UNIVERSITÀ DEGLI STUDI DI MILANO

DOCTORAL PROGRAMME IN NUTRITIONAL SCIENCES

SEAFOOD INSPECTION: CURRENT ISSUES

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Abstract

Abstract

The thesis focuses on quality and safety aspects of different types of seafood by using a multidisciplinary approach, combining different types of analyses (behavioural studies, haemolymph analyses, microbiological and chemical-physical analyses) and regulatory aspects. The thesis consists of a collection of scientific papers and laboratory activities that report the main studies of my PhD research. In the first study the evaluation of the stress level during lobster commercialization was evaluated by analysing eight different haemolymph parameters. The second study faced the potential application of a new Loop-mediated isothermal amplification (LAMP) assay for the detection of *Anisakis* spp. in seafood products. The third study shows the preliminary results of an ongoing investigation that aims at developing a method suitable for the extraction of microplastics from mussels. The fourth study compared the chemical composition in fish roe products from different species in order to achieve a deeper knowledge of the chemical and microbiological composition of fish roe products collected on the Italian market.

Riassunto

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La tesi tratta aspetti relativi alla qualità e alla sicurezza di prodotti ittici e prodotti a base di pesce utilizzando un approccio multidisciplinare, che combina analisi di laboratorio (analisi emolinfatiche, analisi chimiche, fisiche e microbiologiche) e la valutazione degli aspetti normativi. La tesi consiste in una raccolta di articoli scientifici sugli studi affrontati durante la mia attività di ricerca e nella descrizione di un'attività sperimentale ancora in via di sviluppo. Il primo studio riguarda la valutazione dell'effetto della legatura delle chele sul benessere dell'astice americano durante la commercializzazione tramite l'analisi dei parametri emolinfatici. Il secondo studio valuta l'efficacia della tecnica di amplificazione isotermica LAMP nella rilevazione del parassita *Anisakis* spp. in prodotti a base di pesce. Il terzo studio descrive la ricerca preliminare, ancora in corso, finalizzata allo sviluppo e alla validazione di un metodo per l'estrazione di microplastiche nei molluschi bivalvi. Il quarto e ultimo studio confronta la composizione chimica e gli aspetti di sicurezza alimentare di uova di pesce provenienti da diverse specie presenti sul mercato italiano.

Contents

CHAPTER 1

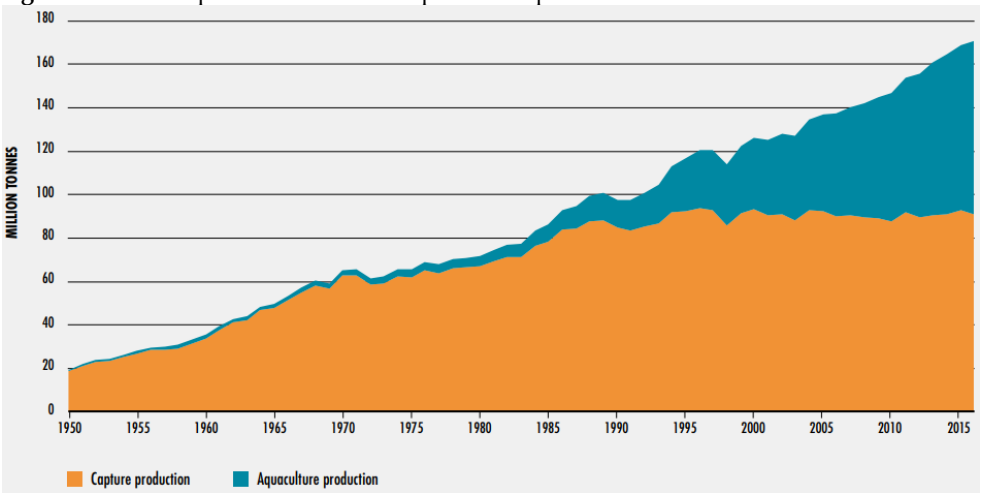
Background

1.1. The global seafood market

1.1.1. Total fish production

Seafood is represented by freshwater and marine animals, excluding mammals, regarded as food for human consumption and represents an important source of food and nutrition for hundreds of millions of people. Fish is one of the most-traded primary product worldwide with more than a half of fish exports by value originating in developing countries (FAO, 2018a). Aquaculture has been responsible for the impressive growth in the supply of fish for human consumption in the last two decades. In 2016, global fish production was as about 171 million tonnes, divided between capture fisheries at 91 million tonnes and aquaculture at 80 million tonnes (Figure 1).

Figure 1. World capture fisheries and aquaculture production



Source: Source: FAO. 2018. The State of World Fisheries and Aquaculture 2018.

Of the global total capture fishery production, 79.3 million tonnes were from marine waters and 11.6 million tonnes from inland waters (Table 1). For marine capture production, China was the major producer followed by Indonesia, the United States of America, and the Russian Federation. Major captured marine species and genera in 2016 were Alaska pollock (*Theragra chalcogramma*), Anchoveta or Peruvian anchovy (*Engraulis ringens*), Skipjack tuna (*Katsuwonus pelamis*) and Sardinellas nei (*Sardinella* spp.) (FAO, 2018a). For inland waters capture production, the major producers were China, India, Bangladesh and Myanmar.

The global aquaculture production in 2016 comprised 54.1 million tonnes of finfish, 17.1 million tonnes of molluscs, 7.9 million tonnes of crustaceans and 938'500 tonnes of other aquatic animals including amphibians. China accounted for 49'244 tonnes in 2016, representing more than 60% of world aquaculture production (FAO, 2018a). Other major producers were India, Vietnam, Bangladesh and Egypt.

Table 1. World fisheries and aquaculture production and utilization (million tonnes)

Category	2011	2012	2013	2014	2015	2016
Production						
Capture						
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total capture	**92.2**	**89.5**	**90.6**	**91.2**	**92.7**	**90.9**
Aquaculture						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total aquaculture	**61.8**	**66.4**	**70.2**	**73.7**	**76.1**	**80.0**
Total world fisheries and aquaculture	**154.0**	**156.0**	**160.7**	**164.9**	**168.7**	**170.9**
Utilization[b]						
Human consumption	130.0	136.4	140.1	144.8	148.4	151.2
Non-food uses	24.0	19.6	20.6	20.0	20.3	19.7
Population (*billions*)[c]	7.0	7.1	7.2	7.3	7.3	7.4
Per capita apparent consumption (*kg*)	18.5	19.2	19.5	19.9	20.2	20.3

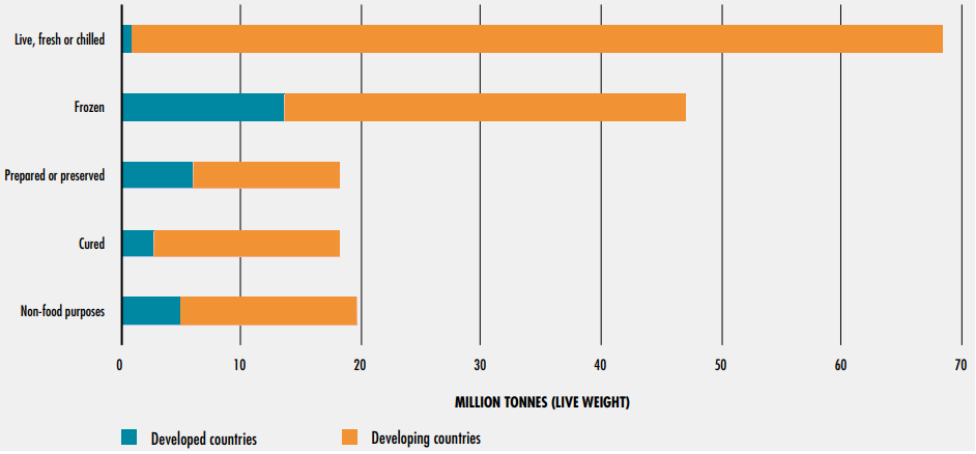
[a] Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants.
[b] Utilization data for 2014–2016 are provisional estimates.
[c] Source of population figures: UN, 2015e.

Source: FAO. 2018a. The State of World Fisheries and Aquaculture 2018.

The world fish production used for direct human consumption was more than 150 million tonnes in 2016 (Table 1). The remaining 20 million tonnes was destined for non-food products, of which 74% was reduced to fishmeal and fish oil and the rest was utilized for other purposes, such as raw material for direct feeding in aquaculture. Since fish and seafood are perishable, they are often processed to conserve their nutritional properties and prolong their shelf-life. In 2016, 45% of the fish for direct human consumption (68 million tonnes) was in the form of live, fresh or chilled fish. The rest of the production for edible purposes underwent some form of processing: 31% (about 46 million tonnes) in frozen form, 12% (18 million tonnes) in prepared and

preserved forms, and 12% (18 million tonnes) in dried, salted, smoked or other cured forms (Figure 2). For longer shelf-life, freezing represents the main method of processing fish, accounting for 56% of total processed fish for human consumption and 27% of total fish production in 2016. However, the utilization of fish and the processing methods vary by continent, region and country.

Figure 2. Utilization of world fisheries production: developed vs developing countries, 2016



Source: FAO. 2018a. The State of World Fisheries and Aquaculture 2018.

Commercialization of live fish has been increasing thanks to technological developments, improved logistics and higher demand. However, live-fish marketing and transportation can be challenging due to health regulations and quality standard. In many developing countries commercialization and trade are based on tradition, while in the European market live fish must comply with requirements concerning animal welfare.

In developing countries, frozen fish increased from 8% in the 1980s to 26% in 2016 (Figure 2). In the same period, the share of prepared or preserved forms also grew from 4% in the 1960s to 9% in 2016. This is due to the major innovations in refrigeration, ice-making and transportation of the recent decades that allowed a growing distribution of fish in fresh and other forms. Despite these technical innovations, developing countries still lack adequate infrastructure and services (e.g. hygienic landing centres, reliable electricity supply, potable water, ice, ice plants, refrigerated transport). These factors, combined with tropical temperatures, lead to high post-harvest losses and quality deterioration. For this reason, fish is commercialized mainly in live or fresh form soon after landing or harvesting or preserved using traditional methods, (e.g. salting, drying and smoking). In 2016, cured forms (dried, smoked or fermented) in developing countries represented 12% of all fish for human consumption in 2016. Processing methods in developing countries are

represented by unsophisticated methods of transformation (e.g., filleting, salting, canning, drying and fermentation) that provide livelihood to many people in coastal areas and remain important components of rural economies. However, fish processing has also evolved in the last decades, ranging from simple gutting, heading or slicing, to breading, cooking and individual quick-freezing, depending on the commodity and market value.

In developed countries, frozen products and prepared/preserved forms represent most of fish for human consumption (Figure 2). Frozen fish has risen from 42% in the 1980s to 58% in 2016, while prepared and preserved forms has remained stable at 26% in 2016. Innovations and changing in food habits are converging on convenience foods, mainly fresh, frozen, breaded, smoked or canned forms, marketed as ready and portion-controlled uniform-quality meals.

1.1.2. Fish utilization

Part of world fisheries production is processed into fishmeal and fish oil, contributing indirectly to human consumption when used as feed in aquaculture. Fishmeal is the crude flour obtained after milling and drying fish, while fish oil is usually a yellow liquid obtained through the pressing of cooked fish.

Fish oil is the richest available source of long-chain highly unsaturated fatty acids (HUFAs), but it mostly goes into aquaculture feeds. Due to the growing demand for fishmeal and fish oil, a growing part of fishmeal is being produced from fish by-products. It is estimated that by-products contribute 25-35% to the total volume of fishmeal and fish oil produced (FAO, 2016). Due to their high price, fishmeal and fish oil are selectively used as strategic ingredients at lower levels and for specific stages of fish production, such as hatchery, brood-stock and finishing diets.

Processing of fish products is creating increasing quantities of offal and other by-products that are not usually on the market due to low consumer acceptance or sanitary regulations that restrict their use. However, fish by-products are gaining attention because they represent an additional source of nutrition (FAO, 2016). Besides, technologies enable efficient fish by-products utilization: heads, frames and fillet cut-offs can be used directly as food or turned into products for human consumption such as sausages, cakes gelatine and sauces. Other by-products are used for production of feed, biodiesel/biogas, dietetic products (chitosan), pharmaceuticals (including oils), natural pigments (after extraction), cosmetics (collagen), and in other industrial processes. Fish viscera and frames

represent a source of protein hydrolysate, a potential source of bioactive peptides, and of proteolytic enzymes, (e.g. pepsin, trypsin, chymotrypsin and collagenases, lipase enzymes). Protease is a digestive enzyme used in the manufacture of cleaners to remove plaques and dirt, and in food processing. Fish bones are a source of calcium that can be used in food, feed or as supplements.

Another category of by-products is shell of crustaceans and bivalves. The increasing mussels and crustaceans' production together with their slow shell degradation rate, make their efficient utilization of remarkable importance. Chitosan, produced from shrimp and crab shell, is used in water treatments, cosmetics and toiletries, food and beverages and pharmaceuticals. Crustacean wastes yield pigments (carotenoids and astaxanthin) used in the pharmaceutical industry. Mussel shells provide calcium carbonate for industrial use and, in some countries, oyster shells are used in building construction and the production of quicklime (calcium oxide). Shells can also be processed into pearl powder (used in medicine and cosmetics manufacturing) and shell powder (a rich source of calcium that serves as a diet supplement in feeding livestock and poultry).

In addition to the above-mentioned fish quantities, in 2016, about 31 million tonnes of seaweeds and other algae were harvested for direct consumption or further processing for food (traditionally in Japan, the Republic of Korea and China) or for use as fertilizer and in pharmaceuticals, cosmetics and other purposes. Several seaweed species have a high nutritional value due to their abundance of natural vitamins, minerals, and plant-based protein. However, seaweeds are characterized by a variable composition, depending on species, collection time and habitat. More research is also exploring the use of seaweed as an alternative to salt in the industrial preparation of biofuel.

1.1.3. Fish consumption

The significant growth in fisheries and aquaculture production has enhanced the world's capacity to consume diversified and nutritious food. A healthy diet has to include sufficient proteins containing all essential amino acids, essential fats (e.g. long-chain omega-3 fatty acids), vitamins and minerals. Fish represent a rich source of these nutrients and provides about 34 calories per capita (daily global average) that can exceed 130 calories per capita in countries that are lacking of alternative protein food and where fish is part of the food habit and tradition (e.g. Iceland, Japan, Norway, the Republic of Korea and several small island States).

In terms of animal protein, 150 g of fish provides about 50–60% of

the daily protein requirements for an adult. Fish proteins represent a crucial component in countries where the dietary pattern reveals heavy dependence on staple food. For example, fish contributes 50% of total animal protein intake in some small island developing States, as well as in Bangladesh, Cambodia, Ghana, Indonesia, Sierra Leone and Sri Lanka. In these countries, fish consumption becomes particularly important in improving the calories/protein ratio. Also, for these populations, fish often represents an affordable source of animal protein because it is cheaper than other animal protein sources and is part of local and traditional recipes.

Fish contribution to nutritional intake changes considerably between and within countries, due to the availability and cost of fish, and to the accessibility of fishery resources, socioeconomic and cultural factors (e.g., food traditions, eating habits, tastes, demand, seasons, prices, marketing, infrastructure and communication facilities). Despite their relatively lower levels of fish consumption, developing countries and LIFDCs (low-income food-deficit countries) have a higher share of fish protein in their diets compared with developed countries. In 2015, fish accounted for about 26% of animal protein intake in developing countries and about 19% percent in LIFDCs, while in developed countries the amount of fish in animal protein intake was 11.4%. World per capita apparent fish consumption increased from an average of 9.0 kg in the 1961s to 20.2 in 2015 (Table 2) (FAO, 2018a). The increase in fish consumption is due not only to the increase in production, but also reductions in wastage, better utilization, improved distribution channels, and growing demand linked to population growth, rising incomes and urbanization. International trade had also an important role in providing a wider range of choices to consumers.

Table 2. Total and per capita apparent fish consumption by region and economic grouping, 2015

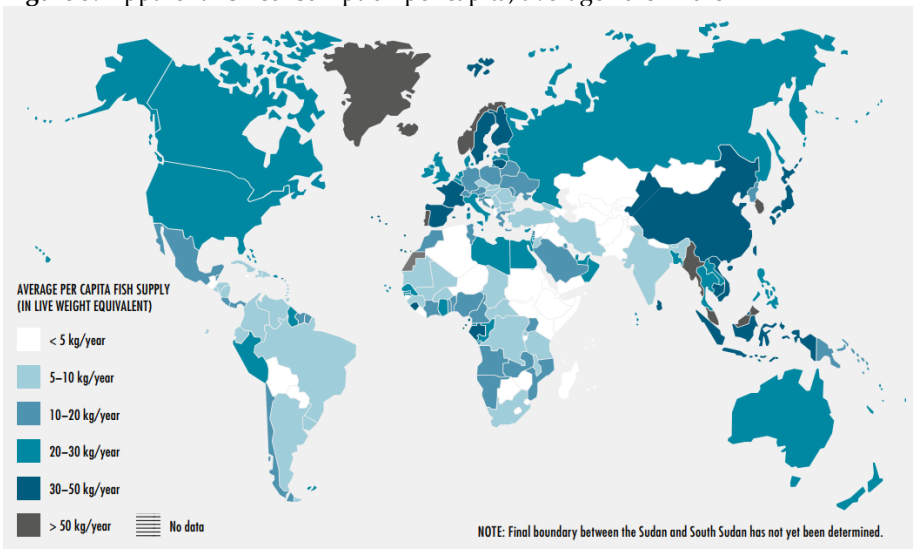
Region/economic grouping	Total food fish consumption *(million tonnes live weight equivalent)*	Per capita food fish consumption *(kg/year)*
World	148.8	20.2
World (excluding China)	92.9	15.5
Africa	11.7	9.9
North America	7.7	21.6
Latin America and the Caribbean	6.2	9.8
Asia	105.6	24.0
Europe	16.6	22.5
Oceania	1.0	25.0
Developed countries	31.4	24.9
Least-developed countries	12.0	12.6
Other developing countries	105.4	20.5
Low-income food-deficit countries	20.8	7.7

NOTE: discrepancies with Table 1, page 9, are due to the impact of trade and stock data in the overall calculation of the FAO Food Balance Sheets (FAO, 2018b). Source: FAO. 2018a. The State of World Fisheries and Aquaculture 2018.

Europe, Japan and the United States of America together accounted for 20% of the world's total food fish consumption in 2015. In 2015 Asia consumed more than two-thirds of the global total of 146 million tonnes (106 million tonnes at 24.0 kg per capita). Oceania and Africa consumed the lowest share (1 million tonnes and 11.7 million tonnes, respectively) (Table 2). This shift is due to the increased fish production in Asian countries, and to the gap between the economic growth rate of mature fish markets and the emerging markets, particularly in Asia. Although consumers in many advanced economies have a wide choice of fish products and are not deterred by price increases, their per capita consumption levels is near the saturation point in terms of quantity.

Annual per capita apparent fish consumption varies from less than 1 kg in one country to more than 100 kg in another (Figure 3). Disparities in fish consumption also exist between the more-developed and less-developed countries. Annual per capita consumption of fish has grown constantly in developing regions (from 6.0 kg in 1961 to 19.3 kg in 2015) and in low-income food-deficit countries (from 3.4 to 7.7 kg), but it is still lower than that in more developed regions (24.9 kg per capita consumption in 2015), even though the gap is narrowing. Fish consumed in developed countries consists mostly of imports, due to steady demand and static or declining domestic fishery production. While in developing countries, where fish consumption is usually based on locally available products, consumption is driven more by supply than demand. However, emerging economies are experiencing a diversification of the types of fish available owing to an increase in fishery imports.

Figure 3. Apparent fish consumption per capita, average 2013 - 2015



Source: FAO. 2018a. The State of World Fisheries and Aquaculture 2018.

Species such as shrimps, salmon, bivalves, tilapia, carp and catfish (including *Pangasius* spp.) contributed to the growth rates of per capita consumption in recent years. Since 2000, average annual growth rates have been significant for freshwater fish (3.1%), molluscs, excluding cephalopods (2.9%) and crustaceans (2.8%). In 2015, global per capita consumption of freshwater fish was 38% of the total, as compared with 17% in 1961.

Although fish producers and marketers are responsive to the evolution of consumers preferences, natural resources limitations and biological considerations influence which species and products can be available to consumers. This characteristic is reflected in the rapid growth of the aquaculture since 1980s, coinciding with the relatively stable fisheries production since 1980s. Aquaculture producers can exercise more control over fish production processes than capture fisheries and are able to address consumer concerns related to sustainability and product origin more easily than fishery producers (FAO, 2018a).

Consumption of fish is being influenced by globalization in food systems and improvements in processing, transportation, distribuition and food technology. They allow lower costs and also wider choices, thanks to imports. Also, long-distance refrigerated transport facilitates the trade and consumption of a major variety of species and product forms. Consumer habits are changing, and their consumption decisions are influenced by topics such as sustainability, health and well-being. World food markets are becoming more flexible, with new products, including value-added products that are easier for consumers to prepare (FAO, 2016).

The rise in fish consumption is also being boosted by modern retail channels (e.g. supermarkets and hypermarkets). This is a major shift from a few decades ago when traditional fishmongers and municipal markets were the main retail outlets in most countries. Retail chains and supermarkets are also increasingly driving consumption patterns, particularly in developing countries, offering consumers a wider choice, reduced seasonal fluctuation in availability and, often, safer food (FAO, 2016).

1.2. Fish in human nutrition and food security

The fisheries and aquaculture sector contribute to improving food security and human nutrition and have an important role in the fight against hunger (FAO, 2018a). "Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (World Food Summit, 1996).

In addition to providing nutrients, fish contributes to the food and nutritional security in developing countries. In fact, given its availability and affordability, it is the main source of animal protein in many developing countries (FAO 2016). Also, it allows livelihood diversification and income generation (Béné et al., 2015; Thompson and Amoroso, 2014). In this contest, FAO assists nations to achieve a successful management of fisheries and aquaculture, enabling a worldwide consumption of freshwater fish species and marine species.

1.2.1. The health benefits of fish

Fish play an important role in human diet because of its high nutritional value. Different fish have different nutritional qualities (e.g. "white fish" and "oily fish") that may vary according to the different aquaculture techniques, such as different types/levels of feeds (HLPE, 2014). All fish species have a specific chemical profile changing with seasons, reproductive phase, geographic origin and aquatic environment characteristics.

***Protein*:** fish is an excellent source of high-quality protein containing several essential amino acids (e.g., lysine and methionine), and bioavailability of protein is approximately 5 to 15% higher than that from plant sources (WHO, 1985). However, without proper hygiene or storage conditions, protein quality is easily destroyed by bacteria and viruses. Therefore, to conserve the high nutritional value of fish protein, proper processing and conservation methods are critical (FAO/WHO, 2012).

***Lipids*:** fish is a rich source of long-chain omega-3 fatty acids (LC-PUFAs) in the form of arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA cannot be synthesized in significant amounts by the human body and must be obtained through the diet. The main source of EPA and DHA is fatty fish (Williams and Burdge, 2006). Among fish species, small pelagic fish such as anchovy and sardine are perhaps some of the richest sources of LC-PUFAs (USDA United State Department of Agriculture, 2011). Another omega-3 fatty acid is alpha-linolenic acid (ALA), that

can be found in plant-based oils. It is a precursor to EPA and DHA but is converted at very low rates in the human body (<10%) (Williams and Burdge, 2006). Intake of long-chain fatty acids of marine origin has been associated with reduced risk of coronary heart disease (CHD) (Konig et al., 2005; Whelton et al., 2004), sudden death (Mozaffarian, 2008; C. Wang et al., 2006) and stroke (Bouzan et al., 2005; He et al., 2004), increased duration of gestation (Nesheim and Yaktine, 2007), and improved visual and cognitive development (Brenna and Lapillonne, 2009; Fleith and Clandinin, 2005; Nesheim and Yaktine, 2007). Some other health effects associated with EPA/DHA consumption include alleviation of colitis (Hudert et al., 2006) and rheumatoid arthritis (Kremer, 2000), reduced cognitive decline, dementia, depression, and suicides (Calon and Cole, 2007; Hibbeln, 2008; Morris et al., 2005; Sontrop and Campbell, 2006; van Gelder et al., 2007), and decreased likelihood of macular degeneration (SanGiovanni et al., 2007). Fish intake also influences the PUFAs levels in the breast milk of lactating women, which contribute to visual and cognitive human development during the first 1'000 days of life (Roos, 2016). These fatty acids are also transferred from the mother to the unborn child, and foetal DHA status of premature infants is positively related to head circumference, birth weight and birth length (Hornstra et al., 1995). PUFAs affect positively learning ability, measured as reading and spelling improvements in 5–12 years old (Richardson and Montgomery, 2005). Human diet in industrialized countries is often characterized by a high consumption of fats, particularly saturated fats, essential n-6 fatty acids and by a low intake of n-3 PUFA. This results in a fatty acid imbalance, high cholesterol serum levels and altered lipid metabolism. Based on the identification and characterization of the health benefits of EPA/DHA intake, various health organizations developed guidance values and nutritional recommendations (Table 4). The Guidelines for a Healthy Diet of the Italian National Research Institute for Food and Nutrition recommend intake of 1 g/day of EPA+DHA (for an healthy adult on a 2000 kcal diet), along with the reduction of the caloric intake, the increase of physical activity and the consumption of vegetables and fruits (FAO, 2016).

Table 4. Health guidance values for beneficial compounds. Source: Hellberg et al., 2012.

Compound	Guidance value	Target population	Source
DHA+EPA	500 mg/d	1a	ISSFAL 2004
	250 mg/d	1c	FAO/WHO 2008
	300 mg/d*	4a	FAO/WHO 2008
	1000 mg/d	5	AHA
	800 mg EPA+DHA	1b	NATO
DHA+EPA/seafood	Two, 113-g servings of seafood	1b	DGAC 2010

	per week, equivalent to about 250 mg/d		
Omega-3 fatty acids from fish	450 mg/d	All	HCN
Long-chain omega-3 fatty acids	500 mg/d 250 mg DHA and 500 mg EPA + DHA	1b 4b	AFFSA 2010
Fish	Two servings of fish per week, especially fatty fish	1b	AHA
PUFA	500 mg/d long-chain PUFA	1b	ADA
ALA: 10% EPA+DHA	1.6 g/d approximately 10% EPA+DHA 1.1 g/d approximately 10% EPA+DHA	2, 3	IOM 2005
n-3 PUFAs	1% to 2% of energy/d	1b	WHO/FAO 2002
DHA	200 mg DHA	4c	WAPM

* Of which at least 200 mg/d should be DHA.
1. (a) Healthy adults; (b) general adult population; (c) adult males and nonpregnant/nonlactating adult females.
2. Adult men.
3. Adult women.
4. (a) Adult pregnant and lactating females; (b) pregnant women; (c) pregnant and lactating women.
5. Patients with cardiovascular disease.

***Micronutrients*:** fish contains vitamins (D, A and B1, B2 and B3, thiamine, riboflavin and niacin) and minerals (including calcium, iodine, zinc, iron and selenium) (Bonham et al. 2009, Roos et al. 2007, Roos, Islam & Thilsted 2003) that reduce the risks of malnutrition and chronic diseases that may co-occur when high energy intake is combined with a lack of balanced nutrition (Allison et al., 2013). Vitamin A is required for normal vision and bone growth; vitamin D is essential for bone growth since it is involved in calcium absorption and metabolism. Thiamin and riboflavin contribute to glucose conversion and niacin guarantee the normal function of the nervous system. Recent research (Kawarazuka and Béné, 2011; Roos et al., 2007b) suggests that fish species that are consumed whole (usually small fish) play a critical role in micronutrient intakes as micronutrients concentrate mostly in bones, heads and viscera. The contribution that fish, even in small quantity, may offer to address multiple micronutrient deficiencies, such as phosphorous deficiency in LIFDCs or B-vitamins deficiency, is being increasingly recognized. Fish should therefore be an integral component of the diet, preventing malnutrition by making macro- and micro-nutrients readily available to the body.

1.2.2. Fish and food safety

The positive health effects of fish are counterbalanced by the potential negative effects associated with contamination/safety risks related to toxins/poisoning from harmful algae, bacteria, virus, and chemical pollutants (Table 5). The most significant health hazards

from marine organisms are heavy metals such as methylmercury, cadmium and organic tin (STAP 2012). Mercury is released into the environment from both natural and anthropogenic sources and is converted into MeHg by aquatic microorganisms (Rasmussen et al., 2005).

