodstuff set by the European Commission, PAH concentration in warty crab were always lower the MLRs established for other types of foods (Commission Regulation (EC) 1881/2006).

The European Union legislation (Commission Regulation (EC) 1881/2006 and its amendment Commission Regulation (EU) 420/2011) on food safety established the MRLs for total Pb in the muscle meat of crustaceans (0.5 µg g^{-1} wet wt.). The results obtained in the current study showed low levels of Pb in crab muscle from both sampling sites and largely below the MRLs established by the European Union legislation (Commission Regulation (EC) 1881/2006).

There is no European or Italian regulation for Cr and As levels in crustaceans and fish products. Furthermore, Cr concentrations in crab muscle were below the threshold concentration suggested by FDA of 1.089 µg g^{-1} wet wt. for human consumption (FDA, 1993).

This research not only seeks to implement data regarding dangerous chemical compounds in a traditional Mediterranean crustacean, but also, wants to highlight the necessity to put more careful attention to official controls and monitoring on toxicological investigation required to guarantee public health. This is essential because our result demonstrate the presence

of multiple PAHs congeners beyond those that need to be investigated by law.

In conclusion, the results of the present study suggested a limited contamination of Pb, Cr and PAHs in the study areas and were indicative of low risk for human consumption. The higher As concentrations in muscle of *E. verrucosa*, especially for smaller specimens, must be well studied in order to well understand the bioaccumulation mechanisms and to identify possible sources of contamination in the these areas. Further studies are also needed in determining the percentage of organic and inorganic As in warty crab tissue.

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Chapter 4

Potential use of seaweeds *Kappaphycus alvarezii* and *Kappaphycus striatum* from Palau Bidong (Malaysia) in animal nutrition: chemical characteristics, toxic and essential elements concentration

Trace elements in *K. alvarezii* and *K. striatum* from Malaysia

Ariano A., Olanrewaju O.S., Velotto G., Genovese A., Guerriero G., Severino L. Levels of Pb, Cd and As in seaweeds *Kappaphycus alvarezii* and *Kappaphycus striatum* from Palau Bidong (Malaysia).
XXVIII SILAE Congress - September 16-20 2019 – La Habana, Cuba.

Ariano A., Olanrewaju O.S., Guerriero G., Musco N., Scivicco M., Genovese A., Piccolo G., Bovera F. and L. Severino. Potential use of seaweeds *Kappaphycus alvarezii* and *Kappaphycus striatum* from Palau Bidong (Malaysia) in animal nutrition: chemical characteristics, toxic and essential elements concentration.
Manuscript submitted

4.1 Abstract

Aims of the present study were to evaluate chemical constituents and to quantify toxic and essential elements in two species of red seaweed *Kappaphycus alvarezii* and *Kappaphycus striatum* collected in Palau Bidong (Malaysia) in order to assess their potential use as additive in animal nutrition and possible health risks related to animal consumption. *K. alvarezii* showed a significantly higher percentage of dry matter and ash, and a significantly lower percentage of nitrogen free extract than *K. striatum*. The relative abundance of trace elements in *K. alvarezii* was Fe > Al > Mn > Cu > Zn > Se > As > Ni > Cd > Hg > Pb while in *K. striatum* was Fe > Al > Zn > Se > As > Cu, Mn > Ni > Cd > Hg > Pb. The total amount of trace elements in *K. alvarezii* was almost double than in *K. striatum*, in agreement to the differences in ash percentages. On the whole, red seaweeds *K. alvarezii* could be used in animal nutrition, but due the low amount of protein on dry matter, it should represent a mineral additive in animals under intensive production that. could contribute to increase animal welfare and sustainability consequently reducing the use of antibiotics.

4.2 Introduction

Nowadays, the increased interest towards natural ingredients to use in animal nutrition for improving health and welfare, is leading to the rediscovery of ancient ingredients. A typical example is the seaweeds used to feed livestock for thousands of years and mentioned in Ancient Greece and in the Icelandic sagas (Makkar et al, 2016). There are many species of seaweeds with different properties: someone is rich in protein and thus can be used as an alternative protein sources in animal nutrition, while others are mainly source of bioactive compounds (Wan et al, 2018).

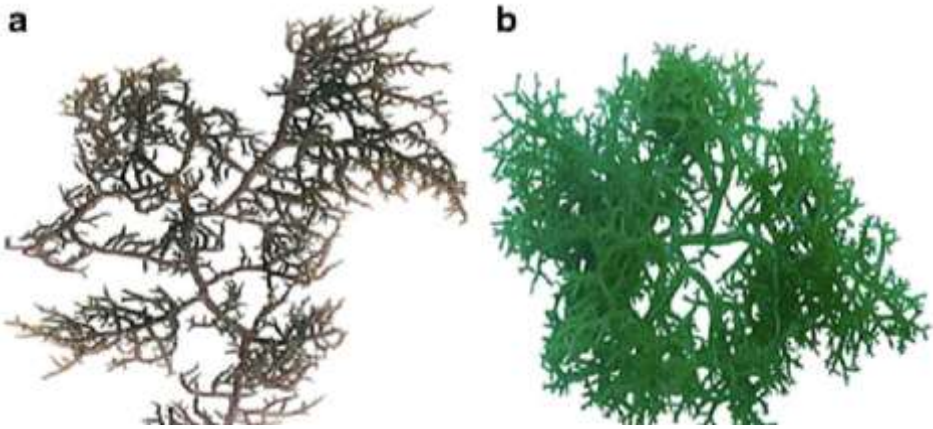

Fig. 4.1 Seaweeds a) Kappaphicus alvarezii b) Kappaphicus striatum.

Among the red seaweeds, *Kappaphycus alvarezii* and *Kappaphycus striatum* are cultivated extensively as a source of carrageenan which have been used for decades in food applications and are generally regarded as safe (add a reference). Seaweeds have the ability to accumulate high amount of minerals from their environment. This characteristic can be considered positive for essential elements (manganese, iron, copper, nickel, zinc and

selenium) and negative for the toxics (cadmium, lead, mercury and arsenic) as their high level is one of the problems limiting the use of seaweeds (Dawczynski et al, 2007; Yong et al, 2017). Therefore, seaweeds could be considered as a mineral additive to animal diets, but, on the other hands, it is possible that an excess of heavy metals can induce toxicological effects on the organism (Watanabe et al, 1997). There are several studies in literature on the chemical-nutritional characteristics and the benefits of the use of seaweeds in animal nutrition (Evans and Critchley, 2014; Al-Harthi et al, 2012; Casas-Valdez et al, 2006), but very few studies are focused on the identification of the toxicological risks related to their use (Yong et al, 2017; Kumar et al, 2008). Therefore, monitoring the concentration of heavy metals is necessary to evaluate seaweed contamination.

In light of this, the aims of the present study were to evaluate the main chemical constituents and to quantify the total concentrations of toxic (Cd, Pb, Hg and As) and essential elements (Mn, Fe, Cu, Ni, Zn and Se) in two species of seaweed *K. alvarezii* and *K. striatum* collected in Palau Bidong, Malaysia in order to evaluate their potential use as additive in animal nutrition and to assess the health risk related to animal consumption.

