SCUOLA DI DOTTORATO
UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Dipartimento di / Department of

Earth and Environmental Sciences DISAT

Dottorato di Ricerca in / PhD program Ciclo / Cycle XXXVI

Curriculum in (se presente / if it is) Terrestrial and Marine Environmental Sciences

"Species identification and geolocalization tools for seafoods products". Enhancement of Italian fish and analysis of food safety through a system of characterization, guarantee of quality and origin of origin based on the use of innovative bio-technological tools.

Cognome / Surname UBALDI Nome / Name PAOLO GIUSEPPE

Matricola / Registration number 869023

Tutore / Tutor: ANDREA FRANZETTI

Coautore / Co-tutor:
(se presente / if there is one)

Supervisor: PAOLO GALLI
(se presente / if there is one)

Cosupervisor: FRANCESCO SALIU
(se presente / if there is one)

Coordinatore / Coordinator: MARCO MALUSA'

ANNO ACCADEMICO / ACADEMIC YEAR 2022/2023

Table of contents

ABSTRACT

Fish products are one of the excellences of the great Italian food and wine culture. Italy, with over 7900 km of coastline, offers a wide variety of fish products with a wide variety of dialect names. Due to their nutrients, rich in proteins of high biological value and lipids containing important fatty acids and vitamins, particularly useful for the prevention of diseases related to the cardiovascular system, they are an important component of the "Mediterranean diet". In contrast to these beneficial effects, due to their biochemical composition and high-water content, they are easily perishable, subject to bacterial and chemical contamination mainly linked to human activity.

This paper examines the fish world in different aspects, starting from the examination of the economics linked to fish products, the European fish market and in particular the Italian one. The intrinsic and extrinsic criticalities of fishery products, the role of traceability as a tool to guarantee their quality, nutritional and nutraceutical properties, eco-sustainability as an issue to which the end consumer shows more and more attention, aspects related to the arrival in the Mediterranean of toxic fish species of Lessepsiana origin, of new emerging fish toxins thanks to the increasing demand for fish products and the consequent increase in globalization of the commercial network, of new emerging environmental contaminants such as microplastics. Finally, some instrumental analysis techniques in use are examined to ensure quality, food safety and the accuracy of the information that the final consumer must know. The experimental part of the work was concerned with verifying whether there was a valid correlation between the techniques for identifying species of an Italian fish excellence such as the Red Shrimp of Mazara del Vallo and its geographical location that increases its economic value. The guarantee of food safety must be ensured through appropriate legislation, the application of appropriate quality assurance systems, versatile, portable analytical instruments that guarantee a rapid and precise response, as required by the fishing industry.

Italian Abstract

I prodotti della pesca sono una delle eccellenze della grande cultura enogastronomica italiana. L'Italia con oltre 7900 km di coste offre una grande di varietà di prodotti ittici con una grande varietà di denominazioni dialettali. Per i loro principi nutritivi ricchi di proteine di alto valore biologico e lipidi contenenti importanti acidi grassi e vitamine particolarmente utili alla prevenzione di malattie legate all'apparato cardiocircolatorio sono una importante componente della "dieta mediterranea". In contrapposizione a questi effetti benefici, per la loro particolare composizione biochimica e l'elevato contenuto di acqua si presentano facilmente deperibili, soggetti a contaminazioni batteriche, e chimiche legate principalmente all'attività antropica.

Il presente lavoro prende in esame il mondo ittico in diversi aspetti, partendo dall'esame dell'economia legata ai prodotti ittici, il mercato ittico europeo e in particolare quello italiano. Le criticità intrinseche ed estrinseche dei prodotti della pesca, Il ruolo della tracciabilità quale strumento per garantirne la qualità, le proprietà nutritive e nutraceutiche, l'ecosostenibilità quale tema a cui il consumatore finale mostra sempre più attenzione, aspetti legati all'arrivo ne Mediterraneo di specie ittiche tossiche di origine lessepsiana, di nuove tossine ittiche emergenti grazie alla sempre maggiore richiesta di prodotti ittici e il conseguente aumento della globalizzazione della rete commerciale, di nuovi contaminati ambientali emergenti quali le microplastiche. Infine, vengono esaminati alcune tecniche strumentali di analisi in uso per garantirne la qualità, la sicurezza alimentare e l'esattezza delle informazioni che il consumatore finale deve conoscere. La parte sperimentale del lavoro si è occupata di verificare se esistesse una valida correlazione tra le tecniche di identificazione di specie di una eccellenza ittica italiana quale è il Gambero rosso di Mazara del Vallo è la sua localizzazione geografica che ne accresce il valore economico. La garanzia della sicurezza alimentare deve essere garantita attraverso una appropriata legislazione, applicazione di appropriati sistemi di certificazione della qualità, strumenti analitici versatili, portatili che garantiscano una risposta rapida e precisa, come richiesto dall' industria ittica.

Preface

Fishery products have always played a significant role in gastronomy and national food culture. Along with their unique taste and organoleptic properties, they provide essential macro and micro elements that are necessary for our body's well-being. The geographical peculiarity of our nation, with over 7,900 kilometers of coastline, has led to the development of an important fishing and aquaculture industry. This industry includes both inland river and lake waters, making it a critical part of our economy and food supply. Consider that one of the symbolic species of the so-called "**pesce azzurro**" King of healthy foods such as the anchovy (*Engraulis encrasicholus*), is called according to the D.M of 22 September 2017 Anchovy or Alice, from the dialectal point of view taking as an example a region like Sicily there are the following denominations: Aléce, Alice, Aliccia, Anciva, Anciova (assonance with the Spanish Anchoa), Ancioja, Anciora, Masculina, Masculinella, Masculinu, Mascolini, 'Nnannata, Sfigghiata (both attributed to juveniles) and finally Anciovilo. These colorful and diversified name differences typical of our national costume have generated confusion over time and sometimes even a pretext for fraud.

In the food sector, fishery products are particularly susceptible to fraud, as throughout history, valuable species were often substituted with less valuable ones. From Lingan (*Molva molva*) or Haddock (*Melanogrammus aeglefinus*) that once threaded and peeled and salted replaced the most valuable Baccalà (*Gadus mohrua*).

From the Sea Swallows (*Hirundichthys rondoletii*) whose pectoral fins were expertly cut and trimmed made them look similar to the most appetizing Mackerel, (*Scomber spp.*) up to a classic fraud of our inland waters, fillet of Perch (*Perca fluviatilis*) replaced with fillets of Scardola (*Scardinius erythrophthalmus*), expertly fried and lying on fake “risotto al pesce persico” risotto with perch fillets.

CHAPTER 1

The Italian seafood market in the global context and the role of Research & Innovation in solving the current seafood market issues.

1.1 The Seafood Market: global trends and issues

Since the beginning of the Covid 19 Pandemic, there has been an increase in spending on the purchase of fishery products, this increases in 2021 increased by 7% compared to the previous year. Spending growth outpaced price growth (+1.5%).

The reason is well understandable, consumers have spent more time at home due to the restrictions imposed by the pandemic and consequently have spent more time cooking and consuming "healthy" products. In addition, according to Euromonitor, consumption has increased even after the pandemic, this increase has also been recorded in catering (+ 15% from 20202 to 2021) (Fish market in the EU 2022). The trade flow of seafood products in the EU increased in 2021, due to the post-pandemic recovery occurred, this increase came to a halt in February 2022 due to the war between Russia and Ukraine. Intra-EU exports for the first time since 2011 exceeded imports from third countries, compared to 2020 the value of trade flows increased by 15% (+ 3.4 million euros). The reasons could be attributed to an increase in EU production and thus to an intensification of intra-EU trade.

Each year, EUMOFA estimates the total supply of fishery and aquaculture products to EU consumers8 (catches + aquaculture production + imports). Subtracting exports, this formula provides an approximation of apparent consumption in the EU. As mentioned above, consolidated data on fish production in the EU are available until 2020, so these estimates have also been compiled up to 2020. From 2019 to 2020, both imports and the production of fish products collapsed, and it was precisely the decrease in domestic production that contributed.

most to the decrease in the total supply of fish products, which in 2020 reached one of the lowest values in ten years. Again, the negative trend is one of the effects that the COVID-19 pandemic has had on the sector. The pandemic has made logistics at different stages of the supply chain and in international flows of goods, and productive activities, especially fishing, more complex. Exports have also fallen; Nevertheless, apparent consumption9 in the EU stood at a ten-year low of 10.41 million tons of live weight equivalent in 2020.

As for per capita consumption, it is estimated a decrease of 1.7 kg of live weight from 2019, which brought it to a total of 23.28 kg (of which 16.79 kg consisting of wild products and 6.49 kg of livestock products). According to estimates by EUMOFA (European Market Observatory for Fishery and Aquaculture Products) and national administrations, Portugal has once again stood out as the EU's largest consumer of fishery and aquaculture products10, despite an apparent consumption lower than in 2019. Contrary to the negative trend at the EU level, some countries reported slight increases in apparent consumption, the highest of which was estimated for Bulgaria (+6%).

The two-year period 2019/2020 showed an increase of 0.1% in fishing and aquaculture activities, going from almost 213.6 million tons to over 213.8 million tons. This increase is largely driven by aquaculture activity (+2%) which offset the decrease in fishing activities (-2%) (Eumofa 2022).

All states recorded an increase in aquaculture activity with the sole exception of Indonesia. Fishing activities have instead suffered a collapse in the U.S. and China, Europe has also suffered a collapse in the production of fish products, falling to 2%. This collapse was determined by the decrease in volumes captured (-7.4%) and in those farmed (-5.1%). More details can be found in **Table 1.1.1** below, with a focus on the main producing countries and their comparison with EU production.

TABLE1.1.1 top 15 producers in 2020 (1.000 tons). Source: Eurostat and FAO. Possible discrepancies in % changes and totals are due to rounding.

	Wild Catches	**Aquaculture**	**Total production**	**% of total**	**% evolution of total production 2020/2019**
China	13.446	70.483	83.929	39	2
Indonesia	6.989	14.845	21.834	10	-7
India	5.523	8.545	14.164	7	7
Vietnam	3.422	8.541	8.037	4	2
Perù	5.675	4.615	5.819	3	16
Russian federation	5.081	144	5.382	3	3
EU-27	**3.869**	**291**	**4.957**	**2**	**-7**
US	4.253	1.088	4.702	2	-11
Bangladesh	1.920	449	4.503	2	3
Philippines	1.912	2.584	4.235	2	-4
Japan	3.215	2.323	4.211	2	1
Norway	2.604	996	4.094	2	4
Republic of Korea	1.375	1.490	3.703	2	-2
Chile	2.183	2.328	3.688	2	-3
Myanmar	1.854	1.505	2.999	1	-1
Others	27.940	1.145	37.584	18	0.1
Total	**91.260**	**122.573**	**213.832**	**100**	

Asia contributes with the top four producing countries in the world, most of their production has aquaculture as its source: over 80% in China, almost 70% in Indonesia, more than 60% in India and almost 60% in Vietnam.

In the rest of the continents the situation is reversed, where in the two Americas, Europe and Africa, only a fifth of total fish production is represented by aquaculture production, The same trend is found in the EU member states. Even smaller is the share of aquaculture in total fish production in Oceania.

In 2020, aquaculture reached 112 million tons, an increase of 2% compared to 2019, while catches decreased in volume to about 48 million tons, a decrease of 3% compared to 2019. Most Asian catches, or a fifth of the total, are bony fish (Osteichthyes).

In aquaculture, China is by far the largest producer and contributor to overall trends at continental and even global level. In 2020, production of almost 70.5 million tons accounted for 58% of world aquaculture production and 63% of Asian production. The two most prominent species are algae, 30% and carp with 26% of total domestic production. China covers almost 60% for algae and 84% for carp. By way of comparison, in 2020 the EU produced 90,000 tons of carp, 90% of which came from aquaculture, and collected 55,000 tons of seaweed, most of which are harvested for non-food purposes, which limits the relevance of comparison with Chinese production. Another limiting factor for the EU was the 35% drop between 2019 and 2020, caused by a decrease in collection activity during the COVID-19 outbreak.

The fish production of the American continent (North, Center, South) is the second most important in the world. With a total volume in 2020 of 22.4 million tons, of which the majority - 18 million tons - came from catches. Among the species caught stands out the capture of "anchoveta" (*Engraulis ringens*) intended for the production of fish meal. The production of which reached 4.4 million tons in 2020 (+ 25% compared to 2019), due to the improvement in the state of the stocks as a result of the increase in biomass that benefited from an improvement in climatic conditions. This species accounts for almost a quarter of the total. In the U.S.A. the most significant captured species was the Alaskan Pollack (*Theraga chalcogramma*) although the 2020 volumes (1.5 million Tons) represent a 3% decrease compared to 2019. It is not possible to make comparisons with other continents, for example, the EU as all the Alaskan pollack consumed in the EU is imported, while anchovy catches in the EU, which reached.

103,651 tons in 2020, include only the species *Engraulis encrasicolus*, intended for human consumption. Aquaculture recorded volumes that stood at 4.4 million tons in 2020. The most representative species were Salmon farmed in Chile (*Salmo salar*) with 992,000 tons in 2020, equal to 23% of the total farming of the continent.

Shrimp and tropical shrimp farmed in Ecuador with over 760,000 tons, which represents 17% of their total aquaculture production in the Americas. The production of the three species has seen an increase in recent years. In 2020, growth was 54% and 80% respectively, compared to five years earlier. They compare the European production of 2020, the salmon farm totaled only 17,250 tons, while that of king prawns and prawns only 143 tons.

Europe, which includes both EU and non-EU countries, ranks third in the production of fishery products. In 2020 the total volume amounted to 17.2 million tons, of which 14 million were represented by catches, the figure compared to 2019 has not changed. EU production totaled almost 5 million tons, covering 29% of European production. Similar quotas can be observed both if catches are considered, for which the EU accounted for 28% of the European total, and aquaculture, for which the EU contribution was 33%.

The five species that account for more than half of total European fish production are:

herring (*Clupea harengus*) with 1.9 million tons produced in 2020,

the Alaskan pollack (*Theragra calchogramma*) with 1.8 million tons caught exclusively by Russia: blue whiting (*Micromestius poutassou*) with 1.5 million tons, Atlantic cod (*Gadus morhua*) with 1.2 million tons mackerel (*Scomber scombrus*) with almost 1.2 million tons.

As regards the production of other main species specifically by EU Member States, herring stood at around 550,000 tons in 2020, surpassing Norway at 527,440 tons and Russia at 504,456 tons. In 2020, production reached 12 million tons, a decrease of 3% compared to 2019.

Catches account for more than 80% of total production. The decrease in volumes is due to the decrease in sardine catches, they fell just below 2 million tons, the lowest level reached in four years, the nation that suffered the greatest drop in catches was Morocco which represents the nation with the highest number of catches. Comparing them with catches made in 2020 in the EU (185,718 tons), the level is still very high. The most farmed species in aquaculture is represented by the Nile Tilapia (*Oreochromis niloticus*) in Egypt. There was also a decrease of 12% from 2019 to 2020, reaching its lowest level in four years, i.e., 955,000 tons. Oceania accounts for only 1% of world fish production, with a figure that settles in 2020 at 1.76 million tons, 86% of which consists of catches. Skipjack (*Katsuwonus striatus*) is by far the most produced species in Oceania, with 625,345 tons of catches recorded in 2020. The most important producers on the continent are Kiribati, Micronesia, and

Papua New Guinea.

EU trade flows in 2021 for fishery and aquaculture products as the sum of imports and exports with third countries were second only to China in terms of values and volumes. In 2020, the EU had the best trade performance. This was partly driven by the decrease in Chinese trade flows in correspondence with the COVID 19 pandemic. In 2021, the trade flow reversed, overtaking China. Consequently, there was a slight decrease (-1%), compared to 2020 it stood at 8.6 million tons, and their value reached 32.6 billion euros, with an increase of 4%. Imports in 2021 also rose again, totaling 25.8 billion euros and 6.2 million tons, recording increases of 6% in value and 1% in volume compared to 2020 due to COVID. The greatest impact of the pandemic had mainly affected the Ho.Re. Ca sector (Hotels, Restaurants, Catering). In 2021, the downward trend in exports continued following the negative trend recorded in 2020, with a decrease of 6% in volume (2.4 million tons). In value, exports fell by 3% to €6.8 billion. Export prices of higher-value species have risen significantly in 2021.

According to the OECD-FAO Agricultural Outlook forecast for 2021, the EU ranked 13th in the world per capita consumption of fish, with consumption accounting for less than half of the forecasts for the top three consumers (Malaysia, Korea, and Norway). According to OECD forecasts, global fish consumption increased from 180.2 million tons to 180.7 million tons from 2020 to 2021. For 2022, an increase of 2% is expected, which would bring it to 184.5 million tons. For the EU, consumption increased by 0.5% from 2020 to 2021, and the OECD forecasts a further increase of 1.3% from 2021 to 2022. As already mentioned, the need for fish products in the EU is met mainly through imports. This is confirmed by the fact that the five most consumed species in the EU, which account for 43% of total apparent consumption of fishery products, are predominantly imported: tuna, salmon, northern cod, Alaskan pollack and shrimp. For these species, EU self-sufficiency was only 11% in 2020.

1.2 The Italian Seafood Market.

In the two-year period 2020/2021 in the EU there was a simultaneous increase in expenditure on the purchase of products This simultaneous growth in demand had never been perceived in the decade between 2020 and 2020. Between 2020 and 2021 there was a further average increase of 7%, 10 of the 27 states of the European Union recorded an increase of more than 10% and six an increase of less than 7%. The value of expenditure reached in 2021 by EU countries reached €58.5 billion. Italy, which is historically the country with the highest total expenditure on seafood in the EU, recorded the most significant increase in absolute terms, with an increase of more than 880 million euros from 2020 to 2021, followed by Spain with 724 million euros and France with 649 million euros.

In terms of per capita consumption expenditure, Italy ranks fourth among EU countries, showing an increase in 2021 compared to 2020 of 8% and recording an annual expenditure of 223 euros Apart from Portugal, which historically has the highest per capita consumption expenditure (285 euros), it has placed behind Spain (EUR 242), and Luxembourg (EUR 226).

Another interesting fact to consider is the consumption of fish products versus meat products. Historically, the volume of consumption and spending on the purchase of meat have always been higher than that of fish, in 2021, it was 231 billion euros for meat and 58.5 billion euros for fish products, In Italy, spending on fish products represents a quarter of that for meat.

The driving reason is that the purchase price of fish products is higher than the price of meat. The reason is that the meat sector consists almost entirely of farmed species, these species have been genetically selected long before the fish species, also the breeding conditions (HEARTH VS. WATER) are more favorable for the species bred on the ground resulting in better weight performance in mammals compared to aquaculture products. In addition, some species of vertebrates (chicken, pork) have very competitive prices compared to fishery products.

The five species most consumed by Italian families in 2021 were : salmon, mussels, sea bream, sea bass, anchovy as you can see the first four species are bred, often their commercial presentation is made in the form of fillets often accompanied by ingredients of vegetable origin (tomatoes, olives, potatoes and spices) packaged in skins or in films and trays that can be cooked directly in the oven or microwave oven and ready-to-eat products cooking and delivered under vacuum (mussels).

In Europe in 2021, extra-EU imports of fishery and aquaculture products increased by 7% compared to 2020 totaling 6.23 million tons for a total value of 25.82 billion euros. Compared to 2020, their value increased by 7%, or 1.59 billion euros, while in terms of volume they

increased by 1%, or 71,876 tons. The graph below shows the extra countries from which fish is imported both in value and volume.

Starting from the late 1990s, the way food products are presented for sale has undergone a change. Sales channels to the public, excluding the HO.RE.CA sector, consist of weekly open-air markets, fish shops, and fish counters in supermarkets and hypermarkets. Weekly open-air markets still sell fish by displaying it on ice, with most of the product being whole and being eviscerated, filleted, or cut into slices according to customer preferences. Fish shops sell whole products, a good percentage of fillets, and some fish-based preparations with other ingredients (vegetables) in small quantities based on sales volume and business turnover. The most significant evolution has occurred in the organized retail sector (GDO). An important evolution took place when offering eviscerated fish, whole fish, and fish fillets packaged in polystyrene trays wrapped in stretch film. This provided the advantage of marketing fish similarly to how it was already done for meat, fruits, vegetables, and cheeses, in addition to increasing convenience and preventing the spread of typical fish odors, improving the hygiene of the products by reducing handling.

This evolution, driven by the increasing number of people (men and women) engaged in daily work activities with reduced time for meal preparation, has continued over the years. As a result, there has been a trend towards offering "service" components in products, presenting the final customer with a product ready for consumption or cooking. Fillets have become more popular than whole fish, which are still consumed but their purchase is concentrated on the weekend when people have more time to cook.

Fillets and fish slices have been prepared in recipes with predominantly vegetable ingredients and packaged in containers ready to be cooked in a traditional oven or microwave. In the early 2000s, another important fish-based preparation made its appearance, to be enjoyed individually or combined with other vegetable components. "Sashimi" (slices of raw fish) and "sushi" (raw fish with rice and other vegetable components and sauces), belonging to the Japanese culinary tradition, originally created for fish preservation, have become some of the most popular trend products. Another evolution is represented by "poke," dishes from the Hawaiian tradition with Japanese influences. This evolutionary trend has not only affected new exotic products but has also contributed to evolving the trade of traditional fish products.

Bivalve mollusks, such as mussels and clams, traditionally sold in nets, have been available in vacuum-sealed packaging for a few years, preserving their vitality by preventing the shells from opening, thus avoiding the release of water and, consequently, oxygen. Another example is the marketing of cod and stockfish, ready for consumption as they are already desalted and rehydrated. Production processes have also evolved with the introduction of production technologies to improve hygiene levels (HPP) capable of inhibiting bacterial growth and packaging with modified atmosphere to preserve some organoleptic aspects (smell and color). Finally, the packaging is made from recyclable materials, reducing its environmental impact.

Through market analysis and sector studies, the GDO has been able to capture these trends, creating a production technology capable of enhancing fish products and offering them safely to the end consumer. Therefore, fish has evolved from being a product relegated to religious traditions for Lent or Fridays to being a product consumed throughout the week. In this context, Italian products must play an important role, as future generations should also learn about Italian excellences through the evolution of gastronomy and presentation for sale.

1.3 Challenges in the quality assurance

Fish products are among the most perishable food products. This is mainly due to their muscle composition, in which the water content in lean fish is around 80%, a percentage that decreases in fatty and semi-fatty fish.

In fish muscle, water is strongly linked by particularly strong chemical bonds that make it difficult to separate from the proteins to which they are bound even when subjected to strong pressure. If structural changes occur due for example to technological preservation processes (freezing or deep-freezing) following prolonged storage times, protein bonds are weakened with consequent loss of water, soluble proteins, water-soluble vitamins, and mineral substances.

The peculiarity of the chemical composition compared to other high-protein foods makes the fish product unique. On the other hand, such a composition affects their perishability. Conservation is highly influenced by the species, lipid content, fishing method, production method (caught, farmed), production operations (evisceration, filleting, peeling). In this context, with the creation of the European Single Market, quality has become one of the main objectives of the common agricultural policy, increasingly oriented towards a quality of agri-food products linked to the environment and the territory, encouraging the use of environmentally friendly and responsible agricultural practices. (Quality, hygiene, and safety in the fish supply chain Orban et al. 2011). Currently the globalized and computerized world fish market needs to have a total food security system. To achieve this goal, it is necessary that food safety must be supported by the traceability of products and the legal and ancillary information of each fish product.