Table 5. Types of fish- and seafood-borne illnesses

Types of illness		**Causative agent**
Infections	Bacterial infections	*Listeria monocytogenes, Salmonella* sp., *Escherichia coli, Vibrio vulnificus, Shigella* sp.
	Viral infections	Hepatitis A virus, Norovirus, Hepatitis E
	Parasitic infections	Nematodes (round worms), cestode (tapeworms), trematodes flukes)
	Toxi-infections	*Vibrio cholerae e Parahaemolyticus, Escherichia coli, Salmonella* sp.
Intoxications	Microbial	*Staphylococcus aureus, Clostridium botulinum*
	Biotoxins	Ciguatera, paralytic shellfish poisoning (PSP), diarrhoeic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), histamine
	Chemical	Heavy metals: mercury, cadmium, lead. Dioxins and polychlorinated biphenyls (PCBs). Additives: nitrites, sulphites. Microplastics

Source: FAO, 2004

Methylmercury is found in small amounts in many fish species and bioaccumulates in the food chain. For this reason the highest levels are reached in predatory fish, with concentrations increasing with age and size (Storelli et al., 2001). Tuna consumption, particularly of the long-lived species, is reason for concern as tuna holds high methylmercury levels. Methylmercury was first recognized as a hazard in seafood after the industrial poisonings in the 1950s in Minimata Bay in Japan, and between 1955 and 1972 in Iraq (the latter was due to the consumption of wheat seeds coated with alkylmercury fungicide). These events demonstrated that organic mercury have neurotoxicological effects and that foetus have a heightened sensitivity to high levels of exposure (Watanabe and Satoh, 1996). Methylmercury also affect the peripheral nervous system in adults and the central nervous system in children, where it can cause impaired cognitive and motor skills (Grandjean et al., 2004).

Metals other than mercury may be found in seafood, although seafood does not seem to be a main route of exposure to these metals (Hellberg et al., 2012). Cadmium may be found in large amounts in invertebrates, such as in squid, in brown meat of crab, and in heads of shrimp. Organic tin compounds are found in bivalves as a result of pollution, especially in harbours since tin compounds were used in antifouling agents for boats (FAO/WHO, 2011).

Dioxins and PCBs represent another significant health hazards.

PCBs are a group of 209 congeners that were widely used commercial and industrial applications. Dioxin is a collective term for a group of toxic chemicals with a similar mechanism of toxicity which includes 7 congeners of polychlorinated dibenzodioxins (PCDDs) and 10 congeners of polychlorinated dibenzofurans (PCDFs). Most human exposure to dioxins and PCBs is through the diet, specifically from animal fats found in meats, seafood, and dairy products (Hellberg et al., 2012). Fat from marine fish is often the most important source of intake of dioxins and PCBs (FAO/WHO, 2011). Their concentrations in marine organisms result from local pollution (FAO/WHO, 2011), and they are a problem in lower organisms such as bivalves because they are less able to convert and excrete PAH (Polycyclic aromatic hydrocarbons) compounds.

CBs and dioxins can bioaccumulate and have been characterized by the United States Environmental Protection Agency (EPA) as likely human carcinogens. Noncancer effects, including changes in hormone levels and foetal development, have been observed at levels of about 10 times above the normal background, by the EPA. As long as maternal exposure to dioxins (from fish and other dietary sources) does not exceed the Provisional Tolerable Monthly Intake (PTMI) of 70 pg/kg body weight, neuro-developmental risk for the foetus is negligible. If maternal exposure to dioxins (from fish and other dietary sources) exceeds the PTMI, neuro-developmental risks for the foetus may "no longer be negligible" (FAO/WHO, 2011). In general, adult men and women have daily TEQ (Total dioxin-like toxic equivalency) intakes of 2.4 and 2.2 pg/kg bw, respectively. About 9% of dietary exposure is from fish and shellfish (Schecter et al., 2001), primarily from fish caught in fresh waters, estuaries, and near-shore coastal waters rather than the open ocean. The European Community has established a maximum level for dl PCBs and dioxins in seafood of 8 pg TEQ per g seafood (ppt TEQ) (European Commission, 2006) and the U.S. FDA has set a tolerance level for PCBs in seafood of 2.0 ppm (FDA, 2011). The greatest risk to human health from POPs occurs when fish is harvested recreationally from contaminated waters and consumed in large amounts by subsistence anglers, pregnant women, and young children. When making risk assessments, the EPA assumes there is no "safe" lower threshold for carcinogenic compounds, signifying any exposure may pose some cancer risk. Limited toxicological information exists on algal toxins.

Another risk to human health is from pathogens. Bacteria in fish products can come from the group *Listeria*, *Salmonella, E. coli, Campylobacter, Yersinia, Shigella* and *Vibrio.* Bacteria increase is due to lack of hygiene during processing operations, and pathogens risk can

be managed through proper hygiene practices, cooking, handling, and storage. Hygiene and processing method also affect the presence of viruses, especially hepatitis A and Norovirus. Filtering molluscs are also known to accumulate bacteria or viruses found in water. Thus, when fish and other seafood are involved in virus-borne diseases, most of the cases are caused by bivalves, especially oysters (Lees, 2000).

In recent years there has been a rapid increase of therapeutic and prophylactic usage of antibiotics/antimicrobial agents in aquaculture, including those important in human therapeutics, to overcome shortcomings in sanitary and unhealthy conditions in fish farming. Scientific evidence suggests that unrestricted use of antibiotics is detrimental to fish, human health and environment, and efforts are needed to prevent antibiotic/antimicrobial resistance in aquaculture to reduce the risk to human health (Cabello, 2006; Cabello et al., 2013; Heuer et al., 2009; Serrano, 2005). For this reason, many countries have put in place stringent regulations on the use of antibiotics/drugs/chemicals in aquaculture/animal food production.

In 2010, FAO and WHO experts emphasized that fish consumption reduces mortality due to coronary heart disease in the adult population and improves the neurodevelopment of foetuses and infants and is therefore important for women of childbearing age, pregnant women and nursing mothers, outweighing the health risks associated with mercury and dioxins. Experts tend to agree that the positive effects of fish consumption overcome the potential negative effects associated with contamination risks among the general population, especially when a variety of fish is consumed at least twice per week (FAO/WHO, 2011; Hellberg et al., 2012; Hoekstra et al., 2013; Mozaffarian and Rimm, 2006). However, together with the potential consumption of high-MeHg seafood by sensitive populations, underconsumption of EPA + DHA is reason of concern: messaging regarding seafood consumption that try to "protect" an at-risk population, can have negative unintended consequences on that group and on the overall population (Hellberg et al., 2012). Studies have shown that messaging reduces fish consumption in general, in both the target and nontarget populations, resulting in an overall reduction in the potential health benefits derived from EPA + DHA. For this reason, messaging must not exclude the benefits of fish consumption, and a more targeted approach is needed to ensure that both non-target and target population consume fish that are low in contaminants but high in omega-3 f atty acids. The majority of research has shown that seafood consumption greatly outweighs the risks, but this field of science is at the incipient stages of determining

how to accurately assess the everyday choices we make in our diet and how these ultimately affect our lives (Hellberg et al., 2012).

1.3. Subjects

1.3.1. Marketing of lobsters: animal welfare and product quality

The European Union's Lisbon Treaty recognizes animals as sentient beings, meaning that they are capable of feeling pleasure and pain. In recognition of this, the European Commission has adopted the EU Animal Welfare Strategy 2012–2015, a new strategy to improve welfare conditions for animals kept on farms, living in zoos and used for experiments (European Commission, 2015).

In applied animal welfare science, it is generally accepted that the quality of life of farmed animals is of paramount importance not only for an ethical reason, but also because it affects quality and safety of animal products (EFSA, 2005). Lack of animal welfare and health can pose risks to consumers, for example, through common food-borne infections (European Commission, 2004). The welfare of food-producing animals depends largely on their management and it is influenced by several factors, such as housing, space and crowding, transport conditions, and slaughter methods (EFSA, 2005; European Commission, 2015).

The global total consumption of seafood products is increasing and there is a growing worldwide market demand for crustaceans. In Europe, lobsters are available throughout the year: *Homarus americanus* is caught especially in North America and its global landing has increased in the last decade, passing from 85'643 tonnes in 2007 to 157'805 tonnes in 2017; *Homarus gammarus* is mainly captured in the North-Eastern Atlantic countries (United Kingdom, Ireland, Channel Islands and France) and its global production reached 5'517 tonnes in 2017 (FAO, 2020). *Homarus Gammarus* catches in the Mediterranean Sea decreased (Phillips and Wahle, 2013), for this reason about 4'387 tons of American lobster are annually introduced in Italy for an economic value of 44 million of euros (Barrento et al., 2009).

Consumer perception on the respect of health, welfare, and quality of lobsters during marketing is increasing (Esposito et al., 2018) and the objectives of food business operators and retailers should correspond to consumers' demands (Sans et al., 2005). Currently, the European legislation lacks specific regulations on crustacean welfare. The current norm is to set minimum standards for animal storage and to provide only guidelines for invertebrate welfare, such as those from the Scientific Panel on Animal Health and Welfare (EFSA, 2005) and

of the Sea Fish Industry Authority (Jacklin and Combes, 2007). According to EU Regulation n. 853/2004, crustaceans that are offered for sale alive are defined as "seafood" and must be kept at a temperature and under conditions that do not adversely affect food safety and their viability. Some species of crustaceans, including lobsters, are marketed alive and stored for long periods in supermarkets or restaurants' aquariums. Keeping these animals alive is considered a good and fair practice by many consumers and gives the best commercial quality, because it decreases the microbiological risk and promotes a better nutritional organoleptic quality (EFSA, 2005; Esposito et al., 2018). Furthermore, these animals are highly perishable, and they can exhibit off-odours and deterioration of organoleptic qualities within a short period after death (Coppola et al., 2019). The European legislation does not fix limits on the parameters of storage, ban on feeding or safety measures for food business operators (e.g., clasped claws). In restaurants and supermarkets, the retail staff usually manage water parameters (temperature, pH, nitrites), while third parties carry out inspections and maintenance of the aquaria twice a month or every six months (Esposito et al., 2018; Sabbioni, 2015). For these reasons, the marketing of live lobsters has been subjected to legal interpretation and the scientific community has been showing a growing ethical concern toward their welfare.

Scientific studies suggest that lobsters show a complex behaviour and physiological responses to noxious stimuli that provide evidence of their capacity to experience pain and stress (Baker, 1955; Horvath et al., 2013; Yue, 2008). Thus, handling and conservation of these animals during marketing are practices that must meet minimum welfare requirements (Candotti, 2007; Liuzzo et al., 2017). Despite the significant contributes to the welfare of live crustaceans (Barrento et al., 2009; Basti et al., 2010; D'Agaro et al., 2014; Lorenzon, 2005; Lorenzon et al., 2007) several objectives have not been considered by international research (e.g., assessment of distress and health status of crustaceans maintained in commercial holding tanks, hygienic status of crustaceans found dead on arrival or in the holding tanks, the most appropriate slaughtering method). Few scientific researches consider crustaceans slaughter, describing the most suitable methods, such as electrical stunning (Neil and Thompson, 2012; Roth and Øines, 2010), anaesthetics (Kildea et al., 2004) and splitting. In 2005, EFSA wrote a report on the protection of animals used for scientific and experimental purposes (EFSA, 2005). The conclusion was that the largest of decapod crustaceans are able to feel pain and distress.

Despite this opinion there is no legislation for crustacean in European Union.

Studies have been conducted to investigate the effect of potential "stressors" (e.g., commercial maintenance methods air exposure vs water immersion; claw ligatures; confinement; starvation) on haemolymph stress parameters of American lobsters (Basti et al., 2010; Bernardi et al., 2015; Coppola et al., 2019; D'Agaro et al., 2014; Lorenzon et al., 2007). The improved knowledge of the haemolymph parameters may help to find the most significant biomarkers of stress in the American lobster. Furthermore, an increased comprehension of the effect of different commercialization methods on physiological modifications may lead to better commercial practices with consequent better survival, animal welfare and food quality. Defining the welfare status of crustaceans is difficult and the identification of reliable welfare indicators represents a challenge, since an accurate evaluation can only be made through the integration and interpretation of several indicators (e.g., haemolymph parameters, short and long-term effects concerning behaviour and pain). Despite their reliability as stress parameters, haemolymph indicators may be of difficult interpretation because stress response is modulated by several factors. Development in technologies such as proteomics, genomics and metabolomics could help to identify stress and/or welfare indicators and understand the mechanisms involved in stress-related process (Alves et al., 2010). Some studies identified metabolic molecular indicators of stress in fish and seafood by using proteomics: 33 different proteins have been identified in Chinese shrimp as a result to stress exposure by hypoxia (Jiang et al., 2009), and potential metabolic molecular indicators of stress were identified in gilthead seabream (*Sparus aurata*) (Alves et al., 2010). It is possible that proteins found by Jiang and Alves may be present in crustaceans and become part of a panel of welfare biomarkers, after appropriate validation studies.

Further research should also aim at investigating microbial ecology and shelf-life of lobsters found dead during transport and housing. A study conducted by (Gornik et al., 2011) provided information about the bacterial load and microbial composition in Norway lobster (*Nephrops norvegicus*) under commercially relevant storage conditions; while (Barrento et al., 2009) studied the proximate chemical composition, energy, fatty acid and amino acid profiles, and cholesterol content in edible tissue of European Lobster *Homarus gammarus* and American Lobster *Homarus americanus*. However, one

specific study (Tirloni et al., 2016) has been conducted so far on the microbiological quality of American lobsters' meat in terms of spoilage microbiota. Results of the study showed that marine *Pseudoalteromonas* spp. and *Photobacterium* spp. are the main bacteria isolated from meat samples. Very limited microbial loads were detected (average < 5 log CFU/g), suggesting a good hygiene quality and the possibility to commercialise these subjects without particular quality faults (FDA, 2013).

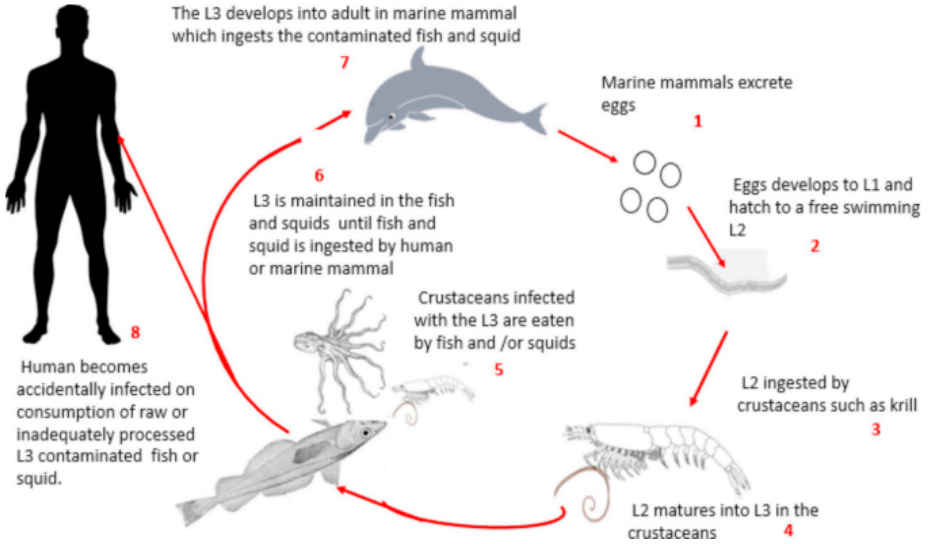
Considering the global context, characterized by a growing population and an increased demand for food, new strategies are necessary to reduce losses and waste all along fish food chains, particularly for fresh fish (HLPE, 2014). Throughout the world, 27% of landed fish is lost or wasted between landing and consumption. When discards prior to landing are included, 35% of global catches are lost or wasted and therefore not utilized (Gustavsson et al., 2011). Despite the increasing role of aquaculture in global fish supplies, the capture sector is expected to remain dominant in the supply of many species, such as lobster, and to be vital for domestic and international food security (FAO, 2016). For these reasons, further studies are needed to investigate the microbial ecology of crustaceans found dead (e.g., after landing, during storage) in order to understand if these products can be commercialized. All of the scientific data obtained may be useful to widen the knowledge on the topic and to provide the basis for the development of a management strategy aiming at reducing useless discards.

1.3.2. Molecular identification of *Anisakis* spp.

1.3.2.1. Anisakiasis in humans

Anisakiasis is an important fish-borne zoonosis caused by members of the family Anisakidae. This family includes the genera *Anisakis*, *Pseudoterranova*, *Hysterothylacium* and *Contracaecum* (Smith and Wootten, 1978). *A. simplex* sensu stricto, *Anisakis pegreffii* and *Anisakis physeteris* and the species *Pseudoterranova decipiens* are reported to be the causative agent of infections in humans (Simonetta Mattiucci and Nascetti, 2008). *Contracaecum* is rarely found in association with gastric/intestinal illness, while *Hysterothylacium* is commonly considered not pathogenic. Anisakis typically parasitizes adult marine mammals. Intermediate and/or paratenic hosts of the larvae are crustaceans, cephalopods and fish. Humans are accidental hosts and become infected after ingestion of raw or undercooked marine fish and cephalopods contaminated with viable L3 stage larva (Fig. 4).

Fig. 4. Anisakis life cycle. Location of the L3 larvae may be gastric, intestinal or ectopic.



Picture source: Aibinu et al., 2019.

After ingestion, the parasite is usually regurgitated or become dead in a few days or weeks. If not, it can cause gastrointestinal infection, which may be classified as acute, chronic, ectopic or allergic reactions (Bucci et al., 2013).

In gastric anisakiasis, parasite penetrates into the greater curvature of the stomach body (Kakizoe et al., 1995; Shimamura et al., 2016) and causes acute gastrointestinal symptoms (e.g., abdominal pain, nausea and vomiting) that mimic gastrointestinal disorders such as gastric ulcer and acute appendicitis (Audicana and Kennedy, 2008a). For intestinal anisakiasis, non-specific symptoms such as nausea, vomiting or diarrhea develop within 5 days after consumption of infected fish (Takabayashi et al., 2014). Gastric anisakiasis represents 95% of the disease burned, with intestinal anisakiasis accounting for the remaining burden (Kojima et al., 2013). However, intestinal anisakiasis is frequently misdiagnosed with other diseases such as inflammatory bowel disease, bowel obstruction, ulcer, acute appendicitis, diverticulitis, ileitis or cholecystitis (Miura et al., 2010) and this might have resulted in the low record of intestinal anisakiasis.

Clinical manifestation of anisakiasis has been associated with allergy reactions in some individuals (Alonso-Gómez et al., 2004; Alonso et al., 1997; Anadón et al., 2009; Audicana et al., 2002; Choi et al., 2009; Guardone et al., 2018; Ivanović et al., 2017; Mazzucco et al., 2018; Rodríguez-Mahillo et al., 2010; Shimamura et al., 2016). The parasite causes an IgE-mediated allergic reaction characterized by urticaria and gastrointestinal response and, in highly sensitized subjects, angioedema, asthma, and anaphylaxis (Baird et al., 2014). The allergic response to Anisakis has been associated with A. *simplex* and

Anisakis pegreffii, with acute urticaria and angioedema affecting 20% of the population, particularly young adults (Kaplan, 1992). This condition is usually self-limiting, however it is unpleasant due to the intense itching, inability to sleep and even disfigurement, when angioedema is present. In 30% of the cases, angioedema is associated with urticaria and it is potentially lethal due to glottic oedema.

The ingestion of dead parasites or fragmented parts of the parasite in contaminated fish might result in allergic symptoms after consumption. Occupational allergies in aquaculture and fishery workers, cooks and fishmongers have also been reported after inhalation or contact with *A. simplex* allergens (Nieuwenhuizen et al., 2006; Nieuwenhuizen and Lopata, 2014, 2013).

Scientific community is still debating on whether live or dead larva is necessary to induce allergic reaction (Audicana et al., 2002). Authors reported that some allergens from *A. simplex* are highly resistant to heat and pepsin treatments, therefore the ingestion of dead parasite or residues might cause allergic symptoms (Caballero and Moneo, 2004; Moneo et al., 2005). A case of anaphylaxis associated with the ingestion of cooked hake (*Merluccius merluccius)* parasitized with the third stage larvae of *A. simplex* has been reported in the North of Spain (Audicana et al., 1995). The patient was not only sensitive to the ingestion but also to the handling of Anisakis parasitized fish. A further 28 cases of people allergic to *A. simplex* have been diagnosed by the same authors.

Other authors reported that allergic sensitivity to *A. simplex* is initiated by an active infection with live parasite (Alonso-Gómez et al., 2004) and challenges studies with thermally treated *Anisakis* larvae revealed that patients did not show any allergic or gastric symptoms during or after the challenge (Pravettoni et al., 2012). For this reason, some authors accept the general assumption that allergic reactions may occur after the primary infection with viable larva and exposure to allergenic proteins in the food (Ivanović et al., 2017; Pravettoni et al., 2012). However, the evidence of variations in people allergic response to Anisakis and the identification of the allergens involved still remains to be clarified.

To date, 14 allergens have been identified in *A. simplex* (Table 6). Humans might be exposed to somatic antigens (from dead larva in food), excretory-secretory antigens (ES) derived from expulsion or surgical removal of the intact larva, or both (Kaplan, 1992). Ani s 1 and Ani s 3 are considered pan-allergens of *Anisakis* (Guarneri et al., 2007). The remaining group includes 4 major allergens (Ani s 1, Ani s 7, Ani s 12 and Ani s 14) and 7 minor allergens (Ani s 4, Ani s 5, Ani s 6, Ani s 8, Ani s 9, Ani s 10, and Ani s 11).

Table 6. WHO/IUIS registered *Anisakis simplex* allergenic proteins

Allergen	Molecular weight (kDa)	Name
Ani s 1	21	Kunitz-type trypsin inhibitor
Ani s 2	100	Paramyosin
Ani s 3	33	Tropomyosin
Ani s 4	9	Cystatin
Ani s 5	15	SXP/RAL-2
Ani s 6	7	Serine protease inhibitor
Ani s 7	139	Ua3 recognized allergen
Ani s 8	16	SXP/RAL-2
Ani s 9	15	SXP/RAL-2
Ani s 10	23	Unknown
Ani s 11	30	Unknown
Ani s 12	33	Unknown
Ani s 13	37	Haemoglobin
Ani s 14	23.5	New major allergen

Anisakiasis is often misdiagnosed because of non-specific symptoms (EFSA, 2010). Direct diagnosis is usually performed through endoscopy or radiological examination, whereas indirect diagnosis uses various immunologic assays such as the skin-prick test, complement fixation test (CFT), immunofluorescent-antibody test (IFAT), immunodiffusion test (IDT), immunoelectrophoretic assays, enzyme-linked immunosorbent assay (ELISA), and radioallergosorbent test (RAST). However, interpretation of the serological tests and immunological assays may be difficult. In fact, serum from unaffected individuals may contain antibodies which can give false-positive results against *Anisakis* antigens. This is due to the cross-reaction of *Anisakis*' allergens tropomyosin and paramyosin (proteins in the muscle and skin of many invertebrates) to other seafood allergens such as shrimp and crustaceans (Reese et al., 1999). Furthermore, anisakiasis patients' serum cross-react with antigens from closely related nematode species (e.g. *Ascaris* and *Toxocara* species).

A scientific opinion from the European Food Safety Authority indicates protection and prevention as priorities and recommends a continuous research in parasites of public health importance in fishery products (EFSA, 2010). Food-borne anisakiasis is considered an emerging health risk and a predominant biological hazard associated to seafood, due to the high prevalence in the main commercially relevant species worldwide (e.g., cod, hake, sea bass, frog fish, ling, blue whiting, saithe, redfish, forkbeard) (Cavallero et al., 2017).

Parasitological studies for *Anisakis* larvae in consumed seafood is crucial for guarantee food safety. The presence of the parasite in the

edible flesh of the fish need to be evaluated as this poses the main hazard to public health (Cipriani et al., 2016). Moreover, due to the possibility of intra-vitam migration of larvae to the fish, the storage temperature after fish capture becomes crucial in the post-mortem motility of Anisakis larvae into the flesh (Cipriani et al., 2016). European regulation (European Commission, 2004) specifies freezing of fish products at -20 °C for 24 h, or at -35 °C for 15 h.

However, it is important to consider that allergenic proteins from Anisakis may still be present in food products after removal of intact worm (Audicana et al., 1997). The existence of *Anisakis* parasites in a variety of mesozooplankton organisms suggests that *Anisakis* species may not be specific to any intermediate host and may use any of the intermediate hosts to move from one habitat to another depending on the seasonal and ecological availability of such intermediate hosts (Gregori et al., 2015). This may result in *Anisakis* species expanding their pathways to find their definitive host. This implicates an increase in occurrence of anisakiasis with a potential increase in anisakiasis and allergic reactions accompanied by cross-reactivity to homologous proteins in other invertebrates.

1.3.2.2. PCR and LAMP as molecular identification methods for *Anisakis* spp.

In food analysis, nucleic acid amplification is a valuable tool for the identification of several potential pathogenic bacteria and parasites like *Salmonella* spp., *Listeria monocytogenes* and *Anisakis* spp. (Hara-Kudo et al., 2005; Tang et al., 2011; Erica Tirloni et al., 2017; Wang et al., 2010, 2012). Different methods are used for the detection of *Anisakis* larva in fish: non-destructive (visual inspection, candling, UV transillumination) and destructive (chloro-peptic digestion). These methods can be ineffective, laborious, expensive, and they are able to detect only the intact larva. For this reason, they are not applicable on processed products (e.g., surimi, cooked food, canned food, salted food). Furthermore, since *A. simplex* has been recognized as a food-borne allergen source than can resist thermic treatments, biomolecular techniques have been developed for its detection (Herrero et al., 2011).

The current immunological methods for *Anisakis* detection (e.g., ELISA) are not feasible because they have low specificity and cross-react with numerous pan-allergens, and also because anisakis larvae contains multiple allergens that can vary in abundance (Poms et al., 2004).

Molecular methods operating on *Anisakis* spp. DNA are widely used. The choice of method depends on the food concerned (availability of specific antibodies/DNA primers and the achievable

detection limit) and on the history of processing involved during food production (Poms et al., 2004). Different screening molecular-based methods (e.g., Real time PCR, SYBR green qPCR and multiplex PCR) are currently used for detecting the presence of *Anisakis* spp. in processed fish products (Cavallero et al., 2017; Herrero et al., 2011; Lopez and Pardo, 2010; Mossali et al., 2010; Zhang et al., 2010). Molecular approaches are relatively stable against environmental and technological influences and have the advantages of being more sensitive and of discriminating between species and genera, according to the specificity of the probes selected. This is particularly relevant in the evaluation of risk related to anisakiasis, given that pathogenic and not pathogenic anisakis species may be present in fish and fish products, and for revealing alimentary frauds as these nematodes may be used as valuable biological tags for stock discrimination (Mattiucci et al., 2014). However, the employment of DNA analysis in allergen detection is controversially discussed, because proteins are the allergenic component and DNA methods' results cannot be linked to any allergen/protein content (Lopez and Pardo, 2010).

As PCR methods require expensive tools and operational skills that can limit their broad applicability in fish products processing industry, Loop-mediated isothermal amplification (LAMP) methods have been developed in recent years. LAMP is considered a powerful innovative gene amplification technique emerging as a simple rapid diagnostic tool for early detection and identification of parasites and pathogens, both in food and in clinical diagnosis of infectious diseases (Goodarzi et al., 2018; Hara-Kudo et al., 2005; Parida et al., 2008). LAMP amplifies DNA at constant temperature (60–65 ◦C) without requiring special reagents and sophisticated temperature control devices, which make it suitable for on-site conditions. In the following, PCR and LAMP techniques are discussed.

PCR method (Polymerase Chain Reaction). PCR-based method is a molecular biology technique that allows exponential amplification of small quantities of target DNA sequences resulting in millions of copies of the original DNA (Walker-Daniels, 2012). PCR amplifies DNA by using Taq polymerase (a DNA polymerase from the thermophilic organism *Thermus acquaticus*), primers (oligonucleotide sequences, usually 15-40 bases long, complementary to the opposite strands of a specific DNA target) and nucleotides. A PCR cycle is characterized by three phases at three different temperatures, repeated 20-40 times.

1. Denaturation: temperature is maintained at 95 °C for 5 minutes and then for 30 seconds. The 30 seconds step is repeated 2-40 times. This

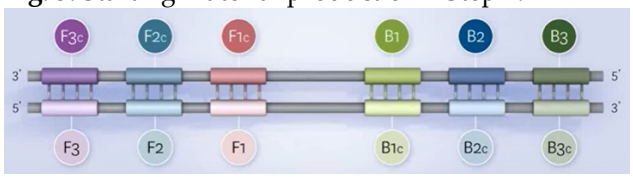
leads to the target DNA strands separation.
2. Annealing: temperature decreases to 60 °C, allowing the primers to anneal to each of the single DNA strand. This temperature is optimized in order to obtain the maximum annealing efficiency.
3. Elongation: Taq polymerase synthetizes DNA complementary to DNA strand template, adding deoxynucleotides at a temperature of 72 ° C, resulting in two double strand DNA copies.