4.3 Results

Table 4.1 shows the chemical characteristics of the two seaweeds used in this trial. *K. alvarezii* showed a higher (P <0.01) percentage of dry matter and ash, and a lower percentage (P < 0.01) of nitrogen free extracts (NFE) than *K. striatum*. No differences were observed between the seaweeds species for the content of crude protein, ether extract and crude fiber, even if crude fiber tended to be higher (P = 0.056) in *K. striatum*.

Table 4.1 Chemical characteristics of the two seaweeds K. alvarezii and K. striatum.

	Dry matter %	Ash % DM	Crude Protein % DM	Ether extract % DM	Crude fiber % DM	NFE % DM
K. alvarezii	88.93^{A}	45.37^{A}	6.81	0.38	4.36	43.08^{B}
K. striatum	83.88^{B}	35.88^{B}	6.95	0.47	5.67	51.03^{A}
RMSE	1.87	2.29	0.12	0.084	0.28	0.95
P	0.0031	0.0088	0.5216	0.1539	0.0560	0.0029

NFE: Nitrogen Free Extracts; RMSE: root mean square error; A, B: P < 0.01

The Table 4.2 shows that *K. alvarezii* shows a higher amount of Al, Fe (P<0.01) and Mn (P < 0.05) in comparison to the other seaweed.

Table 4.2 Trace elements (Al, Cu, Mn, Fe, Ni, Se and Zn) in seaweed K. alvarezii and K. striatum (mg/kg dry weight).

	Al	Cu	Mn	Fe	Ni	Se	Zn
K. alvarezii	0.915^{A}	0.035	0.091^{a}	40.009^{A}	0.033	0.153	0.265
K. striatum	0.307^{B}	0.070	0.070^{b}	21.889^{B}	0.033	0.156	0.216
RMSE	0.262	0.036	0.013	6.515	0.0145	0.039	0.084
P	0.0024	0.1177	0.0224	0.007	0.9991	0.9076	0.3413

RMSE: root mean square error; A, B: P < 0.01

Heavy metals concentration in seaweed *Kappaphycus alvarezii* and *Kappaphycus striatum* are summarized in Table 4.3. No significant differences were found between metal concentrations in analyzed samples of *Kappaphycus alvarezii* and *Kappaphycus striatum*, with the exception of the As, which showed higher ($P < 0.05$) levels in *K. alvarezii*. Table 4.4 shows sea water physical-chemical parameter of sampling site.

Table 4.3 Toxic elements (As, Cd, Pb and Hg) in seaweed K. alvarezii and K. striatum (mg/kg dry weight).

	As	Cd	Pb	Hg
K. alvarezii	0.097^{A}	0.009	0.004	0.006
K. striatum	0.082^{B}	0.010	0.004	0.005
RMSE	0.010	0.004	0.002	0.001
P	0.0343	0.7319	0.9213	0.1992

RMSE: root mean square error; A, B: $P < 0.01$

Table 4.4 Physical-chemical sea water parameters of sampling site.

Wave height (m)	0.2 m
Temperature (°C) sea surface	26.8 °C
Current speed (m/s)	0.8 m/s
Salinity (ppt)	34 ppt
NH_4^+	0.2732 mg/L
NO_2^-	0.0063 mg/L
NO_3^-	1.8061 mg/L
PO_4^{3-}	0.0406 mg/L

4.4 Discussion

The chemical characteristics of the seaweeds in the present research are consistent with previous studies (Yong et al, 2015; Fa Farah Diyana et al, 2015; Matanjun et al, 2010). The greater amounts of ash recorded in *K. alvarezii* in comparison to *K. striatum* could be related to a higher ability to absorb minerals and trace elements from its surrounding environment (Peña-Rodríguez et al, 2011). This suggests that *K. alvarezii* could have a higher potential for metal recovery from waters compared to *K. striatum* (Kumar et al, 2007). Similary, Ahmad et al. (2016) found a higher percentage of ash in *K alvarezii* compared to *K. striatum* collected in Malaysia. The higher amount of ash is also responsible of the higher percentage of dry matter measured in *K. alvarezii*, indicating that the increased dry matter in this case is not tied to a higher amount of nutrients because of, as well known, minerals cannot contribute to the supply of energy or protein for an animal. According to Adharini et al. (2019), crude fiber showed a tendent higher level in *K. striatum*. Carbohydrates in *Kappaphycus* were the second highest component after moisture content: our results are in contrast with that one reported by Adharini et al. (2019) who found higher carbohydrates percentages in *K. alvarezii* compared to *K. striatum*. It is well known that the differences in the chemical composition of *Kappaphycus* depend on several factors, including culturing conditions (stocking density, seawater depth, culture period) as well as the sampling season and geographical location (Syam et al, 2013; Hurtado et al, 2008). In our trial, both seaweeds were collected from the same environment, differences in their chemical composition might thus be species-specific.

The relative abundance of trace elements analyzed in field cultured *K. alvarezii* was Fe > Al > Mn > Cu > Zn > Se > As > Ni > Cd > Hg > Pb while in *K. striatum* was Fe > Al > Zn > Se > As > Cu, Mn > Ni > Cd > Hg > Pb, suggesting differences in their accumulation of the two seaweeds. On the other hand, the total amount of trace elements detected in *K. alvarezii* (42.75 mg/Kg) is almost double than that in *K. striatum* (22.85 mg/kg) and this is in perfect agreement with the differences found for ash percentages. The high minerals content in seaweeds may due to their cell wall polysaccharides

and proteins with anionic carboxyl, sulphate and phosphate groups as excellent binding sites for metal retention (Yong et al, 2017; Tropin et al, 1995). Biosorption capacities of seaweeds were significantly affected by several environmental factors, such as water pH and temperature (Kumar et al, 2007). The concentrations of minerals in the environment do not always reflect their bioavailability: under similar environmental conditions, mineral composition differ greatly among the different families, genera and specie of seaweed and this is in agreement with our findings (Larrea-Marín et al, 2010). In addition, the bio-avalability of heavy metals for seaweeds varies according to their geographical origin and harvesting time and this could be responsible of the differences of our findings in terms of relative abundance in comparison to Chuah and Teo (2016) and Yong et al. (2017).

The higher levels of Fe found in our trial are in line with the results reported by Chuah and Teo (2016) who found the Iron as the most represented heavy metal in *K. alvarezii* (14.9 ppm). Under a nutritional point of view, the abundance of Fe, Cu, Mn, Se and Zn found in the present trial is very important as trace elements are supplied by animals via dietary intake of feeds and are essential for health and immunity (Arthington, 2005; Andrieu, 2008), growth (Hesari et al, 2012; Gressley, 2009), production (Gressley, 2009; Siciliano-Jones et al, 2008; Spears et al, 2008) and reproduction (Andrieu et al, 2008; Boland, et al, 2003).

The amount of Ni available in seaweeds need attention when the ingredients are included in a diet, considering the total amount supplied by the other ingredients. However, a recent opinion of EFSA (2019) confirmed that adverse effects from Ni in feed are unlikely to occur in cattle, pigs, rabbits, ducks, fish, chicken, turkeys, dogs, goats, sheep (EFSA, 2019). Regarding the Al, a ubiquity metal for which the animals has not a specific requirement, it can be rarely toxic when accumulated in the animal organism, including some showing adverse effects of aluminum on the nervous and reproductive systems in animals (Drugă et al, 2010). However, the EFSA (2008) established a Tolerable Weekly Intake of 1 milligram of aluminum per kilogram of body weight.