The challenge of Quality Assurance in this sense is aimed at the global control of all risks related to the environment and human activity. The scientific process that assesses the risk (Risk assessment) in food safety, goes to estimate the probability of damage to human health due to exposure to dangers related to the consumption of foods containing harmful substances. The risk analysis process allows to describe qualitatively and "quantitatively" the probability and potential impact of some risks (risk assessment phase), to choose and adopt the appropriate risk mitigation actions (risk management phase) and to communicate to all stakeholders (including consumers) the results of the risk assessment and the decisions that are suggested to be taken (risk communication phase).

Applied for the first time in the 70s and now introduced in world organizations as a fundamental tool for the control and maintenance of Food Safety.

Risk is a function of the probability and severity of a harmful effect on health resulting from the presence of a hazard; In food safety, it is represented by a biological, chemical, physical agent contained in a food or feed, or condition in which a food or feed is found, capable of causing a harmful effect on health. The EEC Regulation n.178/2002 introduced the concepts of "Risk analysis" and "Risk assessment". The regulation contributed to the creation of the European Food Safety Authority (EFSA). EFSA offers scientific advice, scientific advice and technical assistance on food safety, in this sense, it has become the Community reference point for a correct application of the principles underlying risk analysis.

The risk assessment consists of 4 points.

- Hazard identification
- Hazard characterization
- Hazard exposure assessment
- risk characterization.

Evaluation is the basis for the subsequent choices of the risk management bodies, the next step which consists of examining alternative interventions and, if necessary, making appropriate prevention and control choices.
EU Regulation 2019/1381 has strengthened the transparency of risk assessment in the European Union and consequent complete communication of this.

In the evaluation of an analysis plan for the control of food safety on fish products, it takes into account several factors such as: Microbiological risk Chemical risk Additives, Allergens Pests

The microbiological risk is linked to the type of diet, the life area of the fish products and the anthropic influence in the moments related to fishing and the subsequent processing of fish products and the type of consumption (ready to eat, ready to cook). Bacteria from fish products can be divided into two macro groups Bacteria linked to the environment where aquatic organisms live, among which we remember; *Aeromonas, Clostridium botulinum, Listeria monocytogenes, Plesiomonas shighelloides*, Vibrio *allophiles (Indigenous flora), bacteria originating from anthropic contamination of acues such as Escherichia coli, Salmonella*, *Shighella, Campylobacter, Vibrio cholerae, Staphilococcus spp.* (Non-indigenous flora).

In the construction of an analytical plan of self-control it is important to consider the type of product to be processed, its geographical origin, the production methods (caught or farmed) and the purpose of its consumption (ready to eat, ready to cook etc.). Therefore, it is important to consider the main bacteria and viruses, linked to the various types of products.
Aeromonas spp. Fish especially freshwater fish

Clostridium botulinum. Fish, Mollusks and Crustaceans, localized in some geographical areas, eg: Baltic Sea where type E is particularly widespread, (Hyytia et al. 1998), tropical waters where types C and D abound (Lalitha et al. 2002).

Listeria monocytogenes. Ubiquitous, resistant to salinity and low temperatures. Ready to eat products such as cold-smoked fish products, marinades and cooked products are particularly at risk. The most common serotypes are 1,2,3 rarer than 4 (Gambario et al. 2012).
Plesiomonas shigelloides (Enterobacteriaceae). Widespread in both fresh and salt water, responsible for poisoning due to the consumption of polluted water or undercooked and raw fish, crustaceans and molluscs (Miller et al., 1985; Miller et al., 1986; Shigematsu et al., 2000; Wong et al., 2000; Kirov et al. 2001; Herrera et al. 2006)
Vibrio spp. Widespread in bivalve molluscs, divided into non-halophiles such as V. cholerae *and*

halophiles such as V. parahaemolitycus

Escherichia coli: It is the bacterium that commonly with Salmonella and Vibrio spp is sought in fish products especially in bivalve molluscs (Tusèvliak at al., 2012). The bacterial load from E. coli is normally broken down by the purification systems to which the bivalve molluscs are subjected. Heat treatment (cooking with lid) at 60 ° C for less than one minute ensures the inactivation of the bacterium (Rajkowski, 2012).

Salmonella spp. Not being part of the bacterial population of fish products, their presence in them is due to pollution of water of human or animal faecal origin. Particularly common in bivalve molluscs where in the case of oysters they can persist up to 60 days. The particular virulence allows the bacterium to penetrate and fix itself in the connective tissue of oysters (Morrison et al., 2012). Since 2010, there has been an increase in the spread of the disease (EFSA 2022). This increase is attributable to several factors such as:

- increase in mass food preparations.

- Errors in food storage

- Increased consumption of raw or undercooked foods

- Improvement of hygienic conditions and consequent decrease in resistance to infections
- Increase in international trade.
- Poor hygienic attention in the production phases

Shigella spp. Bacterium widespread in polluted waters, endemic in the poorest areas of the planet in some areas of the planet. Fish products from these areas are the most at risk. Prevention consists in maintaining a high level of hygiene.

Campylobacter. Widespread mainly in cow's milk, in fish products the most at risk are the filter bivalve molluscs to be consumed raw, prevention consists in maintaining a high level of hygiene.

Staphylococcus aureus Bacterium spread in the nasal mucous membranes and human skin. In fish products particularly common in shellfish cooked ready to eat or cooked processed (shrimp pulp, crab etc.) Products in local outdoor markets are more at risk than packaged products sold in supermarkets.

Several preventive factors can intervene to decrease the microbiological risk linked to fish products.

- Supplier selection
- Sending specific guidelines for the various types of fish products
- Training of personnel employed in production areas
- Environmental hygiene
- Personal hygiene
- Product traceability
- Tense and correct production flows

Algal biotoxins Algal biotoxins are toxic substances also called *phycotoxins* that develop in phytoplankton and accumulate in bivalve molluscs, fish, crustaceans, tunicates, echinoderms. Their consumption gives rise to food poisoning, biotoxins are not toxic to aquatic organisms living in the waters where they are widespread. They are thermostable cooking does not eliminate the risk of intoxication. In the presence of favourable environmental conditions, phytoplankton are able to multiply "algal bloom" and pollute the waters and the organisms that live inside them. Currently, biotoxins are classified according to their solubility as shown in the table below.

Other fish toxin

Histamine

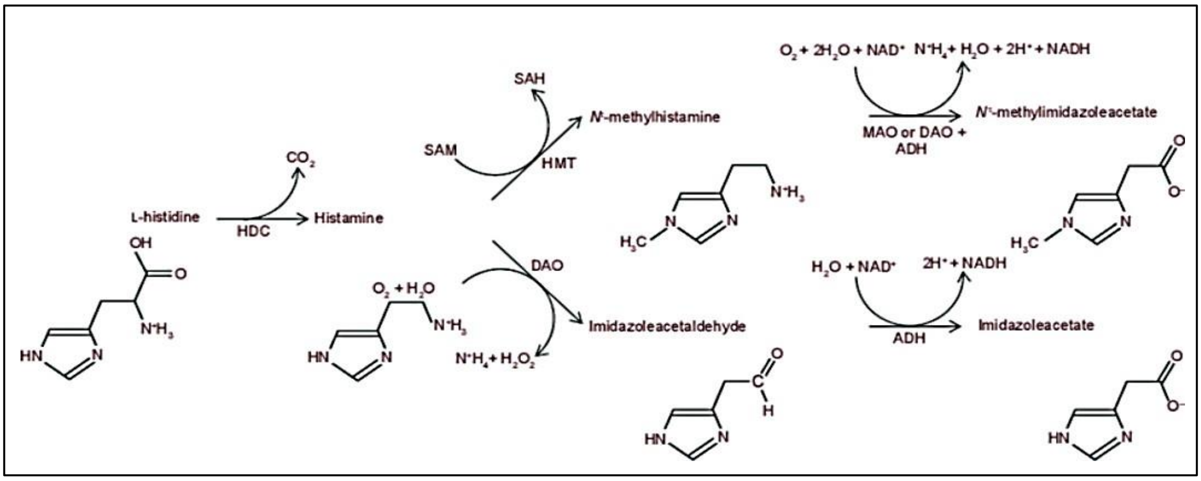
Histamine is a biogenic amine resulting from the decarboxylation of histidine, an amino acid present in some families of fish:

Scombridae (Tuna and Mackerel)
Clupidae (Sardines, Herring)
Engraulidae (Anchovies)
Coryphaenidae (Dolphin fish)
Pomatomidae (Greenhouse fish)
Scomberesocidae (Saury fish)

The origin of histamine derives minimally from tissue autolytic phenomena but derives mainly from the action of various bacterial strains that through enzymatic processes transform histidine into histamine. The high level of histidine may be present in fish tissues or result from degenerative processes related to thermal abuse. Histamine poisoning develops in a short time (from a few minutes to a few hours), histamine is heat-resistant (it is degraded after 90 minutes at 116 ° C), contaminated meats have no abnormal smells, colours or tastes. Violent symptoms cause skin rush, vomiting, headache, dizziness, dyspnoea, tachycardia, gastrointestinal disorders. In extreme cases it can cause anaphylactic shock and consequent fatal resolution. The bacterial strains involved are numerous, including:

Klebsiella spp
Proteus spp.
Enterobacter spp.
Kluyvera spp.
Vibro spp.

DIAGRAM 1.3.3.1 Decarboxylation mechanism



histamine synthesis and catabolism in mammals. Abbreviations: aDh, alcohol dehydrogenase; DaO, diamine oxidase; hDC, histidine decarboxylase; hMT, histamine N-methyltransferase; MaO, monoamine oxidase; sah, S-adenosylhomosysteine; saM, S-adenosylmethionine.

1.3.4 Ciguatera

Ciguatoxins are found in tropical species of the genus, Carangidae, Serranidae, Lutianidae and some genera of Carcharhinus sharks as a result of biological transformation of a toxin produced by a benthic Dinoflagellate (*Gambierdiscus toxicus*). The symptoms of intoxication are borne by the nervous system that give rise to acute or chronic toxicosis. Reg. Ce 853/2004 Sec. VII, cap. V, prohibits the marketing of ciguatoxin fish.

1.3.5 Oil fish

Fish belonging to the genus Gempylidae such as (*Ruvettus pretiosus*) or (*Lepidocybium flavobrunneum*), contain an oil (paraffin esters) with fast diarrheal effects without the formation of intestinal cramps. This feature had in the past prohibited their sale. In 2004, a study conducted by EFSA showed that cooking them makes meat edible. EU Reg. 2074/2005 has thus legislated and regulated their consumption provided that they are marketed packaged and packaged and labelled with precise information on their method of consumption (cooking), identified with their scientific name followed by their common name.

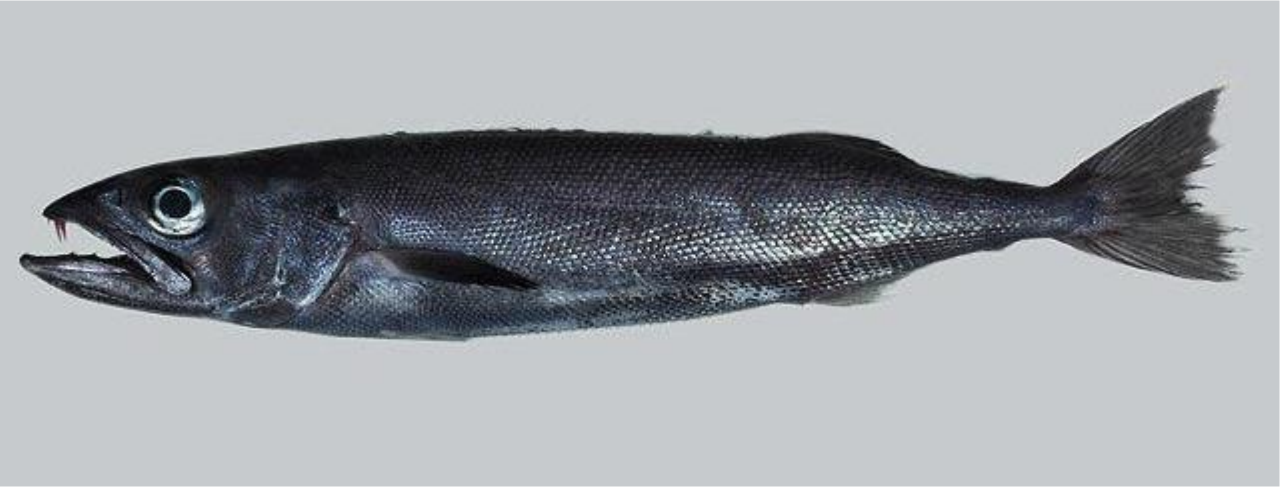


Figure 1.3.3.5.1 Ruvette (*Ruvettus pretiosus*)

Figure 1.3.3.5.2 Tersite *Lepidocybium flavobrunneum*

These photos by Unknown Author are licensed by CC BY

1.3.6 Egg consumption toxicosis: the consumption of freshwater fish eggs, especially during the reproductive period, has given phenomena of enteric intoxication of a lipoprotein biotoxin. The genus involved is that of the Ciprinadae.

1.3.7 Selacean consumption poisoning: Selaceans are among the most consumed marine species, so much so that their survival is put at risk. 37.5% of Shark and Rays species are at risk of extinction.

(Source WWF 2022). Generally, their consumption is not associated with acute pathological phenomena, but rather with their content of environmental contaminants that can cause a bioaccumulation in the human body and constitute a chronic risk. However, under particular conditions some shark species such as the Greenland shark (*Somniousus microcephalus*), consumed dried naturally contains high doses of TMAO (Trimethyl Amine Oxide) deriving from protein catabolism and the particular physiology of the Selaceans that eliminate protein catabolites through the skin. In the human gut, TMAO is reduced to TMA (Halstead 1990) toxic to humansIn the species Madagascar shark (*Carcharhinus leucas*) the presence of two fat-soluble toxins (A and B) was detected, which in 1993 caused the death of 60 people. The toxin acts at the level of the central nervous system causing ataxia and paralysis (Boisier et al. 1995). Further studies have found the presence in juveniles of the species of algal biotoxins that develop after algal blooms of brackish water lakes near the coast that are the place of growth of these sharks.

Specifically, phycotosins (mycrocistine, nodularins, teleocidins) have been detected (M.L.Edwards et al., 2023)

1.3.8 Ichthyohemotoxins

Present in the blood of Eels (*Anguilla anguilla*), Carp (*Ciprynus carpio), Tench (*Tench tench*), Conger (*Conger congrus) *and Moray (Moray* eel). Mucous membranes and wounds are the gateway to penetration into the human body of ichthyotoxin, which is thermolabile and is destroyed by cooking at a temperature above 70 degrees.

1.3.9 Toxic cyanobacteria

Algal blooms similar to marine ones but widespread in brackish and fresh waters that affect freshwater and brackish water species. In case of blooms, fishing activities are suspended.

1.3.10Hallucinogenic fish

Linked to the consumption of fish especially rocks that feed on macroalgae containing a compound similar to LSD (Helfrich and Banner, 1960), (Thuran and Cavas 2019). Intoxication causes gastrointestinal discomfort and hallucinations, dizziness and nausea. Particularly sensitive to the phenomenon are Sails fish *(Sarpa salpa*), precaution to avoid the phenomenon is immediate decapitation evisceration of the fish and elimination of the celomatic membrane.

PICTURE 1.3.3.9 Sails fish (*Sarpa salpa*) photos by Unknown Author are licensed by CC BY

1.3.11 Chemical risk

The risk derives from elements mainly related to human activity that are discharged into fresh water and indirectly or directly into marine waters. The main sources are related to industrial activity or residues related to livestock breeding activities. **Heavy metals** (Hg, Cd, Pb, As), linked to anthropogenic and environmental pollution (Mercury is widely spread on the Earth's crest and d is linked to volcanic activity) and identified with density greater than 5 mg \ cubic centimetres or with atomic weight greater than 20. Heavy metals are conventionally divided into two macro groups, the first consisting of metals with essential nutritional functions (Cu, Zn, Co, Fe, Ni)

The other consists of metals that have no nutritional function but have toxic effects due to their accumulation in the human body. (Fresh and processed fishery and aquaculture products: Gallina, Caburlotto, Arcangeli). Individual sensitivity is influenced by several factors such as diet, age, exposure to other toxic factors. Reg 1881\ 2006.

Lead: Bivalve molluscs are accumulators of this toxic metal especially in the digestive gland ((Trinchella et al. 2012). **Cadmium**: In the fish Trout (*Onchrhynchus mykiss*) and Whitefish (Whitefish clupeaformis) the maximum levels are found, generally the Cephalopod and Bivalve molluscs are accumulators of these metals. Also present in Gastropods and in Lobsters and Crabs in particular in their digestive gland.

Mercury: Target fish, Tuna (*Thunnus spp.)* Swordfish (*Xiphias gladius*), Atlantic Cod (*Gadus morhua*), hake (*Merluccius spp*.), whiting *(Merlnagius merlangius)* Pike *(Exos lucius*).

Arsenic: Technically it is not a metal but a metalloid. Not particularly widespread in fish products except in the Atlantic mussel (*Mytilus edulis*) (Sloth and Julshamn 2008), present in some edible algae (*Hizikia fusiforme*) and in rice (*Oryza sativa)* main ingredient of one of the most popular ready ro eat foods on the planet, sushi.

Dioxins derive mainly from the combustion of industrial and domestic waste, widespread in the environment and linked to the food chain, particularly widespread in adipose tissues.

PCBs: used in various industrial applications (from insulators in transformers to use in lacquers and paints) over one million tons were massively produced and used between 1930 and 1985. This has led to their ubiquitous environmental diffusion the level found in the lake and marine substrate is equal to that found in fish (Demis 1975, Nadeau 1976). Both pollutants accumulate in fat deposits and are particularly in blue fish especially in the Baltic Sea area (Herring, Baltic salmon) and in freshwater fish Pike (*Exosus lucius*), Agony (*Alosa fallax lacustris*). **PAHs (PAHs) Polycyclic aromatic hydrocarbons** Related to combustive phenomena related to food. The highest concentrations are found in fish materials deriving from aquatic organisms grown in environments particularly polluted by these compounds or from foods subjected to cooking processes that involve contact with combustion products (smoke) through the smoking process. Regulation 1881 of 2006 identified benzo(a)pyrene as the exceptional marker for assessing the degree of PAH pollution of food.

1.3.12Veterinary medicinal substances the use of veterinary drugs in aquaculture is regulated by EU REG No. 470/2009. Residues in fish meat can pose a problem for human health. The presence is due to their incorrect use and non-compliance with the suspension times. Their use can be limited or even eliminated by applying breeding methods that use low stocking density that favours the reduction of stress from particularly high food competition in feeding moments that mark the day and the life cycle of aquatic organisms. The incorrect administration of veterinary medicines represents a real risk of environmental pollution, which is also responsible or antibiotic resistance in wild populations of fish and other organisms in the aquatic ecosystem.

1.3.13Additive

They are substances used in the processing of prepared and processed products, but also directly in fishery products such as sulphites used in crustaceans.

The use of these substances is regulated in EC Reg. No. 1333/2008 The
main additives used in fishery products are:

Organic antimicrobials:

Sorbic acid E200
Benzoic acid E 210

Inorganic antimicrobials

Sulfur dioxide E220 and its salts, Sodium sulfite E221, Sodium bisulfite E222, Sodium metabisulphite E 223, Potassium metabisulphite E224, commonly known as sulfites and used in crustaceans.
Carbon dioxide E290, used in mixture with other gases (Nitrogen and oxygen) Sodium nitrate E251
Potassium nitrate E252
Boric acid E 284
Sodium tetraborate or Borax E 285

Acidifying

Acetic acid E 260
Citric acid E 300, Sodium citrate E331, Potassium citrate E332, Calcium citrate E 333, Potassium tartrate E336
Lactic acid E270
Gluconedeltalactone E575

Antioxidants

Ascorbic acid E300

Flavor enhancers

Glutamic acid E620
Monosodium glutamate E621
Monopotassium glutamate E 622

Stabilizers gelling thickeners.

Diphosphates E 450, Triphosphates E 451, Polyphosphates E 452, Sorbitol E 420, Alginates E400, 401 Agar agar E 406 Carrageenan E 407, Andragante rubber E 413

Sweeteners

Dyes
Acidity regulators
Fragrances

1.3.14 Allergens

Regulation 382/2021 legislates on the management of allergens in food and food processes. In production, risk analysis must consider the presence of allergens in food, traces of allergens and production flows. In the production design phase or at the time of introducing a new food reference, the risk of cross contamination of allergens and related traces contained in new ingredients must be assessed.

1.3.15 Parasite

A parasite is defined as any living animal or plant that lives at the expense of other living beings. Parasites present in fish products are classified into.

Protozoa (*Giardia spp.*)

Metazoa (*Anisakis spp, Diphyllobothrium, Opistorchis)*

According to their pathogenicity they are divided into

Zoonotics - transmit disease to humans (*Anisakidae, Diphyllobothrium, Opistorchis*).

Non-Zoonotic - do not transmit diseases to humans but create discomfort and harm because they depreciate the product (*Trypanorhyncha, Brush, Myxosporids, Ceratothoa, Annelids polychaetes*).

ZOONOTIC

The most common zoonotic and dangerous parasite in seafood products is the genus Anisakidae. Generally, the parasite during the life of fish is confined to the visceral package, although it can migrate *intravitae* into muscle tissues. After death, migration occurs especially in the case of Teleosts in the abdominal muscle fascia.

Anisakis' parasites control should be performed by eviscerating marine organisms (with appropriate cutting tools) and carefully checking the bowels and abdominal cavity (picture 1.3.14.1). The control is more effective if it is performed on a light plane by means of candling (picture 1.3.14.2/3).

PICTURE 1.3.14.1

Picture from P.G Ubaldi 2023

PICTURE 1.3.14.2/3 Parasite control trough light plane

Picture from P.G Ubaldi 2023

Anisakidosis is the most serious and dangerous disease linked to the consumption of raw fish products. Anisakis is widespread among wild fish as the food chain involves predation by organisms containing it. Farmed fish and crustaceans do not run this risk as the diet is exclusively based on industrially produced feed that is subjected to production processes that involve their heat treatment at temperatures near to 90° C. In fact, the thermal remediation process (cooking at 60 °C at the core of the product for at least one minute or freezing at -20 °C for at least 24 hours).

The presence in seafood represents.

- Serious danger to health
- presence prohibited by law (Reg CE 853/2004)
- exposes the company to administrative and criminal sanctions.
- exposes the company to customers.
- huge economic danger
- risk of creating disgust in those who buy.
- image risk

1.4 Challenges in Traceability

Traceability and labelling of fishery products can be an effective means of protecting the food safety of the fishery supply chain. Among the food sectors, the fishery products sector is currently the most complex. This fact depends on the high number of species, the capture and breeding systems, their geographical origin and, as we have previously mentioned, its continuous evolution as the main component of preparations and recipes with other ingredients that are mainly of vegetable origin (sushi, pokè, ready-to-cook dishes). To cope with this complexity, the EU has developed a control system based on laws common to all EU member states, capable of providing the final consumer with all mandatory and some optional information. As mentioned at the beginning of the chapter, food safety is guaranteed by traceability, the date of production and especially the expiration date, for example, guarantee the correct management of food. The storage temperature ensures control over the biology of the microorganisms, and the "to be consumed after cooking" method of consumption provides valuable advice to the buyer on the correct way to consume food. The list of ingredients, including allergens (which must be described in a highlighted font or different from the rest of the ingredients field) raises the level of food safety, and finally, the presence of mandatory nutritional tables in the context of products with multiple ingredients provides useful information to the consumer that correctly directs him in the context of his purchases. Through the dictates contained in Regulation (EC) No. 178 of 2002 taken up in a specific regulation on fishery products, Regulation (EC) No. 1379/2013 in which the fundamental concept is that all information on fishery products is properly communicated to the consumer.

