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XXVI CICLO

HEALTH CONCERNS ON FISHERY PRODUCTS: ANTIBIOTIC RESISTANCE AND PARASITE RISK ASSESSMENT IN FISH PRODUCTION VALUE CHAINS

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a Carlo

"Di ciò che è importante nella propria esistenza non ci si rende quasi conto, e certamente questo non dovrebbe interessare il prossimo. Che ne sa un pesce dell'acqua in cui nuota per tutta la vita?"

Albert Einstein

Sono arrivato alla fine di questa esperienza, sono volati tre anni, ed è doveroso spendere qualche parola per ringraziare chi mi è stato accanto.

Ringrazio la mia famiglia che ha dapprima plasmato il mio carattere e la mia persona e che mi ha sempre dato forza e grinta per migliorare e per gettare alle spalle le difficoltà che si sono presentate nei giorni.

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ABSTRACT

Capture fisheries and aquaculture supplied the world with about 148 million tonnes of fish in 2010, of which about 128 million tonnes were utilized as food for people. The fishery market is becoming much more complex and stratified, with greater diversification among species and product forms and for this reason food safety remains a major concern facing the seafood industry. Along fish value chain there are a lot of concern regarding public health and for this reason the aim of this work was to propose an approach to antibiotic resistance and parasites risk assessment in fish production value chains.

The main consequences of the use and the abuse of antibiotics is the development and diffusion of antibiotic resistance that become a serious global problem. The aim of this research was to evaluate the presence of antimicrobial residues and to assess the antimicrobial resistance in bacteria species isolated from different wild caught seawater fish and fishery products. Three antibiotic substances (Oxytetracicline, Sulfamethoxazole and Trimethoprim) were detected (by screening and confirmatory methods) in *Octopus vulgaris, Sepia officinalis* and *Thais haemastoma*. All *Vibrio* strains isolated from fish were resistant to Vancomycin and Penicillin. In *Vibrio alginolyticus*, isolated in *Octopus vulgaris*, a resistance against 9 antibiotics was noted.

Human fishery product-borne parasitic diseases include those caused by cestodes, trematodes and nematodes. These diseases are either caused by an infection following ingestion of viable parasites, or as an allergic (hypersensitivity) reaction against parasite antigens which occurs for nematodes of the family *Anisakidae*.

Parasite risk assessment was evaluated in 3 different steps:

- Different infection indexes in european hakes (*Merluccius merluccius*) belonging from Atlantic ocean (FAO zone 27 IXa) and from Tyrrhenian sea (FAO zone 37.1.2) were assessed identifying and charactering species and populations of parasites. A total of 2990 larval specimens of *A. simplex* (s. l.) from *Merluccius merluccius* captured in the two different fishing grounds were collected: 2836 anisakids in hakes from FAO 27 fishing ground and only 154 anisakids in hakes from 37.1.2 fishing ground. *A. simplex (s. s.)* and *A. pegreffii* co-infected the same individual fish host, in viscera and in the fillets (hypaxial and epaxial) fished from the FAO 27 fishing

ground; only *A. pegreffii* in hakes from FAO area 37.1.2 in viscera and in hypaxial flesh was found.

- The presence of nematodes in 33 fish species belonging from different FAO areas using artificial peptic digestion was evaluated and parasites identification using microscopical techniques and RT-PCR was performed. A total of 10897 anisakid larvae were found and collected in the flesh. The blue ling (*Molva dypterygia*), european conger (*Conger conger)* and Peter's fish (*Zeus faber)* were the most highly infected species respectively. A total of 27,27 % of the species analyzed were anisakid-free; a monitoring tool for the evaluation of parasite infection in fish lots with the aim to assess parasites impact on marketed fish, as EFSA states in a recent report, was applied; 29 % of the evaluated fish lots had a score between 0-3, refering to serious weaknesses in the fish lots.
- The survival of *Anisakis* larvae, naturally present in fish, at different stages of ripening process in traditional salted anchovies was evaluated since a recent EFSA panel on risk assessment of parasites in fishery products stated that research on identification of alternative treatments for killing viable parasites in fishery products are needed. Viability was tested with different methods and the results showed that all larvae were not viable at the 15th ripening day. The dry salting process at mean salt concentration of 24% in all parts of the flesh at the end of the ripening period can be considered an effective method for devitalising anisakids larval forms present in the raw material and obtain a safe product for the consumer.

KEYWORDS: Antibiotic resistance, zoonotic desease, food safety.