Compared to maximum levels of heavy metals set by EU Commission Cd, Pb, As and Hg levels for seaweed always resulted lower than the maximum

values established for feed materials. In fact, the EU regulation establishes the following MLs of heavy metals content in mg/kg (ppm) relative to a feed materials: the Cd, Pb, As and Hg MLs in the feed materials are 1.0 (for feed materials of vegetable origin), 10.0, 40.0 (for seaweed meal and feed materials derived from seaweed) and 0.1 mg/kg respectively (Directive European Commission Number 2002/32 of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed).

Overall, our results indicate that the risk of exposure to heavy metals from consumption of seaweeds is relatively low and in compliance with EU regulations. Although, further studies on a greater number of samples are needed on metals and other pollutants. Concerning Pb, Cd, As and Hg concentrations, these preliminary results support the possibility to use these seaweed species for animal feed with no additional hazards.

4.5 Materials and Methods

4.5.1 Biological material

Samples of red seaweeds *K. alvarezii* (n=6) and *K. striatum* (n=6) were collected from the eastern coast of Palau Bidong, (Malaysia) during the dry season in August 2018. Palau Bidong island is one square kilometer in area and accessible from the coastal town of Merang, located at 05° 36 N and 103° 03 E. The water samples are stored in dark plastic bottles and kept at the sampling no longer that six hours before filtered by hand operated vacuum pump. Sea water analyses were performed using a multiparametric analyzer Micromac (SYSTEA, Italy). After sampling, the seaweeds were rinsed with milliQ water, dried and stored until analyses.

4.5.2 Chemical and instrumental analysis

The chemical composition of our seaweed samples was determined according to the methods proposed by AOAC (2015). Nitrogen free extracts (NFE, as % of dry matter) were estimated as DM–Ash–CP–EE–CF.
For trace elements determination, 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).
Before Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) analysis, the sample (0.5 ± 0.02g) was placed in a Teflon vessel with 5.0 ml of 65% HNO_3 and 2.0 ml of 30% H_2O_2 (Romil UpA). The vessel was sealed and placed in a microwave digestion system (Milestone, Bergamo, Italy). Microwave assisted digestion was performed with a mineralization program for 25 min at 200°C. The vessel was then cooled at 32°C, the digestion mixture was transferred into a 50.0 ml flask and the final volume was obtained by adding Milli-Q water (Moniello et al, 2019).
Concentrations of trace elements were determined by ICP-OES technique using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT. The calibration curve and two blanks were run during each set

of analyses to check the purity of the chemicals. Reference material (CRM DORM-4, NRC, Canada) were also included for quality control. All the values of the reference materials were within certified limits. Instrumental detection limits expressed as wet weight (w.w.) and determined following the protocol described by Perkin Elmer ICP application study number 57 (Barnard et al, 1993).

4.5.3 Quality Assurance

The performance of the method was assessed through participation in interlaboratory studies organized by FAPAS (Food Analysis Performance Assessment Scheme, Sand Hutton, UK)

4.5.4 Statistical Analysis

Data were processed by ANOVA using the PROC GLM (SAS, 2000). The differences between the seaweeds were analyzed by one-way ANOVA according to the following model:

$$Yij = m + Si + eij$$

where Y is the single observation, m is the general mean, S is the effect of the seaweed (i = *K. alvarezii* and *K. striatum*) and e is the error. The comparison between the means was performed by Tukey's test (SAS, 2000). P values < 0.01 were considered as a tendency.

4.6 Conclusions

Considering the chemical-nutritional characteristics, the content of essential elements and the negligible levels of heavy metals, red seaweeds *K. alvarezii* could be used in animal nutrition but, due the low amount of protein on dry matter percentage, their best use is as mineral additive in animal feeding under intensive production. These properties could contribute to improve the immune response and increase animal welfare and sustainability and, consequently, to reducing the use of antibiotics. In addition, the use as nutrient supplement has the advantage to include little amount of seaweeds in the feeds, reducing the risks related to the accumulation of toxic elements.

4.6.1 Acknowledgments

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Chapter 4 - References

Chapter 5

Accumulation of Polychlorinated Biphenyls in Mussels: A Proteomic Study

Proteomic study: PCB in *Mytilus galloprovincialis*

Ambrosio L, Russo R, Salzano AM, Serpe FP, Ariano A, De Tommasi N, Dal Piaz F, Severino L, 2018. Accumulation of Polychlorinated Biphenyls in Mussels: A Proteomic Study. Journal of Food Protection, Vol. 81, No. 2, 2018, Pages 316–324 (doi: 10.4315/0362-028X.JFP-17-148).
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5.1 Abstract

Polychlorinated biphenyls (PCBs) are environmental pollutants of industrial origin that can contaminate food, mainly food of animal origin. Although production of PCBs has been banned in many countries since the 1980s, they are still present in the environment and are considered dangerous pollutants for human health. In fact, they can bioaccumulate in living organisms such as marine organisms because of their chemical and physical properties. New analytical approaches are useful to monitor the presence of such contaminants in seafood products and in the environment. In this work, we evaluate changes in protein expression of *Mytilus galloprovincialis* (Lam.) experimentally exposed to a PCB mixture and identify chemically specific protein expression signatures by using a proteomic approach. In particular, we identify 21 proteins whose levels of expression are sensibly modified after 3 weeks of exposure. The present work shows that a proteomic approach can be a useful tool to study alterations of protein expression in mussels exposed to PCBs and represents a first step toward the development of screening protocols to be used for biomonitoring surveys of fishery products.

5.2 Introduction

Fish, shellfish, and crustaceans are important components of the human diet; however, consumers could be exposed to various contaminants through the consumption of seafood products if the latter have accumulated these toxins in polluted waters. Contamination of the marine environment from organochlorine compounds and other organic pollutants is of great concern because of their toxicological properties and the high frequency of detection of their residues in aquatic ecosystems (Stockholm Convention on Persistent Organic Pollutants, 2009; World Health Organization, 1993, 2001). The consumption of fish, shellfish, and fishmeal feeding contaminated with polychlorinated biphenyls (PCBs) represents an important source of accumulation in both humans and food-producing animals (European Food Safety Authority, 2005; Malisch et al, 2004).

PCBs include a group of 209 different congeners that differ in the number and the position of chlorine atom substituents (Thomas et al, 2008), and they are classified as (i) dioxin-like PCB (12 DL-PCB) and (ii) non–dioxin-like PCB (197 NDL-PCB) congeners. Because of their physicochemical properties, such as chemical stability, low heat conductivity, and high dielectric constants, PCBs were widely used in many industrial applications. Recently, the production and the use of PCBs have been banned in the majority of industrialized countries (Council Directive 85/647/EEC; Council Directive 96/59/EC; Stockholm Convention on Persistent Organic Pollutants, 2009). Nevertheless, they are still present in the environment because of their high stability. Furthermore, because of both their lipid solubility and the absence of adequate metabolic pathways in the organisms, PCBs tend to accumulate in fatty tissues, and they biomagnify along the trophic chain (Thomas et al, 2008; WHO 2001).