Each round of temperature cycling results in two times more target sequence than the prior round. This leads to the exponential amplification of the original template. Then, the amplified PCR product can be visualized in many ways. The most commonly used technique is agarose gel electrophoresis with EtBr (ethidium bromide). EtBr is a fluorescent dye that intercalates into the DNA. Size markers can be electrophoresed on the gel to allow size determination of the PCR product. Another technique for PCR product visualisation is DNA sequencing. Sequencing allows to determine the order of nucleotides in PCR product. The result is later compared with known sequences of microorganisms deposited in international nucleotide databases, in order to identify the genus/species.

LAMP method. Loop-mediated isothermal amplification is a real time DNA-amplification technique firstly described by Notomi et al., 2000. This method amplifies and detect DNA in a single step under isothermal conditions (60 - 65 °C) with a set of four specifically designed primers which hybridize to six different parts of the target DNA sequence. The method uses *Bacillus stearothermophilus* thermostable polymerase (Bst polymerase) with strand displacement activity. Bst polymerase large fragment displaces third strand DNA during primer-initiated polymerization of new DNA leaving a new single stranded matrix DNA for further primer annealing and DNA polymerization (Fig. 5 – 14, HiberGene Diagnostics, 2020). Since Bst polymerase has a very high activity, vast amounts of high molecular weight DNA are produced within short time. The high specificity of LAMP is due to the fact that a set of four primers with six binding sites must hybridize correctly to their target sequence before DNA biosynthesis occurs. A third pair of primers (loop primers) can be added optionally to the reaction in order to further amplify the amount of DNA produced during LAMP (Nagamine et al., 2002) (Fig. 15 – 16, HiberGene Diagnostics, 2020). Once DNA has been amplified, DNA can be detected by using several methods. Mass production of DNA during LAMP leads to high risk of cross contamination of samples by aerosolized product. In order to prevent cross contamination, methods for in-tube detection of DNA amplification

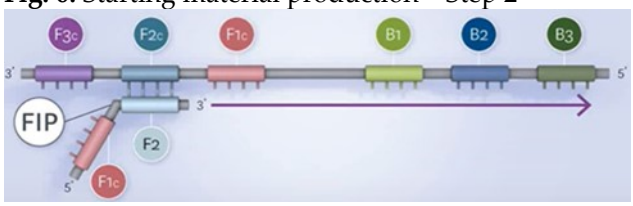
were developed. In-tube detection of amplified product was be achieved by direct staining of double stranded DNA using fluorogenic intercalating dyes (Iwamoto et al., 2003; Maeda et al., 2005; Notomi et al., 2000). Substances such like SYBR green (Noble and Fuhrman, 1998), EvaGreen (Ihrig et al., 2006; W. Wang et al., 2006), Hoechst 33285 (Latt et al., 1975), ethidium bromide (Higuchi et al., 1992), P2 (Yamamoto and Okamoto, 1995) and SYTO9 (Monis et al., 2005; Njiru et al., 2008) have been used.

Fig. 5. Starting material production – Step 1.



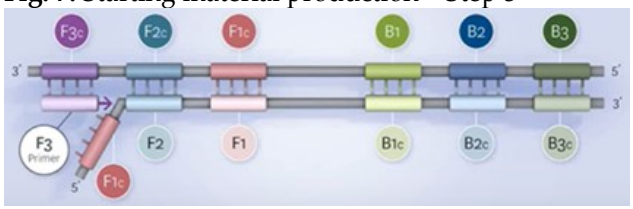
LAMP primers (two outer: F3 and B3; two inner: FIP and BIP) recognise six independent regions of target DNA (F3c, F2c, F1c; and B1, B2, B3).
Picture source: (HiberGene Diagnostics, 2020).

Fig. 6. Starting material production – Step 2



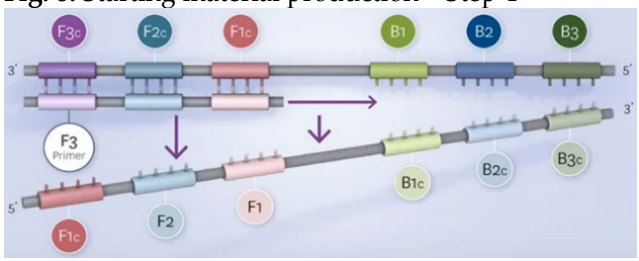
The Forward Inner Primer (FIP) binds to the F2c region of double strand target DNA and synthetises DNA in the 3′ -> 5′ direction by using the DNA polymerase with strand displacement activity.
Picture source: (HiberGene Diagnostics, 2020).

Fig. 7. Starting material production – Step 3



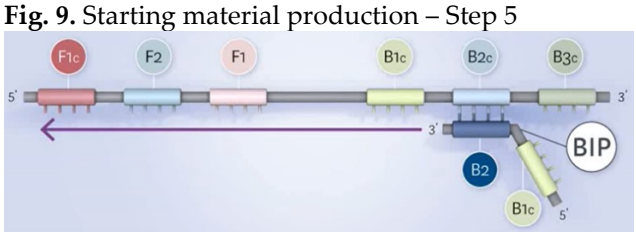
The F3 Primer (F3) binds to a region upstream from the FIP binding site.
Picture source: (HiberGene Diagnostics, 2020).

Fig. 8. Starting material production – Step 4



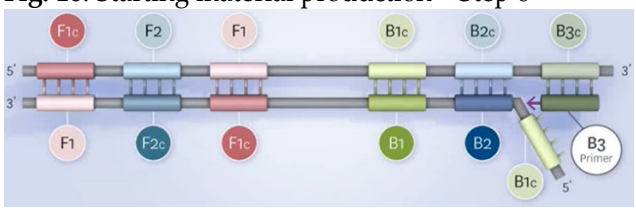
The F3 Primer displaces and releases the new strand formed by the action of the FIP as it synthetises complementary DNA.
Picture source: (HiberGene Diagnostics, 2020).

Fig. 9. Starting material production – Step 5



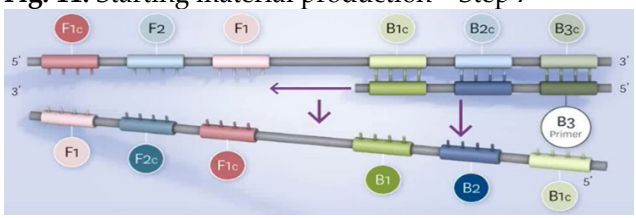
The Backward Inner Primer (BIP) binds to the newly-released complementary strand at the B2c region and synthetizes complementary DNA in the 3′ -> 5′ direction.
Picture source: (HiberGene Diagnostics, 2020).

Fig. 10. Starting material production – Step 6



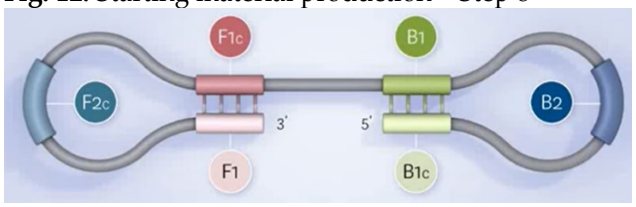
The B3 Primer (B3) binds upstream from the BIP binding site. Picture source: (HiberGene Diagnostics, 2020).

Fig. 11. Starting material production – Step 7



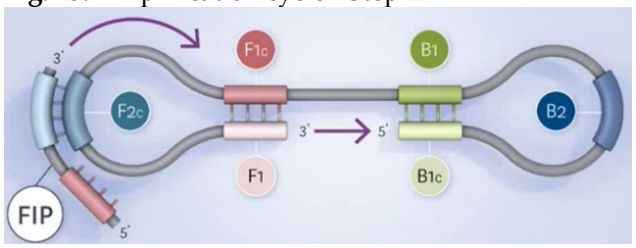
The B3 Primer displaces and releases the new strand formed by the action of the BIP as it synthetizes complementary DNA. Picture source: (HiberGene Diagnostics, 2020).

Fig. 12. Starting material production – Step 8



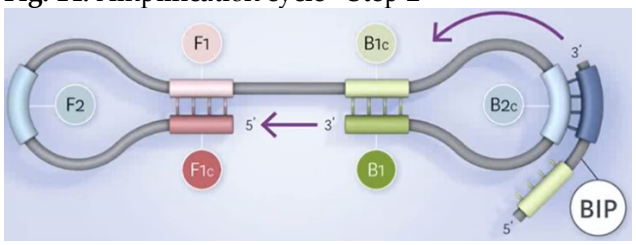
As the strand synthetized contains two complementary regions (F1 and F1c; B1 and B1c), these regions bind to each other, causing a "dumbbell" shape sequence. Picture source: (HiberGene Diagnostics, 2020).

Fig. 13. Amplification cycle - Step 1



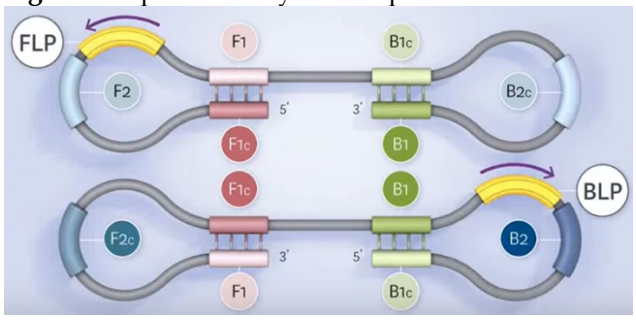
The dumbbell forms the basis for the cycling phase of the LAMP reaction. Binding of the FIP primer at the F2c region and self-priming at the F1/F1c region result in further synthesis.
Picture source: (HiberGene Diagnostics, 2020).

Fig. 14. Amplification cycle - Step 2



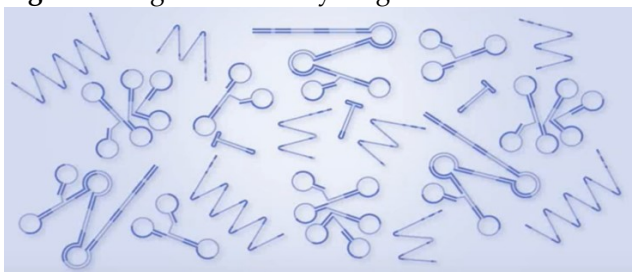
Release of a self-priming single-stranded structure with a sequence complementary to that of the initial dumbbell, and to which the BIP primer can bind. Picture source: (HiberGene Diagnostics, 2020).

Fig. 15. Amplification cycle – Step 3



Additional primers (Loop primers) may be included. Forward Loop Primers (FLP) bind to a sequence between the F1 and F2 target regions; Backward Loop Primers (BLP) bind to a sequence between the B1 and B2 target regions. Picture source: (HiberGene Diagnostics, 2020).

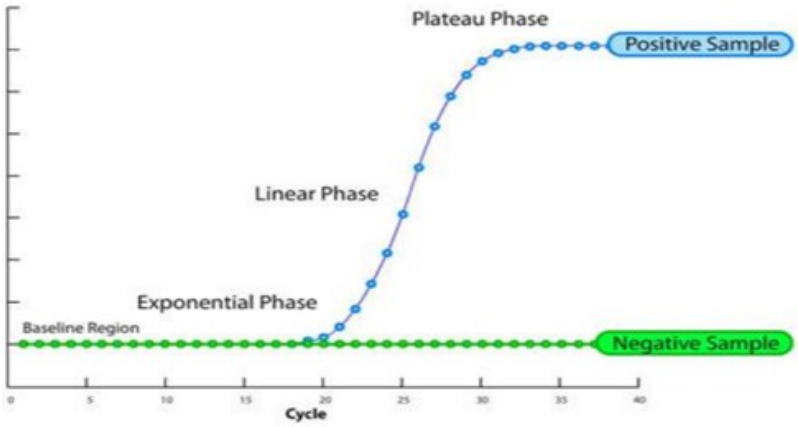
Fig. 16. Elongation and recycling



The ongoing synthesis produces huge numbers of increasingly complex and variously-sized tract of DNA containing multiple repeats of the initial target sequence. Picture source: (HiberGene Diagnostics, 2020).

Since most of these substances substantially reduce productivity of the Bst DNA polymerase during LAMP, they must be added after the reaction has run. However, opening of reaction vessels after the reaction has run will lead to heavy spoiling of the lab environment with aerosolized LAMP product which results in false positive reactions in subsequent LAMP analysis (Niessen et al., 2013). A fluorescently labelled cationic polymer or the use of a specific fluorescently labelled probe have been shown to be an alternative for direct in-tube detection (Mori et al., 2006). Indirect in-tube detection of LAMP product was done using pyrophosphate, a specific by-product of enzymatic DNA synthesis. By monitoring the turbidity of LAMP reactions resulting from magnesium-pyrophosphate precipitation (Mori et al., 2001), LAMP can be used in real-time mode to produce semi-quantitative results (Mori et al., 2004). DNA positive sample is represented by a sigmoid curve, while DNA negative sample is represented by a straight line (Fig. 17).

Figure 17. Real-time PCR. Picture source: (Fuselli, 2015)



1.3.2.3. Advantages of LAMP in food safety testing

LAMP advantages over PCR-based techniques are shorter reaction time (35-60 minutes vs 94 min), no need for specific equipment, high sensitivity and specificity as well as comparably low susceptibility to inhibitors present in sample materials, which enables detection of the pathogens in sample materials without time consuming sample

preparation (Niessen et al., 2013). Compared with PCR reactions, where primers recognise two independent DNA regions, LAMP reactions result in a higher specificity due to the use of six primer binding sites that recognize specifically eight independent DNA regions, enhancing the sensitivity and specificity and decreasing the probability of false-positive results (Li et al., 2012).

The fact that the reaction runs at constant temperature of 65 °C without thermal cycling so that no dedicated and expensive lab equipment is necessary to perform, gives LAMP based diagnostics the advantage of being faster and cheaper as compared to the reference PCR assays used in food safety testing (Niessen et al., 2013).

LAMP seems to be as accurate as the Real-Time PCR method, but more rapid, easy to use, and with a limit of detection up to 10^2 times lower than the RT-PCR method (Cammilleri et al., 2020; E Tirloni et al., 2017). Also, LAMP is suitable for on-site conditions because it does not require special reagents and sophisticated temperature control devices. In fact, LAMP assays typically consist of a kit with ready-to-use reagents and a portable instrument that can help to minimize operator error. These kits are not expensive and require little equipment and technical support. The preservation of the reagents only requires storage at 4 ◦C. For these reasons, Loop-mediated isothermal amplification (LAMP) of DNA has been emerging as an alternative to the use of PCR-based methods for testing food borne pathogens and toxicants (Niessen et al., 2013). However, the usefulness of the LAMP technique has its limits, such cases where only living and multiplying cells of a pathogen pose a hazard to the safety of food, because it cannot readily exclude dead cells from being detected. LAMP-based diagnostics will not replace classical microbiological analysis but may provide a valuable tool in cases where rapid and sensitive identification of a hazard is a priority, in order to screen samples and facilitate decisions about further analysis. Moreover, LAMP provides a valuable tool in applications where presence or absence of the target DNA sequence in a sample needs to be detected in order to analyse for typical hazards and facilitate rapid decision making, e.g. in food quality control and HACCP applications.

1.3.3. Microplastics in seafood: an emerging issue for food safety

1.3.3.1. Source and definition of microplastics

Since the discovery of the first plastic made from synthetic components in the early 1900s (Andrady and Neal, 2009), industry has been exploring new properties and opportunities regarding plastic materials. Plastic materials are extremely versatile due to their low density, low thermal and electric conductivity, resistance to corrosion, while their low price also contributes for their easy and widespread manufacture, where they are used in a wide range of applications, from everyday items such as lunch bags, to more complex medical and technological products and machines (Frias and Nash, 2019). However, contamination of the environment with plastic is one of today's major environmental problems with ubiquitous distribution in land, marine and freshwater ecosystems (Kühn et al., 2015; Wagner and Lambert, 2018; Zeng, 2018). The most common plastic polymer types are polyethylene (PE) and polypropylene (PP), followed by polystyrene (PS), polyamide (PA), polyester (PES), polyvinylchloride (PVC) and polyethylene terephthalate (PET) (GESAMP, 2015; Thompson, 2004). A considerable amount of plastic debris comes from continental sources entering the marine environment mainly through rivers (Lebreton et al., 2017), industrial and urban effluents, and runoff of beach sediments and fields. The other part comes from direct inputs, such as offshore industrial activities (e.g. oil and gas extraction, aquaculture), loss of nets in fisheries and litter released during sea activities, including tourism. Among plastic litter, microplastics are of special concern regarding the environment as well as animal and human health mainly due to their small size, the lack of technology available to quantify the presence of the smallest microplastics in the environment, and their potential to cause adverse effects on the marine biota and humans (Barboza et al., 2018a).

The natural environmental conditions within natural ecosystems, such as such as ocean current dynamics, solar radiation, abrasion and interactions with vessels and organisms, cause the degradation and fragmentation of large plastic items, including synthetic textile fabrics, into smaller particles commonly known as microplastics. Microplastic fragments may also be manufactured and intentionally added to certain products for a specific purpose, such as exfoliating granules prepared for body/face care. Once released into the environment, microplastic fragments may accumulate in organisms such as fish and

crustaceans, and therefore be ingested by consumers further along the food chain. The term *microplastics* was coined in 2004 by (Thompson, 2004) to describe the accumulation of microscopic pieces of plastic in marine sediments and in the water column of European waters. In 2009, Arthur et al., proposed an upper size limit to the initial term and microplastics were known as "plastic particles smaller than 5 mm". This definition was refined in 2011 by (Cole et al., 2011). They distinguished microplastics, according to their origin, into primary (produced to be of microscopic dimensions) or secondary (resulting from degradation and fragmentation processes in the environment). The Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP), defines microplastics as 'plastic particles < 5 mm in diameter, which include particles in the nano-size range (1 nm)' (GESAMP, 2016, 2015) and it helped further spreading the definition worldwide.

The focus on the microplastic issue as a novel pollutant has resulted in an exponential increase of microplastics literature (Frias and Nash, 2019). To date, there is still no consensus on a definition that encompasses all the criteria to describe 'microplastics' and this technicality causes several methodological challenges (Frias and Nash, 2019).

Size: the most used definition is the one proposed by (Arthur et al., 2009). Other authors refer to the lower size limits ranging from 1 to 20 μm (De Witte et al., 2014; Ryan, 2015; Van Cauwenberghe et al., 2015) while the upper size limits used in research range from 500 μm to either 1 mm or 5 mm (Dekiff et al., 2014; Desforges et al., 2014). (Gigault et al., 2018), made a strong contribution to the on-going debate. They defined nanoplastics as "particles resulting from the degradation of plastic objects that exhibit a colloidal behaviour within size ranging from 1 nm to 1 μm", therefore defining a lower size limit to microplastics (1 μm). Despite this contribution and bearing in mind the technological limitations of laboratory processing, the current potential size limit for identification ranges between 20 and 100 μm (Frias and Pagter, 2018), but with technological advances this range will potentially be lowered to 1 μm.

Type and shape*:* the most common types of microplastics recorded in the literature worldwide are pellets, fragments and fibres (Frias and Pagter, 2018), but it should be noted that different countries will use different terminology to classify the same object or plastic type.

***Colour**:* this aspect is not considered to be crucial to defining microplastics, because colour differentiation is subjective, and it cannot contribute to the visual identification of microplastics by itself. However, recording microplastic colour is important for studies concerning aquatic organisms, as some species may potentially ingest microplastics based on a colour preference behaviour (Wright et al., 2013).

Based on (Verschoor, 2015) report, that considers specific microplastic properties, an all-inclusive definition would need to include terms such as 'synthetic solid particle or polymeric matrix'. This would allow for the inclusion of all solid synthetic polymers, in their individual or composite forms. Therefore, (Frias and Nash, 2019) proposed the following definition for microplastics: "*Microplastics are any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water"*. The authors see this definition as descriptively all-inclusive, and helpful for both comparative and monitoring microplastics worldwide.

1.3.3.2. Occurrence of microplastics in seafood

Field studies demonstrate that a wide range of marine organisms across multiple trophic levels (from zooplankton to megafauna) contain microplastics, including those targeted by fisheries (de Sá et al., 2018; Desforges et al., 2015; Foekema et al., 2013; GESAMP, 2015; Hermsen et al., 2018; Kühn et al., 2015; A. Lusher et al., 2017a; Lusher, 2015). Ingestion is considered the most frequent interaction between microplastics and biota (GESAMP, 2015; Hermsen et al., 2018; Kühn et al., 2015; Lusher, 2015). The incidence of ingestion is highly variable, due to ecological, geographical, and methodological differences (Hermsen et al., 2018; Kühn et al., 2015). Filter feeders, deposit feeders and planktonic suspension organisms have been considered the most susceptible to microplastic ingestion, due to the relatively unselective nature of their feeding strategies (GESAMP, 2015; Lusher, 2015).

In the last years, researchers have been considering the potential effects of microplastics on human health along with the occurrence, transport, and distribution of microplastics in the marine environment and their adverse effects on marine life. Research has shown that shellfish (including crustaceans and bivalves), and a variety of commercially important fish species are often contaminated with

microplastics (Barboza et al., 2018a), being a potential route of exposure for human consumers (Bouwmeester et al., 2015; GESAMP, 2015).

Regarding mussels, microplastics were found in *Mytilus edulis* and *M. galloprovincialis* from five European countries (France, Italy, Denmark, Spain and The Netherlands) (Vandermeersch et al., 2015). In commercial mussels from Belgium, the number of microplastic particles varied from three to five fibres per 10 g of mussels (De Witte et al., 2014). Li et al. 2015 reported that the average number of microplastics in commercial bivalves from China (size range 5–5000 μm) varied from 2 to 11 items per gram and from 4 to 57 items per individual bivalve. In five shellfish species (including gastropods and bivalves) of the Persian Gulf, 3.7 to 17.7 particles per individual were found (Naji et al., 2018).

Concerning fish, microplastics were found in the Atlantic cod (*Gadus morhua*), the European hake (*Merluccius merluccius*), the Red mullet (*Mullus barbatus*) and the European pilchard (*Sardina pilchardus*) from several localities (Avio et al., 2015; Bellas et al., 2016; Bråte et al., 2016; Compa et al., 2018; Liboiron et al., 2016; Rummel et al., 2016). Rochman et al. 2015 demonstrated the presence of microplastics (size > 500 μm) in 9% and 28% of the gastrointestinal tracts from fish sold at markets in the USA and Indonesia, respectively, with an average number of plastic pieces of 0.5 per individual fish in the USA samples and 1.4 in the Indonesian samples. Neves et al., 2015 detected microplastics in 19.8% of commercial fish from the Portuguese coast. Moreover, microplastics have been detected in the stomachs of commercially important fish from the Mediterranean (Romeo et al., 2015), and in the gastrointestinal tract and liver of anchovies and sardines that sometimes are totally consumed (i.e. the entire fish) (Avio et al., 2015; Collard et al., 2017; Compa et al., 2018).

The occurrence of microplastics in the gastrointestinal tract of fish does not provide direct evidence for human exposure since this organ is usually not consumed (Wright and Kelly, 2017), but seafood species that are eaten whole (e.g. some molluscs and crustaceans, and small or juvenile phases of fish) may pose a greater threat to seafood contamination. However, the presence of microplastics in the eviscerated flesh (whole fish excluding the viscera and gills) of two commonly consumed dried fish species *(Chelon subviridis* and *Johnius belangerii*) was significantly higher than excised organs (viscera and

gills), evidencing that the evisceration does not necessarily eliminate the risk of microplastics intake by human consumers (Karami et al., 2017). Moreover, the presence of microplastics was also detected in the muscle of commercially important species of fish and of a crustacean (Akhbarizadeh et al., 2018; Koelmans et al., 2014a). These findings raise concerns about possible implications for human consumers.

1.3.3.3. Implications for food safety and human health

Chemical hazard: in the marine environment microplastics may act as vehicles for chemicals, including chemicals additives intentionally added during their manufacturing process and chemical contaminants that may be absorbed during their permanence into the environment, such as styrene, toxic metals, phthalates, bisphenol A (BPA), polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAHs) (Ashton et al., 2010; Bakir et al., 2012; Barboza et al., 2018b; Holmes et al., 2012; Teuten et al., 2009). A wide range of chemical products used in plastic manufacturing are recognized as toxic to animals and humans (e.g. carcinogens, endocrine disruptors, neurotoxic chemicals) (Rochman et al., 2013a; Thompson et al., 2009; Wright and Kelly, 2017). Additionally, chemicals associated with microplastics may accumulate and bio magnify in marine trophic webs (Amiard-Triquet et al., 1993; Kelly et al., 2007), increasing the risk of toxic effects of these chemicals, especially to top predators and humans consuming species contaminated with microplastics or with chemicals released from these particles after their ingestion (Hartmann et al., 2017; Hermabessiere et al., 2017; Koelmans et al., 2016). The ability of microplastics to adsorb toxic metals has been demonstrated in some studies (Ashton et al., 2010; Holmes et al., 2012; Vedolin et al., 2018). Among these metals, mercury is a common contaminant in the marine environment occurring at high concentrations in several regions. Mercury is highly toxic to animals and humans, it can accumulate in high number of organisms, and its organic form, methylmercury, bio magnifies in trophic web (Eagles-Smith et al., 2018). Although there is laboratory evidence that microplastics may increase the effects of chemical contaminants in fish (Barboza et al., 2018b; Pedà et al., 2016; Rainieri et al., 2018; Rochman et al., 2013b), there is little evidence from field studies that the ingestion of microplastics affects the bioaccumulation of pollutants (Lohmann, 2017). To date, at the current observed microplastic

concentrations, there is little evidence that microplastics may increase the chemical contamination of seafood when compared with other environmental sources (i.e., water, sediments, food web) (GESAMP, 2016; Koelmans et al., 2016, 2014b; Lohmann, 2017; Pittura et al., 2018). Considering the high concentrations of additives or contaminants reported in microplastics and their potential release from the microplastics upon ingestion, EFSA Panel on Contaminants in the Food Chain, 2016; Lusher et al., 2017 calculate that microplastics may have a negligible effect on the exposure to some pollutants and additives considering the total dietary exposure of humans. However, given the uncertainties surrounding this issue the contribution of plastic-derived chemicals to the human diet should receive attention in future research (Barboza et al., 2018a).

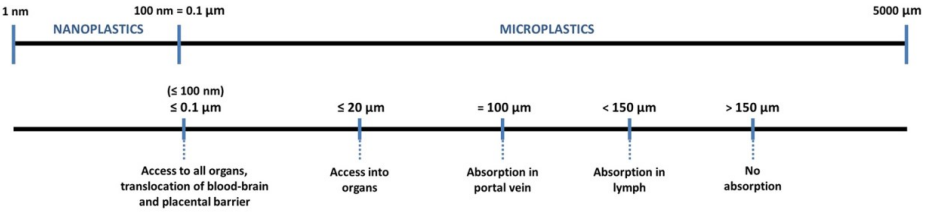
Biological hazard: microbes and other organisms have been found on plastic debris (Zettler et al., 2013) and some of these communities have been found to include pathogenic organisms, such as *Vibrio* spp. (De Tender et al., 2015; Keswani et al., 2016; Kirstein et al., 2016), *Escherichia coli, Stenotrophomonas maltophilia, Bacillus cereus* (van der Meulen et al., 2014) and *Aeromonas salmonicida* (Viršek et al., 2017). It has been suggested that plastic debris may increase the global risk of human and animal diseases via new contamination/infection routes, introduction of pathogens and their vectors into new areas through the environmental spread of microplastics or migrations of organisms contaminated with the pathogens mediated via microplastics (Keswani et al., 2016). The transfer of pathogens from ingested plastics to humans is still speculative. The contribution of plastic debris in the spread of infectious diseases to humans is currently unknown and the survival of pathogenic organisms on plastic debris has not been deeply studied, as well as pathogens transmission via consumption of contaminated seafood.

Scientific evidence demonstrates multiple pathways of microplastic exposure via food, but there is no information available about the fate of microplastics in the human body following ingestion of the particles (Barboza et al., 2018a; Rist et al., 2018; Wright and Kelly, 2017). In this context, adverse effects on human health are still controversial. Human health effects depend on exposure concentrations, but current information to assess the true amount of microplastics humans are exposed to via food is insufficient, due to data gaps in microplastic research. As for seafood, researchers showed that among the 25

species contributing mostly to global sea fishing (FAO, 2016), 11 were found to contain microplastics. Van Cauwenberghe & Janssen 2014 calculated that in European countries with high shellfish consumption, consumers may ingest up to 11000 microplastic particles (size range 5–1000 μm) per year, whereas in countries with low shellfish consumption, consumers ingest an average of 1800 microplastics per year. Considering shrimp consumption only, estimates indicate about 175 microplastic particles (size range 200–1000 μm) per person per year (Devriese et al., 2015).