1.4.1 Flow of information

Primary production

All fisheries-related activities must comply with the principles of the Common Fisheries Policy (CFP) related to the management of fishing fleets and the shelf life of fish stocks, primary producers must commit to exploiting fish stocks in a sustainable way, placing them correctly on the markets and providing all the necessary information to the final consumer. All primary producers must comply with the principles contained in EU Reg. No. 1224/2009 and No. 1379/2013.

1. Possession of a fishing license, which is mandatory to obtain a fishing license.

2. Fishing authorization

3. Compilation of the fishing logbook in which the data of the fishing trip are unequivocally reported, with particular reference to the identification number of the vessel, the species identified by the scientific name in Latin and FAO code alpha 3, fishing zone (FAO area and its divisions and sub-areas for the Mediterranean Sea and the eastern Atlantic Ocean), the date of catch, the date of departure and arrival at the port and the duration of the fishing trip, fishing gear used, quantitative estimates of live weight catches with a tolerance of 10 %, number of fishing operations
4. In addition to this information, the master is required to provide information relating to disembarkation and any transshipment operations.

After unloading, the products are weighed with calibrated and sealed systems by the competent authorities and a label bearing the net weight will be affixed to the containers.

1.4.2 Labelling

EU Regulation 1224/2001 requires that the identification numbers of each batch be reported on the label, consisting of the EU number of the vessel or the number of the aquaculture site,

date of landing/harvest, FAO alpha 3 code of each species. Regulation 1379/2013 provides that the consumer must be provided with the following information:
official trade name in force in the State, scientific name Production
method: caught, farmed.
Catching or rearing area
If the product has been thawed the
date of minimum durability

1.4.3 Placing on the market

It is always made through the transfer to a registered auction centre, to a registered buyer or to registered buyers' associations. Once the batches have been purchased, the producer can divide them into smaller batches using the information received from the previous steps and entering a batch number that identifies the batch and is able to return to the information linked to the primary batch purchased. The transport of the goods, as well as being carried out on appropriate insulated means of transport to maintain the temperature suitable for proper storage. The retailer may sell the product to the final purchaser only if the label affixed to the product contains all the mandatory information required by EC Reg. 1379/2013

To meet these tasks, over the years the sector has equipped itself with a system for carrying over the information mentioned earlier in this chapter.

The most widely used system of information dissemination is still paper through the production of the label that is physically affixed to wooden crates or insulating material such as polystyrene or labels displayed on sales counters or affixed to the packages containing fishery products. The information is obviously managed through software that guarantees the correct dragging and dropping. Various tools can meet this need. Starting with fishing vessels that are equipped with monitoring technologies that allow their location, bait, and transshipment data. Through the use of unit (AIS) automatic identification system the Vessel monitoring systems (VMS) transmit information about the movement of commercial vessels via electronic information exchange with other ships, base stations, or satellites to governmental or private recipients. AIS transceivers are required under the International Maritime Organization's (IMO) International Convention for the Safety of Life at Sea (SOLAS) for all passenger ships and for commercial vessels over 300 gross tons, although fishing vessels are currently exempt from this requirement (SOLAS Annex 17; IMO 2017). AIS transceivers are required, however, by many Regional Fisheries Management Organizations (RFMOs) and the fisheries management agencies of many countries (FAO 2017). This information are highly confidential and tightly regulated, but can be used as a surveillance tool to deter fishing violations as part of a harvest management system. This type of tools has a high potential of use to determine the correct traceability as the data obtained by the system can be compared with the data coming from the logbook, or the capture modules can confirm the data present on the labels of the products placed on the market. In the case, for example, of a traceability test normally carried out during a quality inspection at a supplier's premises, the quality inspectors of large fish processing or sales companies regularly carry out this type of verification starting from the final label of a product to test the robustness of a company's traceability system. In addition, in the most recent widely used quality standards (BRC, IFS, FSSC 2200) this type of check is part of the annual audit process.

1.4.4 Electronic logbooks

They can significantly reduce the reduction of data transcription errors and facilitate the reduction of transmission times by speeding up transmission between vessels and port authorities and customers. Some are equipped with the possibility of being connected to sensors placed on fishing nets that transmit data regarding the depth of fishing and the correct deployment or directly monitor the storage temperatures of the cells used to receive the catch after capture, preventing food safety problems.

1.4.5 Electronic monitoring

In the context of the eco-sustainability of fishery products and the activities through which these products are made available to the market, it is important to ensure the proper conduct of production activities. Over the years, this activity has been carried out through independent observers who, by embarking on the ships, guaranteed what was requested. Monitoring programs are extremely expensive, sometimes dangerous for the observer, and the number of vessels monitored is limited by the fact that few properly trained observers are available. An effective alternative can be, for example, monitoring through electronic cameras that turn on automatically when the nets are set sail, allowing, through the viewing of videos, the identification of captured species and assessing the impact of endangered species captured by catch.

1.4.6 Traceability Software Solutions

As we have learned in this chapter, the best solution for the transmission of traceability data is electronic transmission, which allows for rapid use and minimizes errors. Most of the players in the seafood supply chain use this form of transmission, through the use of scanners for reading barcodes, tags and radio frequencies. Paper transcription is increasingly relegated to artisanal fishing.

CHAPTER 2 The role of the Research &Innovation in solving the seafood market issues

Introduction

Fish products, as discussed in the previous chapter, are among the food products those that, due to their nature and their transformation into complex foods, are those most subject to microbiological risks, the presence of chemical contaminants, drug residues, commercial frauds such as species substitution, fraud regarding their tax status (fresh vs. frozen), fraud or inaccuracies related to traceability and consequently to their labelling. The modern fishing industry requires tools that are accurate, portable, easy to use and have a quick response. For this reason, the research industry and universities must provide this technology at a ready and low cost. The control of food safety and the truthfulness of what is expressed on the labels must be maintained at high levels in order to continue to generate trust among consumers, who have the right to enjoy such a noble food from a nutritional point of view and with nutraceutical properties. The concepts of "Health" and "Eco-sustainable" and "Italian product" must therefore be supported by reliable data. We have previously seen in this work the positive trend that the consumption of fish products is taking (6th place among EU countries), therefore it is important to generate consumer confidence in the Italian product. Our fisheries and aquaculture provide, among other things, fish products that make use of quality marks such as PDO and PGI: these labels enrich the commercial value of the products as they highlight how traditional farming techniques and geographical origin can exert a conditioning and peculiar factor on the quality of food products. This factor is reflected in the organoleptic characteristics (color, smell, texture and flavor) of the final product. These particular characteristics make them products of Italian excellence.

The **DOP mark** indicates the Protected Designation of Origin and is applied to agri-food products whose quality characteristics originate partially or totally from the geographical environment in which they are produced. The natural factors (climate, soil, water, etc.) that characterize a territory and the human factors (e.g. the production and processing techniques typical of a delimited area) influence and characterize the finished food product. An essential condition is that the production cycle must be carried out in the territory itself.

The **IGP mark** defines the Protected Geographical Indication with which agri-food products that have specific qualities and/or characteristics deriving from their geographical origin are identified. At least one of the production phases must take place in a defined geographical area, but not necessarily all of them, as is the case for PDOs.

Regulation (EU) No. 1151/2012 defines that PDO means "*The name of a region, a specific place*

or a country which serves to designate an agricultural product or foodstuff originating in that region, place or country and the qualities or characteristics of which are essentially or exclusively due to the geographical environment, including natural and human factors, and the production of which, transformation and elaboration take place in the demarcated area". PGI means "*The name of a region, a specific place or a country which serves to designate an agricultural product or foodstuff originating in that region, place or country and of which a particular quality, reputation or other characteristic can be attributed to the geographical origin and the production and/or processing and/or elaboration of which takes place in the specified geographical area*". Thus, PGI foodstuffs are defined as "*The name of a region, a specific place or a country which serves to designate an agricultural product or foodstuff originating in that region, place or country and of which a particular quality, reputation or other characteristic can be attributed to its origin*

When we talk about PDO and PGI, we traditionally think of products such as cured meats, cheeses, fruits, vegetables, pasta and bakery products: fish products are never contemplated. Yet there are also ***DOP and IGP*** fish.

In Italy, about twelve hundred different fish species are marketed for a number of references that exceeds ten thousand, in the face of such a considerable volume of products, there are only six PDOs and PGIs of fish products out of a total of 316 PDO and PGI certified food products.

1. Golden humped tench of the Pianalto di Poirino

2. Mussel of Scardovari

3. Anchovy sauce from Cetara

4. Salted anchovies from the Ligurian Sea

5. Arctic char from Trentino

6. Trout from Trentino

DOP products

Tench Golden Humpback of Pianalto di Poirino It was the first Italian PDO fish (2008). Bred in the Pianalto di Poirino area between the provinces of Turin, Asti and Cuneo. Tench (*Tinca tinca*) is characterized by delicate, firm and non-fatty meat with a pinkish white color and the delicate and less incisive taste of sea fish. The ideal time for consumption is their second year of life.

Mussel of Scardovari Obtained in 2013 the **Mussel of Scardovari**, whose production is limited to the homonymous Sacca and in the territories of the hamlets of Cà Mello and Santa Giulia in the municipality of Porto Tolle in Veneto. With rounded valves of 6-8 centimeters in length, it has a meat that is always more than 25% of the total weight and, thanks to its low sodium content, a particularly delicate flavor.

Anchovy sauce from Cetara PDO obtained in (2020) which concerns the sea area in front of the province of Salerno, in Campania, at a maximum distance of 12 miles from the coast. All processing steps of this product must take place exclusively in the province of Salerno: marketed in clear transparent glass containers, they must have indicated, if practiced, the aging on the label with the relative period and the mention "aged". Their goodness also derives from the great ability to fill the wooden barrels used for maturation thanks to the arrangement of the anchovies in neat layers covered with salt.

IGP products

Salted anchovies of the Ligurian Sea: the fishing area includes the entire area of the Ligurian coast. Between 10 and 20 cm in size, thin skin, dry meats, lean meats with a savory taste. It takes two months of salting to fully appreciate its taste. The anchovies (*Engraulis encrasicolus*) of the Ligurian Sea fully express the concept of PGI and the importance of the territory, waters with a low temperature range that translates into the presence of few fats that also have a thermoregulatory action and great salinity of the waters that give a savory flavor further enhanced by the oldest technique of preservation of fish products; Salting

Trentino trout (2013) produced in the autonomous province of Trento and in the municipality of Bagolino in the province of Brescia, Lombardy. White or salmon meats are compact, lean and tender with a mild flavor. Fat content never exceeds 6% and a body index defined between
1.25 and 1.35 grams depending on whether they are less or greater than half a kilo.

Arctic char from Trentino (2013) produced throughout the autonomous province **of Trento** and, as for trout, also in the municipality of **Bagolino**. Its characteristics (index of full bodied, the low-fat, low-fat content and delicate and unmistakable taste) are attributable to the production area and in particular to the peculiarities of the water in which it is bred which, coming from glaciers and perennial snowfields, has high oxygenation, good bio-chemical qualities and low average temperatures.

This type of certification is legislated like other private labels (e.g. fished in Italy, weighed in the Tyrrhenian Sea, etc.) These are just a few examples of clear and easily understandable communication by the end consumer to enhance the national product.

As we have seen in chapter 1, of the five species most consumed by Italians, four are present as species fished and bred in our territory. Creating consumer confidence in the purchase of domestic products means concretely helping the national economy by increasing GDP. Currently, in a period of economic contraction due to the COVID 19 pandemic, we are witnessing customer purchases of products at low prices but with increasing attention to the origin of the products. One of the most frequently asked questions when buying fish by the customer to the department manager is: Is it a domestic product? Is this fish farmed in Italy? This means that the end consumer is increasingly sensitive to factors such as product quality, origin, and eco- sustainability.

Comparing an Italian product also means having a product with a short commercial supply chain that is therefore fresher, with more available organoleptic and nutraceutical characteristics (Omega 3 and Omega 6).

Buying Italian products offers the opportunity to discover or re-evaluate fish products of our tradition. Our food and wine tradition is part of our national culture deriving from 20 centuries of history, already the Romans seasoned their food with "the Garum" a condiment obtained from the fermentation of marine fish such as sardines and anchovies (a probable progenitor of the Colatura di Alici di Cetara). The use of fish products in our cuisine has made it possible, for example, through the use of salted fish products such as anchovies or other similar fish products (sardines, sprats) to consume products with a high biological value of proteins, lipids and vitamins to populations living within the Peninsula who suffered from food shortages, favouring trade and therefore economic development.

The use of analytical tools in order to guarantee food safety, origin and therefore the typicality of our products is the means to ensure the continuous maintenance and development of our food excellence. A precious economic resource that, if properly managed, would promote the economic recovery of our nation.

The following is a set of techniques that support the above. In particular, the work of my PhD performed on the relationship between species identification and geolocation of the fish species under consideration expresses the need to identify not only the species but also the range of distribution of the species. The choice of a cosmopolitan species such as the Red Shrimp (*Aristeomorpha foliacea*) makes this type of approach close to what really happens in the fish market. Same species, with different origin and with different economic value. In this case, one could only think of an economic one in the case of fraud, but there could be risks related to microbiological contamination due to incorrect handling of the fish, which is culturally treated differently in one fishing area than another. Or fishing areas with different risks of pollution from anthropogenic environmental contaminants.

2.1 Application of electronic nose in quality assurance

In light of the increase in the consumption of fish products, both the fishing industry and large- scale distribution show the need to develop innovative rapid and resistant monitoring systems to measure the freshness and organoleptic characteristics of food raw materials, including fishery products. Starting from the knowledge of the biochemical phenomena that occur post- mortem, the most recent studies have turned to the development of this type of tools. The freshness parameters of fish products. Freshness derives from the measurement of a series of parameters that can be evaluated through various indicators that are in turn influenced by different biological factors, specific to production processes, climatic factors that intervene on the physical, chemical, biochemical and microbiological changes that occur post-mortem in fish. An estimated measurement of freshness can be obtained by defining criteria, quantifiable through instrumental and sensory methods, related to changes in organoleptic attributes that currently remain the most used such as the olfactory evaluation of aromas deriving from catabolism, lipid protein and sugars. Therefore, the profile of volatile organic compounds (VOC's) is decisive for characterizing and consequently assessing the conservation status of fish. One of the tools created for the evaluation of the aromatic status of fish products is the electronic nose, which is able to detect the concentration of these aromas and to correlate it through statistical software to other investigation techniques (microbiological and chemical) to the degree of freshness of the product. Conceived and developed by Persaud and Dodd in 1982 (Persaud & Dodd, 1982), the electronic nose has been defined as "an instrument, which includes a series of electrochemical sensors with partial specificity and an appropriate pattern-recognition system, capable of recognizing simple or complex odors". Multisensory technology was created in the 60s when designing the first instruments to act as simulators of human olfactory abilities (Moncrieff, 1961). The development has continued to this day evolving with the production of benchtop E. nose (90s), portable (2000s), the evolution has undergone further growth with the birth of instruments that exploit nanoparticle sensors with greater sensitivity and selectivity (Suzuki *et al.*, 2017). Electrochemical sensors convert volatile compounds present in the studied matrices into electronic signals. The data are recorded and processed through the use of statistical techniques, currently the main need is given by the analysis of matrices with greater complexity, optimizing the diversification of the types of sensors by improving their operating mechanisms, increasing the mastery in the use of data mining and pattern recognition. This evolution has produced a variety of instruments with numerous applications especially in the food industry. Figure 2 shows a typical example of how an electronic nose is structured, practical, manageable ideal as a dual functionality instrument, both in the laboratory and "in the field".

2.2 Micronir application to detecting the histamine value in seafood product

As we have already mentioned in this thesis, the food industry and in particular those that process products with rapid qualitative decay and therefore that can put human health at risk requires analytical tools that give rapid and precise answers. In the fishing sector, one of the analyses carried out during self-control following the dictates of H. A.C.C.P is the determination of histamine. According to EU Reg 2073/2004, the determination of the histamine content in sensitive species is performed in an accredited laboratory with HPLC methodology (References: 1) Malle P., Valle M., Bouquelet S. Assay of biogenic amines involved in fish decomposition. J. AOAC Internat. 1996, 79, 43-49. 2) Duflos G., Dervin C., Malle P., Bouquelet S., Relevance of matrix effect in determination of biogenic amines in plaice (Pleuronectes platessa) and whiting (*Merlangius merlangus)* J. AOAC Internat. 1999, 82, 1097-1101.). For practical reasons, this method can be combined with a rapid methodology through the application of rapid analytical kits that exploit the ELISA (Enzyme-Linked Immunosorbent Assay) technology. The methodical operate on the basis of competition between the horseradish peroxidase (HRP) enzyme conjugate and the analyte in the sample for a limited number of specific binding sites on the precoated microplate. The fast method allows the monitoring of the goods delivered by the supplier. The running time including sampling time is about one hour. This time frame is reasonably fast in the industrial production environment, the kit has a reasonable economic cost and after training the staff can easily perform it. However, sampling has the disadvantage of being destructive to the samples to analyzer, as it is necessary to take a sample of about 50/100 g from each sample. An alternative method that is just as quick but non-destructive is the use of technology named Nir (Near-infrared spectroscopy). This type of analytical approach s based on identification and quantification of several organic molecules from vibrational absorption at specific frequencies. The method allows, for example, to find molecules in quantities expressed in ppm. However, a limitation of the method is that these concentrations are higher than 0.1 ppm, recent studies have shown that this drawback can be avoided by coupling the NIR with pretreatment spectra and selecting the wavelength with chemometric methods (12). NIR technology has been developed and adapted to be reduced to an easily transportable size, creating instruments called the MicroNIR OnSite. This instrument, through a WI-FI connection, interacts with software to transmit, process data and create calibration curves and certificates of analysis.

The two methods described at the beginning of the chapter have the advantage of being fast, easily transportable and carried out by easily trained personnel. However, as shown in Table 2.2.1, there are small but significant differences that may lead to the choice of the two different analytical methods.

2.3 Differentiation of fresh and frozen-thawed fish

The fraudulent sale of frozen fish products is not only a commercial fraud but can pose a serious health risk. As already mentioned, it is right to reiterate that labelling and traceability must clearly indicate whether the product has been frozen, (FAO 1982; JAS 2000). These standards have been transposed and become regulatory requirements in many countries.

Freezing is the main process in use to extend the shelf life of fish by inhibiting microbial growth and reducing biochemical and physical deterioration. On the other hand, it is important to remember that freezing can alter the quality of products, creating physical and chemical changes in protein structures, worsening their structure and consequently their nutritional properties. From the point of view of lipids, there are oxidation phenomena during long-term storage which can lead to organoleptic deterioration (yellowish discoloration and development of rancid odor and taste). In the case of correct freezing, thawing and compliance with storage times that vary from species to species, these phenomena are safely avoided. From the consumer's point of view, frozen fish has the advantage of a cheaper purchase price and easier product management, however, the choice of fish Fresh food is always dictated by health factors to the detriment of the higher price (I eat less but I eat better). The fraud of substituting fresh with frozen is not always easy to spot as the chemical and physical characteristics are very similar. The following is a list of the methods used.

Examination of the opacity of the lens of the eye, the medullary part of the lens becomes opaque during freezing and is transparent in the fresh one (Love*,* 1956), but Yoshioka and Kitamikado (1983) have shown that this change did not occur in all species.

Measurement of electrical resistance based on the measurement of electrical resistance that decreases with damage to cell walls (Duflos *et al.,* 2002; Sakaguchi *et al.,* 1989). good results on fresh whole fish, less so on fillets and saltfish due to the high salt content (Rehbein *et al.,* 1992; Okazaki *et al.*, 2003)., and the freezing method (slow vs rapid) also affects the results.

Determination of hematocrit value and erythrocyte examination. Both methods are based on changes in red blood cells as a result of freezing and thawing. Difficult to apply on bled fish and fillets with little dark muscle (Gadidae, Merluccidae, etc.).

Enzymatic methods exploit the release of enzymes following the destruction of cells and their organelles by freezing (Rehbein *et al.,* 1979; Uddin et al., *2004) , (Rehbein* et al., 1979; Yuan *et al.,* 1988). The techniques have been extensively studied, but it is necessary to expand the number of species and especially of genera to be evaluated.

Spectroscopic analysis represents a very powerful method of investigation of the structure of matter, based on the analysis of the decomposition of the light emitted by matter into its fundamental wavelengths. The decomposition of light into component wavelengths, obtained by passing through a dispersive element such as a prism or a diffraction grating, constitutes the spectrum, i.e. the fingerprint of the light source, which unequivocally identifies its chemical nature and the physical conditions in which it is found. Some NADH spectra gave good discriminating results between fresh and frozen-thawed fish compared to other spectra (tryptophan). Therefore, they can be considered as a promising probe for reliable differentiation between frozen-thawed fish

Nuclear magnetic resonance (NMR) is a technique for investigating matter based on the measurement of the precession of the spin of protons or other nuclei with a magnetic moment when they are subjected to a magnetic field. The variations in NMR parameters are in agreement with histological observations and allow the characterization of structural changes in tissue induced by the freezing process. The NMR technique has shown that most damage occurs at the time of freezing.

NIR microscopy NIR spectroscopy is a physical analytical method based on the absorption of electromagnetic radiation characterized in the near-infrared zone by wave numbers between 12800 and 4000 cm-1 (780-2500 nm). The analytical signal obtained depends on the chemical- physical properties of the sample that is hit by incident radiation during the analysis, which can be absorbed, partly transmitted and partly reflected. As a result, NIR spectroscopy is a very effective analytical technique for determining numerous product properties simultaneously in a short time.

CHAPTER 3 Overview of the State of the Art of Ciguatera Fish Poisoning

3.1 Introduction

Ciguatera fish poisoning (CFP) is a frequent seafood borneillness caused by the bioaccumulation of lipophilic ciguatoxins (CTX) in coral reef fish and invertebrates, and their subsequent consumption by humans. These phycotoxins are produced by tropical epiphytic dinoflagellates belonging to the genera Gambierdiscus and Fukuyoa, which live on a variety of macrophytes, as well as on dead corals and sand. The disease is of significant concern in many tropical areas where it has been known since centuries. Although mortality from CFP is low, morbidity is high, and symptoms may be debilitating and prolonged. Ciguatera produces characteristic gastrointestinal, neurological, and to a lesser extent, cardiovascular symptoms. Though the symptoms are relatively well documented, the disease often goes unreported or misdiagnosed.