Aquatic invertebrates, such as bivalves, often become reservoirs for many environmental pollutants because of their ability to accumulate trace contaminants from the aquatic environment. Mussels of the genus *Mytilus* are among the most commonly used sentinel organisms for the monitoring of biological effects of various contaminants in the marine environment because of their sedentary lifestyle, expressed filter-feeding activity,

capacity to accumulate and tolerate chemicals, and wide geographical distribution (Cocci et al, 2017; Dondero et al. 2006; Fernandez et al, 2013; Manduzio et al, 2005; Moschino et al, 2016; Rodrıguez-Ortega et al, 2003). Among the molluscs, the edible mussel *Mytilus galloprovincialis* (Lam.) is the most consumed in Italy, and these animals come almost exclusively from mariculture (Istituto di Servizi per il Mercato Agrario, 2012), which is a branch of aquaculture involving the cultivation of marine organisms in an enclosed section of the sea.

Because the monitoring of all PCB congeners is not possible, surveillance activities target a limited number of congeners, considered as markers of the overall contamination (EFSA, 2005). Data on occurrence of NDL-PCBs in different food and environmental samples, provided by the European Food Safety Authority (EFSA, 2010), have been reported as the sum of six PCB congeners (PCBs 28, 52, 101, 138, 153, and 180 according to the International Union of Pure and Applied Chemistry) often referred to as indicator PCBs. These PCBs are the most common NDL-PCB congeners found in food (approximately 50%) and are easily quantified, if compared with the other NDL-PCBs (EFSA, 2005, 2010). Although there are different sources for the production of dioxins/furans (PCDD/Fs) and PCBs, including DL-PCBs and NDL-PCBs, a correlation between (i) the occurrence of NDL-PCBs and DL-PCBs and (ii) the occurrence of NDL-PCBs and total PCDD/F+DL-PCBs has been verified (EFSA, 2005). Maximum levels for PCDD/Fs, DL-PCBs, and NDL-PCBs in foodstuffs have recently been laid down in Commission Regulation (EC) No 1259/2011.

According to Commission Regulation (EC) No 589/2014, the use of screening methods is provided for analysis of PCDD/Fs and DL-PCBs in food. A valid screening method should allow the fast analysis of a large number of samples. Moreover, it has to be simple and cheap and sensitive enough to detect PCDD/Fs and DL-PCBs at the level of interest. In this way, suspect samples are identified and undergo further analysis with a confirmatory method. The latter allows the unequivocal identification and quantification of PCDD/Fs and DL-PCBs in a sample, but it is more sophisticated and expensive and requires well-trained operators (Ahmed,

2003; Malisch et al, 2014; ten Dam et al, 2016). It usually features gas chromatography–high-resolution mass spectrometry (GC-HRMS) or gas chromatography–tandem mass spectrometry (GC-MS/MS).

Various screening methods have been used for the determination of DL-PCBs in different environmental and food matrixes, such as enzyme-linked immunosorbent assays, chemical-activated luciferase gene expression bioassays, and several types of sensors (Chobtang et al, 2011; Reiner et al, 2006). In contrast, because screening methods for the determination of NDL-PCBs are not yet widespread, the presence of these contaminants is usually determined by gas chromatographic methods (Commission Regulation (EC) No 589/2014).

In this framework, a novel and cheap screening tool for preliminary screening of NDL-PCBs in fishery products may be useful. To fulfill this goal, one of the more innovative and promising techniques is proteomics. In fact, the proteome of living organisms responds even to the most subtle environmental changes (Monsinjon et al, 2007); therefore, proteomics allows for the measurement and identification of thousands of proteins without any prior assumption on their mechanisms of action (Frohlich et al, 2009). Furthermore, a proteomic analysis elucidates the connection between proteins and toxicant exposure that has not been described previously (Dal Piaz et al, 2013; Gomiero et al, 2006; Lemos et al, 2009). The study of proteome alterations also explains the early molecular events involved in toxic responses, if such study is carried out at toxic concentrations that do not induce significant physiological alterations (Aardema et al, 2002). If applied to environmental toxicology, proteomics may be used to identify chemically specific protein expression signatures (PES). These PES could be used as biomarkers and provide useful molecular descriptions of the state of the cell and of the tissue. Therefore, PES are currently replacing single molecule biomarkers because they may be more robust indicators of stress exposure because of their higher specificity and sensitivity (Poynton et al, 2007). The identification of these PES may overcome the lack of genome information, which is characteristic of many species that are relevant for the environment (Apraiz et al, 2006; Magi et al, 2008). They have been determined for several aquatic organisms exposed to metals (Shepard and

Bradley, 2000), polychlorinated biphenyls (Rodrıguez-Ortega et al, 2003; Shepard et al, 2000), polyaromatic hydrocarbons (Knigge et al, 2004), physicochemical agents (Gardestrom et al, 2007), and even to natural contaminated environments (Romero-Ruiz et al, 2006).

In this work, we evaluate changes in protein expression on *M. galloprovincialis* exposed to a NDL-PCBs mixture. The goal of the study was to identify PES that could characterize PCB exposure through MS. Further studies could allow us to characterize new biomarkers of early exposure by the identification of key proteins altered by the presence of these contaminants in bivalves. Overall, identified proteins, after validation, may represent a starting point for the development of new tools for surveying PCB levels in fish and fishery products, aimed to protect the health of consumers.

5.3 Materials and Methods

5.3.1 Reagents

The following reagents for electrophoresis and proteomic analyses were from GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Sigma (St. Louis, MO): agarose ammonium carbonate; ammonium persulfate; analytical standards of PCBs 138, 153, and 180; bromophenol blue, 3-[(3-cholamidopropyl)-dimethylammonio]-propane-sulfonate (CHAPS); Coomassie brilliant blue; dithiothreitol; formic acid; glycerol; Immobiline DryStrip gels; iodoacetamide; b-mercaptoethanol; pharmalyte, pH 3 to 10; reagent of Bradford; sodium dodecyl sulfate; thiourea; trichloroacetic acid; trypsin; 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris); and urea. The following reagents for chemical analyses were from Carlo Erba (Milan, Italy): diethyl ether, anhydrous Na_2SO_4, petroleum ether, H_2SO_4 (96%), and isooctane. Extrelut NT3 columns were purchased from Merck (Darmstadt, Germany), and Florisil cartridges (1 g) were purchased from Isolute (Uppsala, Sweden). All the reagents were of molecular biology or analytical grade, if not otherwise stated.

5.3.2 Exposure condition of mussels

Adult *M. galloprovincialis* mussels (6 to 7 cm valve size) were collected from a mariculture farm in Pozzuoli, Napoli, Italy. The mussels (n = 200) were transported alive immediately to the Pozzuoli Fish Market laboratory and divided into two equal groups: control (C) and exposed (E). Each group was placed in a 100-L tank for acclimation for 7 days at 15 °C. The tanks were filled with PCB-free seawater and equipped with a continuous water recirculation system. After acclimation, group E was exposed to the three PCB indicators, PCB138, PCB153, and PCB180, contaminating the aquatic environment at a nonlethal concentration of 30 µg L^{-1} for each congener for 3 weeks. This was achieved by adding a mixture of these PCB indicators to PCB-free feed. Group C was kept in seawater and fed with PCB-free feed added with the vehicle. To assess the effects of the contaminated

environment on protein expression, 20 mussels were selected from each group, before (samples E0 and C0) and after (samples E3 and C3) contamination.