It is supposed that microplastics with size bigger than 150 μm are not absorbed while microplastics smaller than 150 μm may translocate from the gut cavity to the lymph and circulatory system, causing systemic exposure. However, the absorption of these microplastics is expected to be limited (≤ 0.3%). Only microplastics with size ≤ 20 μm would be able to penetrate the organs while the smallest fraction (0.1 > 10 μm) would be able to access all organs, cross cell membranes, the blood-brain barrier and the placenta (Fig. 18) (Bouwmeester et al., 2015; Browne et al., 2008; EFSA, 2016; Galloway, 2015; A. Lusher et al., 2017a; von Moos et al., 2012). In vitro studies with cerebral and epithelial human cells evidenced for the first time the potential of micro- (10 μm) and nano-plastics (40–250 nm) to cause cytotoxic effects at cell level in terms of oxidative stress (Schirinzi et al., 2017), reinforcing the scientific assumptions on the possible consequences for human health. The knowledge in this field is still very limited and there is little evidence on the impact on human health from eating microplastics. Also, it is not known the amount of very small microplastics in the water, sediments, organisms and air, and the assessment of biota exposure.

Fig. 18. Fate of micro- and nanoplastics in mammalian bodies (adapted from Lusher et al., 2017).



Humans are vulnerable to exposures other than seafood, such as airborne microplastics (Prata, 2018). In this regard, the potential for human ingestion of fibres resulting from domestic dust during a meal

may be higher than fibre intake through consumption of mussels (Catarino et al., 2018). However, actual exposure is difficult to quantify, and the understanding of human health risks related to microplastics is in the early stages. Adopting food safety risk analysis frameworks to evaluate hazards and risks to consumers posed by seafood contaminated with microplastics is of extreme necessity (A. Lusher et al., 2017a). Microplastics risk assessment should include the dietary exposure from a variety of foods across the total diet (GESAMP, 2016) and the best understanding of parameters such as particle size, polymeric composition, particle shape, surface area, density, persistence, adsorbed pollutants, additive content and toxicological consequences (Hale, 2018).

The subsequent effects of microplastics on human health should be viewed with caution, since there is a large discrepancy between the current knowledge based on scientific evidence of the real implications for human health and the magnitude of the problem that has been addressed by the media (Barboza et al., 2018a; Rist et al., 2018; Wright and Kelly, 2017). Thus, further research is needed to understand the real effects of microplastics on human health.

1.3.3.4. Analytical methods for microplastics analysis in fish

The vast range of different plastic types available on today consumer market makes the qualitative or quantitative analysis of microplastics extremely challenging. No officially standardized methods are currently available. However, researchers have been developing and validating specific methods applicable to diverse matrix types, including fish products. The following sections describe sample preparation, detection/identification methods and contamination preventive measures in microplastics analysis.

1. ***Sample preparation***

The aim of the sample preparation is to remove biological, inorganic or organic materials that would interfere with the analysis of microplastics but will not affect the microplastics. Depending on the sample matrix, a range of different sample digestion techniques can be applied. Three options are commonly considered for eliminating undesirable organic material from analytical samples.

Acidic chemical digestion. The use of acid digestion typically involves oxidative acid treatments with various combinations of HCl, HNO_3,

$HClO_4$ and HClO with and without H_2O_2. Although some polymers types are resistant to such aggressive conditions, reports say that these methods can result in varying levels of modification, damage or total destruction of plastic particulates (Enders et al., 2017).

Alkaline chemical digestion. Alkaline digestion using concentrated solutions of bases such as NaOH or KOH solutions as 10% or in the range 30-40% p/v is effective for hydrolysing complex biological matrices such as animal tissues and organs (Toussaint et al., 2019). (Enders et al., 2017) show that alkaline digestion can be use with a greater range of polymers and present a lower risk of chemically modifying or structurally damaging particulates.

Enzymatic digestion. Enzymatic digestion represents a specific mean of hydrolysing proteins and breaking down tissues. Enzymes such as proteinase K, chitinase and pepsin have been used in microplastic research (Catarino et al., 2017; A. L. Lusher et al., 2017). This type of digestion is non-aggressive towards plastic polymers and safer for laboratory workers, but enzymes are specific to certain types of matrices (proteins, carbohydrates, fats, etc.) and complex samples may require the use of several steps to remove the matrix components. Furthermore, enzymes are sensitive to their environment and their specific activity can change during storage, and the costs of enzymatic digestion can be significantly higher than those of chemical digestion.

2. *Density separation*

Density separation may be useful in studies following digestion. Compared with inorganic materials, majority of polymers have relatively low densities ranging from the lightest materials such as polyethylene (0.85 g/cm^3) up to 2.1 g/cm^3 in the case of Teflon. Within this range, majority of polymer types have densities below 1.5 g/cm^3, which is lower than the density of many inorganic materials that may be mixed in microplastics. This difference is exploited to preferentially extract plastic particulates by floating them out from salt solutions whose density is higher than plastics (Quinn et al., 2017). A variety of solutions may be used depending on the maximum density required. For polymers with densities of up to 1.2 g/cm^3 NaCl solutions may be sufficient while ZnCl (1.7 g/cm^3), NaI (1.8 g/cm^3) or sodium polytungstate (3.1 g/cm^3) solutions may be considered for more dense materials. Depending on the size and density particles, sedimentation/flotation may be achieved using normal gravitation

force but may be accelerated by the appropriate use of laboratory centrifuges.

3. *Filtration*

Filtration is an extra step after the sample digestion in order to retain microplastics. The filtration process needs to match the requirements of the analysis. For example, the initial use of a large pore size can filter out the larger plastics present or can remove other larger debris to prevent blocking of the filter. A range of different filter types and sizes have been evaluated to determine the optimum filter types, such as alumina oxide, glass microfiber, gold coated polycarbonate, polyvinylidene fluoride (PVDF), silver and silicon. The filter diameter influences the sample capacity and the filtration ability and should be kept down to a reasonable size to reduce the time required for the subsequent IR imaging (Robertson, 2018). The pore size will determine the smallest particle size to be retained but should be kept to a size that will not block easily with some of the sample matrices. For compatibility with IR analysis the particles need to be larger than 5 microns, due to the approximate diffraction limit of IR analysis. The relative cost per filter can be important, except for samples where the sample preparation can take hours or days. For laboratories with higher sample throughput it should be an important consideration (Robertson, 2018). The filter type either needs to transmit or reflect IR radiation without any significant absorptions. Figures 20a and 20b show infrared transmission and reflectance spectra for each type of filter analysed by Robertson, 2018. The gold-coated polycarbonate filter has very good energy in reflectance but no transmitted energy, while the PVDF filter shows significant absorption bands in both transmission and reflectance so it is unsuitable.

Figures 19a and 19b. Transmission and reflectance ranges of filter types (adapted from (Robertson, 2018)

4. *Identification*

Microplastics identification requires particle size determination and chemical identification. Depending on the sample type and the instruments available, microplastics can be firstly identified and sorted on the filters by using a microscope and then analysed for polymer identification, or they can be directly identified on the filters by using Fourier Transform infrared FT-IR or Raman spectroscopy.

Visual identification. The visual identification can be conducted using light, polarised or electron microscopy. Criteria for visually identifying microplastics include: the absence of cellular or organic structures; a homogenous thickness across the particles; homogenous colours and gloss. Visual identification is an essential step in classifying microplastic shape and type. However, it must be supported by subsequent polymer analysis. Plastics are classified by their morphological characteristics: size, shape, and colour. Size is typically based on the longest dimension of a particle; size categories can be used where appropriate. When reporting microplastic shape, researchers tend to use five main categories, although the nomenclature used varies between research groups (A. Lusher et al., 2017b). The categories used when classifying microplastic shape are fragments, fibres, beads, foams and pellets.

Colour differentiation is subjective and visual identification of microplastics cannot be based on colour alone. Caution should be given to categorising microplastics suffering embrittlement, fragmentation or bleaching, or encrusted with biota, as this may skew results. Visual identification is rapid, relatively cheap and can be conducted without the need for additional technical staff. However, accurately differentiating microplastics, particularly in the smaller size ranges, requires training and experience. Generally, if no more accurate methods (e.g. FTIR or Raman spectroscopy) are used to verify synthetic polymer origin of microplastics, the visual identification should not be applied to particles <500 μm as the probability of a

misidentification is very high (Löder and Gerdts, 2015). Owing to the difficulties in handling and differentiating microplastics from organic and inorganic matter, error rates could be as high as 70%, increasing with decreasing particle size (Davidson and Dudas, 2016), with incorrect identification most prevalent with microfibres (Hidalgo-Ruz et al., 2012; Remy et al., 2015). To gauge the accuracy of visual discrimination, sub-samples of potential plastics should be chemically analysed.

Polymer verification. The different polymer types, colours, shapes, fragmentation and surface degradation due to weathering and additives make it difficult to assess and classify microplastics by microscopy methods (Shim et al., 2017). Therefore, secondary analyses are used to detect, identity and quantify suspected polymeric material. Plastics are generally submitted to the following analytical techniques: Fourier transform infrared (FT-IR) or Raman Spectroscopy; Fluorescence Microscopy (Fluorescent tagging with Nile Red); Pyr-GC-MS (Pyrolysis-Gas Chromatography combined with Mass Spectroscopy). Each method has specific advantages and limitations (Table 7), for this reason a minimum of two methods should be used (Toussaint et al., 2019). FT-IR and Raman spectroscopy represent the main techniques. They are based on irradiating the sample to stimulate molecular vibrations and to produce an optical spectrum. This spectrum contains a variety of peaks forming a fingerprint that can be compared with spectral libraries to identify the polymer.

Table 7. Advantages and limitations of the methods for characterisation of microplastics (adapted from (Toussaint et al., 2019).

Method	Advantages	Limitations
Micro FT-IR spectroscopy	Small sample amounts Limited sample preparation Size and polymeric identification Images and analysis of the entire filter Non-destructive Localisation of debris in living organisms	Particle size limits 30-50 μm No automated particle analyses My not be reliable on degraded samples or mixed polymer Expensive
Micro Raman spectroscopy	Small sample amounts Limited sample preparation Size and polymeric identification Particle size < 30-50 μm (spatial resolution < 1 μm) Localisation of debris in living organisms	Auto-fluorescence of samples may mask the Raman signal (e.g. organic contamination or additives) Partially destructive (laser can burn/evaporate particles) May not be reliable on degraded samples or mixed polymer Applicable only on small regions of the sample Expensive

Fluorescent microscopy	Detects and quantifies particles 20 μm -1 mm Semiautomated Low cost	Not reliable with weathered samples No polymer identification False positives due to residual organic matter that may become stained
Py-GC-MS	Polymers and organic additives identification Little sample preparation Small sample amounts (< 0.5 mg) Suitable for biological matrices and screening of environmental samples	No data on particle number, size and shape Need an expert operator Manual manipulation of sample that limits lower particle size to fractions of mm Destructive Time consuming

Raman spectroscopy. During the analysis, the sample is irradiated with a monochromatic laser source with a wavelength usually ranging between 500 and 800 nm. The interaction of the laser with the molecules and atoms of the sample results in differences in the frequency of the backscattered light when compared to the irradiating laser frequency. This so-called Raman shift can be detected and leads to substance-specific Raman spectra. Since plastic polymers possess characteristic Raman spectra, this technique can be applied to identify plastic polymers by comparison with reference spectra. Raman spectroscopy is a "surface technique", thus large microplastic particles can be analysed. Accordingly, micro-Raman spectroscopy allows for the identification of small plastic particles below 1 μm (Rocha-Santos and Duarte, 2017; Schymanski et al., 2018). One drawback of Raman spectroscopy is that many samples show fluorescence. Fluorescent samples excited by the laser (e.g. biological residues) generate a signal that can completely covers the signal of the sample, preventing the generation of interpretable Raman spectra. The fluorescence can be minimized by using lasers with higher wave lengths (> 1000 nm), but the lower energy of the laser results in a lower signal of the polymer sample. For this reason, a purification step of samples to prevent fluorescence is recommended prior to measurements (Löder and Gerdts, 2015).

FTIR spectroscopy. Similar to Raman spectroscopy, infrared (IR) or Fourier transform infrared (FTIR) spectroscopy offers the possibility of identification of polymer particles. FTIR and Raman spectroscopy are complementary techniques. In fact, molecular vibrations that are Raman inactive are IR active and vice versa and can thus provide complementary information on microplastic samples (Löder and Gerdts, 2015). FTIR analysis measures the range of wavelengths in the

infrared region that are absorbed by a material through the application of infrared radiation (IR) to samples. The sample's absorbance of the infrared light's energy at various wavelengths is measured to determine the material's molecular composition and structure. Unknown materials are subsequently identified by searching the spectrum against a database of reference spectra. FTIR can also measure levels of oxidation in some polymers as well as quantifying contaminants or additives in materials. Depending on the size of the particle, it is possible to record several spectra at different points on a single particle.

The FTIR surface technique - ATR (Attenuated Total Reflectance) FTIR spectroscopy - can analyse large particles, while FTIR microscopy characterises particles as small as 20 microns, allowing quick and cost-effective identification of unknown particles, residues, films or fibres. In the context of FTIR microscopy, the use of two measuring modes is possible: reflectance and transmittance.

Although micro-FTIR mapping, i.e. the sequential measurement of IR spectra at spatially separated, user-defined points on the sample surface, has been applied for microplastics identification (Levin and Bhargava, 2005), this technique is still extremely time-consuming when targeting the whole sample filter surface at a high spatial resolution because it uses only a single detector element. Harrison et al., 2012 concluded that a highly promising FTIR extension, focal plane array (FPA)-based FTIR imaging (Levin and Bhargava 2005), allows for detailed and unbiased high throughput analysis of total microplastics on a sample filter. This technique enables the simultaneous recording of several thousand spectra within an area with a single measurement and thus the generation of chemical images.

Fluorescent tagging with Nile Red. This technique is used to detect and quantify small microplastics from 20 mμ to 1 mm. This method is inexpensive, uses readily available equipment and can be semi-automated. It requires a sample purification step, fluorescence microscopy and suitable image analysis software. However, the elimination of organic matter is a crucial step, as it could be stained with Nile Red and lead to an overestimation of microplastic amount. Raman spectroscopy and FT-IR can be used to confirm that the fragments stained with Nile Red are only synthetic polymers (Erni-Cassola et al., 2017; Toussaint et al., 2019).

Pyrolysis-Gas Chromatography and Mass Spectroscopy. Py-GC-MS analyses particles by using their thermal degradation properties and can be used to analyse polymer type and organic plastic additives simultaneously (Fries et al., 2013). It is usually coupled with an FT-IR or a mass spectrometry. The limitation of this technique is that is destructive and does not provide data on the size and shape of the plastic particles, it only gives mass concentration results (Shim et al., 2017) and the particles have to be manually placed into the pyrolysis tube, thereby significantly reducing the sample throughput. Also, as thermal decomposition of microplastics occurs at similar temperature (350 °C to 450 °C), separation of the decomposition products is needed (GC-MS).

In a context of method comparison and harmonisation of analytical practices, there is a need for method performance assessment. Such approaches should also be encouraged for spectroscopic methods allowing for better comparison of results from different studies. For example, among the 21 FT-IR studies analysed by Dehaut et al., 2019, only 52% provided a threshold beyond which the identification of the polymer's nature is certain, and this threshold varied from 60% to 85%. While other authors reported a confidence thresholds for spectra matches between 70-75% (Lusher et al., 2016, 2013; Neves et al., 2015). Furthermore, post-visual analyses have shown misidentification of microplastics of up to 70% (Collard et al., 2015; Eriksen et al., 2013; Remy et al., 2015), and errors in identification often include un-matched spectra that could not be assigned with confidence to a known polymer type.

5. *Contamination*

Airborne contamination of samples with synthetic fibres from clothing or atmospheric fallout is a recurrent issue within the literature (Davidson and Dudas, 2016; De Witte et al., 2014; Mathalon and Hill, 2014; Rochman et al., 2015; Santana et al., 2016). Sources of contamination should be eliminated where possible, and otherwise quantified using environmental filters and procedural blanks.

Contamination during field sampling. Marine animal species are often captured/collected by polymer ropes, nets or traps. In these situations, animals should only be exposed for minimal periods and a reference sample of the gear should be retained to exclude contamination during the identification phase (A. Lusher et al., 2017b).

Avoiding contamination in the field is more complex than in the laboratory but remains an important consideration. Furthermore, it must be noticed that marine species can be collected also from farms and commercial fish markets, where the captured method is often unknown. Steps for mitigating contamination include cleaning of all equipment prior to sampling; covering samples and equipment between use; wearing polymer-free clothing or cotton coveralls, and gloves.

Contamination during sample processing and analysis. Researchers should process samples in a laminar flow hood or "clean room" (e.g. non-ventilated or negative flow) with low foot-traffic. Glassware is preferential to plastic consumables. Glassware, benches and equipment should be rinsed with deionized water, (Avio et al., 2015; Lusher et al., 2016, 2013; Mathalon and Hill, 2014; Rochman et al., 2015; Santana et al., 2016; Van Cauwenberghe and Janssen, 2014), ethanol (Cole and Galloway, 2015; Collard et al., 2015) or acetone (De Witte et al., 2014) prior to use. Environmental filters can be placed near equipment to quantify external contamination (Dehaut et al., 2019; Lusher et al., 2016, 2013). Procedural blanks (e.g. controls) are highly recommended quantifying contamination and for identifying aspects of the experimental design where contamination can occur.

1.3.4. Caviar and fish roe product

Fish roe products are extremely valuable and are currently expanding in international and domestic markets. Fish roes obtained from fish of the *Acipenseridae* family are named as Caviar and they are obtained by light salting of roes extracted from sturgeons, separated from their connective tissue (Bledsoe et al., 2003), while fish roe from other fish species than sturgeon are caviar substitutes (Codex Alimentarius, 2010) and must be identified with a qualifying term that includes the common name of the fish used (e.g. salmon caviar).

Together with Caviar, several other products forms are consumed, such as Bottarga, that is generated from processing of mullet and tuna roes, and Japanese preparations such as Ikura, Tarako and Tobiko, that originate from salmon, pollock and flying fish roes, respectively (Shirai et al., 2006). The reduction in the availability of Caspian Sea sturgeon caviar and the limited aquaculture production resulted in increased attention being paid to caviar substitutes. In addition, the popularity of sushi led to the development of new products obtained by manufacturing roes from other fish species and to the expansion of their market. These products are sold at a lower price and they refer to caviar with the aim of exploiting its high appeal among consumers.

Composition of fish roe depends on the fish species, the age of the fish, the stage of maturation of the eggs at the time of harvest, the type of diet and the origin of animals, the microbial condition of the fish and the conditions during processing and preservation (Lopez et al., 2020; Oeleker et al., 2015). Many bacteria traced in caviar are commensals of the fish microbiota itself (Gram and Huss, 2000), however, if a bacterial contamination is present, high loads might occur even under high salt concentrations (Shin et al., 2010). Thus, fish roe products can pose safety risks if not properly processed and handled. Most fish roe products are marketed as a refrigerated food and as shelf-stable products, but the possibility of unintentional thermal abuse is a risk that can occur. Also, since caviar is typically consumed raw, the risk is not reduced via thermalization e.g. cooking.

In order to keep the microbiological quality, raised salt concentrations (3–6 %) and maintenance of cold chain are crucial factors (Shin and Rasco, 2007). To extend shelf life, pasteurization can be performed at mild temperatures to avoid protein denaturation (which generally occurs between 70-80 °C) or preservatives such as benzoic acid (E210) or borax (E285) can be added.

Fish roe products are regarded as high-quality foods since they include important amounts of protein and amino acids. The lipid from fish roe products has presented as a useful food source for maintaining human health (Shirai et al., 2006) It is known that, fish roes contain eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3), (Bledsoe et al., 2003; Kaitaranta, 1980; Tocher and Sargent, 1984) and they play an important role in the prevention and treatment of cardiovascular diseases (Nordøy et al., 2001).

Little data about caviar quality and safety attributes of non-sturgeon species are present. (Tocher and Sargent, 1984) carried out lipid class analyses and fatty acid analyses on ripe roes of herring, cod, haddock, whiting, saithe, sand eel and capelin. They found that total lipid was 10-26% of roe dry weight and that phospholipids accounted for 62–77% of roe total lipid. Phospholipids had high concentrations of (n-3) polyunsaturated fatty acids (PUFA), frequently amounting to 50% of the total egg lipid. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) had similar fatty acid compositions in all species, with an average ratio (n-3)/(n-6) of ca. 20:1. Phosphatidylinositol (PI) had high concentrations of 18:0 and 20:4 (n-6) with an average ratio of (n-3)/(n-6) of 1.8:1.

(Bledsoe et al., 2003) described processing technologies for many fish roe and caviar products together with chemical composition (proximate and lipid composition, cholesterol content, fatty acids composition, wax and steryl esters, vitamin content) and food safety issues associated with roe products.

(Shin and Rasco, 2007) analysed the effect of water phase salt content and storage temperature on *Listeria monocytogenes* survival in chum salmon (*Oncorhynchus Keta*) roe and caviar (Ikura). They found that *L. monocytogenes* did not grow in chum salmon ikura held at 3 °C during 30 d at any salt level tested, while in chum salmon ikura held at 7 °C, *Listeria* counts reached 5 to 6 logs CFU/g at the same salt levels tested. Their results indicate that temperature control is critical for ikura and similar products, but that products with lower salt contents can be safe, as long as good refrigeration is maintained.

(Mol and Turan, 2008) measured and compared three different types of imported black caviar Beluga (*Huso huso*), Imperial (*Acipenser persicus*), and Osetra (*Acipenser gueldenstaedti*), red salmon roe and waxed mullet roe. Specifically, they measured and compared proximate compositions, amino acid compositions and fatty acid

compositions. The authors concluded that fish roes may be considered a high-quality food for human nutrition. In fact, their results showed that the analysed fish roe products represent a source of high-quality protein, with glutamic acid, aspartic acid, lysine, and serine being the major amino acids, and they had an Essential/Nonessential (E/NE) ratio that ranged between 0.93 - 1.23. Also, fish roe products showed a n3/n6 ratio between 2.56–8.06 and contained significantly higher ($p < 0.05$) amount of unsaturated fatty acids (MUFA and PUFA) than saturated fatty acids (SFA) and therefore a valid alternative for normal diets.

Despite the importance of fish roe products in international market, little technical information are available about their chemical composition, food safety, and quality attributes. For this reason, deeper research should be addressed towards these topics.

CHAPTER 2

Objectives

Today, food safety is a major concern facing the seafood industry. The production and consumption of safe food are central to any society, and they have a wide range of economic, social and environmental consequences. The issue of seafood safety is even more important in view of the growth in international fish trade. Before the 80s, food quality and hygiene were guaranteed by controls and analysis on the final product, but in the late 1980s, the European Community needed new strategies to preserve the hygienic quality of food (Orban and Di Lena, 2011). For this reason, in 1987, the International Organization for Standardization published the ISO 9000 family of standards, that modified the quality concept, shifting the attention from the final product to all the processes contributing to its production (Orban and Di Lena, 2011). Later, the International Organization for Standardization developed the ISO 22000 family of standards on food safety management systems, that takes the approach of ISO 9001 as a management system and incorporates the hygiene measures of prerequisite programmes and the HACCP principles and criteria (Ryder et al., 2014).

The public health significance of seafood-borne illnesses depends on the likelihood and the severity of the illness. The concept of "risk analysis" has become the method for establishing tolerable levels of hazards in foods in international trade and, equally, within national jurisdictions. For international fish trade, countries and regions have developed national and regional regulations to control seafood entering or exiting their territories. In the European Union and as the result of a white paper on food safety in 2000, the approach taken in the legislation is to separate aspects of food hygiene from animal health and to harmonize food control across the member countries of the European Union. A key aspect of the legislation is that all food and

feed business operators, from farmers and processors to retailers, have principal responsibility for ensuring that food placed on the market in the European Union (Member Organization) meets the required food safety standards (Ryder et al., 2014).

In this work, different approaches for the evaluation of fish quality and safety are evaluated. In the following part of the thesis are reported the main studies conducted during the PhD period. The first study aims at investigating the effect of claw ligatures on lobsters' welfare by analysing eight different stress haemolymph parameters.
With regard to the second study, the aim is the evaluation of a new Loop-mediated isothermal amplification (LAMP) assay for the detection of *Anisakis* spp. in seafood products.
The third study shows the preliminary results of the current investigation that aims at studying and developing a method suitable for the extraction of microplastic pollutants from mussels.
The fourth study compares the chemical composition and food safety issues in fish roe products from different species, by defining quality, traceability, and food safety.

CHAPTER 3

The effects of claw ligatures in American lobster (*Homarus americanus*) storage: a preliminary study of haemolymph parameters

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3.1. Abstract

American lobsters are crustaceans that are offered for sale live and are stored in controlled temperature recirculating aquaria. During marketing, they are subjected to stressors that can affect their welfare, such as air exposure, confinement, and handling. European legislation does not provide specific criteria or retention requirements, and so their management depends largely on the common sense of food business operators. Claw ligatures before and during storage are not legally required but are recommended because they prevent lobsters from damaging each other and ensure workers' safety. The aim of the present study is to evaluate the effect of claw ligatures on the lobsters' welfare by analysing eight different haemolymph stress indicators, vitality, and weight. The calcium level showed significant differences in the two experimental groups ($P < 0.05$).
Our results suggested that the absence of rubber bands did not offer any significant contribution to the lobsters' welfare.

3.2. Introduction

European legislation (European Commission, 2004) defines

crustaceans as seafood, therefore, they can be commercialized when dead. However, keeping these animals alive gives the best commercial quality and buying live animals is considered a good and fair practice by many consumers. Furthermore, these animals are highly perishable, and they can exhibit off-odours and deterioration of organoleptic qualities within a short period after death. In contrast to the practices of other countries (e.g., Norway, Australia, and New Zealand) where specific guidelines and regulations on animal welfare apply to these decapod crustaceans (Bennison, 2002; Johnston and Jungalwalla, 2005), in Europe the current norm is to set minimum standards for animal storage and provide only guidelines for invertebrate welfare, such as those from the Scientific Panel on Animal Health and Welfare (EFSA, 2005) and of the Sea Fish Industry Authority (Jacklin and Combes, 2007), a public body in the United Kingdom. Stress factors and poor welfare can lead to increased susceptibility to diseases among animals (EFSA, 2005) and this can pose risks to consumers (e.g., food-borne infections) (European Commission, 2004). Thus, handling and conservation of these animals during marketing are practices that must meet welfare requirements (Candotti, 2007).

The Swiss Government took a step forward in acknowledging and promoting invertebrate welfare by modifying the Swiss Animal Protection Ordinance (Swiss Federal Council, 2018). The new regulation (Art. 23) establishes that from 1st March 2018, live decapods cannot be transported on ice or in icy water, and species that naturally live underwater must always be held in this environment. Furthermore, decapods must be stunned before slaughtering and only electric shocks or the "mechanical destruction" of the brain are accepted (Art. 178). Extensive research worldwide has considered the welfare of crustaceans (Basti et al., 2010; D'Agaro et al., 2014; Lorenzon et al., 2007), and the physiological responses of lobsters to external stressors such as confinement, starvation, air exposure and temperature have been evaluated by measuring different biochemical parameters. The sampling of haemolymph is a non-destructive method which is commonly used to quantify physiological stress in crustaceans. Lobsters are solitary animals, and in order to avoid competitiveness, cannibalism and operator injuries, their claws are kept clasped before and during storage. This condition has been considered to be highly restrictive, as lobsters use their claws for locomotion, feeding and defence, but there is a lack of scientific evidence for this. Thus, the aim of the present study was to investigate haemolymph stress parameters, vitality and weight to assess if claw ligatures may influence lobster welfare.