. The toxins are ingested by and accumulate in herbivorous fishes that, when consumed by humans, ultimately cause CFP. CTX has also been found in farmed fish fed with fishmeal obtained from ciguatoxin intoxicated fish. The first report of CFP dates back to the 16th century (1555) by historian Peter Martyr de Anghera, who described the phenome-non in the Indian and Pacific Ocean area. In the Pacific area, cigu-atera intoxication in Spanish sailors was also reported as early as 1606 . However, the best-known case dates back to the 18th century (1774) and involved the crew of Captain Cook's vessel, who became intoxicated after eating fish meat belonging to the Lutja-nidae family. The term "Ciguatera" is of Iberian origin and derives from the name used in the 18th century to indicate intoxication caused by the ingestion of the marine mollusc *Livona pica*, known in the Spanish Antilles with the Cuban name of "Cigua" which means snail. Being phytophagous, this species can accumulate toxins in its meat and transfer them.

along the food chain [9]. The onset of the disease occurs after the consumption of fish caught between 35° north latitude and 36° south latitude, although minor cases have been described after the consumption of seafood fished even outside these coordinates. In 2008, cases were described in the Iberian Peninsula both in the Mediterranean and in the Canary Islands and in the Azores and Madeira [10]. Large fish, once poisoned, can remain poisonous for years, as has been shown in some experiments. The size of the fish is linked to the risk of intoxication because they have the possibility of accumulating the toxin. Contaminated fish weighing more than 5 - 6 kg are considered dangerous. The species of fish involved in CFP are more than 400. Generally, only a few of them are regularly involved in CFP. The most common species are: Carcharhinus leucas (Carcharhinidae), Lutjanus sp. (Lutjanidae), Gymnothorax spp. (Muraenidae), Seriola fasciata (Carangidae), Chlorurus microrhinos (Scaridae), Epinephelus mer-ra (Serranidae), Epinephelus multinotatus (Serranidae), Plectropo-mus leopardus (Serranidae), Variola louti (Serranidae), Epinephelus spilotoceps, (Serranidae), Pagrus pagrus (Sparidae), Balistes vetula(Balistidae), Balistapus undulatus (Balistidae) Sphyraena barra-cuda (Sphyraenidae), Scarus altipinnis (Scaridae), Kyphosus ciner-ascens (Kyphosidae), Liza vaigiensis (Mugilidae), Pterois volitans (Scorpaenidae), Scomberomorus commerson (Scombridae).Also invertebrate species are known to be involved in CFP, in-cluding Tridacna maxima, Tectus niloticus, Octopus cyanea (bigblue octopus), Percnon spp. (nimblespray crab), Dendropoma max-ima, Panulirus penicillatus (lobster), Tripneustes esculentus (sea ur-chin), Ophidia sterophidianus, Marthasterias glacialis, Livona pica.

DistributionThe CFP is specific to tropical regions, from three great oceans: Pacific Ocean (French Polynesia, New Caledonia, Australia, Vanua-tu, Hawaii, Japan), Atlantic Ocean and Indian Ocean. It is also foundn Florida, and recently (2003) it was founded in the Mediterranean Balearian islands, Greek coast (Crete), Atlantic Ocean especially in Madera and the Canary [10]. Within the EU, only countries without almost regions, located in the tropics and very far from the European continent, have been exposed to ciguatera, in particular, Portugal (Azores and Madeira), France (Guadeloupe, Saint Martin, Global warming of the ocean surface is expected by the middle of the 21st century with an increase in temperatures of 0.4 - 1.4°C. causing the increase in the spread of many dinoflagellate genera of tropical and subtropical origin which are harmful to health such as Gambierdiscus, Fukuyoa and Ostreopsis. An exception to this trend will occur in the tropical area where the exceeding of the thermal tolerances will bring the water to a temperature of around 30 -31°C. Above these temperatures, growth and diffusion will suffer a significant slowdown.

Ciguatoxin intoxication had not been described in the West Africa coast until (2004). In 2008 and 2009 two episodes were de-scribed in Canary Island. At the end of the 80' (1989) cases of ciguatera intoxication were reported in Israel [14]. Mechanism of action of toxins The dinoflagellates are widespread on macrophytes and dead corals and are ingested by herbivorous fish or gastropods or crustaceans that accumulate the toxin in their body becoming toxic, these phytophagous or detritivores animals can be consumed by humans who become intoxicated or be preyed upon by carnivorous fish, which in turn become toxic. By eating carnivorous fish, man becomes intoxicated. A mechanism of accumulation of toxins is known which reach toxic levels for humans in fish weighing more than 1.5 kg. The local health authorities of Pacific islands recommend above all to tourists but also to indigenous populations not to consume fish larger than 1.5 kg. The first works on CFP starting in 1958, (1963) , and. (1971,1976). They made a correlation from disc-shaped dinoflagellates in stomach of fish-es (i.e., Surgeonfish *Ctenochaetus striatus*) contents with toxins from viscera extracts. The collection of Dinoflagellates found on calcareous algae and dead corals at the Gambier.

Islands and the subsequent extraction of compounds produced chemical and toxicological similar fractions with those extracted from ciguatoxic fish. The laboratory cultivation of Dinoflagellate samples collected in nature allowed to confirm the benthic bond with ciguatox- ins [17]. Dinoflagellate belonging to the Phyrophyta division has been classified with new or genus and species, Gambierdiscus toxicus. After this discovery, new species Gambierdiscus belizeanus, Gambierdiscus yasumotoi, Gambierdiscus pacificus, Gambierdiscus australes and Gambierdiscus polynesiensis were further found and added to the Genus.

However, a work taking a cue from detailed sequencing of rDNA suggests the hypothesis of a single cosmopolitan species of Gambierdiscus tossicus. Genetic clustering suggests that a wide- ranging complex of multiple cryptic species exists" [18]. Respect from the better known open- water "red-tide" that form big face blooms, Gambierdiscus spp. are often found in sparse population only in particular conditions (i.e. protected areas) form dense populations. Exist a correlation from toxin productions and Genus". CTX phycotoxins are lipophilic neurotoxins, polyethers having a molecular weight of 1110 Da, characterized by 10 to 14 cycles transfused by ether bonds. Exist a regional difference in ciguatera toxin profiles associated with fish and the symptoms reported in outbreaks in the three major ciguatera-endemic regions, (Caribbean Sea, Pacifica and Indian Oceans)". They are divided into three families according to their origin: "The P-Ctxs (Pacific Ocean), C-CTX (Caribbean) and I-CTX (Indian Ocean). Toxins accumulate through the trophic chain, starting from the algae that are con summed by herbivorous fish, carnivorous fish, before reaching humans. More than 400 species have been considered as carriers of these toxins responsible for Ciguatera (CFP). The mechanism of action is the same as that of brevotoxins, binding to the S5 site on the Cnavd (Kd- 0.04-

4 nM). This powerful bond between the toxin and the channel exerts a powerful and direct action on the neuromuscular junctions, a depolarization is induced on the sensory neuronal membranes by inducing the selective opening of the sodium channels in tension dependent on normal resting potential. Symptoms appear within 10 - 30 minutes of ingestion. The syndrome is characterized by several symptoms in combination each other acting mainly at the gastrointestinal level, neurological and cardiovascular. Gastrointestinal disorders usually subside and disappear in 1 - 2 days, like cardiovascular ones which subside in 2 - 5 days. The most persistent seem to be the neurological and sensory ones which act for 2 - 3 weeks. Fatal outcome (death) can occur from direct cardiovascular collapse, hypovolemic shock, or respiratory paralysis. The most typical and tell-tale symptom of the syndrome appears to be the temperature inversion, in which the patient mistakes heat for cold. Human tests involving the biopsy of nerve tissues revealed swelling of Schwann cells with axonal compression and vesicular degeneration of the myelin. The toxin is sexually transmitted, significantly harming the foetus with an often-fatal outcome (abortion) [12]. Another toxin produced by Gambierdiscus dinoflagellate is maitotoxin (MTx) in addition to ciguatoxin is the most common toxin involved in ciguatera fish poisoning (CFP), Maitotoxin (MTx) is one of the potent polyether toxins produced by the dinoflagellate Gambierdiscus toxicus and, besides ciguatoxin, is the most common toxin involved in ciguatera fish poisoning (CFP). There is little evidence that maitotoxin can cause behavioral and morphological changes maitotoxin in fish, and its accumulation in the flesh of surgeonfish or other fish, can result in its death. Despite the widespread occurrence of maitotoxins in the benthos (at the bottom of the sea), maitotoxins are unlikely to cause human poisoning because of their poor accumulation might impose an up-per limit on the levels they can in fish flesh and their relatively low ral potency. Actually, the diagnosis of CFP is based on clinical sign and the recent eating history of the patients. This is due to the fact that don't exist human biomarker for CFP. The main CFP 'symptoms are associated with cardiovascular, gastrointestinal neurological and neuropsychiatric symptoms and signs. Cardiovascular symptoms include: arrhythmia, hypertension, bradycardia emerged in the early phases and when include hypotension and bradycardia may necessitate urgent medical care. Gastrointestinal symptoms include: diarrhoea, nausea, vomiting, abdominal pain. These symptoms usually occur after 6/24 hours fish ingestion and often resolve spontaneously after 1-4 days. Neurological symptoms include: Extremity Paraesthesia, circumoral paraesthesia, tempera-ture, Dysesthesia, Myalgia, Arthralgia, Pruritis, Headache, Vertigo, Weakness (Asthenia), Dental Pain/Feeling like teeth are loose or falling out, Dysuria, Chills/Sweating. They are present within the first few days of illness, after the GI symptoms, particularly in CFP from fish came from Caribbean waters. Exist a neurologic symp-toms variation among patients including

paraesthesia (numbness and tingling) in the extremities of body (feet and hands) and oral region, generalized pruritis (itching), myalgia (muscle pain), ar-thralgia (joint pain), and fatigue. A symptom often reported by many patients is an alteration or "reversal" of hot/cold temperature perception, in which cold surfaces are perceived as hot to the patient, or produce dysesthesia (unpleasant, abnormal sensation). This temperature-related dysesthesia is considered characteristicf CFP, although not all patients report experiencing this symptom.Temperature-related dysesthesia has been reported even in other intoxication from seafood (e.g. Neurotoxic Shellfish Poisoning (NSP). Neuropsychiatric symptoms include depression and memory loss, anxiety, hallucination and giddiness, incoordination or ataxia, coma, these last especially appear to be specific to CFP in Indian and Pacific Ocean. These geographical differences in symp-tom patterns may be attributable to the presence of different suitesof CTX or toxin precursors in these different areas. Gastrointestinal symptoms are predominating in the acute phase (first 12 hours), in the Caribbean, followed by prominence of neurologic, especially peripheral neurologic symptoms. The neurological symptoms and signs predominate in the Pacific area with severe neurologic effects including coma. In the Indian Ocean, CFP is always associated with mental status alterations, neurological disturbs like depression, hallucinations, giddiness, incoordination, loss of equilibrium.

CFP is rarely reported as fatal itself. Death may occur in some cases due to severe and fast symptoms like cardiovascular shock especially in the early initial illness phase, severe dehydration, respiratory failure from paralysis of the respiratory musculature. Last but not least, the environmental factor also contributes to the fatal outcome, due to the lack of supportive health machinery (respiratory), rehydrating solutions, specific medications. Even the parts of toxic fish consumed can contribute to the fatal outcome of intoxication. Viscera and gonads usually contain more toxins than muscles (fillets). Cases of chronicity was recorded, after the acute illness some patients suffer from weakness generally last a few days to several weeks or months. Other neurologic symptoms such as paraesthesia in the extremities, pruritis, and neuropsychiatric symptoms such as malaise, depression, generalized fatigue, headaches. Symptom recurrence and sensitization [20]. The persistence of nervous symptoms (movements a snap, gaze), for irreversible nerve damage. Due to widespread in the Caribbean area. The chronic form of Ciguatera in that area it is believed to occur in part inspired, the popular traditions on the "living dead" (Zombie). Recently also bacteria (genus Pseudomonas, Vibrio, Aeromonas) living in symbiosis withmicroalgae have been considered possible toxinogens. Despite that Ciguatera intoxication is the world most prevalent foodborne disease, with 50,000 to 500,000 incidences per year, themortality rate remains very rare with deaths estimated at < 0.1%.

The popularity for tourism reasons of tropical and sub-tropical regions with seafoods' exportation, make this disease a global health problem. Actually, no antidote is available. Mannitol' treatment re mains symptomatic but is the most effective remedy that leads to a regression of symptoms)]. Even the local medicine (Polynesian area) based on plant extract (*Heliotropium foertherianum*) (Boraginaceae) and rosmarinic acid can be have good effects. Actually, no valid biomarkers exist to confirm exposure to CTX in humans. The current CFP diagnosis is based on history to eat-en reef fish and importantly", the exclusion of other diagnoses that could have common symptoms with CFP, like Paralytic and Neurotoxic Shellfish Poisonings, scombroid and pufferfish toxicity, botulism, enterovirus, and bacteraemia, as well as organophosphate pesticide poisoning, eosinophilic meningitis, multiple sclerosis, nd other neurologic conditions". "Several methods have been developed to screen for the presence of CTXs in fish tissue prior to consumption. In vivo whole-ani mal detection methods are now superseded by in vitro assays that have greater sensitivity, including receptor-binding assays (RBAs), cell-based assays (CBAs), Enzyme-Linked Immunosorbent Assays (ELISA), capillary electrophoresis (CE)-based immunoassays, electrochemical immunosensors (ECS), and liquid chromatographytandem mass spectrom etry (LC-MS/MS). Present methods for CTX analysis, in general, are labour-intensive, time- consuming, and require laboratory facilities with well-trained technicians. To date, these methods have not been properly validated. At present it is difficult to obtain strong standard CTXs as reference calibrants, impeding corroboration and widespread application of these an- alytical techniques [24]. Other diagnostic methods include the cultivation and harvest-ing of CTX in the phase of maximum development, the extraction of toxins that are analysed with immunosensitivity tools with magnetic bead (MB) -based immunosensing tools (colorimetric immu-noassay and electrochemical immunosensor). This method is used for this diagnosis for the first time and allows to determine the presence of toxins and the screening between Gambierdiscus and Fukuyoa strains, but also the ability to discriminate between two series of congeners (CTX1B and CTX3C) [25]. Recently a portable electrochemical immunosensor for the detection of CTXs is pre-sented. It's based on a sandwich configuration type [26].

From November 2012 to January 2015, an EU-funded project called "Ciguatools" was developed which, through the creation of a consortium made up of experts in marine toxins, determined a search and identification of toxins and related profiles widespread in the Union's waters. European Union, consequent study and production of reference standards. Creation of a simple, fast method to determine the presence of CTX in the meat of marine animals tended for human consumption. The phases of the project included: Cultivation under controlled conditions of Gambeirdiscus, relative extraction of toxins from cultures, their purification, characterization as a standard for the toxin and subsequent use for the production of antibodies for an ELISA type kit and development of PCR techniques for the identification of toxins in the water column. With regard to the possible production of ELISA KITs, further investigations will be required.

Conclusion

The RASSF (Rapid Alert System for Food and Feed) site that col-lects all food and no food warnings, registered 5 warnings in the years from 2012 to 2020 (Table 1). The countries involved in this intoxication were France (2 cases) Germany (2 cases), Netherland (1 case). The origin of toxic fish products was India (3 cases) and Vietnam (2 cases) The species most involved was Lutjanus spp. and in particular Lutjanus bohar and Acanthocybium solandri. All five cases triggered a national health alert.

CHAPTER 4 Invasive Species Drive Human Poisoning: The Case of the Silver Cheeked Pufferfish Lagocephalus sceleratus

Introduction

Lessepsian migration is that phenomenon whereby species move from the Red Sea to The Mediterranean Sea through the Suez Canal. This human-made corridor, opened in 1869, enabled the unidirectional passage of entire Indo Pacific complexes among mollusks, crustaceans, and algae as well as more than 100 species of fish. This type of migration owes its name to the French engineer Ferdinand Marie de Lesseps who designed and supervised the building of the Suez Canal. Lessepsian species cause ecological and financial damage not only on marine life but also by affecting the health of the Mediterranean Sea and the number of these species continues to increase. A relevant impact is that of the Lessepsian poisonous fish species, included withithe following families: Tetraodontidae, Siganidae, Dasyatidae, Scorpanidae, Plotosidae, Ostracionidae, and Ariidae [2]. The Tetraodontidae family is composed of 130 species and 19 genera, mainly marine and estuarine [10]. This family is well represented by at least eleven species: Lagocephalus guntheri, Lagocephalusspadiceus, *Lagocephaluslagocephalus, Lagocephalus suezensis, Lagocephalus sceleratus, Torquigener flavimaculosus, Tylerius spinosissimus, Sphoeroides spengleri, Sphoeroides pachygaster, Sphoeroides marmoratus*, and *Ephippion guttiferum* . The species of main concern is *Lagocephalus sceleratus* (Gmelin, 1789) that is considered among the most dangerous Lessepsian species in terms of toxicological, ecological, and economic impacts. It has been blacklisted as one of the 18 worst invasive fish species by the International Union of Conservation of Nature (IUCN) [14]. The chronology of L. sceleratus invasion has been recently reviewed by Azzurro, et al. [15]. L. sceleratus was first recorded in the Mediterranean Sea in 2003 then in the southern central Mediterranean and later in Spain and France [18], recently reaching the westernmost end of the Mediterranean Sea .

It is the most invasive species of the genus Lagocephalusin the Mediterranean Sea, rapidly spreading in the eastern basin and currently moving westwards, confirming its adaptability to different environmental conditions. Furthermore, it is considered one of the most rapidly expanding invasive species due to its high growth and reproduction rate, absence of natural predators, ability to exploit food resources and to tolerate different environmental conditions [20,21].

Lagocephalus sceleratus is native to the Indo-Pacific region. It is 15-60 cm long and is characterized by two separate lateral lines, small spinules on the head and back that extend almost to the caudal fin and on the belly. It also has characteristic black spots of almost equal size on its back.

It usually lives at a depth of 10-50 m and feeds mainly on benthic invertebrates on sandy bottoms. When threatened, it is able to inflate its body by rapidly engulfing water or air [2,22].

L. sceleratus is known to carry high and variable concentrations of the dangerous tetrodotoxin (TTX) and is therefore considered one of the most poisonous fish in the world .

Tetrodotoxin also accumulates in other Mediterranean species including several marine gastropods, oysters, mussels, parrot fish, toads of the genus Atelopus, several species of blue- ringed octopus of the genus Hapalochlaena, several starfish, an angelfish, a polycladic flatworm, several species of arrow worms, several ribbon worms, and many species of Xanthidae crabs [6. However, the aspects listed above about L. sceleratus led us to the choice of the topic of this review. Indeed, given the increasing prevalence of this Lessepsian species and given its potential lethality we thought it would be useful to provide a review of the current state of the art regarding the presence of L. sceleratus in the Mediterranean Sea and the related emerging problem of food poisoning risks to European and Mediterranean consumers.

Tetrodotoxin in Lagocephalus sceleratus L. sceleratus toxin is tetrodotoxin (TTX) (Figure 1), a strong marine neurotoxin. It is a guanidine heterocyclic compound of low molecular weight (319.1 g/M), not thermolabile, colorless, odorless, tasteless, and soluble in water, discovered in 1909 by the Japanese researcher Yoshizumi Tahara from ovaries of globefish . TTX shows maximum concentrations in the liver and ovaries, followed by intestines, muscles, and skin with regional and temporal variations [30,31]. It is very dangerous to human health as it is responsible for paralysis of nerves and muscles, including the diaphragm and intercostal muscles, through selective blockade of voltage gated-sodium channels of the neuron cell membrane . The production of tetrodotoxin in pufferfish is not endogenous but is produced by strains of symbiotic marine bacteria species belonging mainly of the genera Vibrio, Pseudomonas and Pseudoalteromonas. In particular, the genus Vibrio is represented by about ten species and *V. alginolyticus* is the one most involved in the production of TTX. *L. sceleratus* use the toxin to defend themselves or as a weapon of predation and, as in other cases of animals or plants synthesizing toxins, show great resistance to the toxin itself through biological defense systems. The level of TTX is not evenly distributed throughout the body of *L. sceleratus* and is also influenced by sex and seasonality In *Lagocephalus sceleratus* the gonads generally have a TTX level of more than 1000 MU/g, while the intestine has an average TTX level of 100-1000 MU/g and liver, skin, and muscles have an average TTX level of 10-100 MU/g .

The spawning season is another factor influencing toxicity: it generally occurs between late spring and summer, a period when the TTX levels are high, while they tend to decrease in the fall and winter seasons. Other researchers, however, have not noticed such differences.

CHAPTER 5 Food traceability: diet analysis of *Aristaeomorpha foliacea* using DNA high-throughput sequencing (HTS)

Introduciton

The main objective of the project is the characterization of the diet of Aristaeomorpha foliacea (Risso, 1827) caught in two different geographical areas, in order to obtain useful information for food traceability and for the reconstruction of the trophic ecology of the species.

Aristaeomorpha foliacea (common name: giant red shrimp) is a deep-sea bento-pelagic shrimp with a wide geographical distribution in the world (Cau et al. 2002) and represents an important target of commercial fisheries. Due to the economic value of A. foliacea fished in the Mediterranean Sea (in particular, Mazara del Vallo), fraudulent substitutions are frequent with organisms of the same species fished in different geographical areas (e.g. the Indian Ocean) with a lower commercial value. The conventional food traceability techniques currently in use (e.g. DNA barcoding) do not allow the geographical origin of a food product to be verified with certainty. However, modern DNA sequencing techniques can be a powerful investigative tool, allowing to obtain detailed and large-scale information on the organism of interest (e.g. Urban et al. 2022; Siegenthaler et al. 2019).

Starting from the data already obtained thanks to the SeaTraceOmics project, funded under the Bicocca Starting Grants (University of Milano-Bicocca), the project aims to integrate the information on the microbiome and genotyping of A. foliacea with the information deriving from the analysis of the diet. To this end, 80 samples (already collected within the SeaTraceOmics project) from two geographical areas of interest (Mazara del Vallo area, Mediterranean Sea and Mozambique Channel, Indian Ocean) have been selected.

The project used the most modern DNA sequencing technologies (High-Throughput DNA Sequencing techniques, HTS), applying an innovative metabarcoding approach, in order to characterize the food preferences of the organism of interest. The data obtained were used to validate the usefulness of this information in the geographical traceability of the species of interest. Furthermore, new data on the trophic ecology of A. foliacea were provided.

Material and Methods

Mozambique samples were collected from commercial boxes for seafood use. The boxes showed some important information about the samples, such as taxonomic classification and declared size of the seafood, fishing geographical area and date of catch and freezing, which should coincide. We selected samples belonging to different lots. Mazara samples derived both from commercial boxes and from catches by local fishermen in georeferenced sites.

DNA Extraction.

The dissection procedure was performed in sterile conditions under a biological laminar flow cabinet. The frozen samples were thawed at room temperature until each individual internal organs could be removed separately. Entire gut (~0.25 g) was dissected from each shrimp, collected into separate 1.5 ml sterile microcentrifuge tubes and stored at -20 °C until DNA extraction. To avoid cross- contamination, the dissection tools were flame-sterilized with absolute ethanol between each individual dissection.

Gut DNA was extracted using the DNeasy® PowerSoil® Kit (Qiagen), which allows to isolate microbial genomic DNA from all soil types, as well as from other types of environmental samples, using Inhibitor Removal Technology (IRT). All DNA extraction steps were performed in sterile conditions under a biological laminar flow cabinet. The procedure was done following the manufacturers' instructions.

Molecular Marker Selection and Validation

Regarding the molecular marker selection, the most widely used in DNA metabarcoding studies are the nuclear 18S rRNA gene and the mitochondrial COI gene. COI gene shows advantages in terms of species-level resolution, mostly thanks to the higher genetic variability and availability of large reference databases, even if a debate about its suitability for DNA metabarcoding is still open. Several primer pairs are described in literature to amplify these two DNA regions, showing variable amplification success, also according to the target taxa. To select the most suitable primer pairs, we first investigated the possible diet of A. foliacea according to scientific literature. Then, we downloaded a selection of DNA sequences data based on the previous step. After, we performed an in-silico PCR to perform a screening of the primer pairs.

The primer pair which performed best was selected by calculating the species richness and the Shannon index for all the taxonomic ranks considered (Phylum, Class, Order, Family, Genus, and Species).

A Real-Time quantitative PCR (qPCR) amplification test was performed to validate the primer pair selected, verifying the amplification of the metazoan DNA extracted from gut and confirming the absence of reaction inhibitors. The instrument used was the Applied Biosystems 7500 Real-Time PCR System.

Library Preparation, High-Throughput DNA Sequencing, and Bioinformatic Analyses

The library preparation for HTS sequencing was performed following the protocol "16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System". A total of 80 samples were sequenced in one Illumina Miseq v2 2x250 bp run.