5.3.3 Sample preparation and extraction of proteins

The length of the mussels (shell) was measured. Their edible part was collected, completely homogenized, lyophilized, and then frozen at -80 °C. The weights of the whole mussels, shells, fresh edible part, and lyophilized edible part were recorded. Proteins were extracted by suspending 30 mg of lyophilized tissue in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 3% dithiothreitol) to avoid proteolysis. The mixture was centrifuged at 12,000 x g for 15 min. Supernatants were either used immediately for electrophoresis or were stored at -80 °C. The protein concentration of each extract was determined according to the Bradford method (Bradford, 1976).

5.3.4 Chemical analysis of PCBs

Homogenate (5 g) was extracted using a shaking machine for 12 h with 20 mL of diethyl ether. The extract was filtered through anhydrous Na_2SO_4, dried under nitrogen flow, and dissolved in 2 mL of petroleum ether. A twostep cleanup was performed using a diatomaceous earth solid support (Extrelut NT3, Merck) followed by a solid-phase extraction Florisil cartridge (1 g; Isolute). First, the extract was loaded on an Extrelut NT3 (3 g) column that was previously treated with 3 mL of H_2SO_4 (96%). After 20 min at room temperature, the compounds were eluted with 20 mL of petroleum ether, and the extract was cleaned up through a Florisil cartridge. The column was rinsed with petroleum ether (6 mL), and the extract was eluted with petroleum ether (20 mL) and then dried in the bath Rotavapor (Buchi, Assago, Italy) set at 40 °C and dissolved in 1 mL of isooctane. The sample was filtered through a membrane of 0.45µm nylon (Millipore, Billerica, MA) and injected in a gas chromatograph (Autosystem XL, PerkinElmer, Waltham, MA) equipped with an electron capture detector and a 35% phenyl–65% dimethylpolysiloxane–fused silica capillary column (30

m by 0.25 mm by 0.25 µm) (Serpe et al, 2013). The injection volume of the sample was 0.5 µL, the temperature of the injector was 250 °C, and the temperature of the detector was 380 °C. Oven temperature was increased from 100 to 250 °C at a rate of 15 °C min^{-1}, from 250 to 300 °C at a rate of 5 °C min^{-1}, and then was held for 1 min at 300 °C. PCBs in the sample were identified if their retention time (tR) was tR of standard PCB ± 0.5%. PCB concentration in the test solution was calculated by external calibration with a linear calibration curve constructed with three standard solutions: 1.0, 10.0, and 20.0 ng g^{-1} PCB mixture in isooctane (Serpe et al, 2013).

High-resolution two-dimensional electrophoresis. Eighteen- centimeter immobilized pH gradient strips (pH 3 to 10) (GE Healthcare) were passively rehydrated for at least 12 h with 400 µg of protein in 350 µL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol, and 0.5% ampholine, pH 3 to 10). First-dimension isoelectric focusing was carried out at 20 °C by using a MultiPhor II system (GE Healthcare). The experiment was started with an applied potential of 500 V for 8 h. The voltage was gradually increased to 10,000 V for 3 h and finally raised to 10,000 V until a value of 80,000 V-h had been achieved. The immobilized pH gradient strips were first soaked for 15 min in an equilibration solution (50 mM Tris HCl buffer [pH 8.8], 6 M urea, 30% [v/v] glycerol, 2% sodium dodecyl sulfate, and bromophenol blue traces) containing 25 mg mL^{-1} dithiothreitol and subsequently soaked for 15 min in an equilibration solution containing 45 mg mL^{-1} iodoacetamide.

Second-dimension separation was carried out at 20 °C in 12.5% polyacrylamide gels by using an Ettan Dalt twelve gel tank (GE Healthcare) at a maximum output of 25 W per gel. Image acquisition and analysis. Gels were fixed in 40% ethanol, 10% acetic acid for 3 h, stained in 0.1% Coomassie brilliant blue R-250, and destained in 30% ethanol and 10% acetic acid. Gel images were acquired using the Image Scanner III LabScan 6.0 (GE Healthcare) and analyzed using Image Master 2D Platinum 6.0 software (GE Healthcare) to achieve spot detection, quantification, normalization, and matching. For each gel, the number of valid protein spots and the number of proteins matched were determined.

Moreover, qualitative and quantitative differences in the protein patterns between the E and C groups were assessed. The volume of protein spots from the E3 and the C3 groups was measured using the Image Master 2D Platinum 6.0 software; the achieved intensities were compared using the same software to perform Student's t tests. Spots showing a statistically significant difference (95% confidence level, $P \leq 0.05$) and a fold change $\geq$ 1.8 underwent further analysis.

Identification of proteins. Interesting stained protein spots underwent gel digestion with trypsin; the resulting peptide mixtures were analyzed by high performance liquid chromatography–nano-electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS) on a quadrupole-time of flight mass spectrometer (Waters, Milford, MA) coupled with pumps and autosampler under the following standard conditions: capillary temperature of 90 °C and source voltage of 3.5 kV. Argon was used as collision gas. The digests were separated by reverse-phase LC by using a 3-Å ethylene bridged hybrid column (0.3 by 100 mm) in a nanoACQUITY LC system. Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrilewater (80:20, v/v). The digest (5 µL) was injected, and the organic content of the mobile phase was increased linearly from 5% B to 40% in 60 min. In the survey scan, MS spectra were acquired for 0.5 s in the mass-to-charge (*m/z*) range between 500 and 2,000.

The most intense peptide ions were sequenced. The collisioninduced dissociation energy was set according to the (*m/z*) ratio and charge state of the precursor ion. Raw data MS/MS spectra were converted in PKL format with the ProteoLynxs data analysis software (Waters).

Subsequent protein identification was carried out against the National Center for Biotechnology Information nonredundant protein database through the MS search algorithm on the Mascot search engine. Search parameters were set as follow: MS tolerance, 50 ppm; MS/MS tolerance, 0.25 Da; fixed modifications enzyme specificity, trypsin; one missed cleavage permitted; fixed modification, carbamidomethylation of cysteine; variable modification, methionine oxidation; and significance threshold, $P < 0.05$ and score > 50.

The taxonomy was limited to other metazoan species. The MS/MS spectra were used to perform a homology search in the National Center for Biotechnology Information database with the Basic Local Alignment Tool (BLAST) code (Ahmed, 2003).

5.4 Results

Mussels belonging to group E were exposed to a PCB mixture for 3 weeks. Then, protein expression profiles of mussels belonging to group E and group C were analyzed and compared. Moreover, a complete comparative physical analysis was performed, showing for both groups (i) normal distributions of shell lengths, (ii) similar weights for both shell and homogenized edible parts, (iii) regular liquid inside half shells, and (iv) closed valves. The rate of mortality for both groups during the experiment was less than 5%.