3.3. Materials and Methods

3.3.1. Animals and experimental design

American lobsters were transported by air at refrigeration temperatures (-3 to -4 °C) in cardboard boxes containing ice gel packs. Upon arrival at the Italian distribution platform, the subjects were evaluated for vitality by applying the Vitality Index (Crustasea, 2006) and for sanitary status. Only animals with a Vitality Index of 5 (strong) were included in the trial, defined by the following criteria: animals had very strong claws and walking legs, showed strong uropod movement, rigid uropod, aggressive or defensive attitude and reactive antennae (sick or suspected animals were thus excluded). The use of live animals was necessary in the *in vivo* stages of this work, and they were treated with due care, minimizing discomfort and distress. According to the 3Rs principle (Replacement, Reduction, Refinement), the number of animals used was the minimum necessary for obtaining satisfactory scientific results. The trial was conducted in the Lodi Aquaculture Research Centre at the University of Milan. A total of 24 American lobsters of both sexes, with live masses of 485.11 ± 10.77 g (mean weight ± S.D.), were assigned randomly to one of two experimental groups: a control group (C), in which subjects were maintained free in the tank with tied claws, and a treatment group (T) where the subjects were held in single plastic cages (30 cm length × 18 cm width × 10 cm height) with free claws. The animals were transferred to a commercial aquarium (148 cm length × 58 cm width × 70 cm height - Adriatic Sea International, Ravenna, Italy) containing artificial seawater and equipped with a recirculating system. The animals were maintained unfed throughout the trial. Haemolymph samples were collected from the ventral abdominal sinus (arthrodial membrane covering the articulated base of the 5th walking leg) of each subject at arrival before the immersion (T0) and after 12 h (T1), 36 h (T2), 60 h (T3) and 108 h (T4) of immersion, as described by Bernardi et al. 2015.

The Vitality Index and weight were measured at the same sampling times. Water temperature and dissolved oxygen were recorded daily with a Hach HQ 30d portable meter, (Hach Lange, Düsseldorf, Germany). The water temperature was 6.0 ± 0.2 °C and the oxygen concentration was near the saturation, with a minimum recorded value of 87%. Water salinity was measured by gravity and it remained stable at a value of 1020. Total ammonia and nitrites, measured in each aquarium at every sampling event with a Hach 2800 Portable Spectrophotometer (Hach Lange, Düsseldorf, Germany), were below 0.05 mg·l-1, except for an increase recorded at 12 h, when the ammonia was 0.45 mg·l-1 for group C and 0.51 mg·l-1 for group T, probably due

to the high amount of ammonia released by lobsters when they were returned to the water after transport.

3.3.2. Determination of haemolymph parameters

Eight different physiological parameters were determined for each sample: glucose, total protein, lactate, ammonia, urea, chloride, calcium and magnesium concentrations. The ammonia content was quantified with the Ammonia Checker II (Menarini Diagnostics, Florence, Italy) and commercial kit test strips Ammonia test Kit II (Menarini Diagnostics, Florence, Italy). Glucose, total protein, lactate, chloride, calcium, magnesium and urea were determined using the COBAS MIRA biochemistry analyser (Roche Diagnostic, Monza, Italy). The following kits were adopted: Glucose Quantitative Colorimetric Assay Kit (Biochemical Enterprise, Milan, Italy), Total Protein Quantitative Colorimetric Assay Kit (Biuret method) (Biochemical Enterprise, Milan, Italy), Lactate Dry-Fast Enzymatic Colorimetric Assay Kit (Sentinel Diagnostics, Milan, Italy), Chloride Quantitative Colorimetric Assay Kit (mercuric thiocyanate method) (Biochemical Enterprise, Milan, Italy), Calcium Colorimetric Assay Kit (o-cresolftalein complexone method) (Hagen Diagnostica, Florence, Italy), Magnesium Colorimetric Assay Kit (Xylidyl Blue - I Method) (AdipoGen Life Sciences, Liestal, Switzerland), Urea Quantitative Kinetic Assay (Biochemical Enterprise, Milan, Italy).

3.3.3. Statistical analyses

A three-factor (experimental group, time and sex) analysis of covariance (the covariates were the vitality index and weight) was conducted to analyse the data. The weight effect was of interest while sex was added to the model as a known confounding factor. The data analysis was generated using RStudio Software, Version 0.98.1103. *P* values of less than 0.05 were considered significant.

3.4. Results

The experimental values (mean ± S.D.) of the studied variables are reported in Table 1. During the trial one treated lobster and one control lobster died after 108 h of storage in the tank, so they were not sampled.

The mean vitality at arrival was similar in both groups (4.58 ± 0.51 in group C; 4.50 ± 0.67 in group T), and it reached 5.00 ± 0.00 after 12 h and remained stable in all individuals until 108 h. Vitality significantly increased after 12 h in group C and T. No difference between the control group and the treated group was detected.

Weight increased during the sampling times. On average, the control

group gained 22.46 g (108 h mean weight: 514.80 ± 45.68 g) and the treatment group 23.22 g (108 h mean weight: 501.11 ± 32.54 g). Weight recovery after 12 h was significant, while no significant differences were recorded between the two groups.

The average glucose concentration at arrival was 3.54 ± 2.92 mg dl^{-1}. Both treated and control animals showed significant decreases in haemolymph glucose after 12 h. Minimum variations were recorded during the subsequent sampling times.

Differences in glucose levels between the groups were not significant ($P = 0.20$). A higher concentration of lactate upon arrival was found in all the individuals (average level 9.91 ± 10.74 mg·dl^{-1}).

Lactate concentration significantly decreased after 12 h, but it showed no significant difference between control and treated subjects ($P = 0.62$).

The average protein value at arrival was 1.78 ± 0.54 g·dl^{-1} and the subsequent measurements showed a similar trend. An exception is the protein values after 108 h, which increased and almost returned to those at the arrival time. Significant differences were detected in protein concentration after immersion in tank, while the differences between the groups were not significant ($P = 0.06$).

A significant difference in ammonia concentration was found between arrival time and after 12 h of storage. A higher concentration at arrival was found (average level at T0 vs. T1: 2.75 ± 1.08 mg·dl^{-1} vs. 1.27 ± 4.27). Haemolymph ammonia also increased after 36 h, and after 108 h it reached levels close to those of arrival. Ammonia *P* value between the groups was not significant ($P = 0.09$).

Urea levels between T0 and T1 were significantly different. A higher urea level was found at arrival. The parameter increased after 36 h of storage and variations were recorded during the subsequent sampling times. Urea levels were not significantly different between the groups ($P = 0.14$).

The average chloride concentration at arrival was 451.46 ± 10.93 mmol·l^{-1} and it decreased to 439.58 ±14.05 mmol·l^{-1} after 12 h. This difference was significant, while chloride *P* value between the groups was not significant ($P = 0.44$).

Calcium concentrations at arrival were significantly higher ($P < 0.001$) compared to the other sampling times. The subsequent detections showed a similar pattern. Calcium levels showed a significant difference ($P < 0.05$) also between the treatment and control group.

The mean magnesium value at arrival was 6.23 ± 0.21 mg·dl^{-1} in the control group and 6.10 ± 0.24 mg·dl^{-1} in the treatment group. At the subsequent times, minimum variations in both groups were highlighted. The present study did not show a significant modification

of magnesium concentrations either between T0 and T1, or between the groups (P = 0.12).

Table 1. Haemolymph parameters in the control (C) and treatment group (T) at arrival and after 12, 36, 60 and 108 h of tank storage. Values are expressed as means ± standard deviation.

Parameter	Arrival		12 h (n = 12)		36 h (n = 12)	
	Group C	Group T	Group C	Group T	Group C	Group T
Weight (g)	492.34 ± 44.15	477.89 ± 36.59	509.92 ± 44.00	492.95 ± 39.03	510.58 ± 45.42	497.46 ± 34.73
Vitality	4.58 ± 0.51	4.50 ± 0.67	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00
Glucose (mg·dl^{-1})	3.50 ± 3.58	3.58 ± 2.27	1.92 ± 1.83	3.00 ± 1.41	1.09 ± 1.22	3.18 ± 2.36
Lactate (mg·dl^{-1})	9.91 ± 15.44	9.91 ± 6.05	4.16 ± 5.58	2.65 ± 2.00	0.40 ± 0.37	0.31± 0.60
Protein (g·dl^{-1})	1.66 ± 0.61	1.90 ± 0.48	1.66 ± 0.63	1.98 ± 0.55	1.48 ± 0.68	1.91 ± 0.50
Ammonia (mg·dl^{-1})	2.71 ± 1.13	2.80 ± 1.03	1.33 ± 3.35	1.21 ± 5.20	1.55 ± 1.11	1.63 ± 1.21
Urea (mg·dl^{-1})	2.42 ± 1.51	2.08 ± 1.31	1.33 ± 1.30	1.00 ± 1.15	2.73 ± 2.20	2.00 ± 2.32
Chloride (mmol·dl^{-1})	451.67 ± 8.35	451.25 ± 13.51	432.92 ± 16.8	446.25 ± 11.31	489.17 ± 42.90	469.55 ± 14.22
Calcium (mg·dl^{-1})	81.00 ± 5.51	81.79 ± 14.42	68.13 ± 5.69	68.08 ± 6.57	78.36 ± 18.29	67.64 ± 7.83
Magnesium (mg·dl^{-1})	6.23 ± 0.21	6.10 ± 0.24	5.80 ± 0.71	6.28 ± 1.55	6.57 ± 2.41	6.82 ± 3.76

Parameter	60 h (n = 12)		108 h (n = 10)		Sig[a]	Sig[b]
	Group C	Group T	Group C	Group T		
Weight (g)	508.06 ± 44.43	498. 57 ± 33.06	514.80 ± 45.68	501.11 ± 32.54	***	
Vitality	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00		
Glucose (mg·dl-1)	1.08 ± 0.79	1.78 ± 0.97	1.27 ± 0.65	1.82 ± 0.98	***	
Lactate (mg·dl-1)	0.18 ± 0.25	0.12 ± 0.23	0.18 ± 0.33	0.04 ± 0.15	***	
Protein (g·dl-1)	1.14 ± 0.42	1.74 ± 0.36	1.58 ± 0.62	1.82 ± 0.73	**	
Ammonia (mg·dl-1)	1.85 ± 3.16	1.60 ± 4.57	2.34 ± 2.53	1.85 ± 4.68	***	
Urea (mg·dl-1)	3.25 ± 2.14	1.78 ± 1.39	3.27 ± 2.15	2.91 ± 1.14	**	
Chloride (mmol·dl-1)	473.33 ± 28.55	467.73 ± 9.32	471.82 ± 16.32	473.18 ± 12.90	***	
Calcium (mg·dl-1)	76.42 ± 7.38	70.50 ± 2.97	81.59 ± 7.22	72.14 ± 4.01	***	*
Magnesium (mg·dl^{-1})	5.69 ± 0.29	5.72 ± 0.17	5.82 ± 0.22	5.71 ± 0.17		

[a]Significant differences recorded by ANOVA analysis using time as factor. *P value < 0.05 **P value < 0.01 ***P value < 0.001.[b]Significant differences recorded by ANOVA analysis using treatment as factor. *P value < 0.05 **P value < 0.01 ***P value < 0.001.

3.5. Discussion

3.5.1. The effect of immersion in the tank

Significant differences were detected in the vitality, weight and haemolymph parameters between arrival (T0) and after 12 h of immersion (T1) in both groups.

The increase in vitality and weight detected after 12 h and during the subsequent times may be due to the effects of water loss during transport (exposure to air) and the subsequent water intake during storage, as others have also reported (D'Agaro et al., 2014).

The haemolymph glucose content is an indicator of distress status: crustacean hyperglycaemic hormone (cHH) increases in response to air exposure and high temperature (Lorenzon et al., 2007). High glucose concentration at arrival may be related to air exposure, which leads to glucose mobilization for anaerobic metabolism, while the glucose decrease after 12 h may be due to the reduction of stress linked to air exposure. In the present study, the glycaemia remained under the resting value reported by Lorenzon et al. 2007 (12.07 ± 3.42 mg·dl^{-1}).

The lactate concentrations observed upon arrival differ from those obtained in previous studies (Bernardi et al., 2015; Lorenzon et al., 2007), where it was found that the mean lactate concentrations at arrival were 24.41 ± 17.65 mg·dl^{-1} and 52.23 mg·dl^{-1} ± 12.30, respectively. This difference could be related to the batch variability in the studies: subjects chosen in the aforementioned trials probably had

already higher lactate concentrations prior to T0. The increase of lactate concentration during aerial exposure is indicative of anaerobic metabolism and may be due to an inadequate supply of oxygen to tissues. The lactate decrease after 12 h is equivalent to the results of Lorenzon et al. 2007, in which it decreased after 12 h in storage tanks, presumably due to a better level of oxygenation that reduced anaerobic metabolism and subsequent lactate production.

The mean protein value at arrival is equivalent to the results of Bernardi et al. 2015. Haemolymph protein in lobster is influenced by molting and nutrition, and it increases when lobsters are subjected to high temperature and hypoxia: 80–90% of protein is represented by hemocyanin (Brouwer et al., 2004; Chausson et al., 2004; Lee and Chen, 2003). This increases the amount of oxygen that can be delivered to the tissues, which may explain the low protein concentrations obtained in the present study, as the temperature remained constant during the trial and air exposure was only present during transport.

The death of two animals after 108 h may have increased the lobsters' metabolic activity, resulting in a higher demand for protein as an energy store. However, we were not able to verify this assumption.

Nitrogen is mainly excreted as ammonia in aquatic crustaceans, either by diffusion or Na^+/NH_4^+ exchange across the gills (Evans and Cameron, 1986). The ammonia values observed at arrival were comparable with those found by Bernardi et al. 2015 (2.92 ± 0.32 mg·dl^{-1}). The higher ammonia concentrations at arrival may be due to gill dysfunction during air exposure interfering with the catabolite diffusion. However, gill irrigation during the immersion allowed for catabolite exchange and led to a decrease in ammonia after 12 h. The reason for the increment detected after 36 h and 108 h is not currently known.

Urea is linked to toxicity status and directly increases with salinity (Lee and Chen, 2003). High urea concentrations at arrival were due to the physiological response to air exposure: toxic compounds, as they cannot be eliminated through the gills, are then converted to urea, which is less toxic. The same results were highlighted by Bernardi et al. 2015 who found that urea production increased in *Homarus americanus* subjects maintained exposed to air. The reason for the increase of urea detected after 36 h remains unclear.

Hypoxia and high temperatures generally reduce chloride concentrations in crustaceans (Cheng et al., 2003). These conditions increase anaerobic metabolism and lactate production with the subsequent activation of the $Cl^-/HCO3^-$ exchange system, which leads to haemolymph chloride reduction. Despite the aerial exposure, chloride values at arrival were close to the physiological level (449.29

± 3.57 mmol·l^{-1}) reported by Lorenzon et al. 2007. The low chloride concentration after 12 h suggests that aerial stress at arrival negatively influenced the recovery time in the tank. In the present study a positive correlation between chloremia and immersion was found, starting from 36 h.

Calcium concentrations at arrival were found to be equivalent to those of Bernardi et al. 2015 (our study vs. that of Bernardi et al.: 81.39 vs. 83.43 ± 1.40 mg·dl^{-1}), although their study showed no significant difference between T0 and T1. The calcium levels in crustaceans fluctuate in response to ecdysis, water salinity and lactate concentration. Due to the high lactate concentration, the calcium concentrations at arrival were significantly higher than those after 12 h.

Calcium is mobilized from the exoskeleton as $CaCO^3$, and acts as a buffer against further acidosis (Taylor and Whiteley, 1989) and improves the affinity of hemocyanin for oxygen under anaerobic conditions (Bridges, 2001; Dove et al., 2005). However, there is only indirect evidence that this Ca originates in the exoskeleton (Wheatly, 1996). The subsequent detections showed a similar pattern, despite the calcium levels remaining higher than the physiological level of 64.4 mg·dl^{-1} (Lorenzon et al., 2007).

Magnesium in crustaceans contributes to the maintenance of high hemocyanin affinity for oxygen and can be used as a compensatory mechanism for coping with hypoxia (McMahon, 2001; Truchot, 1975). However, the present study did not show a significant modification of magnesium levels between T0 and T1. This result is also in contrast with that of Bernardi et al. 2015 who found a significant difference between arrival and immersion (12 h) times.

3.5.2. The effect of claw ligature

Significant differences between group C and T were detected exclusively in calcium values. The constant pattern observed in haemolymph calcium concentrations suggests that calcium is highly regulated in lobsters, which is consistent with other findings on their calcium metabolism (McMahon, 2001; Wheatly, 1996). Nevertheless, with our current level of knowledge, we are not able to assess the relation between haemolymph calcium and claw ligature. In conclusion, the results of the present study show that claw ligature in American lobsters did not have a significant effect on vitality, weight and haemolymph parameters except for calcium, and currently we are not able to explain why and how the treatment affected this parameter. Therefore, further investigations are required. The significant effect of immersion on all of the parameters confirms what previous studies

have demonstrated, and particularly the importance of storage in tanks with high quality artificial seawater, to reduce metabolic distress. As our results suggested that the rubber bands did not affect the haemolymph parameters linked to stress, and since the absence of claw ligatures can enhance aggressive behaviour among subjects and difficulties in animal handling by food business operators, the habit of keeping lobster's claw blocked can be considered a good operating practice in the trade of live lobsters. However, our study focused only on haemolymph parameters and for a limited time, so the final decision on the effects of claw ligatures on lobsters' welfare cannot be made without further research on both the short and long-term effects, especially concerning behaviour and pain. Future investigations should also be considered regarding haemolymph parameters as useful indicators of poor lobster welfare and/or stressful conditions.

CHAPTER 4

Evaluation of a New Loop-Mediated Isothermal Amplification (LAMP) Assay for the Detection of *Anisakis spp.*

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4.1. Abstract

Objective of the present study was to test the performances of a real-time LAMP-based field-friendly tool system for the detection of *Anisakis* spp., with particular focus on fish products. The specificity of the method was evaluated on *Anisakis* spp. larvae from internal collection. 100% of the *Anisakis* spp. strains tested were recognized, while, correctly, no amplification occurred for non-pathogenic *Hysterothylacium* spp. The sensitivity was evaluated in three independent trials conducted on intentionally infested, at several intensities, salmon fish fillets homogenate, on seabream commercial baby food and on domestic seabream baby food. Results obtained showed a detected minimum intensity of 20 larvae/kg in the first trial (infected salmon fillets homogenate), while in intentionally infected commercial and homemade seabream baby foods this minimum intensity was 4 larvae/kg, in agreement with the limit suggested by the Codex Alimentarius for instruments intended for the identification of the presence of larvae in fishery products (5 larvae/kg). The system did not give the same performances in the equivalent matrixes after thermal treatment inactivation. This LAMP

method can be considered a very useful tool for the application to fish raw matrixes as it is a cost-effective and easy-functioning method, while in the detection of inactivated larvae for the prevention of possible allergic reactions, other studies should be performed.

4.2. Introduction

Parasitic nematodes of the genus *Anisakis* are all over the world considered as one of the biological hazards of major concern in seafood products (EFSA, 2010). *Anisakidae* larvae, at their third larval stage (L3), may be responsible for the zoonotic human infection known as anisakidosis, with *Anisakis* and *Pseudoterranova* as the two genera
associated with this disease. Among the nine species of the genus Anisakis, *A. simplex complex* and *A. pegreffii* were identified as responsible of several human infections, with *A. pegreffii* recognized as the most widespread in Italian waters (Mattiucci and Nascetti, 2008). Human ingests the parasite through the consumption of infected raw or lightly cooked seafood products. The subsequent migration of larvae through the gastrointestinal walls can determine the formation of an eosinophilic granuloma in the mucosa with severe non-specific symptoms (Oshima, 1987); for this reason, the disease is often misdiagnosed.
Moreover, *A. simplex* is the only parasite associate with fish consumption that could cause clinical allergic responses (EFSA, 2010). The main allergic response reported is the gastro-allergic anisakiasis (GAA), an IgE-mediated generalized reaction in which allergic manifestations (urticaria, angioedema-anaphylaxis, eosinophilic gastroenteritis, rheumatological and dermatological symptoms) are generally associated to the acute gastric symptomatology. The symptom generally arises after the consumption of raw or undercooked fish products that contain live larvae; in some cases, the gastric symptoms are mild or completely absent. In addition, in
some cases, dead or inactivated larvae were previously described as cause of sensitization and immunoglobulin E (IgE)-dependent hypersensitivity in humans (Audicana and Kennedy, 2008a). Furthermore, other immunoglobulin isotypes (IgG4) or non-immunological event were also reported by (Daschner et al., 2012). As showed by Caballero and Moneo (2004) (Caballero and Moneo, 2004), the cause of reactions and symptoms after the ingestion of well-cooked or canned fish was likely due to the presence of heat-resistant and/or pepsin-resistant allergens derived from *A. simplex*. Episodes of allergy and GAA were reported in several countries including Spain, Croatia, Italy and Morocco (Abattouy et al., 2012; Audicana and Kennedy,

2008b; Barbuzza et al., 2009; Del Rey Moreno et al., 2006; Mazzucco et al., 2012; Mladineo et al., 2014). Moreover, gastro-allergic reactions due to the presence of *Anisakis pegreffii* have been reported in recent years also in Italy (Mattiucci et al., 2013).
Currently, two methods for the evaluation of the presence of larvae are used: the non-destructive one based on visual inspection of fish fillets, and the destructive one based on artificial chloric-peptic digestion of fish muscle.
In recent years, many different screening molecular-based methods including SYBR green qPCR and multiplex PCR have been developed. Thanks to the potential high sensitivity and the ability to discriminate pathogenic from non-pathogenic nematodes larvae, different systems have been developed and evaluated for the detection of anisakis nematodes in fish and fish products (Cavallero et al., 2017; Fang et al., 2011; Godínez-González et al., 2017; Herrero et al., 2011; Lopez and Pardo, 2010; Mossali et al., 2010). Loop-mediated isothermal amplification (LAMP) is a relatively new real time DNA amplification technique, first described by (Notomi et al., 2000). The main advantage of LAMP consists in the absence of complex thermal cyclers and its results could be obtained within few hours (Mori et al., 2001; Nagamine et al., 2002). Thus, in recent years, the LAMP technique has been deeply studied and used as an easy, rapid and economic field-friendly tool for the identification of several potential pathogenic bacteria like *Salmonella* spp. and *Listeria monocytogenes* (Hara-Kudo et al., 2005; Tang et al., 2011; Erica Tirloni et al., 2017; Wang et al., 2010, 2012). Anyway, only one preliminary study, focused on the suitability of LAMP technology when applied for the detection of *Anisakis simplex* (Tirloni et al., 2017). The aim of the present study was to evaluate the performances of a LAMP-based method for the detection of *Anisakis simplex* in fish-based matrix with attention to baby food, where heat-inactivated larvae could be present and responsible for allergic reactions in infants.

4.3. Materials and methods

4.3.1. Evaluation of the specificity of the method

8 *Anisakis* spp., three *Anisakis simplex sensu strictu*, four *Anisakis pegreffii*, one *A. ziphidarum* and 1 *Hysterothylacium* spp. were used to evaluate the specificity of method. These specimens belonged to the internal laboratory collection and were previously isolated from fish and identified by PCR technique. Parasites were kept at -80 °C until use. The producer declared that the instrument was able to detect the following species: *Anisakis simplex, Anisakis pegreffii, Anisakis physeteris, Anisakis typica* and *Anisakis ziphidarum.*

4.3.2. Protocol of Lamp diagnostic test for Anisakis spp.

For the tests, the system "ICGENE mini" (Enbiotech, Palermo, Italy) was used. The system is based on a LAMP technology, and is composed by a fluorescence amplifier, able to detect the fluorescence produced by the samples (it allows the simultaneously analyses of 12 samples), and a device that worked with Radio Frequency Identification technology (RFID), that guides all the activities step by step and can interpret in real-time the results obtained. For this research, specific kits named "ICGENE Anisakis" were tested. The operative protocol of the instrument, provided by the producer, is described in the sections below.

4.3.3. DNA extraction

An aliquot of about 1 mg of each parasite was collected and suspended in extraction buffer and immediately homogenized by vortex to allow the extraction of nucleic acid by chemical lysis. The homogenate was then incubated at room temperature for at least 10 minutes and newly homogenized in the same conditions.

4.3.4 Amplification (LAMP) phase

3 µl of homogenate were inserted into primer mix tubes with 22 µl of LAMP mix (Enbiotech, Palermo, Italy) containing a lyophilized primer and a master mix of reagents useful to carry out the test (enzyme, Magnesium chloride, nucleotides and reaction buffers) and 30 µl of mineral oil. Then, tubes were vortexed with the aim to obtain a homogenous solution and inserted into the amplifier for 60 minutes at 65 °C; the positivity was assessed graphically by the development of sigmoid curve by the instrument. Positive and negative DNA control included in the kit were also used.

4.3.5 Experimental infestations

a. Harvest of Anisakis spp. larvae

Larvae used for the intentionally infection were isolated from commonly consumed fish purchased in retail stores in Milan (Italy); in particular, *Anisakis* was obtained from mackerels (*Scomber scombrus*), caught in Atlantic Ocean (FAO zone 27). To isolate the larvae, body cavity and belly flaps were examined by visual inspection and the larvae isolated were observed by light microscopy in order to identify the genus (Berland, 1961) (6320D spectrophotometer, Jenway, Staffordshire, UK) and the preserved in saline solution until use as reported below.

b. Evaluation of the sensitivity of the method on fish matrix

For the evaluation of the sensitivity of the LAMP method, farmed salmons were chosen at this stage to avoid the presence of the parasite in fish muscle. Anyway, salmon samples were checked before use for absence of larvae. Salmon fish muscle (Sa) was intentionally infected with 8 known larvae concentrations: 3000 larvae/kg (Sa3000), 2000 larvae/kg (Sa2000), 200 larvae/kg (Sa200), 100 larvae/kg (Sa100), 50 larvae/kg (Sa50), 20 larvae/kg (Sa20), 10 larvae/kg (Sa10) and 4 larvae/kg (Sa4); then infected fish muscle was homogenised. Afterwards, to assess the sensitivity of the technique, LAMP analyses were carried out in triplicate on each matrix. Control samples (not infected homogenised fish muscle) were also tested.

c. Evaluation of the sensitivity of the method on homemade baby food

At this stage, farmed seabreams (farmed in Greece) were purchased. Fish muscle was homogenised and homemade seabream baby food (Hs) was produced following a common recipe [200g of fish muscle, 250 g of vegetables (potatoes and carrots in equal concentration), 400 mL of water, 2 g of oil, 30 g of rice flour].

Fish muscle was then intentionally infected with larvae at 6 known concentration: 200 larvae/kg, 100 larvae/kg, 50 larvae/kg, 20 larvae/kg, 5 larvae/kg and 1 larva/kg, obtaining a final infection intensity in baby food equal to 40 larvae/kg (Hs40), 20 larvae/kg (Hs20), 10 larvae/kg (Hs10), 4 larvae/kg (Hs4), 1 larva/kg (Hs1)

and 0.2 larvae/kg (Hs0.2), respectively. All the baby foods infected at each intensity were then dispensed in 80 g glass boxes and afterwards, LAMP analyses were carried out on each matrix in triplicate. Control samples (not infected homogenised fish muscle) were also tested in triplicate

d. Evaluation of the sensitivity of the method on seabream commercial baby food

At this stage, to evaluate the sensitivity of the LAMP method, a commercial seabream baby food (Cs), was used. Each of baby food samples was intentionally infected with the larvae isolated from other fishes, as reported previously (Section a. Harvest of Anisakis spp. Larvae); 6 known intensity of larvae infection were tested: 40 larvae/kg (Cs40), 20 larvae/kg (Cs20), 10 larvae/kg (Cs10), 4 larvae/kg (Cs4) and 1 larvae/kg (Cs1). Afterwards, to assess the sensitivity of the technique, LAMP analyses were carried out on each matrix at different infection intensities in triplicate. A control sample (not infected baby food) was also tested in triplicate.

e. Evaluation of the sensitivity of the method on homemade and commercial sterilized baby food fish-based matrix

At this stage, to evaluate the sensitivity of the LAMP method, sea bream-based baby foods were chosen. Each of these baby foods was intentionally infected with the larvae isolated from other fishes; 5 known intensity of infection were considered: 40 larvae/kg (Css40), 20 larvae/kg (Css20), 10 larvae/kg (Css10), 4 larvae/kg (Css4) and 1 larva/kg (Css1). Afterwards, baby food was sterilized at 105 °C for 75 minutes. Ten LAMP analyses were carried out on each matrix at different infection concentration in triplicate. Control samples (not infected homogenised fish muscle) sterilized were also tested in triplicate.

Homemade baby food was produced as described before. Fish muscle was then intentionally infected with larvae at 6 known intensity: 200 larvae/kg, 100 larvae/kg, 50 larvae/kg, 20 larvae/kg, 5 larvae/ kg and 1 larva/kg, final infested intensity in baby food equal to 40 larvae/kg (Hss40), 20 larvae/kg (Hss20), 10 larvae/kg (Hss10), 4 larvae/kg (Hss4), 1 larva/kg (Hss1) and 0.2 larva/kg (HssA0.2), respectively. Baby foods infected were sterilized at 105 °C for 75 minutes. Afterwards, LAMP analyses were carried out on each matrix in triplicate. Sterilized control samples (not infested homogenised fish muscle) were also tested in triplicate.