The raw paired-end FASTQ reads were imported into the Quantitative Insights Into Microbial Ecology 2 program (QIIME2, ver. 2020.8; Bolyen et al., 2019). Primers were trimmed and sequences were filtered at quality filter threshold = 35, with cutadapt plugin. Vsearch was used to merge, dereplicate, cluster and remove chimaeras and borderline. Feature-classifier (classify-consensu-vsearch) was used to classify reads. Taxonomy assignment was performed using COI DNA sequences downloaded from NCBI. In the first case, we downloaded a selection of the TAXID of Crustacea, Actinopterygii, Foraminifera, Mollusca, and Polychaeta groups, according to the scientific literature investigation reported in the previous section. In the second case, we included all the COI DNA sequences assigned to Metazoa.

In both cases, sequences assigned to Mitochondria, Chloroplast, and A. foliacea were removed. The results of the taxonomy assignment were analysed considering the percentage of rank assigned at different levels (Phylum, Class, Order, Family, Genus, Species).

Result and discussion

According to the reported literature survey about A. foliacea feeding habits, the molecular investigation focused on the following taxa: Crustacea, Actinopterygii, Foraminifera, Mollusca, and Polychaeta (Rainer 1992, Bello & Pipitone 2002, Chartosia et al. 2005, Kapiris et al. 2010, Kapiris 2012). Indeed, these taxa theoretically include most of the organisms identified as voluntary or involuntary preys of the giant red shrimp. Different primer pairs amplifying regions of the 18S rRNA and COI genes (Table 5.1) were tested in silico for the amplification of sequences of the selected potential prey taxa.

Table 5.1. List of markers and primers with information regarding target primer sequence, amplicon length and references.

Gene	Primer name	Primer sequence (5'-3')	Amplicon length (bp)	Reference
18S rRNA	1391F (F)	GTACACACCGCCCGTC	145	Lane 1991
	EukBR (R)	TGATCCTTCTGCAGGTTCACCTAC		Medlin et al. 1988, Edgcomb et al. 2011
COI	mlCOIintF (F)	GGWACWGGWTGAACWGTWTAYCCYCC	313	Leray et al. 2013
	jgHCO219 8 (R)	TAIACYTCIGGRTGICCRAARAAYCA		
	mlCOIintF-XT (F)	GGWACWRGWTGRACWITITAYCCYCC	313	Wangensteen et al. 2018
	jgHCO219 8 (R)	TAIACYTCIGGRTGICCRAARAAYCA		
	BF2 (F)	GCHCCHGAYATRGCHTTYCC	421	Elbrecht et al. 2017
	BR2 (R)	TCDGGRTGNCCRAARAAYCA		
	BF2 (F)	GCHCCHGAYATRGCHTTYCC	322	Elbrecht et al.

		2017
BR1 (R)	ARYATDGTRATDGCHCCDGC	

The best primer pair resulted to be BF2/BR1 (Elbrecht et al. 2017), showing the highest amplification performance for all selected taxa (Figure 5.1).

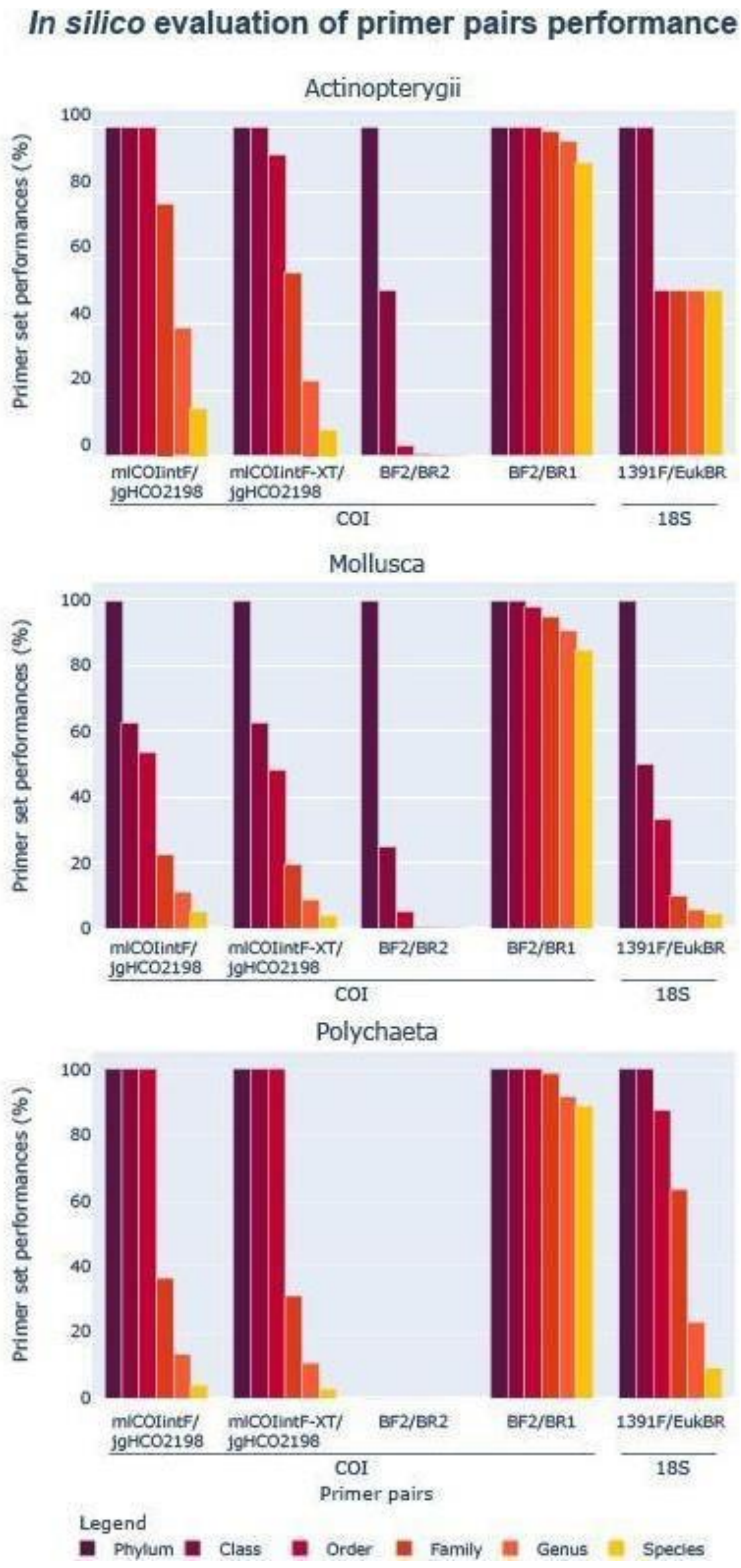


Figure 5.3.1. Amplification performance of the tested primer pairs for Actinopterygii, Mollusca, and Polychaeta groups. Different colours of the bar chart bars reflect the amplification rates at

each taxonomic rank, from the phylum to the species level, as shown in the legend. qPCR Validation A Real-Time quantitative PCR (qPCR) amplification test was performed to validate the primer pair BF2/BR1. All the 80 samples tested showed the amplification of the metazoan DNA extracted from the gut and confirmed the absence of reaction inhibitors.

Therefore, the primer pair BF2/BR1 was selected to perform the high throughput sequencing of the DNA extracted from A. foliacea guts in order to characterize its diet in the two different localities. DNA-Based Characterization of the Diet of A. foliacea

All 80 samples were successfully sequenced, obtaining a total of 11,955,975 forward + 11,955,975 reverse reads. After quality filtering, merging reads, clustering, and chimaera removal, a total of 9,727,190 COI reads were obtained, corresponding to 37,122 features.

Specifically, eight metazoan phyla were identified, here reported in descending order:: Arthropoda
Echinodermata
Chordata
Mollusca
Annelida
Cnidaria
Nemertea
Priapulida

Overall, samples from Mazara showed a higher abundance of unassigned reads, when compared to Mozambique samples. However, in both areas the vast majority of reads were assigned to unidentified arthropods belonging to the order Decapoda (Figure 2, 3). This confirms that crustaceans are a major food source for A. foliacea, as already shown in previous morphology-based studies (Rainer 1992, Bello & Pipitone 2002, Chartosia et al. 2005, Kapiris et al. 2010, Kapiris 2012).

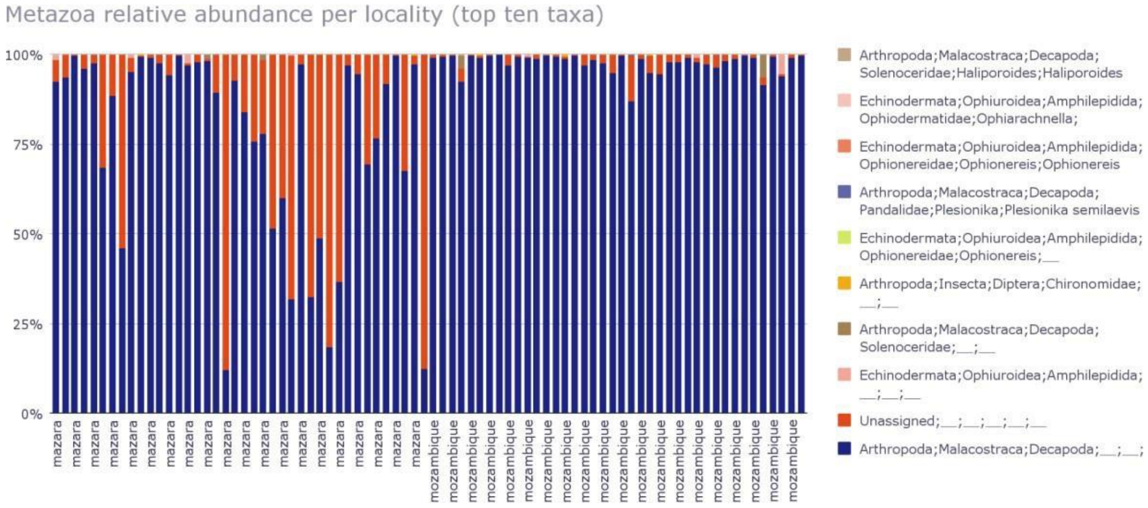


Figure 5.3.2. Relative abundance of the taxa recovered in the 80 samples through COI metabarcoding sequencing.

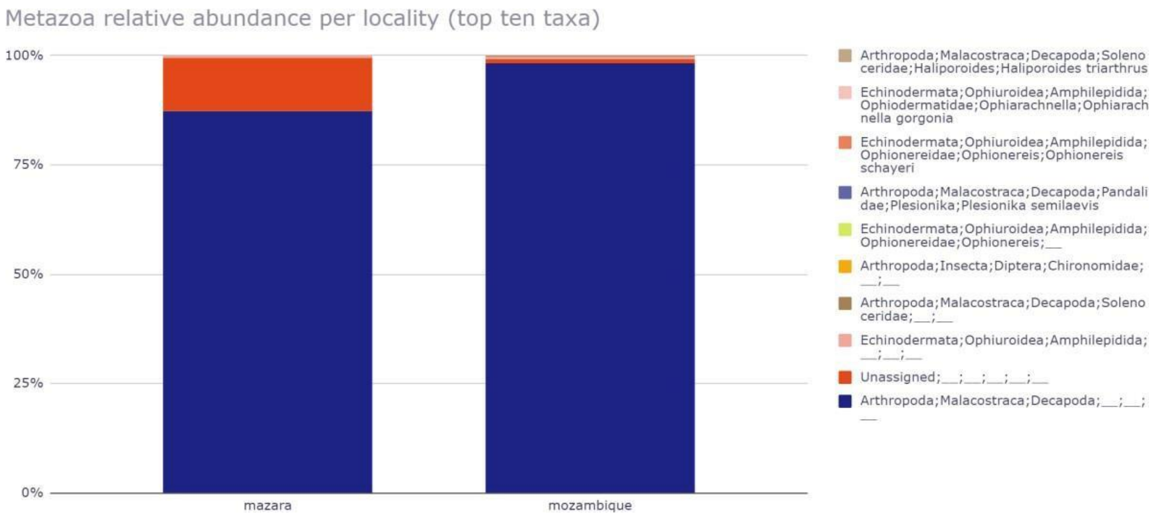


Figure 5.3.3. Per locality, aggregated relative abundance of the top ten taxa recovered in the 80 samples through COI metabarcoding sequencing.

To better observe differences in the A. foliacea diet composition between the two localities, the taxon 'Decapoda' was also removed from the dataset (Figure 4, 5). In this case, most of the reads remained unassigned and arthropods (including Decapoda taxa identified at a lower taxonomic level) were most present, followed by Echinodermata taxa, mostly represented by brittle star (Ophiuroidea) taxa.

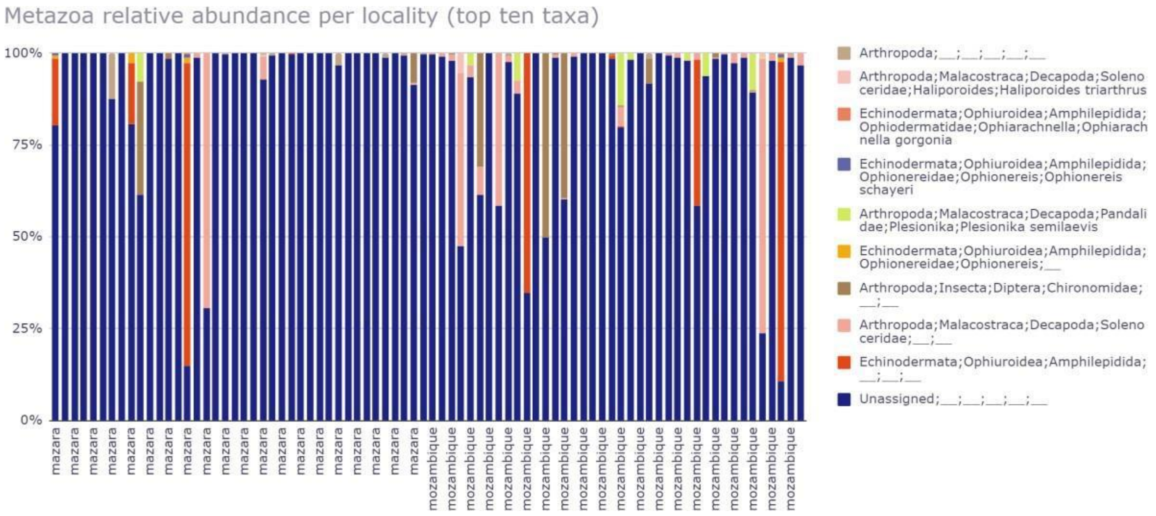


Figure 5.3.4. Relative abundance of the taxa recovered in the 80 samples through COI metabarcoding sequencing, after removing the category "Decapoda.

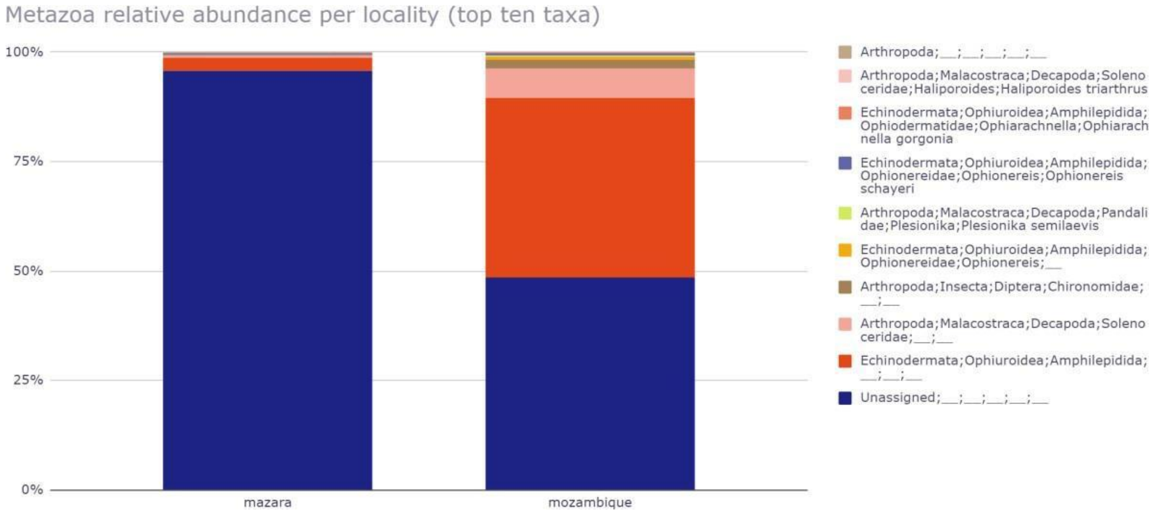


Figure 5.3.5. Per locality, average relative abundance of the top ten taxa recovered in the 80 samples through COI metabarcoding sequencing, after removing the category “Decapoda;_;_;_”.

All other taxa were represented by a few reads, confirming nevertheless the morphology-based identifications of A. foliacea preys consisting in molluscs, polychaetes, and fishes (Rainer 1992, Bello & Pipitone 2002, Charcosia et al. 2005, Kapiris et al. 2010, Kapiris 2012). Notably, some taxa previously unreported as prey item of A. foliacea were also recovered, even if they were represented by a few reads, namely, Anthozoa, Scyphozoa, Oligochaeta, Priapulida, and Nemertea, thus widening the knowledge about A. foliacea diet.

Foraminifera, previously reported as part of the A. foliacea diet (Rainer 1992, Bello & Pipitone 2002), were not found in our analyses, due to the fact that only a few, recently deposited COI sequences are available in public databases and that these sequences only overlap for about 200 bp with the sequences generated in our analysis (Macher et al. 2022).

According to our results, no clear differences could be detected between the two localities, suggesting similar feeding habits for A. foliacea inhabiting the Mediterranean Sea and the Indian Ocean. The impossibility of assigning the unidentified reads to any known taxon likely depends on the incompleteness of public databases of genetic data. Therefore, these reads may belong to species that are still to be genotyped, at least in the COI region, and may, in the future, add additional information on the trophic ecology of A. foliacea and reveal possible differences between the two localities.

Given our initial, in silico approach to find the best suitable primer pair to perform metabarcoding sequencing, we also used a dataset composed of the initial target taxa (Crustacea, Actinopterygii, Foraminifera, Mollusca, and Polychaeta) to perform the taxonomic assignment of the obtained reads. The obtained results are, as expected, similar to those obtained using the 'all Metazoa' dataset, with the exception that the Echinodermata, Cnidaria, Nemertea, and Priapulida phyla could not be retrieved (Figure 6, 7). The employed primer pair was developed to universally amplify and sequence a large set of freshwater macroinvertebrate organisms (Elbrecht et al. 2017), and according to our results it is also able to amplify marine invertebrate and vertebrate (fishes) DNA, making it a powerful tool for biodiversity surveys.

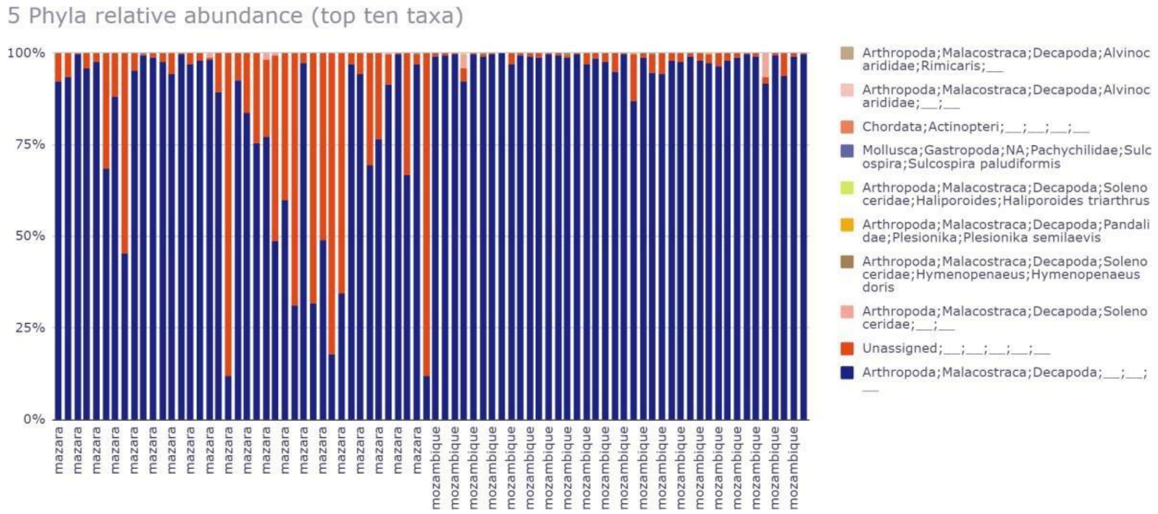


Figure 5.3.6. Relative abundance of the taxa recovered in the 80 samples through COI metabarcoding sequencing (taxonomic assignment with the hypothesised target preys).

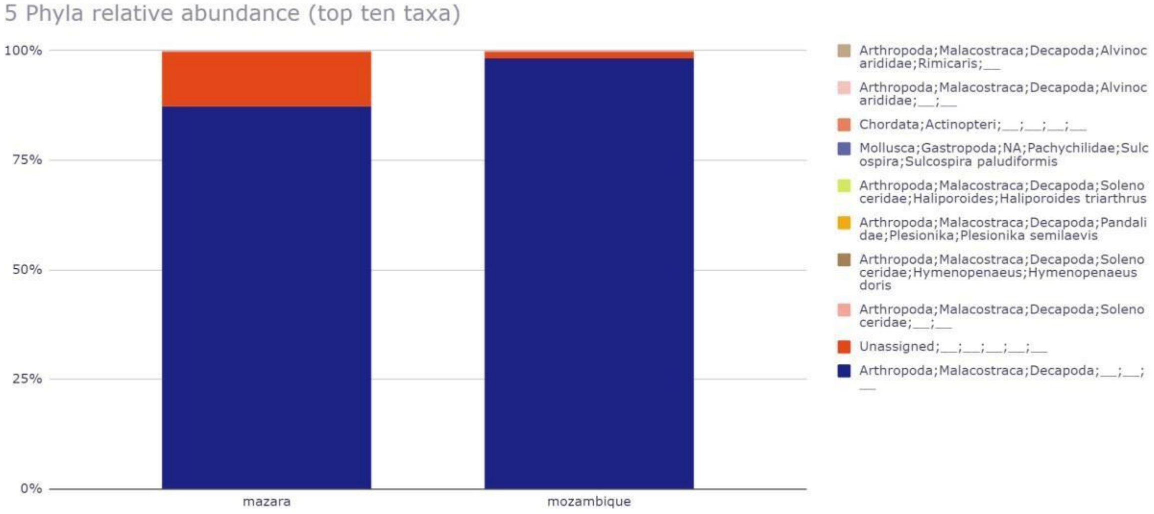


Figure 5.3.7. Per locality, average relative abundance of the top ten taxa recovered in the 80 samples through COI metabarcoding sequencing (taxonomic assignment with the hypothesised target preys).

5.5 Conclusion

Using a metabarcoding sequencing approach 80 gut samples belonging to A. foliacea shrimps from two localities (Mediterranean Sea and Indian Ocean) were successfully characterized, in order to gain information on the trophic habits of the species in the two environments. Results showed that the most represented prey item is Decapoda, in accordance with previous morphology-based studies. Also, other preys already found in the gut of A. foliacea were recovered, namely molluscs, polychaetes, and fishes. Additionally, the metabarcoding approach allowed to describe other preys, likely difficult to identify with a morphological approach, namely priapulids, nemerteans, anthozoans, and scyphozoans. Therefore, the use of a DNA-based approach to characterize the feeding habits of A. foliacea revealed to be more precise and accurate than methods based on morphology alone. Finally, no relevant differences were observed between A. foliacea individuals from the two investigated localities, suggesting that the two populations have similar feeding habits.