A quantitative chemical analysis of the extracts obtained from group E mussels was performed to assess the concentration of PCBs. For this purpose, a novel validated method was adopted (Serpe et al, 2013). The measured concentrations in exposed mussel tissues were 76.41 ng g^{-1} for PCB 138, 80.42 ng g^{-1} for PCB 153, and 50.86 ng g^{-1} for PCB 180.

These results confirmed uptake of the three PCBs. Furthermore, these concentrations are comparable to the maximum allowed concentration listed in Commission Regulation (EC) No 1259/2011. We calculated the bioconcentration factor (BCF) of each PCB ($BCF_{PCB\ 138}$ = 2.5; $BCF_{PCB\ 153}$ = 2.7; $BCF_{PCB\ 180}$ = 1.7) through the following equation: BCF = organism concentration/environment concentration. These values indicated that time of exposure and contamination through feed were efficient. A classical two-dimensional difference gel electrophoresis based on a proteomic approach was performed to obtain a proteomic map of each sample (Gao, 2014). Protein concentration into the different extracts was measured and ranged from 18 to 24 µg μL^{-1}. The quality of the achieved gels was determined considering the following set of parameters: resolution, definition, homogeneous distribution, morphology and clarity of the spots, minimum background, streaks or veined bands, and clear separation of proteins. Spots were almost homogeneously distributed across the entire isoelectric point range. Moreover, they were clear and well defined and had morphologies ranging from circular to oval. A few clusters formed by highmolecular-weight proteins were observed. In this regard, poorly defined regions, such as those including (i) spots at the boundaries of gels, (ii) overlapping proteins, and (iii) areas containing aggregates, were discarded. The number

of spots detected on gels varied between 963 and 1,195 because of our choice to count only clearly defined spots.
Proteins with a molecular mass > 200 kDa could not be observed because of the limited capacity of large proteins to be introduced into the gel of first dimension (Tsuji et al, 1999).

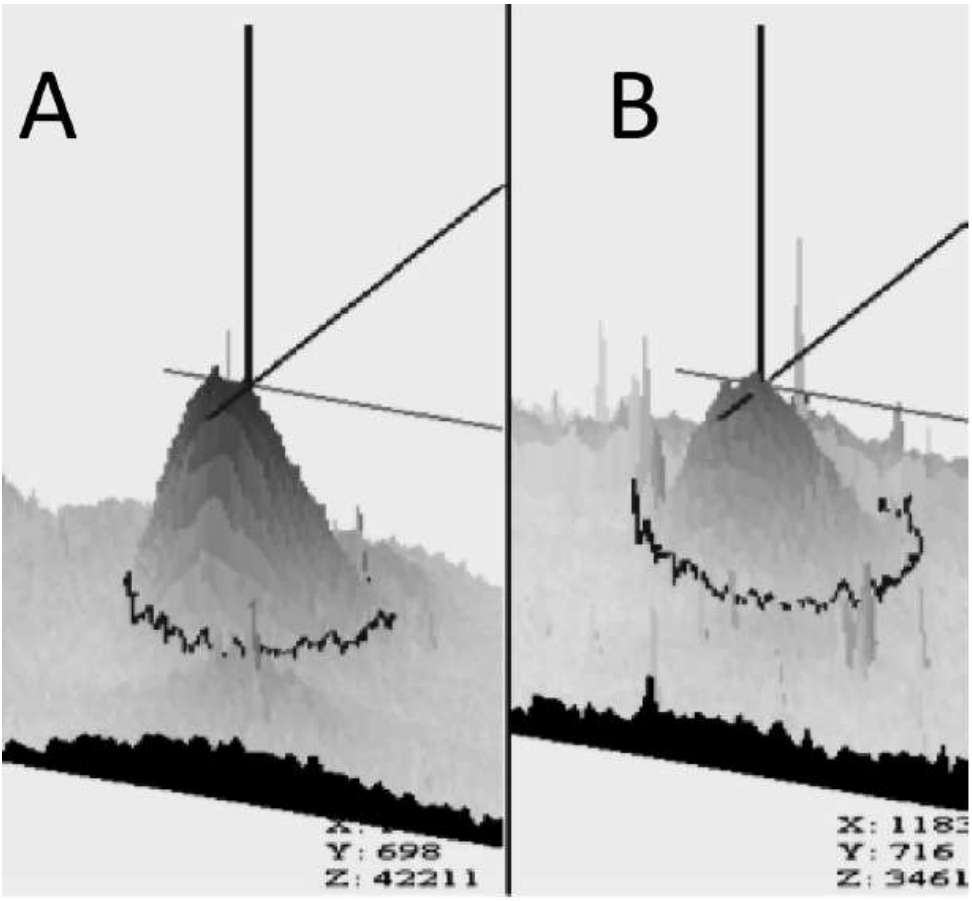

Fig. 5.1 Comparison between volume of spot 1117 in the twodimensional gel of sample C3 (A) and sample E3 (B).

A detailed comparison between protein maps acquired for different samples was carried out, taking into account the volume of the corresponding spots (Fig. 5.1). First, no statistically significant differences were observed for the

proteomic profiles of the C0 and E0 groups; therefore, a comparison between the proteomic profiles of E3 and C3 was performed. According to our threshold, differences ($P < 0.05$) in the volume of 30 spots were observed. In particular, upon exposure, 8 spots showed an increased volume and 22 spots featured a smaller volume.

These spots were excised from the stained gels and underwent trypsin digestion. The resulting peptide mixtures were analyzed through HRMS. Twenty-one proteins were identified from the investigated spots (5 upregulated and 16 down-regulated) (Table 5.1 and Fig. 5.2). Notwithstanding the good quality of the MS data, other proteins were not identified. Indeed, because *M. galloprovincialis* is a nonmodel organism, databases do not include most of the sequences of the proteins expressed by this organism.

The proteins with modified levels of expression, upon exposure of the mussels, are mainly involved in the regulation and the maintenance of cell morphology (Miura et al, 2005; Rodrıguez-Ortega et al, 2003) (β-actin, raminin receptor, rootletin-like protein, gelsolin, collagen α-1(XII) chain, tropomyosin, paramyosin, elongation factor 1-β, myosin regulatory light chain A, myosinase-I) or in energy metabolism (Dorts et al, 2001) (electron transfer flavoprotein subunit α, voltage-dependent anion channel 2, enolase, EP protein, cathepsin L, malate dehydrogenase, guanine nucleotide–binding protein). Furthermore, the identification of many proteins associated with cell survival and stress response (glutathione *S*-transferase, proliferating cell nuclear antigen, 14-3-3 protein, Rho GDP dissociation inhibitor) was significantly affected by the exposure to PCBs. All the variations of volume value fall in the same range (Table 5.1 and Fig. 5.3) and variations higher than fivefold have not been recorded, regardless of the absolute abundance of the protein.

5.5 Discussion

PCBs are toxic compounds that can contaminate the environment (e.g., seawater and soil), and food, particularly products of animal origin. Because of their liposolubility and stability, PCBs tend to bioaccumulate along trophic chains where they are stored in fatty tissue. Therefore, their presence in food and environmental matrices has been monitored for many years in European countries. Among these matrices, fish products including mussels are subjected to analytical control for the presence of PCBs. Mussel is the common name used for members of several families of bivalve molluscs from saltwater and freshwater habitats.