4.4. Results

4.4.1. Evaluation of the specificity of the method

By using the LAMP system, positive amplification was obtained for all *Anisakis* species tested (*A. simplex sensu strictu, A. pegrefiif, A. ziphidarum* and *A. simplex*/*A. ziphidarum*). *Hysterothylacium* spp., correctly, was not identified by the LAMP system. Times of amplification ranged from 16 to 25 minutes.

4.4.2. Evaluation of the sensitivity of the method on fish matrix

Results obtained in infected fish fillets are reported in Table 1. The kit was able to amplify the parasites when present from 2000 to 20 larvae/kg (SaB, SaC and SaF: 3/3 positive samples; SaD: 2/3 positive samples; SaE: 1/3 positive samples), while no amplification was reported for samples characterized by infestation intensities of 3000 larvae/kg, probably due to the great amount of DNA present in the sample, and for samples below 20 larvae/kg (10 and 4 respectively). Amplification times varied from 16 to 35 minutes.

Table 1: Sensitivity of LAMP technique in intentionally infested raw salmon fish muscle (Sa) at different intensities.

	SaA	SaB	SaC	SaD	SaE	SaF	SaG	SaH
Trial 1	-	+	+	-	+	+	-	-
Trial 2	-	+	+	+	-	+	-	-
Trial 3	-	+	+	+	-	+	-	-

SaA=3000 larvae/kg; SaD=100 larvae/kg; SaG=10 larvae/kg;
SaB=2000 larvae/kg; SaE=50 larvae/kg; SaH=4 larvae/kg
SaC=200 larvae/kg; SaF=20 larvae/kg;

4.4.3. Evaluation of the sensitivity on homemade baby food

Results obtained in infested homemade fish-based baby food are reported in Table 2. The kit was able to amplify the parasites when present from 40 (1/3 positive samples) to 4 larvae/kg of baby food (1/3 positive samples), while no amplification was reported for samples characterized by infestation intensities of 1 and 0.2 larva/kg of baby food. Amplification was not successful for the baby food infested with intensities equal to 10 larvae/kg, probably due to the variability during homogenization of the larvae. Amplification times varied from 20 to 35 minutes.

Table 2: Sensitivity of LAMP technique in intentionally infested homemade seabream baby food (Hs) at different intensities

	HsA	HsB	HsC	HsD	HsE	HsF
Trial 1	+	+	-	-	-	-
Trial 2	-	+	-	+	-	-
Trial 3	-	+	-	-	-	-

HsA= 200 larvae/kg fish= 40 larvae /kg baby food;
HsB= 100 larvae/kg fish = 20 larvae /kg baby food;
HsC= 50 larvae/kg fish = 10 larvae /kg baby food;
HsD= 20 larvae/kg fish = 4 larvae /kg baby food;
HsE= 5 larvae/kg fish = 1 larvae /kg baby food;
HsF= 1 larva/kg fish = 0.2 larvae /kg baby food.

4.4.4. Evaluation of the sensitivity on commercial baby food

Results obtained in infested commercial fish-based baby food are reported in Table 3. The kit was able to amplify the parasites when present from from 40 (2/3 positive samples) to 4 larvae/kg (1/3 positive samples), while no amplification was reported for samples characterized by infestation intensities of 0.2 larvae/kg baby food. Amplification times varied from 20 to 35 minutes.

Table 3: Amplification by LAMP technique in intentionally infested commercial seabream baby food (Cs) at different intensities.

	CsA	CsB	CsC	CsD	CsE	CsF
Trial 1	+	+	+	+	+	-
Trial 2	+	+	+	-	-	-
Trial 3	-	-	+	-	-	-

CsA= 40 larvae /kg baby food;
CsB= 20 larvae /kg baby food;
CsC= 10 larvae /kg baby food;
CsD= 4 larvae /kg baby food;
CsE= 1 larvae /kg baby food;
CsF= 0.2 larvae /kg baby food.

4.4.5. Evaluation of the sensitivity on sterilized homemade baby food and sterilized commercial baby food

The kit was never able to amplify the parasites in homemade or commercial baby food at each of the six intensities considered when sterilization happened after infestation at fixed intensities (data not shown).

4.5. Discussion

About 20,000 human anisakiasis cases were diagnosed worldwide before 2010; over 90% of notifications came from Japan, where around 2000 cases were described per year (EFSA, 2010). Also, in Italy, 144 number of cases were stated in the period 2009-2013 (Istituto Superiore di Sanità (ISS), 2016): in all the cases, the patients reported the consumption of raw or marinated fish. The incidence of this zoonosis is considerably increased in the last years due to the growth of the international market of fish and fish products and the development of new diagnostic tests that have proven anisakiasis as a worldwide zoonosis. Human anisakiasis is commonly frequent especially in those countries where culinary habits provide the consumption of large amounts of raw or undercooked fish (sushi, sashimi, ceviche or carpaccio). In the Mediterranean countries, human anisakiasis is related also with the consumption of traditional preparations (marinated fish, especially anchovies).

Food business operators and competent authority are particularly sensitive to "Anisakiasis" thematic, and, as the presumptive presence of larvae in many commonly consumed fish is considered a natural condition, they feel the need of specific, fast and easy to use diagnostic systems, able to identify the presence of larvae of *Anisakis* in fish matrixes and fish-based products.

In the present study, a LAMP based system was evaluated with the aim to provide useful information in terms of specificity and

sensitivity in different fish-based matrixes; this real time, rapid and cost-effective field-friendly tool, may be convenient for public and private laboratories as results on the presence of larvae in a product may be available in a short time (less than 1 hour).
Specificity tests demonstrated that, the system was able to detect correctly all *Anisakis* species, without amplification of non-pathogenic *Hysterothylacium* spp.
In terms of sensitivity, the system showed good performances: in the first experiment, where larvae were added to homogenate salmon fish fillets, the minimum intensity detected by the system was 20 larvae/kg. Other study evaluated the performances of real time PCR assay with limit of detection of 1 larva in 25g of fish tissue, corresponding to 40 larvae in 1kg of fish (Lopez and Pardo 2010). The minimum intensity determined in the first experiment, was slightly higher if compared to the limits suggested by Codex Alimentarius (5 larvae/kg fish) and to the suggested British commercial limit (3 larvae in 3,2 kg of fish).
When applied to homemade baby foods, the minimum infestation detected was 4 larvae/kg of whole baby food (20 larvae/kg in the fish muscle as it represents 20% of a baby food), in agreement with the limits reported above. Results obtained from commercial baby food were generally in agreement with those reported above with a minimum intensity detected established at 4 larvae/kg of product, although in one of the three replications, the test was able to amplify the concentration equal to 1 larva/kg of baby food. Positively, the system tested, did not show any interference or inhibition due to the presence in the food matrix of fat, minerals, polysaccharides, enzymes, glycogen, effects reported by previous studies on PCR assays (Richards, 1999).
Furthermore, the attention of the medical community has been moved because of the possible implications caused by *Anisakis* allergy in hypersensitive individuals. (Kasuya et al., 1990) in 1990, reported the first case of urticaria not related to a direct allergy to fish but due to sensitization to *Anisakis simplex*. *A. simplex* is, by now, the only species described to be able to cause allergic responses after consumption of fish products (EFSA, 2010).
As already stated by (Ivanović et al., 2017), food allergies are increasing faster if compared to the other allergic syndromes worldwide and due to the seafood industry globalization, the risk of acquiring anisakiasis in developed countries is miscalculated (Ivanović et al., 2017). In addition, as reported by (Moneo et al., 2017), several patients with no clear symptoms are underdiagnosed in endemic areas; this underestimation should be further investigated by

the research community (Moneo et al., 2017). To match this goal, a constant and continue deepening especially in the development and the evaluation of tools useful for the research of these parasites in fish products, is strategical for the knowledge of the prevalence and intensity of alive, but also dead parasites in fish products.
The second part of the test was performed on the same concentrations of infested larvae in domestic and commercial baby foods submitted to a sterilization process. In this case, the thermal treatment that was mimicked by our sterilization process (105°C for 75 minutes), determined the DNA degradation that impeded the amplification, and dead larvae were not recognized by the system. This fact was already reported for other systems for samples submitted to other thermal treatments (Hird et al., 2006).

4.6. Conclusions

The performances in terms of specificity and intensity of infection detected by the LAMP based system evaluated, indicated its appropriateness in the use in infected raw fish products with the aim to be a quality control point of care system advantageous for food operators and competent authority with the aim to avoid the infection with parasites. The detection of *Anisakis* genetic material in thermally treated products can be still considered a challenge for the performance of PCR-based methods, due to the modifications of DNA molecules, and the food industries should consider the control of the raw matter used for the production as a key phase in their control programs.

CHAPTER 5

Development of a protocol for the extraction of microplastic fibres from bivalve tissues

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5.1. Abstract

Microplastics are a widespread pollutant impacting aquatic ecosystems across the globe. Environmental sampling has revealed synthetic fibres are prevalent in seawater, sediments, and biota. However, microplastic fibres are rarely used in laboratory studies. The aim of the present study is the development of a protocol suitable for the extraction of microplastic fibres from bivalve tissues. For this purpose, in the first phase of the present study, standard microfibres were prepared by using surgical synthetic fibres monofilament, paraffine and a microtome and the digestive solution was evaluated for its compatibility with polymers (assessed through recovery rate and morphological changes). In the second phase the digestion efficacy of mussel tissues (*Mytilus galloprovincialis, Chamelea gallina, Ruditapes philippinarum*) and the recovery rate of microfibres from spiked samples was evaluated. The preliminary results suggested that the digestion protocol was able to remove biological materials without affecting the polymer composition of the microfibres. However, standardisation of microfibres lengths was not always possible, preventing the differentiation between field microfibres and standard microfibres. The method proposed is promising, but optimisation and validation is needed. The use of replicable and reproducible methods for preparing microplastic fibres of a size appropriate to the test organisms could allow the inclusion of microfibres into microplastic laboratory testing.

5.2. Introduction

Microplastics (plastic particles ranging from 1 μm to 5 mm in size) are an anthropogenic pollutant that can enter the environment from both primary and secondary sources. Primary sources include particulates which are intentionally manufactured to be of microscopic size (e.g., resin pellets, microbeads incorporated in cosmetic products and beads for abrasive blasting of surfaces). Secondary sources include microplastics originated from the degradation of larger plastic items. Small plastics were first discovered as a contaminant in the marine environment in the early 1970s (Buchanan, 1971), but the term "microplastics" was coined in 2004 by Thompson, 2004. Since then, the topic of microplastics in the marine environment have attracted attention in the scientific community, due to their potential impact in sea organisms (de Sá et al., 2018, 2015; Kühn et al., 2015; von Moos et al., 2012; Wright et al., 2013) and humans (Barboza et al., 2018a; Catarino et al., 2018; Weis et al., 2015; Wright and Kelly, 2017). Microplastics have been detected in seafood sold for human consumption (Bellas et al., 2016; Bråte et al., 2016; Hermabessiere et al., 2019; Karami et al., 2017; Li et al., 2015; A. Lusher et al., 2017b; Rochman et al., 2015; Toussaint et al., 2019; Van Cauwenberghe and Janssen, 2014) and this evidence raised concerns regarding the potential ingestion of microplastics by humans and their effects on health and the food web. Within marine fisheries, mussels deserve a special focus because they represent a potential pathway of exposure to humans: as filter-feeders, they are easily exposed to microplastics, and they are consumed at whole. Also, mussels are suitable indicator for microplastic pollution because of their wide distribution, vital ecological niches, susceptibility to microplastic uptake and close connection with marine predators and human health (Li et al., 2019). The concentrations and types of plastic sampled can vary considerably among literature, with spatial and temporal variances attributed to a suite of biotic and abiotic factors, and the sampling method implemented (Clark et al., 2016; Cole, 2016; Hidalgo-Ruz et al., 2012). Fibres are the most prevalent types of microplastic debris observed in the natural environment. The synthetic microfibres are typically manufactured from nylon, polyethylene terephthalate (polyester, PET), or polypropylene (PP) (Andrady, 2011; Hidalgo-Ruz et al., 2012) and their presence is commonly attributed to the release of synthetic fibres from garments during washing (Browne et al., 2011), degradation of cigarette butts leading to the release of cellulose acetate fibres (Wright et al., 2015), and fragmentation of maritime equipment (e.g. ropes and nets). Owing to their myriad sources, the size of microplastic fibres identified in environmental samples is

understandably variable (Cole, 2016). In the marine environment a number of invertebrates have been observed to ingest fibrous microplastics (Desforges et al., 2015; Mathalon and Hill, 2014; Murray and Cowie, 2011). Qu et al., 2018 showed that fibres were prevalent in mussels from field investigation while beads were most ingested by mussels after five days of indoor exposure, probably because fibres in mussels result from long-term accumulation in the marine environment, while beads are more easily ingested by mussels in short time periods. Another explanation is that beads could be egested more quickly than fibres, since synthetic fibres were detected in mussels after gut clearance period (De Witte et al., 2014). von Moos et al., 2012 demonstrated that mussels can ingest and accumulate microplastics ranging from 0 to 80 μm in digestive system epithelial cells, while (Van Cauwenberghe et al., 2015) found that only microplastics of the smallest size (10 mμ), was detected in mussels although three sizes (10 mμ, 30 mμ, 90 mμ) of microplastics were used in the exposure experiment. Fibres are environmentally prevalent (Phuong et al., 2016), however, according to Cole, 2016 , spherical microbeads have been used as representative plastics in the majority of microplastics studies, while synthetic fibres have been underrepresented. This environmental-laboratory mismatch is largely due to the availability of microplastics suitable for laboratory use. Synthetic microscopic fibres (MFs) are not available for purchase, while microbeads are readily purchased from scientific suppliers, allowing researchers to conduct replicable, reproducible and robust experiments. Existing methods for preparing MFs are limited to cutting or cryogenically grinding synthetic cord, resulting in relatively large fibres (>500 μm in length) with a wide size distribution (Au et al., 2015; Graham and Thompson, 2009; Hämer et al., 2014; Murray and Cowie, 2011; Watts et al., 2015). The subjective nature of these methods limits reproducibility of test fibre, for this reason (Cole, 2016) developed and validated a cryogenic microtome protocol for preparing standardised microfibres that provides a consistent method for preparing standardised fibrous microplastics. In this context, the aim of the present study is the development of a protocol suitable for the extraction of microplastic fibres from bivalve tissues. For this purpose, microfibres were prepared and protocol suitability was determined by assessing the digestion efficacy of mussel samples and the recovery rate of microfibres from dosed samples. Costs, potential hazards associated with the reagent and time spent in extracting microplastics from mussels were also considered.

5.3. Materials and methods

5.3.1. Cross-contamination risk reduction measures

Working area decontamination. all the working surfaces were rinsed with ethanol 70% (CAS n° 64-17-5). The materials were washed with a laboratory glassware washer, intensively rinsed with ultrapure water (Millipore Helix 5) and dried up-side-down in a desiccator, in order for airborne microplastics not to accumulate in them.

Controlled environment. All solutions were filtered prior to use. Ethanol (CAS n° 64-17-5), ultrapure water (Millipore Helix) and KOH (CAS n° 1310-58-3) were filtered through cellulose acetate filter, pore size 0,45 mμ. Filters were previously checked under a dissecting microscope to exclude any potential contamination derived from the fabrication process. A reduced number of people worked on each task and the colour of the clothes of each person was wearing underneath their lab coat was recorded on datasheet. White cotton lab coat and blue nitrile gloves were worn during the entire analyses. Nitrile gloves were washed with filtrated ultrapure water to remove any potential particle. The analysis (preparation of the samples and filtration) were carried out under a laminar flow cabinet.

Control samples. Two types of control samples were prepared according to (Dehaut et al., 2019), the Atmospheric Control (AC) and the Positive Control (PC). AC consisted of glass microfiber filter (Whatman GF/A, 90 mm, pore size 1,6 μm) left opened in Petri dishes during sampling and filtration phases to monitor airborne particles; the PC consisted of a flask containing 100 ml of KOH 52% and two prepared microfibres (3-0) that followed the same steps of the sample treatment, being the main difference the fact that it was run without the sample itself (mussel soft tissues and intervalvular liquid).

5.3.2. Microfibres preparation protocol

Three types of microfibres were prepared by using reels of surgical monofilament synthetic fibres. Fibres were selected to encompass polymers commonly identified in marine samples: nylon (polyamide), polyethylene terephthalate (polyester, PET) and polypropylene (PP) with a range of filament diameters (Table 1).

Table 1. Synthetic fibres used to prepare microfibres.

Polymer	Calibre	Colour	Length (μm)	Manufacturer	Product #
Polyamide 6	3-0	Green	4	Ethicon	669
Polyester PET	6-0	Green	4	Covidien	D1722
Polypropylene PP	8-0	Blue	4	Ethicon	8740

Three filaments were cut into 6 segments of 5 mm lengths (2 segments per filament). The microfibres were placed in tissue embedding cassettes for paraffin inclusions and frozen. The cassettes were secured to a microtome mount (Leica RM2125RT) and sectioned continuously at pre-determined length (4 µm). The paraffine sections obtained were subsequently collected, placed under a stereomicroscope (x10-x57 objective; Olympus SZX9) and analysed. Only paraffine sections containing at least two microfibres were selected for the following step.

Corrosiveness of the digestion solution against microfibres. Paraffine sections selected for the digestion test are shown in Table 2. The resistance of plastic fibres against the selected digestion treatment was evaluated by measuring the polymer recovery rates. For this purpose, paraffine sections containing the microfibres were digested in a 250 ml beaker with 100 ml KOH solution (52%, d = 1.5 g/ml). KOH solution was selected based on literature data: alkaline digestion can be used with a greater range of polymers because it has a lower risk of chemically modifying or structurally damaging polymers.

The selected KOH density was higher than the common polymers in order to float microplastic fibres and recover them from the surface. The solution was then covered with aluminium foil and digested in agitation for 48 h in oven (Ikamag RCT) at 40 °C. After 48 h of digestion, the digestate was removed from the oven and placed under a laminar flow cabinet. The solution was transferred to a 100 ml falcon tube and centrifugated 6000 rpm per 10 minutes. The supernatant was vacuum filtered through a glass fibre filter (Whatman N° 541 - 1,6 µm pore size) and falcon tube was rinsed three times with ultrapure water to recover all the microfibres. Filter was then placed in a Petri glass and left to dry in a desiccator for 24h. Microscopical examination of the filters was performed using a dissecting microscope (x10-x57 objective; Olympus SZX910). Microplastic abundance, size, shape, and colour were recorded.

Table 2. Paraffine sections subjected to the digestion test.

Name of the section	Type of polymer	Calibre	n° of MFs in the section
PET- A	Polyester PET	6-0	4
PET - B	Polyester PET	6-0	2
PO - A	Polyamide 6	3-0	2
PO - B	Polyamide 6	3-0	2

5.3.3. Spiked bivalve samples experiment

To assess the digestion protocol in a practical situation, mussels were separately spiked with the prepared microfibres in duplicate. The

protocol was chosen based on literature data and preliminary tests, as the most economic, timesaving and hazardousness method.

Sample preparation. Three species of mussels were purchased from a fish market in Milan: *Mytilus galloprovincialis*, *Chamelea gallina* and *Ruditapes philippinarum*. Samples were analysed in duplicate. The collecting/capture area, packaging and storage method of each sample was registered (Table 3). Before the digestion step, mussels were rinsed with distilled water (Millipore Helix 5) to remove sand and other potential external particles.

Table 3. Information on mussel samples subjected to the extraction protocol.

Species	Production method	Production area	Packaging and storage method
Mytilus galloprovincialis	Dredges	Castro Bay	Net and frozen
Chamelea gallina	Dredges	FAO 37.2.1.	Net and frozen
Ruditapes philippinarum	Dredges	FAO 37	Canned, precooked, and frozen
Ruditapes philippinarum	Farmed	Goro	Vacuum packaged and refrigerated

KOH digestion. 10 ± 1 g of mussel (soft tissue and intervalvular liquid) were placed in a 250 ml beaker with 1:10 w/v KOH solution (52%, d = 1.5 g/ml). KOH solution was selected based on literature data: alkaline digestion is used successfully to digest bivalves in microplastic studies and can be used with a greater range of polymers because it has a lower risk of chemically modifying or structurally damaging polymers. The selected KOH density was higher than the common polymers in order to float microplastics out from salts and sand. The mixture was then covered with aluminium foil and digested in agitation for 48 h in oven (Ikamag RCT) at 40 °C. After 48 h of digestion, the digestate was removed from the oven and placed under a laminar flow cabinet.

Recovery test. Based on digestion test results, microfibres were prepared from polyamide 6 monofilament (3-0) as explained in section 5.3.2. Paraffine sections containing the embedded microfibres were used to spike the bivalve samples before the digestion with KOH (Table 4).

Table 4. Spiked bivalve samples incubated with digesting solution.

Sample	Species	N° of microfibres	Size range (μm)
1a	*Mytilus galloprovincialis*	2	200 – 500
1b	*Mytilus galloprovincialis*	2	200 – 500
2a	*Chamelea gallina*	4	200 – 500
2b	*Chamelea gallina*	3	200 – 500
3a	*Ruditapes philippinarum*	3	200 – 500
3b	*Ruditapes philippinarum*	3	200 – 500

4a	*Ruditapes philippinarum*	3	200 – 500
4b	*Ruditapes philippinarum*	3	200 – 500

Centrifugation and filtration. The digestate was transferred to a 100 ml falcon tube and centrifugated 6000 rpm per 10 minutes. The beaker was rinsed three times to recover all the biological material. The supernatant was vacuum filtered through a glass fibre filter (Whatman N° 541 - 1,6 µm pore size) and falcon tube was rinsed three times with ultrapure water to recover all the particles. Filter was then placed in a Petri glass and left to dry in a desiccator for 24h.

5.3.4. Microscopical visualisation

Microscopical examination of the filters was performed using a dissecting microscope (x10-x57 objective; Olympus SZX910). Microplastic abundance, size, shape, and colour were recorded by using the criteria of (Frias and Nash, 2019; Gigault et al., 2018; Hidalgo-Ruz et al., 2012; Van Cauwenberghe et al., 2015):

–Absence of cellular and organic structures.

–Size: ranging from 1 µm to 5 mm.

–Shape: fibre is frayed, folded over, equally thick, not taper towards the end and has a three-dimensional bending.

–Dimension: only microfibres equal or longer than 200 were considered in order to the visual identification should not be applied to particles <500 µm were considered in order to reduce the probability of a misidentification.

5.4. Results and discussion

5.4.1. Microfibres preparation protocol

Microfibres were not standardised, with size ranging from 180 µm to 2.9 mm for green polyester (PP) filament 6-0 and from 200 to 500 µm for green polyamide 3-0 filament. This could be the result of fibres misalignment into the paraffine cassettes. Also, the production of microfibres from blue polypropylene (PP) monofilament 8-0 was not possible due to the high length variability that prevent microfibres detection and size classification during microscopical analysis. Prepared microfibres were not always detectable in paraffine sections and, if any were present, the quantity varied among sections. This is probably due to a carryover effect of the microtome during sectioning. For these reasons, paraffine sections containing less than two microfibres were excluded from further analysis. Paraffine sections contaminated with air-borne microplastics were also excluded. Contaminating microplastics were recognised based on colour and dimension. However, an accurate differentiation between

contaminants microfibres and prepared microfibres was possible only in two microfibre samples (PO-A and PO-B), thanks to the size range homogeneity of the prepared microfibres.

5.4.2. Microfibres digestion test and recovery from spike samples

Paraffine with embedded microfibres was completely dissolved by the digesting solution, allowing the filtration of the solution and the subsequent microscopical visualisation of the microfibres. 100% of microfibres were recovered in 3 out of 4 samples after digestion test, and 75% of the microfibres were recovered in 1 sample (Table 5).

Table 5. Recovery of prepared microfibres and number of contaminating microfibres after digestion test.

Sample	n° of MFs before digestion	N° of MFs after digestion	N° and colour of contaminating MFs
PET-A	4	3	2 (red)
PET-B	2	2	4 (red and blue)
PO-A	2	2	3 (red and blue)
PO-B	2	2	2 (red and blue)

However, contaminants microfibres were detected in all the filters. In this phase, differentiation between contaminants and prepared microfibres was only based on dimension, as the colour of microfibres after the digestion test seemed to be affected by digestion treatment. In fact, green polyamide (PO) and green polyester (PET) microfibres appeared blue-coloured at the microscopical visualisation. Differentiation between prepared and contaminants microfibres was possible only in two microfibres samples (PO-A and PO-B), thanks to the size range homogeneity of the prepared microfibres.

For this reason, polyamide 6 monofilament 3-0 was selected as the most suitable for preparing standard microfibres and used for the recovery test in bivalve samples. However, recovery rate in spiked bivalve samples (Table 6) was extremely low (48%). In fact, the lack of fibres standardisation, together with the external contamination, prevent an accurate and precise microfibres differentiation. Also, differentiation based on colour was not possible because the initial microfibres colour was modified by the digestive solution.

Table 6. Type and number of microfibres recovered from bivalve samples after the extraction protocol.

Sample	Species	N° of microfibres before digestion	N° of microfibres after digestion	N° of contaminants MFs
1a	*M. galloprovincialis*	2	2	3
1b	*M. galloprovincialis*	2	0	7
2a	*C. gallina*	4	2	4
2b	*C. gallina*	3	1	2
3a	*R. philippinarum*	3	2	5

3b	*R. philippinarum*	3	2	2
4a	*R. philippinarum*	3	1	4
4b	*R. philippinarum*	3	2	3

5.4.4. Control samples

The microscopical visualisation of the Atmospheric Control filter revealed an external contamination (3 microfibres). The positive control PC was not contaminated and 100% of the prepared microfibres were recovered, suggesting the compatibility of the digestion protocol with the used polymers (polyamide 6 monofilament). The presence of air-borne particles suggests that contamination preventive measures were not sufficient to prevent samples from being contaminated, as also reported by (Dehaut et al., 2019).

5.5. Conclusions

The adapted microplastic fibres preparation protocol proved ineffective in the creation of standard microfibres. Microfibres size was not standardised and microfibres were not always detectable in paraffine sections. Lack of standardisation also prevent an accurate and precise microscopical identification of fibres and their differentiation from contaminating microfibres. For this reason, a specific procedure that guarantee microfibres alignment in paraffine and their stillness during sectioning must be introduced in further research.

The use of fibres that are bright-coloured may facilitate standard microfibres' identification. For this purpose, microfibre colour must not be affected by the digestion solution.

Furthermore, measuring the polymer recovery rates is not sufficient to evaluated corrosiveness of the selected digestion method towards microfibres. For this reason, resistance of microfibres against the digestion treatment should be evaluated by measuring also molecular alterations through Raman spectroscopy of IR spectroscopy.

The presence of air-borne particles suggested that contamination preventive measures were not sufficient to not prevent samples contamination. However, as external contamination cannot be 100% ruled out, the use of cleaning procedure and different controls remains mandatory in order to minimise the risk of contamination and to collect contamination data that can be incorporated into results.

CHAPTER 6

Comparison of chemical composition and safety issues in fish roe products: application of chemometrics to chemical data

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6.1. Abstract

Processed fish roes are acquiring considerable importance in the modern food market, entering more and more often as an ingredient in food preparation and as caviar substitutes. In this study, we defined quality, traceability and safety issues related to processed fish roe products from different species. The results obtained allowed to distinguish eggs originated from different fish species and to discriminate between fish roes and caviar samples obtained from four different sturgeons species. We observed that roes showed a trend of grouping according to ecological and reproductive habits of fish species. We highlighted the differences between eggs originated by farmed and freshwater fish, enriched in n6 polyunsaturated fatty acids (PUFAs), and all the others, in which n3 PUFAs were prevalent. In addition, we evaluated processed fish roes under a food safety point of view, combining microbiological analysis with the determination of organic acids, used in some products as authorized preservatives. Microbiological characterization has proved a general good hygienic level for these products. Organic acids determination showed values in compliance with European Union (EU) regulations in almost of samples; in some cases, we found a mismatch between the organic

acids detected and what was reported in labels. Processed fish roes could be considered a safe product that can provide to human nutrition a valuable content of essential fatty acids.