5.6 REFERENCES

Bello, G., Pipitone, C. (2002). Predation on cephalopods by the giant red shrimp Aristaeomorpha foliacea. J. Mar. Biol. Assoc. U. K. 82, 213–218.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., & Asnicar, F. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857.

Cau, A., Carbonell, A., Follesa, M.C., Mannini, A., Norrito, G., Orsi-Relini, L., Politous, C.-H., Ragonese, S., Rinelli, P. (2002). MEDITS-based information on the deep water red shrimps Aristaeomorpha foliacea and Aristeus antennatus (Crustacea: Decapoda: Aristeidae). Sci. Mar. 66, 103–124.

Chartosia, N., Tzomos, T., Kitsos, M.-S., Karani, I., Tselepides, A., Koukouras., A. (2005). Diet comparison of the bathyal shrimps, Aristeus antennatus (Risso, 1816) and Aristaeomorpha foliacea (Risso, 1827) (Decapoda, Aristeidae) in the eastern Mediterranean. Crustaceana 78, 273–284.

Edgcomb, V., Orsi, W., Bunge, J., Jeon, S., Christen, R., Leslin, C, Hiolder, M., Taylor G. T., Suarez, P, Varela, R., Epstein, S. (2011). Protistan microbial observatory in the Cariaco Basin, Caribbean. I. Pyrosequencing vs Sanger insights into species richness. ISME J. 5, 1344–1356.

Elbrecht, V., Leese, F. (2017). Validation and Development of COI Metabarcoding Primers for Freshwater Macroinvertebrate Bioassessment. Front. Environ. Sci. 5, 11.

Kapiris, K. (2012). Feeding Habits of Both Deep-Water Red Shrimps, Aristaeomorpha foliacea and Aristeus antennatus (Decapoda, Aristeidae) in the Ionian Sea (E. Mediterranean), IntechOpen.

Kapiris, K., Thessalou-Legaki, M., Petrakis, G., Conides, A. (2010). Ontogenetic shifts and temporal changes in the trophic patterns of the deep-sea red shrimp, Aristaeomorpha foliacea (Decapods: Aristeidae), in the Eastern Ionian Sea (Eastern Mediterranean). Mar. Ecol. 31, 341–354.

Lane, D.J. (1991). 16S/23S rRNA sequencing. Nucleic Acid Tech. Bact. Syst. 115–175.

Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudelo, N., Ranwez, V., Boehm, J.T., Machida, R.J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front. Zool. 10, 34.

Macher, J. N., Bloska, D. M., Holzmann, M., Girard, E. B., Pawlowski, J., Renema, W. (2022). Mitochondrial cytochrome c oxidase subunit I (COI) metabarcoding of Foraminifera communities using taxon-specific primers. PeerJ 10, e13952.

Medlin, L., Elwood, H.J., Stickel, S., Sogin, M.L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71, 491–499.

Rainer, S.F. (1992). Diet of Prawns from the Continental Slope of North-Western Australia. Bull. Mar. Sci. 50, 258–274.

Siegenthaler, A., Wangensteen, O.S., Soto, A.Z., Benvenuto, C., Corrigan, L., mariani, S. (2019). Metabarcoding of shrimp stomach content: Harnessing a natural sampler for fish biodiversity monitoring. Mol. Ecol. Resour. 19, 206–220.

Urban, P., Præbel, K., Bhat, S., Dierking, J., Wangensteen, O.S. (2022). DNA metabarcoding reveals the importance of gelatinous zooplankton in the diet of *Pandalus borealis*, a keystone species in the Arctic. Mol. Ecol. 31, 1562–1576.

Wangensteen, O.S., Palacín, C., Guardiola, M., Turon, X. (2018). DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. PeerJ 6, e4705.

CHAPTER 6 Detection of microplastics and phthalic acid esters in sea urchins from Sardinia (Western Mediterranean Sea)

Introduction

Sea urchin gonads are very appreciated in the south of Italy and especially in Sardinia for their taste that recalls the aromas and flavors of the sea. Known since ancient times, they were widely consumed by the Romans, as indicated in various written record i.e. they were included as an ingredient created in recipes described by the famous Roman gastronome Marco Gavio Apicius (De re coquinaria). Nowadays, in Italy the product is mostly consumed raw: in the season in which the gonads are most abundant from September to April, with a peak in January and February, the freshly fished sea urchins are opened with a special tool designed for depriving their apical part, and the gonads are eaten at the seaside accompanied with bread, “focaccia” or “taralli”. In the restaurant or at home, the most famous recipe is “Spaghetti con i ricci di mare”, where the pulp becomes a precious dressing for pasta. For commercial products available in the market, the pulp is extracted, sterilized, and preserved in water and salt and sealed in plastic or glass recipients. Outside Italy, sea urchin eggs are popular in Japan to make sushi known as “Uni Nigiri” and in some other European countries i.e. Portugal, Spain, Greece, and France. Here the gonads are mostly eaten raw. In France, three recipes are famous that have sea urchin as ingredient: “l'Oursinado” a fish soup similar to “Bouillabaisse”, in which the sea urchin is used to prepare the accompanying sauce, the “Daurade `a la cr`eme d'oursin” in which the cooked sea bream is served with a Dutch sauce enriched with sea urchin eggs and the “Omelette d'oursins” made with sea urchin eggs. As indicated by a recent study, the perception of sea urchin as a novel and trendy product is growing worldwide (Bai˜ao et al., 2021), and the aquaculture production is fast developing since stocks faced overfishing in the last years in several geographical areas (including Sardinia). According to FAO data, the world consumption of sea urchins (live weight) in 2018 amounted to 58.58 tons (Bai˜ao et al., 2021). Together with gastronomic, economic, and ecological considerations, unfortunately also marine pollution is becoming a new issue to be included in the overall evaluation of seafoods. The exponential growth of plastic production worldwide and the mismanagement of plastic waste are leading this human made material to be ubiquitous in the marine environment (Browne et al., 2007). Analysis of 60-years' time series confirms in fact that a significant inC rease of the open ocean plastics occurred in the last decades (Ostle et al., 2019). Concern has been mainly addressed to micro and nano particles (Andrady, 2011), which may derive both from the photo oxidative degradation of the larger plastic items (secondary micro plastics) floating on the ocean surface, or by the directly discharge in the waterways of plastics that has been tailored at the microscale by the producer to exert specific functions (primary microplastics). All these particles may threat marine biota by direct physical inter action, i.e. by the entanglement or blocking of the digestive tract after ingestion (Wright et al., 2013), by acting as vectors for alien rafting species, microbial communities, and diseases (Zettler et al., 2013; Lamb et al., 2018) or by leaching out toxic substances associated to the plastic material (Teuten et al., 2007; Koelmans et al., 2013; Atugoda et al., 2021). Since MPs occupy the same size fraction of sediments and of some planktonic organisms, they are potentially bioavailable to a wide range of organisms, actively ingested by low trophic suspension, filter and deposit feeders, detritivores and planktivores. To date, MPs have been found in a large variety of wild marine animals, such as foraging sea birds (Wilcox et al., 2015), marine mammals (Fossi et al., 2016; Lusher et al., 2015), fish (Boerger et al., 2010; Foekema et al., 2013), crusta ceans (Devriese et al., 2015; Murray and Cowie, 2011), worms and mollusks (Van Cauwenberghe et al., 2015), reef building corals (Raguso et al., 2022) and even deep-sea inhabitants (Taylor et al., 2016). Recently, it has been also highlighted that microplastic ingestion fre quency is slightly higher in grazers and omnivores (33 %) with respect to filter feeders (17 %) and carnivorous and detritivores (21–23 %) (Avio et al., 2020). Besides their physical impact, the finer fractions of MPs and nanoplastics may display an intrinsic toxicity correlated to their capacity to overcome biological barriers (Lai et al., 2022). Specifically, Mattsson and coauthors showed that nanoplastics are transferred through a three-level food chain and can cause brain damage in top consumers, pene trating their blood-to-brain barrier (Mattsson et al., 2017). Toxicity could also arise from the leaching of the other constituents of the plastic material (that are present in the formulation with the plastic polymer) such as residual monomers and plastic additives, or by environmental pollutants concentrated onto the MPs surface, since plastic displays the partition on the plastic particle surface may be greatly enhanced by the larger surface area to volume ratio. Under this view, MPs represent a possible and alternative route of exposure of organic micro-pollutants to marine organisms (Wright et al., 2013). Among the plastic ingredient that may be leached from MPs, phthalic acid esters (PAEs) have recently gained attention. PAEs are used as plasticizers (e.g. to increase the flexibility, transparency, and longevity of the final material) and may represent up to 60 % of the total plastic product weight (Teuten et al., 2007).

Since they are not covalently bound to the plastic polymers, they can be released from the plastic material to the surrounding environment, becoming bioavailable to the marine organisms (Fossi et al., 2018; Saliu et al., 2019). As for other plastic ingredients, their release may be greatly enhanced during the generation of secondary MPs by weathering induced breakdown of the larger plastic debris (Yuan et al., 2022). Recent toxicological assessment showed that PAEs may display endocrine disruptor activity, induce oxidative stress and immunotoxicity in various aquatic organisms (Oehlmann et al., 2009; Zhang et al., 2021); surveys highlighted their occurrence in the marine biota from plankton (Browne et al., 2007) to organisms at the higher level of the trophic chain (Net et al., 2015), including marine mammals and large filter feeders (Fossi et al., 2014; Fossi et al., 2018; Saliu et al., 2022). It was also shown that the concentration of PAEs may display correlation with the concentration of MPs and of larger plastic items, suggesting that PAEs may serve as plastic tracers (Fossi et al., 2018; Paluselli et al., 2018; Saliu et al., 2019; Panio et al., 2020). However, at the present time little is known about the influence of the various environmental factors (e.g. temperature, pressure, microbial colonization) on the kinetic of the release of plastic ingredients, especially in the benthic environment (Fauvelle et al., 2021; Isa et al., 2022). Thus, caution in postulating the correlation between MPs and PAEs is required. The purple sea urchin Paracentrotus lividus (Lamarck, 1816) (Echinodermata: Echinoidea) is a keystone species playing a central role in the Mediterranean coastal benthic habitats and trophic cascade (Giakoumi et al., 2012), regulating the organization and structure of shallow macroalgal assemblages (Boudouresque and Verlaque, 2020). Sea urchins are voracious herbivores and are capable of ingesting large amounts of macroalgal biomass, thus acting as an energetic link from shallow water macroalgae to benthic communities (Dethier et al., 2019). It also displays an important commercial value and it is widely exploited as seafood in the Mediterranean and Atlantic Europe, owing its worldwide fame to its gonads, a delicacy generally indicated as “roe” in the fishery and sushi market (Carboni et al., 2012; Furesi et al., 2016). Being a sensitive species, P. lividus is exposed and damaged by several anthropogenic factors that affect the marine environment, such as temperature rise, ocean acidification, toxic algal bloom, and pollution (Migliaccio et al., 2016, 2019; Yeruham et al., 2015; Sukhn, 2010). As reported by recent investigations, the abundance of P. lividus wild stocks is declining due to a combination of several factors such as overexploitation, cascading effects related to the overfishing of predators (Ceccherelli et al., 2022; Farina et al., 2022; Grech et al., 2022) and introduction of new contaminant entities in the environment, lately including MPs (Yeruham et al., 2015; Fern´andez-Boo et al., 2018). Regarding the possible impacts of MPs onto sea urchins, it was recently shown by exposition experiments in seawater that they accumulate more MPs in the 45 μm size class in the digestive system (61.39 ± 27.90 particles/g) than in the gonads (18.17 ± 8.27 particles/g), while smaller plastic particles (10 μm) are mostly stored in the water vascular system, specifically in the ring canal (28 ± 7.34 particles/g) and in the stone canal (33 ± 8.98 particles/g) (Murano et al., 2020). Effects of MPs (and nanoplastics) on P. lividus embryonic development (Gambardella et al., 2018; Martínez-G´omez et al., 2017; Messinetti et al., 2018; Piccardo et al., 2020; Thomas et al., 2020) and immune system (Murano et al., 2020, 2021) have been documented, but very limited information is currently available on their occurrence on native wild specimens populating Mediterranean coastal areas. The few available reports point to 1.0 MPs/individual in the Adriatic Sea, almost exclusively represented by synthetic polymers as PE, PP, PS and PA (Avio et al., 2020), 26 ± 19 MPs/individual in the Aegean Sea (Greece), mainly represented by fibrous particles (97 %) (Hennicke et al., 2021) and 2.6 fibers/individual in the Gulf of Naples, where 67 % was cotton-based and 33 % synthetic polymers (polyester) (Murano et al., 2022). Starting from this basis, in this work we aimed to assess the occurrence of MPs and PAEs in wild sea urchin specimens sampled in the west coast of Sardinia (Italy), where the sea urchin is an appreciated gourmet delicacy, and its gonads are largely exploited in the local seafood market. Our goal was to determine possible correlation in the occurrence of both these contaminants, and more specifically: a) to investigate the levels of exposure to MPs of P. lividus through ingestion in his natural habitat b) to determine the possible contribution of MPs ingestion to the contamination of P. lividus gonads by leaching of PAEs c) to establish the PAEs exposure levels in humans caused by consumption of fresh sea urchin gonads. In our experimental work, we decided to focus on the occurrence of MPs in the digestive tract because it is generally accepted that ingestion is one of the main pathways of MPs uptake: many marine organisms exert limited selectivity between particles and capture anything of appropriate size, while organisms in the higher levels of the trophic chain could passively ingest micro-plastics during normal feeding activity or mistake the plastic particles for their natural prey (Moore, 2008). In the current literature it was already hypothesized that P. lividus may get in contact and accumulate MPs at least through two different pathways: food ingestion and water vascular system. Moreover, it was also verified by a lab exposition experiment in seawater that the digestive tract is the organ displaying the higher MPs accumulation for the larger sizes (Murano et al., 2020). However, at the present time the contribution of MPs ingestion to the accumulation of PAEs in sea urchin tissues due to leaching (the possible role of MPs as "Trojan Horse" for PAEs) has still scarcely considered in the literature.

Under this view, we decided to also research the occurrence of PAEs in the gonads. In fact, these organs display the higher concentration of lipids among sea urchin tissues (Montero-Torreiro and Garcia-Martinez, 2003) related to their dual function as storage tissue and reproductive organ (Hughes et al., 2006), and consequently they are expected to be the main target for the accumulation of PAEs as lipophilic contaminants (Sugni et al., 2007). Moreover, being gonads consumed by humans, they may represent a possible mean of transport of PAEs into humans and the possible associated risk must be carefully assessed, by starting from the evaluation of the dietary intake.

Material and methods

Sampling activities were carried out by scuba diving in July 2020. Specifically, 22 specimens of wild purple P. lividus were sampled across five sites along the west coast of Sardinia (Italy), representative of its common rocky habitat. A map of the sampling area is reported in Fig. 1, while additional information regarding the sample stations is reported in Table 1. The specimens were divided into two classes according to their test diameter (TD) and the threshold established by Italian national regulation that clearly identify sea urchins that may be consumed by those that may be not: individuals of commercial size (diameter without spines, $TD \geq 50$ mm, briefly considered "large" in our work) and the undersized individual ($30 \leq TD < 50$ mm, briefly considered "small" in our work). More specifically, the size of the sampled sea urchins ranged between 32 mm and 59 mm indicating specimens that have lived in their respective site approximately from 3 to 5 years (Loi et al., 2017). In all the specimens the developmental status was sufficient to collect >0.1 g of gonad sample and perform the SPME extraction, indicating that the individuals were in the most advanced gonad maturation stage.

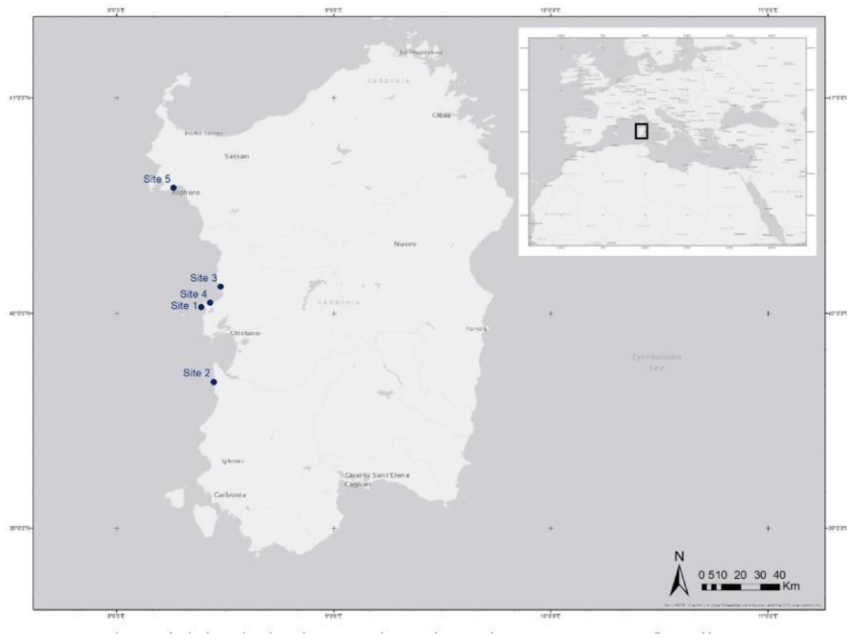


Figure 7.1. Area of study. The map shows the sampling stations in Sardinia (Putzu Idu = 1; Torre dei Corsari = 2; Santa Caterina = 3; Su Pallosu = 4; Alghero = 5).

The sample preparation workflow was performed in dedicated controlled air ISO 6 cleanroom laboratory (temperature: 22 ◦C; SAS pressure: +15 Pa; SAS brewing rate: 30 vol/h; lab pressure: +30 Pa; brewing rate: 50 vol/h) equipped only with glass and metal apparatus and furniture. The use of plastic material was carefully avoided, and all personnel wore only cotton clothes. From each sea urchin individual, the digestive system and gonads were removed and processed separated one from the other. During the gonads dissection particular care was taken to avoid contamination from surrounding organs. The digestive tract was submitted to MPs isolation as described further in this paragraph. Gonads were submitted to PAEs extraction as described in Section 2.4. The choice of processing only these two organs and with two dedicated procedures was dictated by the need of controlling the external contamination in the matrices. According to our experience, extensive handling of the samples and application of different procedures on the same matrix display high risk of contaminating it (Saliu et al., 2020; Raguso et al., 2022). Specifically, the isolation of MPs from the digestive tracts was carried out by following the protocol described by Avio and coauthors (Avio et al., 2015) with slight variations. Di-methyl phthalate (MEP), di-ethyl phthalate (DEP), di-butyl phthalate (DBP), benzyl butyl phthalate (BBzP), bis(2-ethylhexyl) phthalate (DEHP) were researched in the sea urchin gonads by applying a SPME-LC-MS/MS method previously described in Saliu et al., 2020 with slight variations. C18 SPME fibers (purchased from Supelco) were inserted into the gonads and left for 30 min to enable the extraction of the analytes, running the procedure at the pre-equilibrium condition of the time extraction curve. After that the fibers were placed into a glass vial insert containing 100 µL of methanol: water 90:10 and stirred for 40 min at 40 °C in order to perform the elution of analytes. Then, the fiber was taken out from the vial and the vial was directly transferred into the instrument autosampler for the subsequent analysis. LC-MS/MS analysis was performed by using a ThermoScientific TSQ quantum access max instrument. A ThermoFisher Accucore C18 column was used for performing the chromatographic separation of the phthalates, by eluting at 0.6 mL/min with gradient program from 85 to 96 % of methanol (solvent B) and acidified water (solvent A, 0.1 % of formic acid). Injection volume was set up at 20 µL. PAEs were detected by applying a selected reaction monitoring (SRM) of the target ions, with one qualifier and quantifier for each phthalate. Calibration of the system was obtained by using standard calibration mixture and labeled internal standards as described in Saliu et al., 2021. 2Statistical analysis Statistical analyses were performed using SPSS ver. 28 (IBM, New York). All data are expressed as mean ± standard error (SE). Shapiro–Wilk test of normality highlighted the not normally distribution of microplastic concentrations. Kruskal-Wallis test was applied to explore differences in particle concentration across classes (sites and sizes). Spearman correlation was performed to investigate the relationship between plastic particles, five PAEs (DBP, BBzP, DEP, DEHP, DMP) and their total (Σ5PAEs).

Results

Table 2 reports the results of the MPs analysis carried out on the 22 specimens of Paracentrotus lividus surveyed in this study. MPs were found in 9 specimens with concentration up to 4 particles/individual, while 13 resulted not contaminated. Specifically, a total number of 23 particles in the size range 28–803 µm were observed, determining an average of 1.0 ± 0.30 particles for individuals in the whole data set and of 11 ± 4.2 particles for g of digestive tract. Size distribution of the detected particles is reported in Fig. 3. The 25–50 µm class was the most abundant in the whole dataset and it's easily visible that the more the size class increases, the more the number of detected particles decreases. Moreover, particles larger than 250 µm were present only in "big" specimens, while particles in the class range 25–50 µm were most abundant in "small" individuals (Fig. 4). By shape, 22 % of the particles were fibers and 78 % were non-fibers. Specifically, 56 % were fragments, 13 % were foams and 9.0 % were films. Four different colors were observed: grey (65 %), brown (22 %), black (9.0 %) and blue (4.0 %). Considering the constituting polymers, 52 % of the plastic particles resulted to be made of polyolefin (PE/PP), 13 % of synthetic rubber (EPDM), 8.0 %, polyurethane (PU). A same percentage equal to 9.0 % resulted in polystyrene (PS), polyamide (PA), and rayon/cellulose. In addition to the plastic particles, we identified some mineral particles (mostly calcite and smithsonite), probably derived from the grazing activity of sea urchins (as they were found in the digestive tract). Fig. 5 reports the distribution of MPs obtained by considering the total individuals for each different site and the distribution of MPs considering the two size classes adopted in this survey. Specifically, site 2 named Torre dei Corsari displayed the highest level of contamination (2.4 ± 0.68 items/individual), while Su Pallosu resulted the least impacted site with no MPs found in any of the sampled sea urchins. Considering the concentration as particles per gram of digestive tract, Su Pallosu was confirmed the least polluted, whereas the highest concentration was found in Putzu Idu (23 ± 16 items/g). However, no statically significant differences were highlighted among the sites both in terms of items/ individual and items/g (Kruskal-Wallis H test = 8.1, df = 4.0, $\varrho = 0.09$; Kruskal-Wallis H test = 7.5, df = 4.0, $\varrho = 0.11$). Considering the different size of the specimens, the "small" sea urchins (under the commercial size) showed an average concentration of 1.4 ± 0.45 items/individual and of 16 ± 7.1 items/g, while the "big" specimens (in the commercial size) showed an average concentration of 0.60 ± 0.40 items/individual and of 3.7 ± 2.5 items/g. However, the statistical analysis did not highlight any significant difference for these two size classes both in terms of plastic particles per gram of digestive tract and per individual (Mann Whitney U test, $\varrho = 0.09$ and $\varrho = 0.12$). The results of the analysis carried out on the sea urchins by SPME-LC/MSMS are reported in Table 2 and Fig. 6. PAEs were detected in all the samples with an average concentration of 32 ng/g, $\sigma = 5.3$.

Table 2 Presence of microplastics in the surveyed sea urchin specimens. (size: big= more than 4,8 cm, small=less than 4,8 cm; polymers: PU = polyurethane, PE = polyethylene, PS = polystyrene, PA = polyamide, PP =polypropylene).