Some species of mussels are edible; in fact, humans have used mussels as food for thousands of years. One of the most commonly consumed species is *M. galloprovincialis*. It is widespread in European countries, especially in costal areas of France, Belgium, Holland, and Italy, particularly in the southern regions (Istituto di Servizi per il Mercato Agrario, 2012).

Moreover, *M. galloprovincialis* was chosen as a bioindicator of marine pollution because it fulfills most of the criteria required for an acceptable bioindicator (Adami et al, 2002; Licata et al, 2004). In fact, it is sedentary, widespread, easy to collect, and able to accumulate large concentrations of pollutants. Furthermore, its life cycle is long enough to accumulate contaminants, and it possesses amounts of tissue that are sufficient for chemical analysis.

In the present work, we used *M. galloprovincialis* as an aquatic model by exposing it to a mixture of three NDL-PCBs to evaluate changes in protein expression. We chose three indicators considered as markers of the overall contamination (EFSA, 2005), and we used proteomic technology because of its ability to assess biochemical changes at the physiological conditions of the organisms.

Tab 5.1 Proteins identified by LC-MS in the gel spot whose intensity in sample E3 was significantly different from that observed in sample C3 (see Fig. 5.3).

Spot	National Center for Biotechnology Information accession	Identification	Organism	Level change ratio (E3/C3)
677	gi|14161517	Enolase	*Cryphalus abietis*	2.3
700	gi|58306751	Collagen a-1(XII) chain-like	*Anolis carolinensis*	0.5
746	gi|72089178	Gelsolin	*Strongylocentrotus purpuratus*	0.4
790	gi|33469507	b-Actin	*Euprymna scolopes*	0.4
921	gi|126697324	Raminin receptor	*Haliotis discus discus*	1.8
950	gi|73656269	Cytosolic malate dehydrogenase	*Mytilus trossulus*	0.3
999	gi|34304719	EP protein precursor	*Mytilus edulis*	0.3
1004	gi|121014	Guanine nucleotide-binding protein subunit b	*Euprymna scolopes*	0.3
1030	gi|212815279	Tropomyosin	*Mytilus galloprovincialis*	0.4
1050	gi|145895072	Proliferating cell nuclear antigen	*Litopenaeus vannamei*	0.2
1051	gi|58307490	Electron transfer flavoprotein subunit a	*Megachile rotundata*	0.5
1081	gi|310706696	14-3-3 protein	*Chlamys farreri*	0.4
1101	gi|145883962	Cathepsin L	*Pinctada fucata*	0.5
1113	gi|145896447	Voltage-dependent anion channel 2	*Haliotis diversicolor*	0.5
1116	gi|58306972	Myosinase-I	*Todarodes pacificus*	0.4
1117	gi|42559342	Paramyosin	*Mytilus galloprovincialis*	0.4
1129	gi|291239961	Rootletin-like	*Saccoglossus kowalevskii*	4.0
1135	gi|223027747	Elongation factor 1-b	*Danio rerio*	1.8
1178	gi|22094809	Glutathione S-transferase	*Mytilus galloprovincialis*	3.5
1198	gi|149382257	Rho GDP dissociation inhibitor	*Schistocerca gregaria*	0.5
1359	gi|127163	Myosin regulatory light chain A	*Placopecten magellanicus*	0.4

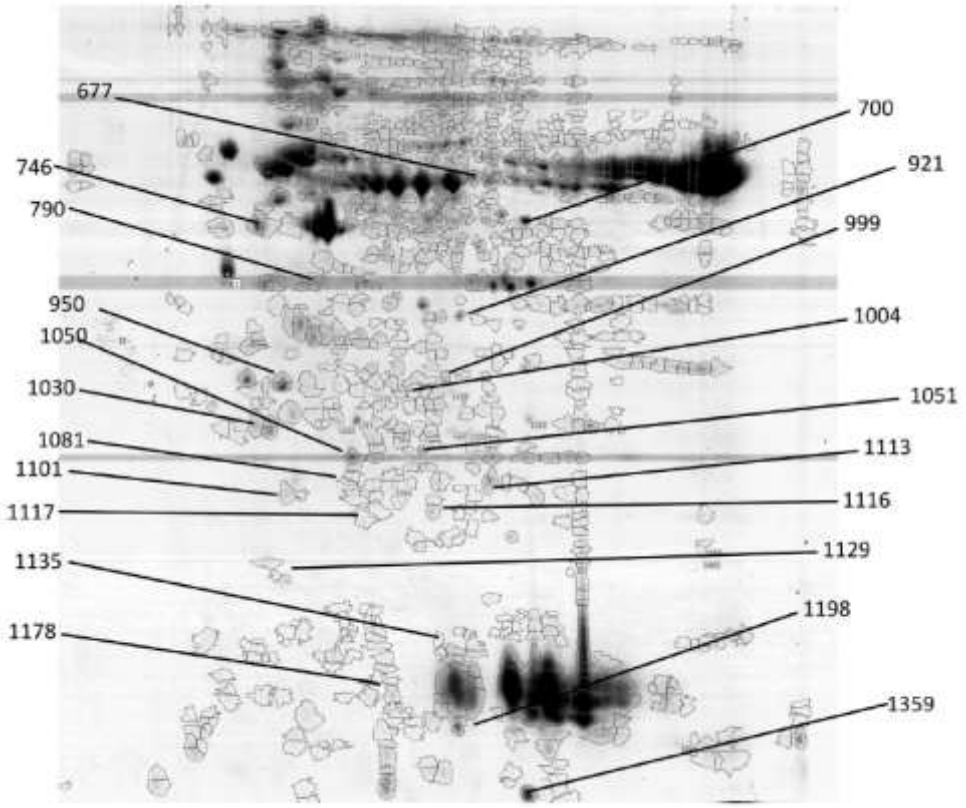

Fig. 5.2 Gel spots whose intensity in sample E3 were different from that observed in sample C3 that were successfully identified by LC-MS analysis (see Table 5.1).