Keywords: fish roes; fatty acids; organic acids; linear discriminant analysis; caviar

6.2. Introduction

Fish roe products has been historically present in many food cultures for a long time. The processing of mullet and tuna roes generates the Bottarga, a typical cured product of Mediterranean countries, while Ikura, Tarako and Tobiko are typical Japanese preparations that originate from salmon, pollock, and flying fish roes, respectively (Shirai et al., 2006). The most precious fish roe product is Caviar, obtained by light salting of roes extracted from sturgeons, separated from their connective tissue (Bledsoe et al., 2003). Caviar traditionally originated from the Russian and Persian empires and nowadays it is one of the most expensive luxury food products, appreciated all over the world. According to the Codex Alimentarius (Codex Alimentarius, 2010), only the product coming from the processing of roes obtained from fish of the *Acipenseridae* family can be named as Caviar. Fish roe from other fish species than sturgeon can be defined as caviar substitutes. They are sold at a lower price, but they refer to caviar with the aim of exploiting its high appeal among consumers. This reference is evidenced by the habit that some producers have when they confer the black colour to products that naturally are differently coloured, in order to resemble as much as possible to sturgeon roe (Bronzi and Rosenthal, 2014). In some cases, with traditional products like lumpfish roes, fish processed roes have a specific niche market that is not in competition with caviar market, but, on the contrary, they can lead new consumers to approach the consumption of fish eggs, starting with the cheapest products and then moving towards the product of excellence represented by caviar (Bronzi et al., 2019). During last years, a great increase in the market of caviar substitutes occurred since (i) the availability of natural-sourced Caviar declined, due to the block of catch quotas granted in 2006 by the CITES (Convention on International Trade in Endangered Species) related to the steady decline of world sturgeon wild populations; (ii) the amount of Caviar produced by aquaculture was limited. In addition, the popularity of sushi led to the development of new products obtained by manufacturing roes from other fish species and to the expansion of their market. The estimated global market of processed fish roes covers 60,000 tons, while real caviar production does not reach 500

tons (Bronzi and Rosenthal, 2014; Sicuro, 2019). In 2000, European Union (EU) countries imported 5000 tons of frozen roes and 1000 tons of raw chilled roes from non-EU countries. At the same time, many EU countries (mainly in Denmark, Spain, and United Kingdom) also imported 7500 tons of prepared fish roe, valued at 119 M€ (Monfort, 2002).

Egg processing techniques are different depending on the features of the raw material. The separation of eggs from the connective tissue could be achieved differently, according to egg dimensions. As an evidence, salmon and trout eggs are obtained by the manual separation, usually performed by a stainless-steel grid (Shin and Rasco, 2007), while smaller fish roes, such as capelin, herring, and pollock roes are automatically processed in drum filters separators. Enzyme separation, using collagenase and pepsine, has also been studied (Fernandes, 2016; Vilhelmsson, 1997). Eggs are then salted, generally by a saturated salt solution, then maintained in barrels at refrigerated temperatures in order to decrease the water activity, thus allowing their shelf stability. Other additives or ingredients, like sugar, could be added in these processing phases (Bledsoe et al., 2003). After a suitable maturation period, eggs are packaged in glass or aluminium cans for the retail. In addition, eggs may be pasteurized at mild temperatures, in order to avoid the denaturation of proteins, as happens with lumpfish roes (Johannesson, 2006). All fish roes are generally ready-to-eat products and they are sold under refrigerated conditions. Nevertheless, organic acids, like citric acid or benzoic acid, are usually added in fish roe as antimicrobials (Bledsoe et al., 2003) although no studies aimed to investigate their presence are available in literature (Pereira da Costa and Conte-Junior, 2015).

Since usually fatty acid analysis performed by mean of analytical chemistry leads to the yield of large amount of data, chemometrics methods appear to be necessary in order to investigate the structure of large datasets, especially when samples have different origins and can be divided into groups. This is the case of Principal Component Analysis (PCA), where the significant dimensionality of data matrices is strongly reduced, allowing the analyst to more easily understand the structure of chemical data, still retaining a good amount of the original information (Defernez and Kemsley, 1997). Moreover, qualitative chemometrics methods can be also very useful to create a classification model and to assign samples included in a dataset to a determinate group. In this scenario, multivariate methods appear to be very powerful, usually leading to a good fit of analytical data to a classification model. Particularly, the aim of Linear Discriminant Analysis (LDA) is to obtain an optimal separation among existing

groups and, further, to maximize the prediction power in order to classify such samples whose membership is not known, on the basis of the distance of each observation from each group mean (Defernez and Kemsley, 1997). However, when the number of variables in the matrix is higher than the number of samples analysed, as often occurs with fatty acid analysis, it appears very advantageous to employ a PCA/LDA combined approach, in which firstly the dimensionality of raw data is reduced and then the discriminant model is built to a subset of the original variables (Defernez and Kemsley, 1997).

Despite the importance of fish roe products in international market, there is a little technical information available about their chemical composition, food safety, and quality attributes. The aim of the present work was to achieve a deeper knowledge of the chemical composition, the fatty acid profile, and the organic acids content of several fish roe products collected on the Italian market. Moreover, we aimed to verify if a discrimination was achievable between caviar and fish roes by mean of a chemometric approach. Finally, microbiological properties of the products and the assessment of the conformity with the label information have been investigated and discussed.

6.3. Materials and methods

6.3.1. Sampling

A total of 38 fish roe samples were purchased during the spring of 2016 (Table 1).

Table 1. Fish roes samples collected in the study with the additives listed according to what was reported in their labels.

Species	Samples		Different Preparations					
	Total Number	Number Per Producer	NaCl *	Stabilizers	Preservatives	Antioxidants	Color Additives	Others
Salmon *Oncorhynchus keta*	12	3	2.5%	ND	ND	ND	ND	ND
		3	3.5%	ND	ND	ND	ND	ND
		3	2.5%	ND	ND	ND	ND	ND
		3	ND	ND	ND	ND	ND	ND
Lumpfish *Cyclopterus lumpus*	11	2	ND	E413	E211	E330	E120, E160c	Chartamus
		3	ND	E413	E211	E330	E150d, E151	
		3	ND	E412, E422	E202, E211	E331	E141, E150d, E163, E151	E621 (flavour enhancer)
Capelin *Mallosus villosus*	3	3	ND	E412, E422	E202, E212	E330	E120, E160c	E622 (flavour enhancer)
		1	ND		E202, E211	E330	E150d	Sugar
		1	ND	E413	E202, E211	E330	E160a, E120	Sugar, soy
Rainbow trout *Oncorynchus mykiss*	3	3	<6%	ND	E200	ND	ND	ND
Pike *Esox lucius*	3	3	ND	E415	ND	ND	ND	ND
Cod *Gadus morhua*	3	3	ND	ND	E200	ND	ND	ND
Alaska Pollock *Theragra chalcogramma*	3	3	ND	ND	E200	ND	ND	ND

* NaCl was present in all samples, table shows the amount as reported on the labels. Legend for the ingredients: ND: Not declared; E 412 Guar gum; E413: Tragacanth gum; E415 Xanthan gum; E422 Glycerol; E200: Sorbic acid; E202: Potassium sorbate: E211; Sodium benzoate: E212; Potassium benzoate; E330: Citric acid; E331: Sodium citrate; E102: Tartrazine; E120: Carminic acid; E133: brilliant blue FCF; E141: Chlorophylls and chlorophyllins, copper complexes; E150d: Sulfite Ammonia Caramel; E151: Brilliant Black BN; E160a: Carotenes; E160c: paprika oleoresin; E163: Anthocyanins; E621: Monosodium L-glutamate; E622: Monopotassium L-glutamate.

The survey involved 12 samples of Chum salmon (*Oncorhynchus keta*) eggs coming from 4 different producers; 11 samples of lumpfish (*Cyclopterus lumpus*) roe purchased from 2 different producers; 3 samples of rainbow trout (*Oncorhynchus mykiss*) roe; 3 samples of pike (*Esox lucius*) roe; 3 samples of cod (*Gadus morhua*) roe, 3 samples of Alaska pollock (*Theragra chalcogramma*) roe, and 3 of capelin (*Mallotus villosus*) roe, one black coloured, one orange coloured, and one green coloured. All samples were packaged in glass jars or metallic cans and sold refrigerated. Samples were transported to laboratory under ice in polystyrene boxes, and then they were opened for the microbiological analyses. The remaining portion of samples was stored at −20 ◦C until chemical analyses.

6.3.2. Chemical analyses

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy).

Before chemical analyses, three groups of eggs coming from each jar or can were weighed. Individual average weight of egg was calculated dividing the total weight with the number of eggs weighed.

The pH was measured using five grams of roes, blended with 20 mL of distillate water with an Ultraturrax for 30 s and pH was registered by a digital pH-meter (Amel Instruments, Milan, I) standardized at pH 4 and 7. Proximate composition and NaCl content were determined using standard methods (Cunniff and Chemists., 1996). The extraction and determination of total lipids were performed according Folch et al., 1957 method with chloroform: methanol mixture (2:1, v/v), using 0.7–1.0 g of sample, depending on its lipid content. The preparation of fatty acid methyl esters was performed according to Christie and Han, 2012. The sample was dissolved in 1 mL hexane and 1 μL sample was injected into the gas-chromatograph. Fatty acid analysis was carried out on an Agilent gas-chromatograph (Model 6890 Series GC) fitted with an automatic sampler (Model 7683) and FID detector (Agilent Technologies, Santa Clara, CA, USA) using helium as carrier gas. A HP-Innowax fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA) was used to separate fatty acid methyl esters. The oven temperature program for separation was from 100 to 180 ◦C at 3 ◦C min^{-1}, then from 180 to 250 ◦C at 2.5 ◦C min^{-1} and held for 10 min. Fatty acids were identified relative to known external standards (Supelco 37 FAME Mix, code CRM47885, Marine source, code 47,033 and Menhaden fish oil code 47085-U, Supelco, Bellafonte PA, USA) and were expressed as percentage of total fatty acids.

6.3.3. Microbiological analysis

For the microbiological determinations, 10 g of product were 10-fold diluted in chilled sterile diluent solution (0.85% NaCl and 0.1% peptone) and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). In some cases, due to the limited amount of material, pools of more than one sample were performed. Appropriate 10-fold dilutions of the homogenates were prepared in chilled saline solution. Total mesophilic viable count (TVC) was determined onto Plate Count Agar (International Organization for Standardization, 2013). Lactic acid bacteria were enumerated on De Man Rogosa Sharpe (MRS) agar (International Organization for Standardization, 1998), *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (International Organization for Standardization, 2004), yeasts and moulds were counted on Sabouraud Agar (International Organization for Standardization, 2008), coagulase positive staphylococci were counted on Baird Parker Agar (International Organization for Standardization, 2018), presumptive *Bacillus cereus* was enumerated onto PEMBA, *Enterococci* were enumerated onto Slanetz agar, (Merck, Darmstadt, Germany). Spores of reducing sulphite Clostridia were counted after pasteurization of the samples at 80 ◦C for 10 min onto Tryptose Sulfite Cycloserine, then incubated in anaerobiosis at 37 ◦C for 48 h. Detection and enumeration of *Listeria monocytogenes* were performed according to the method AFNOR (Association Française de Normalisation (AFNOR), 1998). All the culture media were purchased from Biogenetics (Ponte San Nicolò, Italy).

6.3.4. Determination of organic acids

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy).

The amounts of citric, lactic, and acetic acids were determined by Reverse phase High Performance Liquid Chromatography (HPLC) analysis with ion-exchange chromatography and UV detection (Tirloni et al., 2019). Briefly, 1 g of roe was added to 5.0 mL of water and vigorously shaken by a vortex for 20 s. After centrifugation, the supernatant was filtered through a 0.20 μm regenerated cellulose membrane filter. The HPLC system consisted of two pumps (510 by Waters S.p.A., Milano, Italy), an auto-sampler (717 plus by Waters S.p.A., Milano, Italy) and a UV-VIS detector (484 by Waters S.p.A., Milano, Italy) set at 210 nm. The separation was performed on a Rezex ROA column 300 mm × 7.8 mm, 8 μm (Phenomenex, Torrance, USA). The mobile phase (0.5 mL min^{-1} in isocratic mode) was 0.005 N sulphuric acid. External standards (acetic acid, code 1005706, Sigma

Aldricht, lactic acid, code 252476, Sigma Aldricht and citric acid code 251275, Sigma Aldricht, Steinheim, Germany) were used for identification and quantification of acetic, citric, and lactic acid. The limit of detection (LOD: 0.076, 0.23, and 0.24 mM for acetic, citric, and lactic acid, respectively) and limit of quantification (LOQ: 0.39, 0.43, and 0.77 mM for acetic, lactic, and citric acid, respectively) were determined. LOD and LOQ of organic acids were calculated from the residual standard deviation of the regression line (SDrl) of the calibration curve and its slope (b) in accordance to the following equation: LOD = 3.3 (SDrl/b) and LOQ = 10 (SDrl/b) (European Medicines Agency, 1998).

Concentration of benzoic and sorbic acids were determined by reverse phase HPLC method (Burana-Osot et al., 2014). Briefly, 1 g of roes was homogenized with 10 mL of methanol:water (60:40 v/v) by an ultra-turrax and filled up to 20 mL with the extraction solvent. The mixture solution was placed in an ultrasonic bath for 30 min to complete the extraction, after centrifugation 1 mL of supernatant was filtered through a 0.45 μm regenerated cellulose (RC) membrane filter and fill up to 5 mL with the extraction solvent. The chromatographic analysis was carried out in an Alliance 2695 HPLC system equipped with a PDA 996 (Waters S.p.A., Milano, Italy) diode array detector. The separation was performed at a flow rate of 1 mL/min throughout a C18 Chromolith column, 100 × 4.6 mm, 5 μm, (Merck Millipore, Burlington, Massachusetts, USA). The mobile phase consisted of 5 mM ammonium acetate buffer pH 4.2 and methanol (70:30 v/v) and the eluent was monitored at 228 nm for benzoic acid and 260 nm for sorbic acid. External standards (sorbic acid code 47,845 Sigma Aldricht and benzoic acid, code 242,381 Sigma Aldricht, Steinheim, Germany) were used for identification and quantification of benzoic and sorbic acids. The limit of detection (LOD: 0.52, 0.50 mM for benzoic and sorbic acid, respectively) and limit of quantification (LOQ: 1.59 and 1.51 mM for benzoic and sorbic acid, respectively) were determined.

6.3.5. Statistical analysis

Since data distribution was characterized by unequal variances within the groups (Levene test), statistical analysis was performed by mean of non-parametric tests (Welch test and Steel-Dwass post-Hoc test), declaring a significance when $p < 0.05$. A multivariate analysis was then performed by mean of Principal Component Analysis (PCA) on a 38 × 28 matrix, including fatty acid data, in order to reduce the dimensionality of data matrix and to detect similarities among samples and correlations among variables, according to Scano et al., 2010. Variables were selected when PC loadings score were > |0.5|.

Then, a Linear Discriminant Analysis (LDA) was performed using the variables previously selected, in order to verify if they were satisfying in highlighting the differences among samples coming from different species. Since the distribution of the scores for Canonical-1 and Canonical-2 were confirmed for their normality and homoscedasticity, comparison among groups centroids scores were performed by mean of ANOVA and the Tukey-HSD test. Furthermore, in a successive step, thirty-seven caviar samples (Borella et al., 2016; Lopez et al., 2020) were included in the dataset. The model was developed using roes from fish species analysed in this study as training set, including in the new matrix the variables selected in the previous steps, and caviar samples as validation set. Statistical analysis was performed using JMP Pro 14.0.0 (SAS Institute Inc., Cary, NC, USA).

6.4. Results and discussion

6.4.1. Nutritional quality and authenticity of fish roes

Weights and proximate composition of eggs are presented in Table 2.

Table 2. Weight (mg), proximate composition (g/100 g), and salt (g/100 g) content of fish roes. Data are expressed as mean ± standard deviation.

Fish Species	Salmon $n = 12$	Lumpfish $n = 11$	Capelin $n = 3$	Trout $n = 3$	Pike $n = 3$	Cod $n = 3$	Alaska Pollock $n = 3$
Weight mg	217.3 ± 31.33	6.0 ± 1.07	0.7 ± 0.09	54.7 ± 7.36	4.7 ± 0.50	0.2 ± 0.04	0.2 ± 0.02
Moisture g/100 g	53.5 ± 1.68	79.7 ± 0.92	81.7 ± 0.77	59.2 ± 0.03	64.0 ± 0.83	71.6 ± 0.55	73.5 ± 0.42
Protein g/100 g	29.6 ± 0.85	10.8 ± 0.40	8.1 ± 0.56	23.8 ± 0.07	19.4 ± 1.12	19.6 ± 0.27	19.2 ± 0.50
Lipid g/100 g	12.8 ± 0.85	4.3 ± 0.58	4.5 ± 0.23	12.5 ± 0.08	12.7 ± 0.57	3.2 ± 0.17	2.8 ± 0.10
Ash g/100 g	4.2 ± 0.79	5.2 ± 0.89	5.7 ± 0.02	4.6 ± 0.05	3.9 ± 0.04	5.5 ± 0.11	4.4 ± 0.05
NaCl g/100 g	2.7 ± 0.64	2.7 ± 0.64	2.5 ± 0.32	2.3 ± 0.06	3.3 ± 0.00	3.1 ± 0.06	2.8 ± 0.04

Egg size is influenced by the fish species of origin and by their reproductive behaviour. Generally, fish with demersal spawning have bigger eggs if compared to species with planktonic eggs (Duarte and Alcaraz, 1989). Biological features connected with the spawning site can also influence the chemical composition of roes that vary according to the maturation of fish from which they are obtained (Tocher and Sargent, 1984). The protein content of processed fish roes analysed in this study ranged from 8.1% to 29.6%, with salmon and trout eggs reaching the highest values (29.6% and 23.8%, respectively), lumpfish and capelin eggs showing the lowest values (10.8% and 8.1%, respectively), and pike, cod, and Alaska pollock eggs located in the middle (around 19%). According to their lipid content, samples could be divided in two groups. Salmon, trout, and pike roes recorded a

lipid content > 12%, while all the other species showed a lipid content < 5%. An explanation for this phenomenon could be linked to the ecological habits of the various fish species. Fish that spawn in an environment that is poor of nourishment, like salmon and trout, produce eggs rich in lipid and protein, in order to guarantee an adequate nutrients' supply to new borne generations for a prolonged period. Conversely, marine fish eggs (lumpfish, capelin, cod, Alaska pollock) contain minor reserves of nutrients, probably because the natural sites of spawning of these species are more able to easily provide food to newly hatched larvae. Ash content of roes ranged from 5.7%, recorded in capelin eggs, to 3.9%, recorded in pike eggs. The ash content is largely influenced by the amount of salt added to raw fish roes during their processing. Generally, our results agree with data previously reported by other authors, particularly regarding the species more commonly sold on the market, like salmon and lumpfish. Particularly, salmon roes analysed in this work showed protein and lipid contents comparable to those reported by Bladsoe et al. in their review (Bledsoe et al., 2003), whereas the lipid content was slightly lower than the one found in 2006 by Shirai et al., 2006. Proximate composition of lumpfish roes in our samples showed a lower protein content and a similar lipid content when compared with those reported by several authors in previous studies (Basby et al., 1998; Johannesson, 2006; Kalogeropoulos et al., 2012). In salmon and lumpfish, the economic value of roes is higher than the one of meat (Bledsoe et al., 2003) thus fish from these species are specifically harvested for roes production and their caught is planned when the roes reach the optimal maturation. Conversely, capelin, pollock, and cod roes are considered a valuable by-product of fish meat industry, so their composition could vary according to the harvesting season that is not strictly controlled as occurs for salmon and lumpfish. In capelin roes, Tocher and Sargent, 1984 reported a lipid content of 26.3%, corresponding to 7.89% on wet weight, which is superior to the value of 4.5% reported in the present paper. Regarding trout roes, we found a protein content slightly lower if compared with those reported by other authors (Al-Sayed Mahmoud et al., 2008; Gürel İnanli et al., 2010; Kalogeropoulos et al., 2012; Schubring, 2004; V'uorela et al., 1979) and a similar (Al-Sayed Mahmoud et al., 2008; Schubring, 2004) or higher (Al-Sayed Mahmoud et al., 2008; Bledsoe et al., 2003; Kaitaranta and Ackman, 1981; Kalogeropoulos et al., 2012) lipid content. For pike roes, Bledsoe et al., 2003 reported a protein content ranging from 14 to 27%, in agreement with the value of (19.4%) found in the present study; on the contrary they reported a lipid content between 1.5–2.4%, lower to the value (12.7%) of samples

analysed in the present survey. Proximate composition of cod roes characterized in our work agreed with data reported by Bledsoe et al., 2003 for protein content, whereas lipid content agreed with data presented by Tocher and Sargent, 1984, recalculated as % of wet weight. Finally, Alaska pollock roes, used for the preparation of the typical Japanese fish preparation Tarako, showed a lipid content lower than the one reported by Shirai et al., 2006 and a protein and lipid content similar to the one reported by Chiou et al., 1989, recalculated on % wet weight.

Furthermore, in a previous study, we analysed the chemical composition of caviar obtained from four different sturgeon species (Borella et al., 2016). Caviar samples presented the mean moisture content of 54.71 ± 2.66 g/100 g and a protein content of 24.31 ± 1.51 g/100 g, while lipid content resulted 17.27 ± 2.81 g/100 g and ash content 3.70 ± 0.56 g/100 g. An interesting difference between caviar and its substitutes can be observed in the ash content that in caviar was around 3.7 and 3.9%, while in roes from other fish species was higher, up to 5.7%. Since the ash content is linked to the amount of salt and additives added to raw material during processing, a lower ash content demonstrated the tendency of using as less salt as possible in caviar production, related to the aim to obtain a high quality product.

Concerning protein content, salmon roes are the only analyzed roes that presented a higher amount of protein if compared with caviar, while trout roes showed a similar content.

Fatty acid (FA) composition in fish eggs is not directly related to the fatty acid composition of other fish tissues, as there is a certain selective fatty acid uptake in the ovarian tissues of female fish. As an evidence of this phenomenon, in 2017 Johnson et al., 2017 tested the ability of Coho salmon (*Oncorhynchus kisutch)* of storing essential fatty acids, especially arachidonic acid (ARA) and docosahexaenoic acid (DHA), in the ovary during the secondary oocyte growth. The authors demonstrated that salmons incorporated the essential fatty acids supplied by the diets into the ovarian tissue rather than into muscle tissue. At the same time, it seems that fish are able to reallocate the essential FA stored in muscles to the ovary, if necessary, in order to satisfy the FA requirements of larvae, as reported by Zhu et al., 2019.

All samples of fish roes analysed in this study (Table 3) showed a prevalence of polyunsaturated fatty acids (PUFA), reaching amounts up to 52.4%, on monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). PUFA are present in fish eggs in large amounts since they represent an optimal nutritional supply for the growth of the embryo and the larva (Lopez et al., 2020). Particularly, we recorded a relevant proportion of n3 series FA on n6 series FA in all samples. This

proportion, commonly represented by the n3/n6 ratio, is considered fundamental by a nutritional point of view, either for the reproductive performance of fishes, and consequential larval growth, or for the nutritional quality of fish roes as favourable food product. The results obtained on the n3/n6 ratio in roes analysed in this study largely varied among the species considered. The highest value was recorded in lumpfish eggs (30.63), followed by capelin (17.35), salmon (15.14), cod (13.20), and Alaska Pollack (12.78) eggs, while the lowest values were found in pike (4.26) and trout (2.55) eggs. The significant difference of the n3/n6 values among pike and trout roes and roes from the other species could be explained by the fact that trout and pike are freshwater species, living in an environment with lower n3 FA and higher n6 FA (Tocher, 2003). Moreover, trout roes originated from farmed fish; the use of plant derived oil in fish feed formulation involves an enrichment with n6 FA, mainly linolenic acid (LA, 18:2 n6), in farmed fish products (Turchini et al., 2009). As a consequence, trout roes showed the highest amount of LA, reaching 9.35% of total fatty acids. The trend found in n3 and n6 series FA in roes from these two species was similar to that found by Saliu et al., 2017, who analysed the fatty acid content of roes extracted from European catfish (*Silurus glanis*), a freshwater predatory fish similar to pike for the habitat and the feeding habits. The authors found the prevalence of n3 FA on n6 FA but a lower n3/n6 ratio than in eggs of marine fish, recording a value of 3.4–3.8, comparable to the values we found in trout and pike roes.

Table 3. Fatty acid composition (g/100 g of total fatty acids) of fish roe from different species purchased in the Italian market. Data are expressed as mean ± standard deviation.

Fish Species	Salmon $n = 12$	Lumpfish $n = 11$	Capelin $n = 3$	Trout $n = 3$	Pike $n = 3$	Cod $n = 3$	Alaska Pollak $n = 3$
14:0	3.44 ± 0.51 ^{b}	1.37 ± 0.11 ^{c}	4.54 ± 0.01 ^{a}	1.58 ± 0.04 ^{c}	1.76 ± 0.03 ^{c}	1.47 ± 0.04 ^{c}	1.68 ± 0.19 ^{c}
15:0	0.54 ± 0.11^{a}	0.27 ± 0.03 ^{bc}	0.36 ± 0.00 ^{bc}	0.19 ± 0.00 ^{c}	0.27 ± 0.00 ^{bc}	0.30 ± 0.01 ^{bc}	0.38 ± 0.11 ^{b}
16:0	10.63 ± 0.43 ^{d}	14.19 ± 0.37 ^{c}	17.07 ± 0.09 ^{ab}	10.85 ± 0.20 ^{d}	16.05 ± 0.12 ^{b}	17.59 ± 0.81 ^{a}	17.44 ± 0.56 ^{ab}
16:1 n7	4.83 ± 0.53 ^{c}	1.82 ± 0.25 ^{e}	10.59 ± 0.10 ^{b}	2.93 ± 0.10 ^{d}	13.15 ± 0.30 ^{a}	4.89 ± 0.21 ^{c}	4.93 ± 0.14 ^{c}
16:2 n4	0.14 ± 0.03 ^{c}	0.02 ± 0.06 ^{d}	0.36 ± 0.00 ^{b}	0.13 ± 0.02 ^{c}	0.70 ± 0.03 ^{a}	nd	nd
17:0	0.51 ± 0.10 ^{a}	0.41 ± 0.07 ^{ab}	0.08 ± 0.13 ^{d}	0.19 ± 0.00 ^{bcd}	0.40 ± 0.01 ^{abc}	0.25 ± 0.29 ^{bcd}	0.14 ± 0.24 ^{cd}
16:3 n4	0.85 ± 0.33 ^{a}	0.02 ± 0.06 ^{c}	nd	0.05 ± 0.04 ^{bc}	0.53 ± 0.02 ^{ab}	0.08 ± 0.13 ^{bc}	nd
18:0	4.76 ± 0.23 ^{a}	4.37 ± 0.19 ^{b}	2.29 ± 0.01 ^{b}	3.90 ± 0.06 ^{c}	2.36 ± 0.04 ^{de}	2.83 ± 0.18 ^{d}	2.90 ± 0.28 ^{d}
18:1 n9	18.25 ± 1.55 ^{c}	17.07 ± 1.81 ^{c}	11.21 ± 0.11 ^{d}	27.11 ± 0.22 ^{a}	21.65 ± 0.20 ^{b}	12.50 ± 0.60 ^{d}	12.82 ± 0.59 ^{d}
18:1 n7	2.85 ± 0.40 ^{b}	4.02 ± 0.45 ^{b}	4.43 ± 0.04 ^{ab}	3.28 ± 0.06 ^{b}	7.67 ± 0.14 ^{a}	4.25 ± 3.68 ^{b}	2.09 ± 3.62 ^{b}
18:2 n6	1.32 ± 0.11 ^{d}	1.04 ± 0.21 ^{e}	1.72 ± 0.01 ^{c}	9.35 ± 0.19 ^{a}	3.50 ± 0.04 ^{b}	0.53 ± 0.03 ^{f}	0.61 ± 0.04 ^{f}
18:3 n3	1.22 ± 0.14 ^{c}	0.39 ± 0.06 ^{e}	0.78 ± 0.01 ^{d}	3.53 ± 0.04 ^{a}	2.20 ± 0.11 ^{b}	nd	nd
18:4 n3	1.07 ± 0.11 ^{c}	0.88 ± 0.26 ^{cd}	3.08 ± 0.02 ^{a}	0.66 ± 0.02 ^{de}	1.73 ± 0.05 ^{b}	0.46 ± 0.03 ^{e}	0.47 ± 0.04 ^{e}
18:4 n1	0.34 ± 0.10 ^{a}	nd	nd	0.19 ± 0.00 ^{b}	nd	nd	nd
20:1 n11	0.43 ± 0.15 ^{c}	nd	nd	nd	nd	1.69 ± 0.03 ^{b}	2.01 ± 0.23 ^{a}
20:1 n9	0.85 ± 0.27 ^{d}	4.06 ± 0.92 ^{ab}	5.10 ± 0.08 ^{a}	2.85 ± 0.06 ^{c}	0.41 ± 0.02 ^{d}	2.79 ± 0.05 ^{c}	3.05 ± 0.17 ^{bc}
20:1 n7	0.32 ± 0.03 ^{b}	0.54 ± 0.20 ^{a}	nd	0.03 ± 0.05 ^{cd}	0.26 ± 0.01 ^{bcd}	0.34 ± 0.02 ^{abc}	0.12 ± 0.21 ^{bcd}
20:2 n6	0.32 ± 0.05 ^{a}	nd	nd	1.64 ± 0.03 ^{a}	0.36 ± 0.00 ^{a}	nd	0.16 ± 0.28 ^{c}
20:3 n6	0.12 ± 0.05 ^{c}	nd	nd	0.75 ± 0.02 ^{a}	0.42 ± 0.00 ^{b}	nd	nd
20:4 n6	1.42 ± 0.23 ^{c}	0.64 ± 0.16 ^{d}	0.68 ± 0.00 ^{d}	1.40 ± 0.04 ^{c}	2.34 ± 0.02 ^{b}	3.07 ± 0.16 ^{a}	3.07 ± 0.19 ^{a}
20:4 n3	2.51 ± 0.19 ^{a}	0.97 ± 0.15 ^{b}	0.73 ± 0.00 ^{bc}	0.94 ± 0.02 ^{bc}	0.62 ± 0.01 ^{c}	0.12 ± 0.22 ^{d}	nd