Sample	**Size**	**Sampling site**	**g digestive tract**	**Microplastics**	**Polymer, shape, color**
p.idu 1	small	Putzu Idu	0,09	2	1 PU fiber, grey 1 PE film, grey
p.idu 2	small	Putzu Idu	0,11	1	1 EPDM fiber, brown
p.idu 3	small	Putzu Idu	0,05	4	1 PE film, brown 1 PE film, grey 1 PE film, grey 1 EPDM film, grey
p.idu 4	small	Putzu Idu	0,23	0	/
p.idu 5	big	Putzu Idu	0,17	0	/
t.cors 1	small	Torre dei	0,12	2	1 EPDM film, grey 1 PP foam, grey

		Corsari			
t.cors 2	small	Torre dei Corsari	0,10	4	1 PU fragment, brown 1 PE film, grey 1 PE film, grey 1 PE film, grey
t.cors 3	small	Torre dei Corsari	0,15	3	1 PE film, grey 1 PS film, brown 1 PE fragment, black
t.cors 4	big	Torre dei Corsari	0,20	3	2 Rayon fiber, grey 1 PA fiber, grey
t.cors 5	small	Torre dei Corsari	0,10	0	/
s.cat 1	big	Santa Caterina	0,13	0	/
s.cat 2	big	Santa Caterina	0,16	0	/
s.cat 3	big	Santa Caterina	0,09	0	/
s.cat 4	big	Santa Caterina	0,12	0	/
s.cat 5	big	Santa Caterina	0,10	0	/
s.cat 6	small	Santa Caterina	0,14	1	1 PP foam, blue
p.usu 1	small	Su Pallosu	0,15	0	/
p.usu 2	small	Su Pallosu	0,10	0	/

Sample	**Size**	**Sampling site**	**g digestive tract**	**Microplastics**	**Polymer, shape, color**
p.usu 3	small	Su Pallosu	0,11	0	/
al.ro 1	big	Alghero	0,14	3	1 PA foam, brown 1 PE film, grey 1 PS film, black
al.ro 2	big	Alghero	0,09	0	/
al.ro 3	big	Alghero	0,19	0	/
TOT				23	
Mean ± SE				1,04 ± 0,31 Mps/individual	

Table 7.3 Concentration of PAEs in the gonads of the sea urchin surveyed specimens (site 1=Putzu Idu, site 2=Torre dei Corsari, site 3=Santa Caterina, site 4=Su Pallosu, site 5=Alghero).

Sample	Site	DEP (ng/g)	DBP (ng/g)	BBzP (ng/g)	DEHP (ng/g)	MEP (ng/g)	Sum (ng/g)
1	1	0	1,6	0	43,8	0	45,4
2	1	0	0	0	47,5	0	47,5
3	1	0	5,6	0	0	0	5,6
4	1	10,8	39	0	15,8	13,5	79,1

5	1	8,8	34	0	19,8	7,8	70,4
6	2	0	5,6	0	18,2	0	23,8
7	2	4,1	10	0	0	0	14,1
8	2	3,6	3,4	0	0	0	7
9	2	11,2	28	0	19,8	1,8	60,8
10	2	13,9	12	0	0	0	25,9
11	3	0	1,7	0	33,4	0	35,1
12	3	3,4	0	0	0	0	3,4
13	3	0	4,1	0	30,1	0	34,2
14	3	7,9	12	0	0,5	0	20,4
15	3	5,4	33	3,5	2,7	7	51,6
16	3	3,3	15	0	4	0	22,3
17	4	0	0	0	37,4	0	37,4
18	4	0	6,2	0	0	0	6,2
19	4	0	4,6	0	0	0	4,6
20	5	0	4,6	0	72,6	0	77,2

21	5	3,2	5,7	0	20,1	0	29
22	5	0	4,4	0	0	0	4,4

The maximum concentration resulted 77 ng/g and was found in a sample from site 5 (sample 20). The most abundant congeners were DEHP with an average of 17 ng/g, $\sigma = 4.3$ and DBP with an average of 10 ng/g, $\sigma = 2.5$. Maximum concentration of DEHP was found in sample 20, site 5 with 73 ng/g whereas the maximum concentration of DBP was found in sample 15, site 3 with 33 ng/g. Statistical analysis did not highlight any significant differences among the different sites for the concentration retrieved of the surveyed congeners (DEP: Kruskal-Wallis H test = 6.3, df = 4.0, $\varrho = 0.18$; DBP: Kruskal-Wallis H test = 1.3, df = 4.0, $\varrho = 0.86$; BBzP: Kruskal-Wallis H test = 2.7, df = 4.0, $\varrho = 0.62$; MEP: Kruskal- Wallis H test = 3.3, df = 4.0, $\varrho = 0.50$; DEHP: Kruskal-Wallis H test = 3.3, df = 4.0, $\varrho = 0.51$; ΣPAEs: Kruskal-Wallis H test = 3.5, df = 4.0, $\varrho = 0.48$). Significant differences were instead highlighted for the relative concentration of each congener (i.e. MEP, DEP, BBZP, DBP and DEHP) considering the sampling sites. No significant differences were found among the PAEs concentration detected in small and big individuals (DEP: Kruskal-Wallis H test = 0.50, df = 1.0, $\varrho = 0.48$; DBP: Kruskal- Wallis H test = 0.16, df = 1.0, $\varrho = 0.69$; BBzP: Kruskal-Wallis H test = 1.2, df = 1.0, $\varrho = 0.27$; MEP: Kruskal-Wallis H test = 1.3, df = 1.0, $\varrho = 0.26$; DEHP: Kruskal-Wallis H test = 1.0, df = 1.0, $\varrho = 0.31$; ΣPAEs: Kruskal-Wallis H test = 0.74, df = 1.0, $\varrho = 0.39$). 3.3. Multivariate statistical analysis on MPs and PAEs dataset To highlight a possible leaching of PAEs from ingested MPs into the sea urchin tissues, we tested by rho Spearman the correlation between PAEs and MPs in all the surveyed individuals. No correlation was found for the sum of PAEs and the total MPs concentration. Significant correlation wash highlighted between DEHP and fiber concentration (rho = 0.44, alpha = 0.05) instead. Moreover, correlation between DMP, DEP and DBP was highlighted (rho = 0.70, 0.67, 0.61 respectively) and between BBzP and MEP (rho = 0.43). In addition, the datasets including the PAEs and MPs concentration retrieved from the 22 specimens were submitted to principal component analysis (PCA). The variables related to the polymer and size distributions were also considered in additional runs (all the data were expressed as counts/g for uniformity). As reported in Fig. 7, the two principal components of the PCA explained 43 % of the total variability. Specifically, the PC1 and PC2 scatter plot (Fig. 7) displayed a similar pattern among big and small specimens. The large part of the small individuals (samples 10,17, 16, 18, 19) showed high values of BBzP and was mostly characterized by items blue in coloration, as well as most of the big individuals (samples 15, 12, 22, 11, 13, 21, 14). Concerning the different sites, PCA highlighted that site 1 was mostly influenced by PC1 where major contribution was given by MEP and PU concentrations (variable contribution 33 % and 49 % respectively) and was characterized by presence of non-fibers within the size range 51–150 µm. Even site 2 showed higher influence from PC1 and major contribution was given by BBzP (31 %), EPDM (35 %), PP (12 %) and PE (89 %) concentrations and displayed microplastics mainly fragments and foam in shape. Site 3, 4 and 5 resulted to be very similar, being characterized by high value of BBzP and presenting mostly blue items. Samples 3 and 9 were well separated to the others, characterized by low levels of BBzP; particularly sample 3 presented high value of EPDM and particles in the 25–50 μm class range, while sample 9 was characterized by high presence of particles larger than 250 μm and rayon. three times as fast as older ones and the discharge from their body can be prevented by the smaller anus (Moore and McPherson, 1965; Feng et al., 2020). Considering that the commercial size of Paracentrotus lividus is limited by law to specimens larger than 50 mm diameter Sardinia (TD > 50 mm), from November to April the most contaminated sea urchins should not be consumed by humans. However, despite regional decrees concerning fishing periods issued to safeguard the stocks and avoid the population collapse (that in the last year became even more tight with a completely ban of the sea urchin fishing till 2025), the illegal collection and sale of undersized specimens is unfortunately frequent and largely documented together with the occasional removal by recreational fishermen (Pais et al., 2007). Regarding the size of the particles, we noticed that in the digestive tract, particles ranged between 801 and 25 μm with most of them detected in the 51–150 μm range (Fig. 3). The distribution does not follow the classical 1D-fragmentation model expected in the marine environment (the slope in the logarithmic scale does not follow a linear trend) and it is observed a substantial shortage of particles in the smaller sizes compared to the distribution commonly observed in the surface seawater (C´ozar et al., 2014), especially for the larger specimens (Fig. 4). This is in agreement with the results obtained by Murano and coauthors (Murano et al., 2020), indicating a mayor accumulation of the smaller particles (10 μm) in the water vascular system, and a lack of these particles in the digestive system. According to the authors, these differences in the uptake could be related to a potential sorting action of the madreporite, facilitating the penetration, as well as excretion mechanism, of the smaller particles but also to the dynamics of the retention and egestion processes and the capability of organs to retain the particles of different size. It must be pointed out that at the present time data regarding the transfer of MPs from the digestive tracts to the other tissues (rates and amounts) are not available. In our study, since we used μFITR for MPs identification, due to physical limitation of the infrared radiation, we were not able to identify particles smaller than 20 μm. However, data still clearly confirm the lack of smaller size, which may indicate a possible active excretion and/or translocation processes. Finally, from the plastic polymers identification additional consideration may be drawn. We found that polyolefin (PE/PP) were the most abundant polymer, corresponding to 52 % of the total encountered. They are characterized by low density (0.90 g/cm3) and expected to float in the seawater, therefore, since sea urchins are benthic organisms, it is reasonable that mechanisms such as biofouling, marine snow or turbidity currents

brought them on the seabed (Kvale et al., 2020; Pohl et al., 2020). In addition, an important role as pathway for MPs may be played by seaweeds and seagrass: for instance, the collection of suspended MPs and transfer to the marine benthic herbivore Littorina littorea was described (Gutow et al., 2016). In this case it was asserted that the snail does not distinguish between algae with adherent microplastics and clean algae without microplastics. To date, the role of seaweeds and seagrass in determining the sea urchin MPs contamination in environmental condition has still not been totally elucidated. By comparing Paracentrotus lividus with Psammechinus miliaris, a sea urchin with strong omnivore dietary habits, Suckling recently highlighted the species-specific response to MPs in sea urchins (Suckling, 2021), and how P. lividus, being a strong herbivore and consuming softer items (e.g. biofilms, algae), results more likely sensitive to MPs. With the current available data, the possibility that some selection is operated during the grazing activity cannot be excluded as well as the fact that also the development stage may play an important role. Under this respect, recently Thomas et al. (2020) showed in a feeding study carried out in vitro with 1–230 μm particles that the larvae of Paracentrotus lividus more readily ingested polymethylmethacrylate than polystyrene particles. It must be pointed out that, to the best of our knowledge, at the present time no data obtained by feeding experiments with adult P. lividus individuals are available, and most of the information is related to experiment carried out with specimens exposed to MPs in seawater but not fed. 4.2. Correlation between MPs and PAEs As reported in Section 3.3, our data did not show a clear correlation between the total concentration of MPs in the digestive tract and the levels of PAEs in the gonads, and thus did not confirm the contribution of MPs by leaching to the contamination of sea urchin tissues. On the other hand, a correlation between the concentration of fibers and of DEHP was highlighted. In addition, MEP, DEP, BBzP, DBP resulted correlated, but not correlated with MPs. This may suggest that the concentration of some PAEs in the sea urchin tissues might be more influenced by the environmental background levels (concentration in the seawater), whereas it is possible that DEHP is carried out by the ingested fibers and act as "Trojan Horse" for PAEs. Even if we did not collect seawater samples during our sea urchin monitoring activities, several reports indicated that DEHP is the most abundant congener found in the seawater in this area: e.g. de Lucia et al. (2014) reported concentrations of DEHP ranging from 9.00 ng/to 13.74 ng/g in surveys carried out in July 2012 and July 2013, Fossi et al. (2012) levels of DEHP equal to 23.42 ±
32.46 ng/g; data collected by Paluselli et al., 2018, in the northwestern Mediterranean Sea along the whole water column till 30 m depth displayed values for ΣPAEs ranging from 130 to 1330 ng L 1 (av. 520 ng L 1 ± 313.3 ng L 1) with maximum near the bottom and the lowest values at the sea surface. In this case DEHP (9.3–91.6 %) was the most abundant PAE, followed by DiBP (3.8–78
%) and DnBP (2.1–44.8 %), while DMP, DEP and BzBP were the least abundant (0.2–2 %).

Considering the partition coefficient of DEHP and the reported concentration, the expected levels of DEHP in the sea urchin gonads (assuming an average lipid content in the gonad of 20 %) should be several orders of magnitude higher than the average concentration found in our study. This might indicate that passive diffusion is not the mean of PAEs contamination in sea urchin, that possible mechanisms of PAEs excretion and metabolism are occurring, and that the contribution of fibers in sequestering PAEs from the seawater and/or the occurrence of leaching when fibers are ingested might be relevant. All these hypotheses must be carefully examined in future lab feeding experiments. Under this view, also the residence time of MPs in the digestive tract and kinetic rates in the metabolization of PAEs are important variables that may cause the differences observed in the MPs and PAEs profiles. In this context also the choice of the analytical procedure for the extraction of PAEs is highly relevant. In fact, by SPME only the "free and unbounded" fraction of PAEs is extracted, thus the detected PAEs are those that accumulated in the tissue (in our specific case in the gonads) due to the uptake from seawater or by leaching after ingestion (this second hypothesis needs confirmation by feeding experiments). Differently, when solvent extraction is used, a strict correlation is more easily highlighted, since PAEs might be chemically extracted directly from the MPs occurring in the sample matrix. However, in this case the analysis does not demonstrate the occurrence of the leaching process and ultimately the role of MPs as Trojan horse for PAEs in the marine organism. As underlined in the introduction, at the present time, for *Paracentrotus lividus* there are no data available that support the hypothesis of the leaching of PAEs from ingested MPs to the other organs. Also, the toxicity and the synergistic effect of MPs and PAEs have still not been elucidated. To the best of our knowledge, only a very recent investigation carried out by Beiras and coauthors (Beiras et al., 2021), highlighted how the leaching of contaminants might be the key factor in determining toxicity in sea urchin. In this study the authors compared the toxicity measured by the sea-urchin embryo test (SET) of plastic manufactured with conventional oil based and alternative formulation. Among plasticizers they only tested Diisodecyl-phthalate (DIDP) at 2.5 g/L concentration level but they found no toxicity associated to the leaching (EC 50 < 100 mg/L). In this study, we didn't perform analysis to distinguish among male and female specimens and neither to determine the gonad maturation stage because we wanted to avoid an excessive sample manipulation that may easily ends up in external contamination and a possible over- estimation of MPs and PAEs (Saliu et al., 2020). For the same reason, we did not perform analysis of the phospholipid content in the gonads and of the related fatty acids profiles. This analytical determination requires in fact the use of organic solvent for the extraction, and the use of considerable amounts of the available biological material. Instead, SPME is carried out solventless and with the direct contact of the SPME fiber with the gonad matrix in a non-destructive manner (Saliu et al., 2019). Other analytical techniques may be adopted to obtain a simultaneous determination of all these different molecules classes (polymers, lipids and PAEs) in the same chromatographic run e.g. pyrolysis-GC/MS (Saliu et al., 2022), however a method for the sea urchin gonads is not available in the current literature and must be implemented and validated. As suggested by an anonymous reviewer, the information regarding the sex of the sea urchin specimens and the maturation stage of the gonads may be relevant to explain the patterns observed in the PAEs profiles. To comment the data obtained in the here presented study it is possible to rely on the information provided by recent study carried out in the same area, that highlighted a sex ratio of almost 1:1 for male and female (Loi et al., 2017). Thus, we may consider realistic an equal distribution in our samples. Moreover, we carried out the sampling activities in May–June before summer spawning season identified to be recurrent in this area (Loi et al., 2017). Moreover, all the individuals submitted to analysis showed the presence of large amounts of gonad matrices available for the SPME extraction and this information might indicate an advanced maturation stage of the gonads. On the other hand, it must be pointed out that the seasonal and environmental factors influencing the number of spawning events occurring along the year and the lipid evolution in sea urchin gonads are debated issues in the literature (Siliani et al., 2016). Also, the sex-induced differences in fatty acid profiles in the gonads are reported in some studies (Rocha et al., 2019) while other studies did not highlight any statistically significant gender-related relationship (Carboni et al., 2013). Thus, it will be highly relevant to establish in further studies the temporal variability of the PAEs profile in the gonads together with sex and gonad maturation.

Discussion

According to our measurements carried out on specimens not submitted to PAEs analysis, about

0.70 g of gonads were obtained from a sea urchin in the Italian commercial size which displayed an average weight of 80 g. Since the gonads contained an average value of PAEs of 32 ± 5.3 ng/g, considering a consume of 10–20 gonads for serving corresponding to around 7.0–14 g, this accounts for a total of 0.2–0.4 µg of PAEs. This content is lower compared to that found in other seafood, i.e. salmon fillet, tuna, raw shrimp, chopped clams, and sardines, where mean values from

0.30 to 32 µg/g have been reported (Schecter et al., 2013). Cheng (2013) also reported PAEs values from 2 to 425 µg/kg in fishes from the Hong Kong market while in Norwegian frozen and fresh seafood products levels from beyond the quantification limit to 55 µg/kg have been found (Sakhi et al., 2014), thus the possible level of exposition for a 100 g serving is comprised between 0.2 and

43.5 µg. In a recent study on cultured seabass and rainbow trout, we evaluated that with an average fillet portion of 150 g, humans could be exposed to an average of 0.3–9.4 µg of PAEs (Panio et al., 2020). Of course, the consumption of sea urchin gonads must be considered occasional, but as already pointed out is depending on the traditional habits; in Sardinia it has been reported that an annual pro-capita consumption is about 1.1 kg (Carboni et al., 2012). According to a study by Serrano et al. (2014), in which they examine the per capita total DEHP dietary intake for eight food groups (dairy, meat, egg, fish, grain, vegetable, fruit, fat) in average diets of US infants, adolescents and females of reproductive age, dairy intake was reported at the highest rate (30.8 µg/kg-day in infants, 3.9 µg/kg-day in adolescents, 5.7 µg/kg-day for female of reproductive age 13–49), while fish and egg consumption was minimal (fish: 0.05 µg/kg-day in infants, 0.02 µg/kg-day in adolescents, 0.03 µg/kg-day in females of reproductive age; egg: 0.03 µg/kg-day in infants, 0.01 µg/kg-day in adolescents and 0.01 µg/kg-day in females 13–49 years old) (Serrano et al., 2014). Therefore, based on actual dietary patterns, for women of reproductive age the greatest contribution to DEHP exposure (47.2 %) derived from dairy products and being the total dairy intake for DEHP equal to 5.7 µg/ kg-day, for a 70 kg woman, this translates to a total exposure of 399 µg/ day (Serrano et al., 2014), which is significantly higher than the intake derived by occasionally consuming sea urchin gonads. Similarly, Sakhi et al. (2014) investigated the exposure to 10 PAEs (DMP, DEP, DiBP, DnBP, BBzP, DEHP, DCHP, DnOP, DiNP, DiDP) in the Norwegian adult population, analyzing foods and beverage commonly consumed in a typical Norwegian diet. They found that the food items with the highest concentrations for Σ10PAEs were buns (835.59 µg/kg fresh weight), chocolate spreads (472.5 µg/kg fresh weight) and margarine (441.1 µg/ kg fresh weight). Considering the estimated daily dietary exposure, they found levels under the TDI values established by EFSA, with the larger relative contribution from grain and meat (seafood excluded). DEHP with 384 ng/kg bw/day and DiNP with 402 ng/kg bw/day DiDP resulted the highest contributor among the ten congeners surveyed. In this study it was also evaluated the difference in the dietary intake of other countries worldwide, and the importance of including country specific data for PAEs dietary exposure calculations (e.g. the author found similar level of exposition worldwide except Germany where the intakes were estimated to be 10 times higher). In summary, all this data confirms that the relative intake of PAEs due to the occasional consumption of a sea urchin serving must be considered negligible if compared to the average dietary intakes. For MPs exposition it must be considered that the preparation of gonads for human consumption necessarily requires breaking the sea urchin, dissecting the alimentary tract, and removing the gonads from the inner side of the arterial body. It is therefore highly likely that during the preparation, plastic particles are released from the digestive tract onto the gonads. In our study we found up to 4 particles for individuals and thus determining a possible gonad contamination up to 6 particles gonads were provided by Murano and coauthors (Murano et al., 2020) and indicate concentration around 5.5–19.0 particles/g thus comparable with our estimation. Since at the present time, there are no indications of processes capable to remove the residual plastic particles from the digestive tract (i.e. longer starvation), as pointed out by other studies (Suckling, 2021), it is advisable to implement in the industrial processing a washing stage with filtered water aimed to remove excess of MPs.

Conclusion

In conclusion, the impact of MPs and PAEs on the benthonic sea urchin community living in Sardinia (West Mediterranean Sea), and the level of exposition to humans of these associated contaminants due to the consumption of sea urchin gonads as gourmet delicacy were assessed. MPs were found in 40 % of the analyzed specimens with concentration up to 4 particles for individual, and with the maximum concentrations found in individuals under the commercial size. PAEs were detected in all the samples, with DEHP resulting the most represented congener and resulting significantly correlated to the presence of the microfibers in the digestive tract (but not with fragments). According to our evaluation, the intake of PAEs associated to a sea urchin serving is one-two orders of magnitude lower than the intake associated to the average fish fillet serving. This is mostly due to the smaller quantity consumed.

Final conclusion

The current needs of the average consumer have been oriented towards "healthy or fit" products and "service" foods which, in addition to the basic value linked to goodness or the highest commercial value of the species, must offer a "ready to eat" and / or "ready to cook" product able to meet the minimum preparation needs. It is therefore essential that these biotechnological techniques will have to be refined over time to discern, especially in the case of such processed or prepared products, the presence of other ingredients, additives, and preservatives, pollution (e.g. microplastic) that in the diagnostic phase can interfere more or less markedly on the final result. In the future, this need for speed in analytical response will become increasingly important to ensure the food security of this important food resource at the base of the Mediterranean diet and at the basis of our food and wine tradition

BIBLIOGRAPHY

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62,1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030.

Atugoda, T., Vithanage, M., Wijesekara, H., Bolan, N., Sarmah, A.K., Bank, M.S., You, S., Ok, Y.S., 2021. Interactions between microplastics, pharmaceuticals and personal care products: implications for vector transport. Environ. Int. 149, 106367 https:// doi.org/10.1016/j.envint.2020.106367.

Avio, C.G., Gorbi, S., Regoli, F., 2015. Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. Mar. Environ. Res. 111, 18–26. https://doi. org/10.1016/j.marenvres.2015.06.014.

Avio, C.G., Pittura, L., d'Errico, G., Abel, S., Amorello, S., Marino, G., Gorbi, S.,

Regoli, F., 2020. Distribution and characterization of microplastic particles and textile microfibers in adriatic food webs: general insights for biomonitoring strategies. Environ. Pollut. 258, 113766 https://doi.org/10.1016/j. envpol.2019.113766.

/Beiras, R., Verdejo, E., Campoy-L´opez, P., Vidal-Li˜n´an, L., 2021. Aquatic toxicity of chemically defined microplastics can be explained by functional additives. J. Hazard. Mater. 406, 124338 https://doi.org/10.1016/j.jhazmat.2020.124338.
Bellotti P., Del Bono G.,Ebbli L., Fazio G., Gatta A., Guglielmetto E., Neirotti F., Pasquale L., Starnini L.,

Save your heart with italian mediterranean diet. U.O Cardioligia ASL 2Savonese Coop Tipograf Savona 2008

Boerger, C.M., Lattin, G.L., Moore, S.L., Moore, C.J., 2010. Plastic ingestion by planktivorous fishes in the north pacifc central gyre. Mar. Pollut. Bull. 60, 2275–2278. https://doi.org/10.1016/j.marpolbul.2010.08.007.