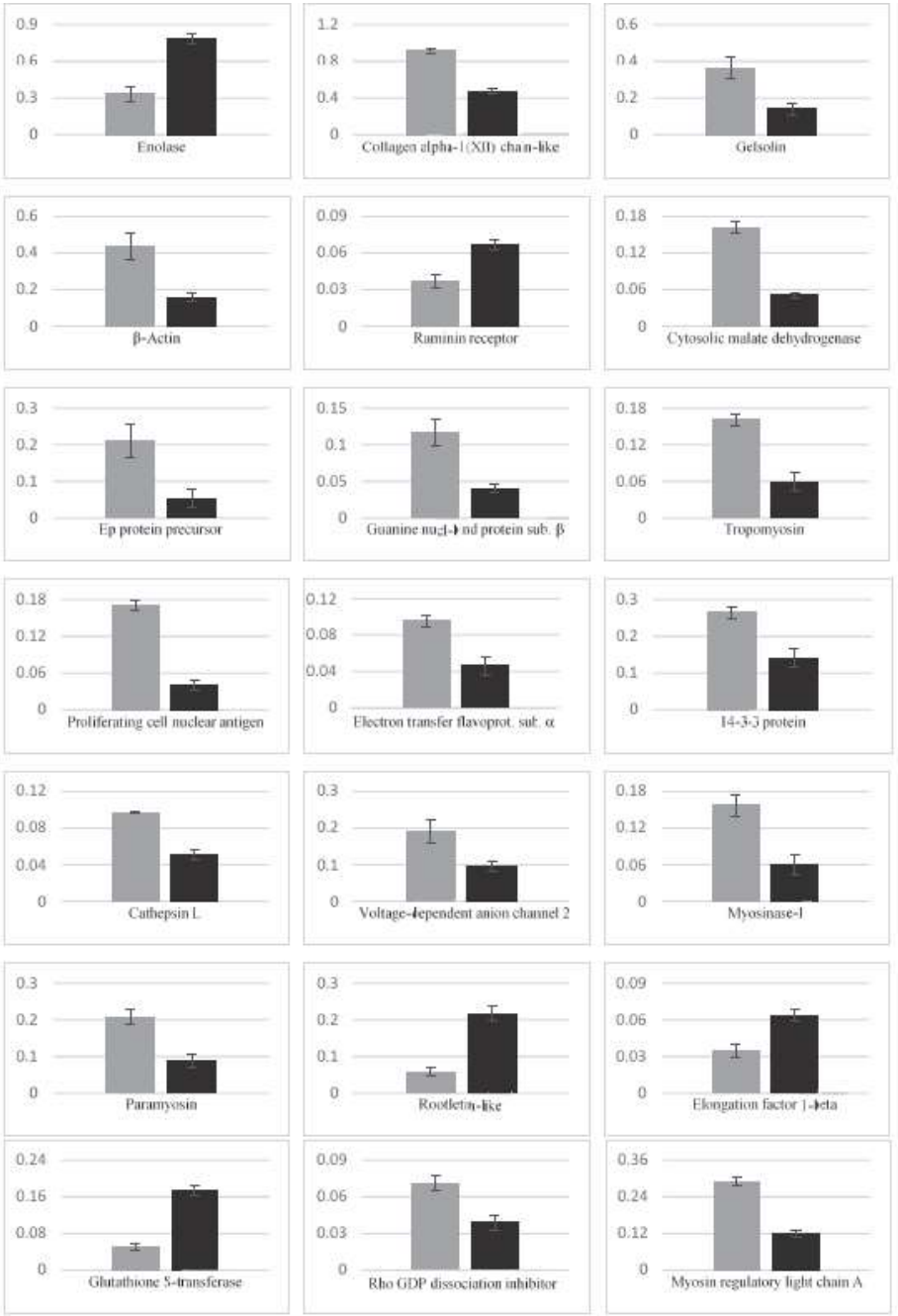

Fig. 5.3 Comparison of spot volume of proteins between C3 (grey bar) and E3 (black bar) samples.

Even if proteomic studies are still limited in the food control and ecotoxicological fields (Rodrigues et al, 2012), some marine organisms, such as fish and molluscs, have already been used for proteomic investigations (Chora et al, 2010; Manduzio et al, 2005; Riva et al, 2011).
In the present study, we were able to identify 21 different proteins whose expression levels were significantly modified after prolonged exposure of *M. galloprovincialis* to a mixture of three NDL-PCBs. Our results indicated that exposure had a mainly down-regulating effect on the expression of protein, probably reflecting the potent inhibitory action of PCBs toward several biotransformingand detoxifying cellular systems (Alzieu, 2000).
Interestingly, some of these proteins were found to be affected by exposure to specific pollutants also in different species. For example, it is well known that the expression levels and the activity of glutathione *S*-transferase significantly change because of environmental contamination by different toxic agents (Cariello et al, 2015; Sotomayor et al, 2015; Vidal-Linan et al, 2015, 2016). Moreover, cathepsin L RNA levels have been demonstrated to be affected by PCB contamination in different marine species (Carlson et al, 2019). At variance, other proteins have never been suggested as possible exposure biomarkers, indicating that they could be selectively affected by the exposure to PCBs. The identification of both novel and well-known biomarker proteins indicates that the results presented in this work represent a promising starting point to define new PES for the exposure of marine organisms to PCBs.
Because different classes of contaminants are present in the environment, further experimental characterization is required to investigate the specificity of this approach that may also be used in conjunction with other analytical techniques. Nevertheless, protein patterns, composed by more than 10 different protein spots, have already enabled distinguishing organisms grown in unpolluted and polluted areas, thus proving the effectiveness of PES determination (Amelina et al, 2007). In fact, PES are currently replacing single molecule biomarkers because they may be a more robust indicator of stress exposure because of their higher specificity and sensitivity to mixed pollutants (Poynton et al, 2007). Because this approach involves the simultaneous measurements of changes in hundreds of proteins,

it provides multiple endpoints. A multi-endpoint analysis is robust against external factors, such as age, season, or abiotic factors, other than the given stressor (Gomiero et al, 2006). PES have been determined for aquatic organisms exposed to several pollutants (Knigge et al, 2004; Rodrıguez-Ortega et al, 2003; Shepard and Bradley, 2000; Shepard et al, 2000), and the proteins that compose them were different for each case, showing the specificity of this approach.

The proteins with altered expression profiles identified in our study are related to the structure and function of cytoskeleton, which has been proposed as one of the first targets of oxidative stress (Miura et al, 2005; Rodrıguez-Ortega et al, 2003). Cytoskeletal proteins are related to plasma membrane through which pollutants enter the cell. Therefore, our results suggest membrane labilization as a major cellular biomarker of environmental pollution (Gomez-Mendikute et al, 2002). However, PCB exposure also affected other biological processes, such as the general stress response and energy metabolism. Disruption of energy metabolism has been associated with exposure to xenobiotics (Dorts et al, 2011). To date, toxicological studies of the responses of metabolic enzyme activities to chlorinated compounds remain limited. Nevertheless, the exposure of some common aquatic species to different pollutants led to the downregulation of genes encoding proteins that were mainly involved in energy metabolism and oxidative phosphorylation (Dorts et al, 2011). Overall, the identified proteins, after an adequate procedure of validation, could be used for the development of a screening method for the analysis of NDL-PCBs in fishery products.

In conclusion, the present results provide further evidence on the suitability of the proteomic approach in toxicology and food control. A challenge of proteomics is to correlate biological response with environmental quality conditions, and this work suggest that proteomic analysis can be used to identify species PES in response to pollutants.

Therefore, this approach can be considered a valuable and promising tool for biomonitoring surveys of marine pollution and chemicals accumulation in mussels and other animals.

The identification of the obtained PES can represent a starting point in the search of new specific molecular biomarkers and may serve as the basis for future investigations aimed to further characterize and develop tools for the rapid screening of chemical contamination in *Mytilus* spp. as well as other fishery products during surveys and monitoring programs. Moreover, this method could enable elucidation of possible mechanisms of toxicity of xenobiotics in mussels, organisms that are used worldwide as sentinels in environmental monitoring.

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