20:5 n3	15.41 ± 0.53 ^{b}	18.93 ± 0.77 ^{a}	16.07 ± 0.07 ^{b}	6.42 ± 0.02 ^{d}	9.03 ± 0.03 ^{c}	17.80 ± 1.07 ^{a}	18.18 ± 0.56 ^{a}
22:1 n9	0.19 ± 0.12 ^{b}	0.49 ± 0.18 ^{a}	nd	nd	nd	nd	nd
22:5 n3	5.56 ± 0.47 ^{a}	1.70 ± 0.16 ^{c}	1.64 ± 0.02 ^{c}	2.02 ± 0.01 ^{bc}	2.38 ± 0.01 ^{b}	1.90 ± 0.15 ^{bc}	1.90 ± 0.07 ^{bc}
22:6 n3	22.04 ± 1.20 ^{b}	26.81 ± 1.53 ^{a}	19.27 ± 0.39 ^{c}	20.01 ± 0.37 ^{bc}	11.91 ± 0.29 ^{c}	27.14 ± 1.21 ^{a}	28.05 ± 1.09 ^{a}
SFA	19.88 ± 0.41 ^{e}	20.61 ± 0.63 ^{d}	24.33 ± 0.18 ^{a}	16.70 ± 0.22 ^{f}	20.84 ± 0.17 ^{cd}	22.44 ± 1.05 ^{bc}	22.54 ± 1.24 ^{b}
MUFA	27.72 ± 1.71 ^{cd}	28.00 ± 1.78 ^{d}	31.34 ± 0.23 ^{c}	36.21 ± 0.29 ^{b}	43.14 ± 0.24 ^{a}	26.47 ± 2.83 ^{d}	25.02 ± 2.82 ^{d}
PUFA	52.40 ± 1.66 ^{a}	51.39 ± 1.28 ^{a}	44.33 ± 0.36 ^{b}	47.09 ± 0.47 ^{b}	36.02 ± 0.40 ^{c}	51.09 ± 2.51 ^{a}	52.44 ± 1.79 ^{a}
n3	47.88 ± 1.43 ^{a}	49.68 ± 1.06 ^{a}	41.57 ± 0.37 ^{b}	33.57 ± 0.34 ^{c}	28.17 ± 0.41 ^{d}	47.43 ± 2.44 ^{a}	48.60 ± 1.71 ^{a}
n6	3.19 ± 0.31 ^{d}	1.67 ± 0.35 ^{e}	2.40 ± 0.01 ^{d}	13.15 ± 0.22 ^{a}	6.62 ± 0.03 ^{b}	3.59 ± 0.18 ^{c}	3.84 ± 0.47 ^{c}
n3/n6	15.14 ± 1.26 ^{b}	30.63 ± 5.19 ^{a}	17.35 ± 0.24 ^{b}	2.55 ± 0.05 ^{c}	4.26 ± 0.07 ^{c}	13.20 ± 0.18 ^{b}	12.78 ± 1.53 ^{b}

a, b, c, d = Value within the same raw not sharing a common letter are significantly different ($p < 0.05$); SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. nd = not detected.

n3 series FA in fish roes are mainly represented by eicosapentaenoic acid (EPA, 20:5 n3) and docosahexaenoic acid (DHA, 22:6 n3), particularly in marine species, reaching higher amounts than in fish flesh because of the higher phospholipids content of eggs (Shirai et al., 2006). Actually, long chain FA of the n3 series are conserved in roes at the expense of other FA, since they are valuable essential components of the biological membranes to be preserved during critical periods of larvae development (Turchini et al., 2009). According to this, we found the highest content of both EPA and DHA in roes from lumpfish (18.93% and 26.81%), Alaska pollack (18.18% and 28.05%), capelin (16.07% and 19.27%), salmon (15.41% and 22.04%), and cod (17.80% and 27.14%), usually obtained after the caught of wild fish in natural stocks. At the same time, cod and Alaska pollack roes showed also the highest amount of arachidonic acid (ARA, 20:4 n6), another FA considered fundamental during starvation of marine fish larvae (IZQUIERDO, 1996). Capelin eggs showed high levels of the monounsaturated FAs palmitoleic acid (16:1) and gondoic acid (20:1 n-9). Nevertheless, the sum of eicosanoic fatty acids did not reach the levels found by Cyprian et al., 2017, who analysed the lipids from the whole capelin fish and found eicosanoic acids content near to 20% of total fatty acids. It can be suggested that these fatty acids do not have an essential role in larval metabolism, and for this reason, they are not stored in capelin roes. Pike eggs represented the only group in which MUFA were predominant (43.14%) on PUFA (36.02%). The prevalence of MUFA in pike eggs was mainly due to the higher content of 16:1 n7 (13.15%) and 18:1 n7 (7.67%) in samples if compared with eggs originating from other species. However, the most representative among MUFA in all analysed samples was oleic acid (OA, 18:1 n9). This fatty acid, together with the SFA palmitic acid (16:0), is known to represent the primary energy source for fish larvae in many species (Meeren et al., 1993). We found the highest amount of oleic acid in trout roes (27.11%). As stated before, trout is a farmed species and, thus, its diet is strongly enriched in vegetable oils that represent a source of many FA, less representative in natural aquatic trophic chains, such as oleic acid. As for LA, an increase in the ingestion of OA could have led to the higher stocking of this fatty acid in trout organs

and eggs, mainly in the storage fraction represented by triacylglycerols (Turchini et al., 2009). Salmon eggs were rich of 18:3 n3 and DHA, even if they presented values of n3 fatty acids lower than other marine species as lumpfish, cod, and Alaska pollock. This aspect could be linked to the anadromous behaviour of the species that partially lived in fresh waters during growing ages and during spawning season, but also to the feeding habits of the salmon species used to obtain roes. As a matter of fact, the Chum salmon (*O. keta*) feed mainly on copepods and euphausiids or gelatinous zooplankton, which have lower nutritional value than other prey (Qin and Kaeriyama, 2016).

In order to visualize the distribution of samples and eventual correlations between variables, a Principal Component Analysis was performed on the dataset including fatty acid data. The first two Principal Components (PC-1 and PC-2) introduced by mean of this multivariate technique explained the 60% of the total variance. The PCA loading plot (Figure 1) showed that 16 variables over the original 28 were related to a loading > |0.5|, thus highly influencing the variability of data and their distribution in the new bi-plot delimited by PC-1 and PC-2. In the loading plot, we can observe that the first PC, which explained the 37.4% of the variance, was mainly described by fatty acids typical of the marine habitat (EPA, DHA, n3/n6) in the positive direction and by fatty acids coming from the farm feeding system (oleic acid, linoleic acid, linolenic acid) in the negative direction. PC-1 significantly separated three groups: (i) pike and trout roes, inversely corelated with the n3/n6 ratio and positively correlated to fatty acids typical of freshwater environment or vegetable oils, such as oleic, linoleic and linolenic acid; (ii) lumpfish, Alaska Pollock and cod roes, from pure marine species, positively correlated with EPA and DHA and, consequently, with the n3/n6 ratio; (iii) salmon and capelin roes, in an intermediate position. The second PC, which explained the 24.7% of the total variance in the dataset, was mainly described by palmitic and palmitoleic acid in the positive direction and by stearic acid, oleic acid, 20:4 n3, and docosapentaenoic acid (DPA) in the negative direction. PC-2 was able to separate four groups: (i) capelin roes, positively correlated to palmitic and palmitoleic acid and saturated fatty acids in general; (ii) salmon and trout roes, positively correlated to 20:4 n3, DPA and stearic acid; (iii) lumpfish roes and (iv) pike, cod, and Pollock roes, in an intermediate position. However, the combination of both PC-1 and PC-2 in a multivariate system allowed distinguishing groups that would have not been recognizable if considering just one over the two direction, as demonstrated also by another study based on processed fish roes

(Caredda et al., 2018).

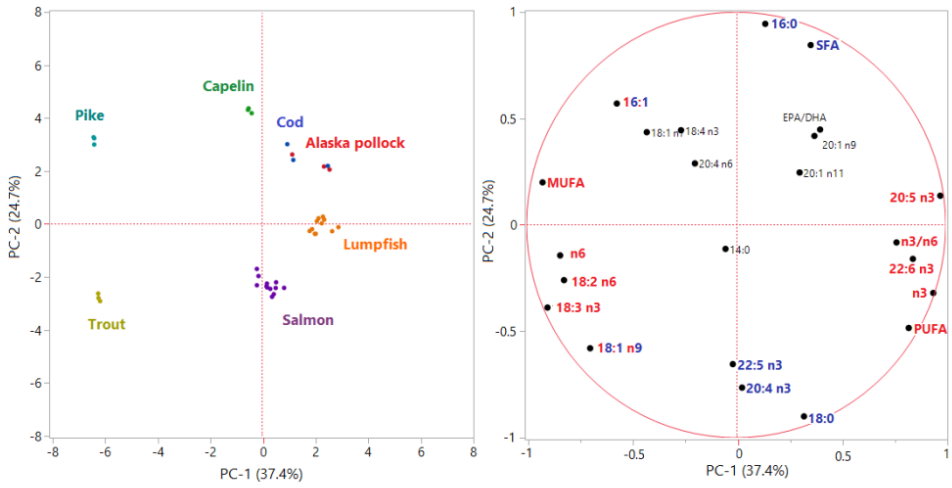

Figure 1. Principal Component Analysis score plot (left) and loading plot (right). In the loading plot, variables with loadings > |0.5| on PC-1 are in red, whereas variables with loadings > |0.5| on PC-2 are in blue.

The variables selected by mean of PCA were then employed in the construction of a Linear Discriminant Analysis (LDA) model, performed in a new 38 × 16 matrix (38 samples and 16 fatty acids). The canonical plot obtained after the performance of the LDA is presented in Figure 2 whereas in Table 4, the Mahalanobis distances matrix is shown.

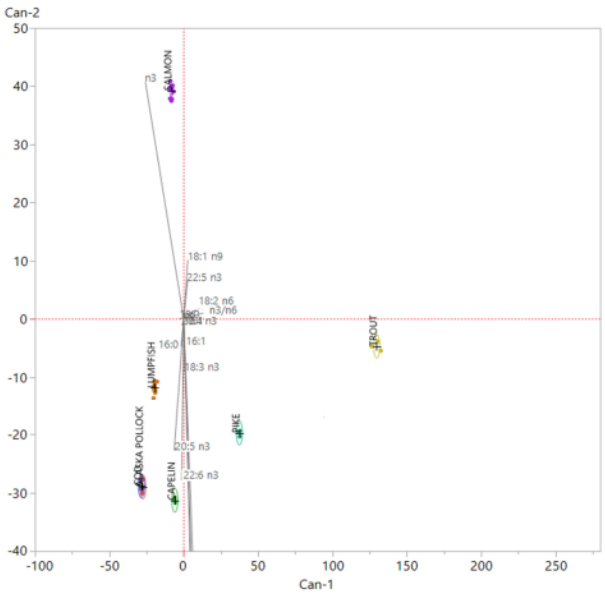

Figure 2. Linear Discriminant Analysis canonical plot. Each group is related with a 95% confident region density ellipse. In this graph, the scoring coefficients of the variables have been standardized and then multiplied by a scaling factor corresponding to 4.5, in order to better fit the canonical plot.

Table 4. Mahalanobis distances calculated as Euclidean distances among groups centroids in the canonical plot defined by Canonical-1 and Canonical-2 on the x- and y- axis, respectively.

	Salmon	Lumpfish	Capelin	Trout	Pike	Cod
Lumpfish	52.3					
Capelin	70.6	23.7				
Trout	144.9	149.4	138.3			
Pike	74.7	57.4	44.9	93.6		
Cod	71.0	19.3	22.7	160.1	66.6	
Alaska pollock	17.3	6.9	21.6	157.2	64.9	0.9 *

* Comparison among group means on Canonical-1 and Canonical-2 scores did not show any statistical difference between Cod and Alaska Pollock centroids ($p > 0.05$).

Sixteen variables were included in the model, in which Canonical 1 (Can-1) and Canonical 2 (Can-2) explained 83% of the total variance, so increasing the discriminant power if compared to the PCA previously performed. In order to investigate the influence of the variables on the construction of the discriminant model, their scoring coefficients were standardized and ordered by their weight on Can-1 and Can-2, respectively. In such LDA model, factors that most affected groups separation were 18:2 n6 and the n3/n6 ratio on Can-1; 18:1 n9, 22:5 n3, 22:6 n3 on Can-2; the total SFA, total MUFA, total PUFA, and total n3 FA, plus 20:5 n3, on both the canonicals. All roe samples coming from the different fish species analysed in this study were clearly distinguishable by mean of the model built on the basis of fatty acid data, with the exception of cod and Alaska pollack roes that showed a strong overlapping among groups. As a matter of fact, the LDA model allowed a proper classification of all samples even if one Cod sample was assigned to the proper group with an uncertainty corresponding to 24%. The superimposition between Cod and Alaska Pollock samples is also observable in Table 4, where the Mahalanobis distance of Cod and Alaska Pollock centroids revealed that they were not significantly different ($p > 0.05$) when testing group means. These outcomes reflected the similarity observed by mean of univariate analysis on fatty acids composition. The two species, cod and Alaska Pollock, belong to the same Family, the *Gadidae*. They share the same environment represented by the cold waters of the northern hemisphere, the same reproductive habits and the same feed substrate; all these biological similarities resulted in a very close composition of their roes, not allowing our analyses to make a clear distinction between samples coming from their eggs. In the canonical bi-plot, samples belonging to groups cod, Alaskan pollock, lumpfish and capelin, pure marine species, were allocated in the same dial of the LDA chart related to n3 FA, particularly EPA and DHA, while pike and trout, freshwater and farmed species were in the opposite dial. Salmon showed a peculiar behaviour, distancing itself from both

marine and freshwater species. Salmon was the species that showed a higher value of n3 fatty acid, having the highest value of n3 fatty acid different from EPA and DHA, like eicosatetraenoic acid (ETA 20:4 n3) and docosapentaenoic acid (DPA 22:5 n3).

Finally, to ascertain whether the model would be able to distinguish among fish roes from these species, considered as caviar substitutes, and sturgeon caviar, we included in the dataset 37 samples of caviar previously analysed for fatty acid composition (Borella et al., 2016; Lopez et al., 2020). Then, we performed the same investigation, by mean of LDA, on the new matrix (75 × 16); the canonical plot obtained is showed in Figure 3. We can observe that all roe samples coming from the various fish species analysed in this study were clearly distinguishable from caviar. Caviar samples showed a more intra-group spread if compared to other fish roes samples. This phenomenon is due to origin of caviar samples, that were obtained from four different sturgeon species, which presented significative differences in their fatty acid composition (Borella et al., 2016). Caviar showed a collocation in the plot close to trout roes samples. This phenomenon is linked to the origin of this samples; all of them came from farmed fish and their fatty acid signature is modified by the fatty acid of plan origin that characterize aquafeed, like linolenic acid (LA) and OA (Lopez et al., 2020).

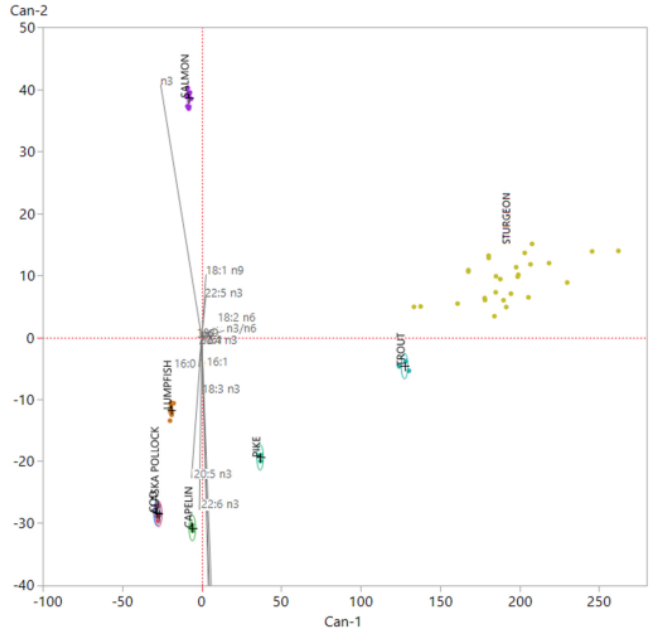

Figure 3. Canonical plot obtained after the employment of the linear discriminant analysis (LDA) model using fish roes from various species as training set and including 37 sturgeon caviar samples as validation set.

6.4.2. Food safety of fish roe products

According to the information found on labels placed on packaging showed (Table 1), salmon roes was the only product where no additive

were added during processing. Pike, capelin, and lumpfish roes have been treated with thickeners, probably to give a more consistent texture to eggs. Capelin and lumpfish eggs were also treated to confer red, black, and green colours to these products, which naturally have a pale-yellow colour, being unattractive for consumers. Preservatives are generally added to fish roes in order to lower the pH, to prevent lipid oxidation, and to make eggs an unfavourable substrate for the growth of microorganisms. The decrease of the pH value contributes to maintaining the quality of fish roes during processing and storage (Lapa-Guimarães et al., 2011). During the extraction, fish roes should be considered sterile; however, they rarely remain sterile as microorganisms present on the surface of fish could be transferred to the roes, as well as the screening stage of eggs could contribute to contamination (Bledsoe et al., 2003). Thus, good manufacturing practices and a correct refrigeration maintenance are crucial to avoid bacterial replication during storage (Lapa-Guimarães et al., 2011).

All the products analysed resulted safe as the presence of L. monocytogenes was never found. According to Reg. UE 2073/2005 (European Commission, 2005), for ready-to-eat food with more than 5 days of shelf-life and able to support the *L. monocytogenes* growth, a tolerance level of 100 CFU/g is indicated during their shelf-life. Although fish roes have a short shelf-life, they are considered to support the growth of this potential pathogenic microorganism. As reported by Miya et al., 2010, *L. monocytogenes* was found able to grow at 22 ◦C in 6 h; in the same study and differently from our findings, *L. monocytogenes* was found present with prevalence equal to 5.7% in salmon roes and 9.1% in cod roes, respectively. In a previous study, a prevalence of *L. monocytogenes* from 10 to 11.4% was found in fish roe products (Handa et al., 2005).

As shown in Figure 4 the total Viable Count (TVC) was generally very low, with 18 out of the 25 whole samples analysed (72%) showing values below the detection limit (2 Log CFU/g). Three out of the five samples of lumpfish roes analysed were countable with mean values equal to 2.26 Log CFU/g and all the capelin roes samples were countable with mean value equal to 3.14 Log CFU/g. The highest value of TVC detected was 5.00 and it was revealed in a sample of pike roes. The threshold limit of 6 Log CFU/g, frequently used for food products to designate the end of shelf-life of a fish product (Olafsdottir et al., 2006), was never exceeded. Moreover, values of total viable count above 7-8 Log CFU/g are often associated to sensory rejection. In agreement with our data, Oeleker et al., 2015 evidenced a good microbiological situation with 54% of fish roes analysed in which total viable count below 2 Log CFU/g, while 84% loads below 4 Log CFU/g.

The loads obtained in our study were in general lower if compared to those obtained by Altug and Bayrak, 2003 for caviar from Russia and Iran, where TVC was from 3 to 6.41 Log CFU/g, and those obtained by Himelbloom and Crapo, 1998 in pink salmon ikura with loads from 3.48 to 6.48 Log CFU.

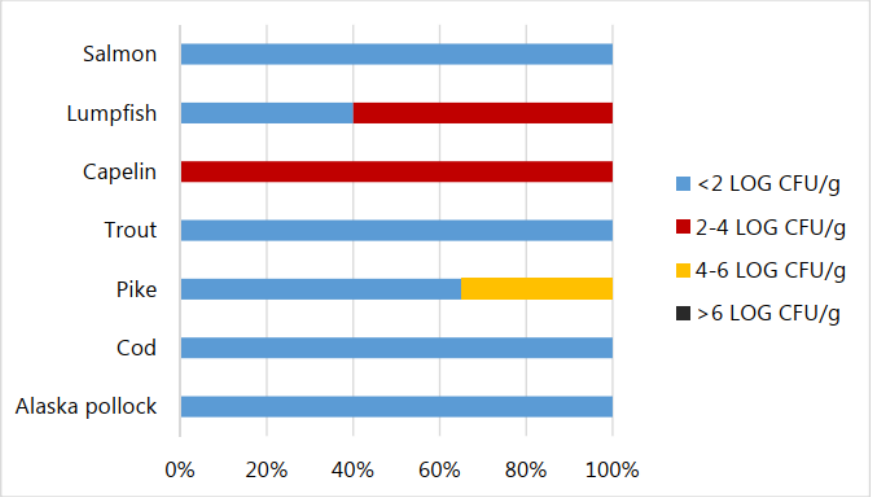

Figure 4. Total Viable Count of fish roe samples.

Lactic Acid Bacteria, *Enterobacteriaceae*, *B. cereus*, and *Enterococci*, resulted always below the detection limit (2 Log CFU/g). In all samples, Coagulase Positive Staphylococci resulted below the detection limit, except for a sample of lumpfish roes with a load of 3.88. Loads that are considered risky in terms of toxin production are generally set from 4 to 5 Log CFU/g, thus in our case, no particular concern resulted from their presence. Additionally, Oeleker et al., 2015 evidenced a single sample containing Coagulase Positive Staphylococci where the load resulted just above 3 Log CFU/g.

Clostridia were always below the detection limit in all the samples analysed: as in fish roe, sterilization could not be applied due to the protein irreversible denaturation process, particular attention should be used to avoid the germination of possible spores present in the product, especially those belonging to *C. botulinum*. The maintenance of low refrigeration temperatures according with salt content are key factors in order to produce a safe product, able to inhibit *C. botulinum* germination. FDA suggests a Water Phase Salt (WPS) above 3.5%; in the products analysed, lumpfish roes and capelin roes resulted to be characterized by a WPS below this limit (3.1 and 2.9%, respectively), thus the intrinsic characteristics of these two products could not guarantee the safety of the final product.

All pH values ranged from 5.12 to 6.28: at this pH range, benzoic acid may be effective against moulds and yeast in the range of 100–500

ppm (Jay, 2000). It is fundamental to maintain a certain pH range in food products in order to inhibit the metabolic activity of spoilage microorganisms. Salmon and pike roes were found to be permissive for moulds replication since they had low benzoic acid and sorbic acid concentrations (<LOD equal to 52 ppm and 41 ppm, respectively) (Table 5). Moulds were present in all the samples of pike roes in very limited concentration (values from 2.00 to 2.60 Log CFU/g). At the same time, trout, cod, and Alaska Pollock roes were found to be permissive for mould replications even if high levels of sorbic acid were found in these samples. Even if in limited concentrations, we found moulds in all the samples of trout roes (all values equal to 2.00 Log CFU/g), in one sample of cod roes and in two samples of Alaska Pollack roes (loads equal to 2.00 Log CFU/g). Despite the fact that in a pH range between 4.0 and 6.0 sorbic acid is more effective than benzoic acid, due to their different pK (pK of sorbate = 4.80 vs. pK of benzoate = 4.20), sorbic acid has higher undissociated ratio in products with low acidity if compared to benzoic acid. For this reason, we isolated moulds and yeasts also in products characterized by the presence of both sorbic and benzoic acid but a low acidity, like capelin roes. In two samples of capelin roes, we found yeasts with loads corresponding to 2.00 and 2.30 Log CFU/g: high values of yeasts >5 Log CFU/g are recognized to cause organoleptic spoilage that in our samples was never detected. Sorbic and benzoic acids maximum level permitted in EU (2000 ppm) is applicable to both of them, with the final levels expressed as free acid (European Commission, 2011). Cod and pollack roes were characterized by sorbic acid concentrations that exceed the limit, whereas trout roes had sorbic acid concentration close to the maximum. All the other products were compliant to the EU regulation. Six out of eleven lumpfish products declared potassium sorbate (E202) in the list of ingredients, but sorbate salt was not detected in those samples. Cod roes did not declare benzoic acid (E211–E212) on the labels, however they all contained this acid, even if in low concentration.

Table 5. pH and organic acids (mg/kg) content of fish roes. Data are expressed as mean ± standard deviation.

Fish Species	Salmon $n = 12$	Lumpfish $n = 11$	Capelin $n = 3$	Trout $n = 3$	Pike $n = 3$	Cod $n = 3$	Alaska Pollock $n = 3$
pH	6.28 ± 0.08	5.77 ± 0.09	6.19 ± 0.06	5.95 ± 0.03	5.88 ± 0.05	5.96 ± 0.06	5.95 ± 0.03
Citric acid ppm	nd	nd	1356 ± 68	898 ± 75	2695 ± 192	<LOQ	<LOQ
Lactic acid ppm	676 ± 261	5343 ± 2472	1546 ± 172	10,812 ± 746	232 ± 7	11,072 ± 370	11,752 ± 2168
Acetic acid ppm	263 ± 92	nd	nd	713 ± 50	<LOD	875 ± 75	1177 ± 56
Sorbic acid ppm	nd	nd	607 ± 70	2021 ± 35	<LOD	2050 ± 118	2573 ± 491
Benzoic acid ppm	<LOD	858 ± 347	598 ± 120	<LOD	<LOD	285 ± 68	<LOD

LOD: Limits of Detection were 216 ppm for citric acid, 16 ppm for lactic acid, 47 ppm for acetic acid, 41 ppm for sorbic acid, and 52 ppm for benzoic acid. LOQ: Limits of Quantification were 722 ppm for citric acid, 52 ppm for lactic acid, 157 ppm for acetic acid, 151 ppm for sorbic acid, and 159 for benzoic acid.

Other organic acids such as citric, lactic, and acetic acid and relative salts are permitted in fish roes with the principle of quantum satis. These organic acids are usually used to lower the pH in food. The order of their effectiveness towards pathogens is acetic > lactic > citric. As was the case for sorbic and benzoic acid, some of the labels did not match our results. Citric acid was found in trout and pike roes (898 ppm and 2695 pmm, respectively) but it was not reported on the labels. Citric and lactic acid (E270) was found in all the samples, ranging from 232 ppm (pike) to 11,752 ppm (Alaska pollock), but none of the products declared lactic acid on the label. Acetic acid (E260) was found in salmon, trout, cod, and Alaska pollock, but even in this case the preservative was not declared. The presence of these undeclared organic acid is not a safety concern but an unfair commercial practice. In Alaska pollock, cod, and trout, the organic acids mixture seem very effective against microbial growth. No data about organic acids in fish roes are available in literature; however, these organic acids were used to build predictive model for *L. monocytogenes* in lightly preserved and ready-to-eat seafood (Mejlholm and Dalgaard, 2009).

6.5. Conclusions

This study was undertaken to provide the seafood industry and the consumers with information on nutritional and safety properties of fish roe preparations on the market, using a chemometric approach. Proximate composition showed that these products are extremely variable and that their composition depends on the biology of fish, their reproductive environment, and their food habits. Furthermore, this study clearly indicates that the combination of fatty acid composition along with a chemometric approach can be successfully applied to give more information on the original fish species of roes. Generally, fish roes are very rich in essential fatty acid, especially EPA and DHA, and they could be considered a good source of these

substances in human diets. Moreover, all the product analysed were considerable safe for human consumption, since the low microbial loads detected, probably related to the presence of organic acids added to the products during manufacturing processes, were associated with a very limited replication of the present microorganisms.

CHAPTER 7

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