Boudouresque, C.F., Verlaque, M., 2020. Paracentrotus lividus. In: Developments in Aquaculture and Fisheries Science. Elsevier, pp. 447–485. https://doi.org/10.1016/ B978-0-12-819570-3.00026- 3.

Boukid, F., Baune, M. C., Gagaoua, M., & Castellari, M. (2022). Seafood alternatives: assessing the nutritional profile of products sold in the global market. *European Food Research and Technology*, *248*(7), 1777-1786.

Browne, M.A., Galloway, T., Thompson, R., 2007. Microplastic – an emerging contaminant of potential concern? Integr. Environ. Assess. Manag. 3, 559–561.

https://doi.org/10.1002/ieam.5630030412.

Carboni, S., Vignier, J., Chiantore, M., Tocher, D.R., Migaud, H., 2012. Effects of dietary microalgae on growth, survival and fatty acid composition of sea urchin Paracentrotus lividus throughout larval development. Aquaculture 324–325, 250–258. https://doi.org/10.1016/j.aquaculture.2011.10.037.

Carboni, S., Hughes, A.D., Atack, T., Tocher, D.R., Migaud, H., 2013. Fatty acid profles during gametogenesis in sea urchin (Paracentrotus lividus): Efects of dietary inputs on gonad, egg and embryo profles. Comp. Biochem. Physiol. Mol. Integr. Physiol. https://doi.org/10.1016/j.cbpa.2012.11.010.

Cataudella S., Bronzi P. 2001, Acquacoltura responsabile. Uniprom

Piazzi, L., 2022. Sea urchin harvest inside marine protected areas: an opportunity to investigate the effects of exploitation where trophic upgrading is achieved. PeerJ 10–12971. https://doi.org/10.7717/peerj.12971.

Cheng, Z., 2013. Risk assessments of human exposure to bioaccessible phthalate esters through market fish consumption. Environ. Int. 6 https://doi.org/10.1016/j. envint.2013.04.005.

C´ozar, A., Echevarría, F., Gonz´alez-Gordillo, J.I., Irigoien, X., Úbeda, B., Hern´andez-Le´on, S., Palma, A.T., Navarro, S., García-de-Lom, J., Ruiz, A., Fern´andez-de-Puelles, M.L., Duarte, C.M., 2014. Plastic debris in the open ocean. Proc. Natl. Acad. Sci. 111, 10239–10244. https://doi.org/10.1073/pnas.131470.

Cenci Goga B. Ispezione e controllo degli alimenti. Point veterinarie Italie 2018.

D'Amico, P., Armani, A., Castigliego, L., Sheng, G., Gianfaldoni, D., & Guidi, A. (2014).

Seafood traceability issues in Chinese food business activities in the light of the European provisions. *Food Control*, *35*(1), 7-13.

De Lucia, G.A., Caliani, I., Marra, S., Camedda, A., Coppa, S., Alcaro, L., Campani, T., Giannetti, M., Coppola, D., Cicero, A.M., Panti, C., Baini, M., Guerranti, C., Marsili, L., Massaro, G., Fossi, M.C., Dethier, M.N., Hoins, G., Kobelt, J., Lowe, A.T., Galloway, A.W.E., Schram, J.B., Raymore, M., Duggins, D.O., 2019. Feces as food: the nutritional value of urchin feces and implications for benthic food webs. J. Exp. Mar. Biol. Ecol. 514–515, 95–102. https://doi.org/10.1016/j.jembe.2019.03.016.

Devriese, L.I., van der Meulen, M.D., Maes, T., Bekaert, K., Paul-Pont, I., Fr`ere, L., Robbens, J., Vethaak, A.D., 2015. Microplastic contamination in brown shrimp Crangon crangon, linnaeus 1758) from coastal waters of the southern North Sea and channel area. Mar. Pollut. Bull. 98, 179–187. https://doi.org/10.1016/j.marpolbul. 2015.06.051.

EUMOFA (European market observatory for fisheries and aquaculture products):
The EU FISHMARKET 2022

Farina, S., Ceccherelli, G., Piazzi, L.D., Panzalis, P., Navone, A., Guala, I., 2022. Protection effectiveness and sea urchin predation risk: the role of roving predators

beyond the boundaries of a Marine Protected Area in the Western Mediterranean Sea. Aquat. Conserv. Mar. Freshwat. Ecosyst. 1–14. https://doi.org/10.1002/qc.3819.

Fauvelle, V., Garel, M., Tamburini, C., Nerini, D., Castro-Jim´enez, J., Schmidt, N., Paluselli, A., Fahs, A., Papillon, L., Booth, A.M., Semp´er´e, R., 2021. Organic additive release from plastic to seawater is lower under deep-sea conditions. Nat. Commun.12, 4426. https://doi.org/10.1038/s41467-021- 24738-

w.

Feng, Z., Wang, R., Zhang, T., Wang, J., Huang, W., Li, J., Xu, J., Gao, G., 2020. Microplastics in specific tissues of wild sea urchins along the coastal areas of northern China. Sci. Total Environ. 728, 138660 https://doi.org/10.1016/j. scitotenv.2020.138660.

Fern´andez-Boo, S., Pedrosa-Oliveira, M.H., Afonso, A., Arenas, F., Rocha, F., Valente, L. M.P., Costas, B., 2018. Annual assessment of the sea urchin (Paracentrotus lividus) humoral innate immune status: Tales from the north Portuguese coast. Mar. Environ. Res. 141, 128–137. https://doi.org/10.1016/j.marenvres.2018.08.007.

Foekema, E.M., De Gruijter, C., Mergia, M.T., van Franeker, J.A., Murk, A.J., Koelmans, A.A., 2013. Plastic in North Sea fish. Environ. Sci. Technol. 47, 8818–8824. https://doi.org/10.1021/es400931b.

Fossi, M.C., Panti, C., Guerranti, C., Coppola, D., Giannetti, M., Marsili, L., Minutoli, R.2012. Are baleen whales exposed to the threat of microplastics? A case study of the Mediterranean fin whale (Balaeno pteraphysalus). Mar. Pollut. Bull. 64, 2374–2379. https://doi.org/10.1016/j.marpolbul.2012.08.013.

.Fossi, M.C., Ped, C., Compa, M., Tsangaris, C., Alomar, C., Claro, F., Ioakeimidis, C., Galgani, F., Hema, T., Deudero, S., Romeo, T., Battaglia, P., Andaloro, R., Caliani, I., Casini, S., Panti, C., Baini, M., 2018. Bioindicators for monitoring marine litter ingestion and its impacts on Mediterranean biodiversity. Environ. Pollut. 237, 1023–1040. https://doi.org/10.1016/j.envpol.2017.11.019.

Furesi, R., Madau, F.A., Pulina, P., Sai, R., Pinna, M.G., Pais, A., 2016. Profitability and sustainability of edible sea urchin fishery in Sardinia (Italy). J. Coast. Conserv. 20, 299–306. https://doi.org/10.1007/s11852-016-0441-0.

Galli A., Bertoldi A., Franzetti L. Igiene degli alimenti e HACCP. EPC editore 2017.

Gallina A., Caburlotto G.,Arcangeli G., Prodotti della pesca e dell'acquacoltura freschi e lavorati, qualità, salubrità, e analisi di laboratorio. Istituto zooprofilattico sperimentale delle Venezie 2013.

Giakoumi, S., Cebrian, E., Kokkoris, G.D., Ballesteros, E., Sala, E., 2012. Relationships between fish, sea urchins and macroalgae: the structure of shallow rocky sublittoral communities in the Cyclades, eastern Mediterranean. Estuar. Coast. Shelf Sci. 109, 110. https://doi.org/10.1016/j.ecss.2011.06.004.

Grassi, S., Benedetti, S., Magnani, L., Pianezzola, A., & Buratti, S. (2022). Seafood freshness: e-nose data for classification purposes. *Food Control*, *138*, 108994.

Grech, D., Mandas, D., Farina, S., Guala, I., Brundu, R., Cristo, B., Panzalis, P.A., Salati, F., Carella, F., 2022. Vibrio splendidus clade associated with a disease affecting Paracentrotus lividus (Lamarck, 1816) in Sardinia (Western Mediterranean). J. Invertebr. Pathol. 192, 107783 https://doi.org/10.1016/j.jip.2022.107783.

Guglielmetti, C., Manfredi, M., Brusadore, S., Sciuto, S., Esposito, G., Ubaldi, P. G., & Mazza, M. (2018). Two-dimensional gel and shotgun proteomics approaches to distinguish fresh and frozen-thawed curled octopus (*Eledone cirrhosa*). *Journal of Proteomics*, *186*, 1-7.

Gutierrez, A., & Thornton, T. F. (2014). Can consumers understand sustainability through seafood eco-labels? A US and UK case study. *Sustainability*, *6*(11), 8195-8217.

Gutow, L., Eckerlebe, A., Gim´enez, L., Saborowski, R., 2016. Experimental evaluation of seaweeds as a vector for microplastics into marine food webs. Environ. Sci. Technol. 50, 915–923. https://doi.org/10.1021/acs.est.5b02431.

Hennicke, A., Macrina, L., Malcolm-Mckay, A., Miliou, A., 2021. Assessment of microplastic accumulation in wild Paracentrotus lividus, a commercially important sea urchin species, in the Eastern Aegean Sea, Greece. Reg. Stud. Mar. Sci. 45, 101855. https://doi.org/10.1016/j.rsma.2021.101855.

Hughes, A.D., Kelly, M.S., Barnes, D.K.A., Catarino, A.I., Black, K.D., 2006. The dual functions of sea urchin gonads are reflected in the temporal variations of their biochemistry. Mar. Biol. 148 (4), 789–798. https://doi.org/10.1007/s00227-005-0124-0.

Isa, V., Saliu, F., Bises, C., Vencato, S., Raguso, C., Montano, S., Lasagni, M., Lavorano, S., Clemenza, M., Galli, P., 2022. Phthalates bioconcentration in the soft corals: interand intra-species differences and ecological aspects. Chemosphere 297, 134247. https://doi.org/10.1016/j.chemosphere.2022.134247.

Issenberg S. 2007 L'onda del suhi Sperling & Kupfer.

Jacobs, S., Sioen, I., Marques, A., & Verbeke, W. (2018). Consumer response to health and environmental sustainability information regarding seafood consumption. *Environmental research*, *161*, 492-504.

Koelmans, A.A., Besseling, E., Wegner, A., Foekema, E.M., 2013. Plastic as a carrier of POPs to aquatic organisms: a model analysis. Environ. Sci. Technol. 47, 7812–7820. https://doi.org/10.1021/es401169n.

Kvale, K.F., Friederike Prowe, A.E., Oschlies, A., 2020. A critical examination of the role of marine snow and zooplankton fecal pellets in removing ocean surface microplastic. Front. Mar. Sci. 6, 808. https://doi.org/10.3389/fmars.2019.00808.

Lai, H., Liu, X., Qu, M., 2022. Nanoplastics and human health: Hazard identification and biointerface. Nanomaterials 12, 1298. https://doi.org/10.3390/nano12081298.

Lamb, J.B., Willis, B.L., Fiorenza, E.A., Couch, C.S., Howard, R., Rader, D.N., True, J.D., Kelly, L.A., Ahmad, A., Jompa, J., Harvell, C.D., 2018. Plastic waste associated with disease on coral reefs. Science 359, 460–462. https://doi.org/10.1126/science.aar3320.

Lin, P. Y., & Su, K. P. (2007). A meta-analytic review of double-blind, placebo-controlled trials of antidepressant efficacy of omega-3 fatty acids. *Journal of Clinical Psychiatry*, *68*(7), 1056-1061.

Loi, B., Guala, I., Pires da Silva, R., Brundu, G., Baroli, M., Farina, S., 2017. Hard time to be parents? Sea urchin fishery shifts potential reproductive contribution of population onto the shoulders of the young adults. PeerJ 5, 3067. https://doi.org/10.7717/peerj.3067.

Lusher, A.L., Hernandez-Milian, G., O'Brien, J., Berrow, S., O'Connor, I., Officer, R.,

2015. Microplastic and macroplastic ingestion by a deep diving, oceanic cetacean: the True's beaked whale Mesoplodon mirus. Environ. Pollut. 199, 185–191. https:// doi.org/10.1016/j.envpol.2015.01.023.

Maffioli G. 1976; Storia piacevole della gastronomia Bietti editore.

Malandra r., Renon P, 1998; Le principali frodi dei prodotti della pesca.

Maldini, M., Marzano, F. N., Fortes, G. G., Papa, R., & Gandolfi, G. (2006). Fish and seafood traceability based on   AFLP markers: Elaboration of a species database. *Aquaculture*, *261*(2), 487-494.

Manzoni P. Enciclopedia illustrata delle specie ittiche marine di interesse commerciale aventi denominazione   stabilita dalla normativa italiana. De Agostini Novara 1987.

Manzoni P.; Tepedino V. 2008; Grande Enciclopedia illustrata dei Pesci Eurofishmarket.

Manzoni P. 2010 Grande Enciclopedia illustrata dei Crostacei dei Molluschi e dei Ricci di mare Eurofishmarket

Martínez-G´omez, C., Le´on, V.M., Calles, S., Gomȧriz-Olcina, M., Vethaak, A.D., 2017. The adverse effects of virgin microplastics on the fertilisation and larval development of sea urchins. Mar. Environ. Res. 130, 69–76. https://doi.org/10.1016/j.marenvres.2017.06.016.

Matiddi, M., 2014. Amount and distribution of neustonic micro-plastic off the western Sardinian coast (Central-Western Mediterranean Sea). Mar. Environ. Res. 7 https://doi.org/10.1016/j. marenvres.2014.03.017.

Mattsson, K., Johnson, E.V., Malmendal, A., Linse, S., Hansson, L.-A., Cedervall, T., 2017. Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. Sci. Rep. 7, 11452. https://doi.org/10.1038/ s41598-017-10813-0.

Maurizi D. Etichettatura degli alimenti e informazioni ai consumatori. Commento tecnico e pratico al Reg. 1169/2011 e alla normativa verticale di settore. EPC editore 2015.

Messinetti, S., Mercurio, S., Parolini, M., Sugni, M., Pennati, R., 2018. Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. Environ. Pollut. 237, 1080–1087. https://doi.org/10.1016/j. envpol.2017.11.030.

Migliaccio, O., Castellano, I., Di Cioccio, D., Tedeschi, G., Negri, A., Cirino, P., Romano, G., Zingone, A., Palumbo, A., 2016. Subtle reproductive impairment through nitric oxide-mediated mechanisms in sea urchins from an area affected by harmful algal blooms. Sci. Rep. 6, 26086. https://doi.org/10.1038/srep26086.

Migliaccio, O., Pinsino, A., Maffioli, E., Smith, A.M., Agnisola, C., Matranga, V., Nonnis, S., Tedeschi, G., Byrne, M., Gambi, M.C., Palumbo, A., 2019. Living in future ocean acidification, physiological adaptive responses of the immune system of sea urchins resident at a CO2 vent system. Sci. Total Environ. 672, 938–950. https://doi. org/10.1016/j.scitotenv.2019.04.005.

Montero-Torreiro, M.F., Garcia-Martinez, P., 2003. Seasonal changes in the biochemical composition of body components of the sea urchin, Paracentrotus lividus, in Lorb´e (Galicia, North-Western Spain). J. Mar. Biol. Assoc. U. K. 83, 575–581. https://doi. org/10.1017/S0025315403007501h.

Moore, C.J., 2008. Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. Environ. Res. 108, 131–139. https://doi.org/10.1016/j.envres. 2008.07.025.

Moore, H.B., McPherson, B.F., 1965. A contribution to the study of the productivity of the urchins Tripneustes esculentus and Lytechinus variegatus. Bull. Mar. Sci. 15, 855–871.

Murano, C., Agnisola, C., Caramiello, D., Castellano, I., Casotti, R., Corsi, I., Palumbo, A., 2020. How sea urchins face microplastics: uptake, tissue distribution and immune system response. Environ. Pollut. 264, 114685 https://doi.org/10.1016/j. envpol.2020.114685.

Murano, C., Donnarumma, V., Corsi, I., Casotti, R., Palumbo, A., 2021. Impact of microbial colonization of polystyrene microbeads on the toxicological responses in the sea urchin Paracentrotus lividus. Environ. Sci. Technol. 55, 7990–8000. https://doi.org/10.1021/acs.est.1c00618.

Murano, C., Vaccari, L., Casotti, R., Corsi, I., Palumbo, A., 2022. Occurrence of microfibres in wild specimens of adult sea urchin Paracentrotus lividus (Lamarck, 1816) from a coastal area of the Central Mediterranean Sea. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2022.113448.

Murray, F., Cowie, P.R., 2011. Plastic contamination in the decapod crustacean Nephrops norvegicus (Linnaeus, 1758). Mar. Pollut. Bull. 62, 1207–1217. https://doi.org/ 10.1016/j.marpolbul.2011.03.032.

Net, S., Semp´er´e, R., Delmont, A., Paluselli, A., Ouddane, B., 2015. Occurrence, fate, behavior and ecotoxicological state of phthalates in different environmental matrices. Environ. Sci. Technol. 49, 4019–4035. https://doi.org/10.1021/ es505233b.

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., Van Look, K.J.W., Tyler, C.R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos. Trans. R. Soc. B Biol. Sci. 364, 2047–2062. https://doi.org/10.1098/rstb.2008.0242.

Orban E., Poli B.M ., & Emilio, G. (2011). Fish production chain: safety and quality for consumers. In *The state of Italian marine fisheries and aquaculture* (pp. 589-610). S. Cautadella e M. Spagnolo, Ministero delle politiche agricole alimentari e forestali.

Ostle, C., Thompson, R.C., Broughton, D., Gregory, L., Wootton, M., Johns, D.G., 2019. The rise in ocean plastics evidenced from a 60-year time series. Nat. Commun. 10, 622. https://doi.org/10.1038/s41467-019-09506-1.

Pais, A., Chessa, L.A., Serra, S., Ruiu, A., Meloni, G., Donno, Y., 2007. The impact of commercial and recreational harvesting for Paracentrotus lividus on shallow rocky reef sea urchin communities in North-western Sardinia, Italy. Estuar. Coast. ShelfSci. 73, 589–597. https://doi.org/10.1016/j.ecss.2007.02.011.

Palombi A., Santarelli M. 1968. Gli animali commestibili dei mari d'Italia. Hoepli.

Paluselli, A., Aminot, Y., Galgani, F., Net, S., Semp´er´e, R., 2018. Occurrence of Phthalate Acid Esters (PAEs) in the Northwestern Mediterranean Sea and the Rhone River. https://doi.org/10.1016/j.pocean.2017.06.002.

Panio, A., Fabbri Corsarini, S., Bruno, A., Lasagni, M., Labra, M., Saliu, F., 2020. Determination of phthalates in fish fillets by liquid chromatography-tandem mass spectrometry (LC-MS/MS): a comparison of direct immersion solid phase microextraction (SPME) versus ultrasonic assisted solvent extraction (UASE). Chemosphere 255, 127034. https://doi.org/10.1016/j.chemosphere.2020.127034.

Pezzolato M. L'uso dell'istologia nel controllo degli alimenti di origine animale con particolare riferimento ai prodotti ittici., 2011.

Pohl, F., Eggenhuisen, J.T., Kane, I.A., Clare, M.A., 2020. Transport and burial of microplastics in deep-marine sediments by turbidity currents. Environ. Sci. Technol. 4, 4180–4189. https://doi.org/10.1021/acs.est.9b07527.

Procter, J., Hopkins, F.E., Fileman, E.S., Lindeque, P.K., 2019. Smells good enough to eat: dimethyl sulfide (DMS) enhances copepod ingestion of microplastics. Mar. Pollut. Bull. 138, 1–6. https://doi.org/10.1016/j.marpolbul.2018.11.014.

Repetto N., Rossi S. 2013, Le ragioni del tonno, storia, biologia, pesca e tutela Sagep editore

Sakhi, A.K., Lillegaard, I.T.L., Voorspoels, S., Carlsen, M.H., Løken, E.B., Brantsæter, A.L.,Haugen, M., Meltzer, H.M., Thomsen, C., 2014. Concentrations of phthalates and bisphenol in Norwegian foods and beverages and estimated dietary exposure in adults. Environ. Int. 73, 259–269. https://doi.org/10.1016/j.envint.2014.08.005.

Schecter, A., Lorber, M., Guo, Y., Wu, Q., Yun, S.H., Kannan, K., Hommel, M., Imran, N.,Hynan, L.S., Cheng, D., Colacino, J.A., Birnbaum, L.S., 2013. Phthalate concentrations and dietary exposure from food purchased in New York state. Environ. Health Perspect. 121 (4), 473–494. https://doi.org/10.1289/ehp.1206367.

Serrano, S.E., Braun, J., Trasande, L., Dills, R., Sathyanarayana, S., 2014. Phthalates and diet: a review of the food monitoring and epidemiology data. Environ. Health 13, 43. https://doi.org/10.1186/1476-069X-13-43.

Siliani, S., Melis, R., Loi, B., Guala, I., Baroli, M., Sanna, R., Uzzau, S., Roggio, T., Addis, M.F., Anedda, R., 2016. Influence of seasonal and environmental patterns on the lipid content and fatty acid profiles in gonads of the edible sea urchin Paracentrotus lividus from Sardinia. Mar. Environ. Res. 113, 124–133. https://doi. org/10.1016/j.marenvres.2015.12.001.

Suckling, C., 2021. Responses to environmentally relevant microplastics are speciesspecific

with dietary habit as a potential sensitivity indicator. Sci. Total Environ. 751, 142341 https://doi.org/10.1016/j.scitotenv.2020.142341.

Sugni, M., Mozzi, D., Barbaglio, A., et al., 2007. Endocrine disrupting compounds and echinoderms: new ecotoxicological sentinels for the marine ecosystem. Ecotoxicology 16, 95–108. https://doi.org/10.1007/s10646-006-0119-8.

Teuten, E.L., Rowland, S.J., Galloway, T.S., Thompson, R.C., 2007. Potential for plastics to transport hydrophobic contaminants. Environ. Sci. Technol. 41, 7759–7764. https://doi.org/10.1021/es071737s.

Ubaldi P.G. Impieghi degli oli di pesci in alimentazione animale ed umana, con particolare riferimento all'acquacoltura 1996.

Wilcox, C., Van Sebille, E., Hardesty, B.D., 2015. Threat of plastic pollution to seabirds is global, pervasive, and increasing. Proc. Natl. Acad. Sci. 112, 11899–11904. https://doi.org/10.1073/pnas.1502108112.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492. https://doi.org/10.1016/j.envpol.2013.02.031.

Yeruham, E., Rilov, G., Shpigel, M., Abelson, A., 2015. Collapse of the echinoid Paracentrotus lividus populations in the eastern Mediterranean-result of climate change? Sci. Rep. 5, 13479. https://doi.org/10.1038/srep13479.

Yuan, Z., Nag, R., Cummins, E., 2022. Human health concerns regarding microplastics in the aquatic environment-from marine to food systems. Sci. Total Environ. 823, 153730.

Zhang, Y., Jia, Y., Li, Z., Tao, Y., Yang, Y., 2021. Hazards of phthalates (PAEs) exposure: a review of aquatic animal toxicology studies. Sci. Total Environ. 771, 145418. https://doi.org/10.1016/j.scitotenv.2021.145418.