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CHAPTER 1. BACKGROUND

Capture fisheries and aquaculture supplied the world with about 148 million tonnes of fish in 2010, of which about 128 million tonnes was utilized as food for people (Table 1 and Figure 1).

World fisheries and aquaculture production and utilization

	2006	2007	2008	2009	2010	2011
			(Million tonnes)			
PRODUCTION						
Capture						
Inland	9.8	10.0	10.2	10.4	11.2	11.5
Marine	80.2	80.4	79.5	79.2	77.4	78.9
Total capture	**90.0**	**90.3**	**89.7**	**89.6**	**88.6**	**90.4**
Aquaculture						
Inland	31.3	33.4	36.0	38.1	41.7	44.3
Marine	16.0	16.6	16.9	17.6	18.1	19.3
Total aquaculture	**47.3**	**49.9**	**52.9**	**55.7**	**59.9**	**63.6**
TOTAL WORLD FISHERIES	**137.3**	**140.2**	**142.6**	**145.3**	**148.5**	**154.0**
UTILIZATION						
Human consumption	114.3	117.3	119.7	123.6	128.3	130.8
Non-food uses	23.0	23.0	22.9	21.8	20.2	23.2
Population (*billions*)	6.6	6.7	6.7	6.8	6.9	7.0
Per capita food fish supply (*kg*)	17.4	17.6	17.8	18.1	18.6	18.8

Notes: Excluding aquatic plants. Totals may not match due to rounding. Data for 2011 are provisional estimates.

Table 1: World fisheries and aquaculture production and utilization

With sustained growth in fish production and improved distribution channels, world fish food supply has grown dramatically in the last five decades, with an average growth rate of 3,2 % per year in the period 1961–2009, outpacing the increase of 1,7 % per year in the world's population.

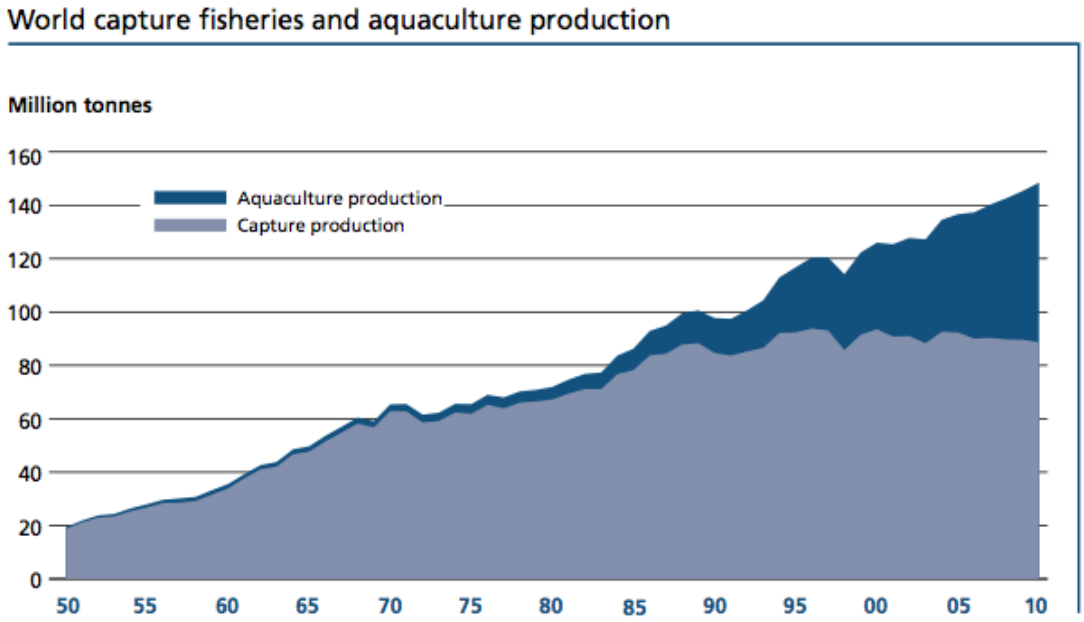

Figure 1: World capture fisheries and aquaculture production

World food fish supply pro capite increased from an average of 9,9 kg (live weight equivalent) in the 1960s to 18,6 kg in 2010 (Table 1 and Figure 2). Of the 126 million tonnes available for human consumption in 2009, fish consumption was lowest in Africa (9,1 million tonnes, with 9,1 kg pro capite), while Asia accounted for two-thirds of total consumption, with 85,4 million tonnes (20,7 kg pro capite), of which 42,8 million tonnes was consumed outside China (15,4 kg pro capite).

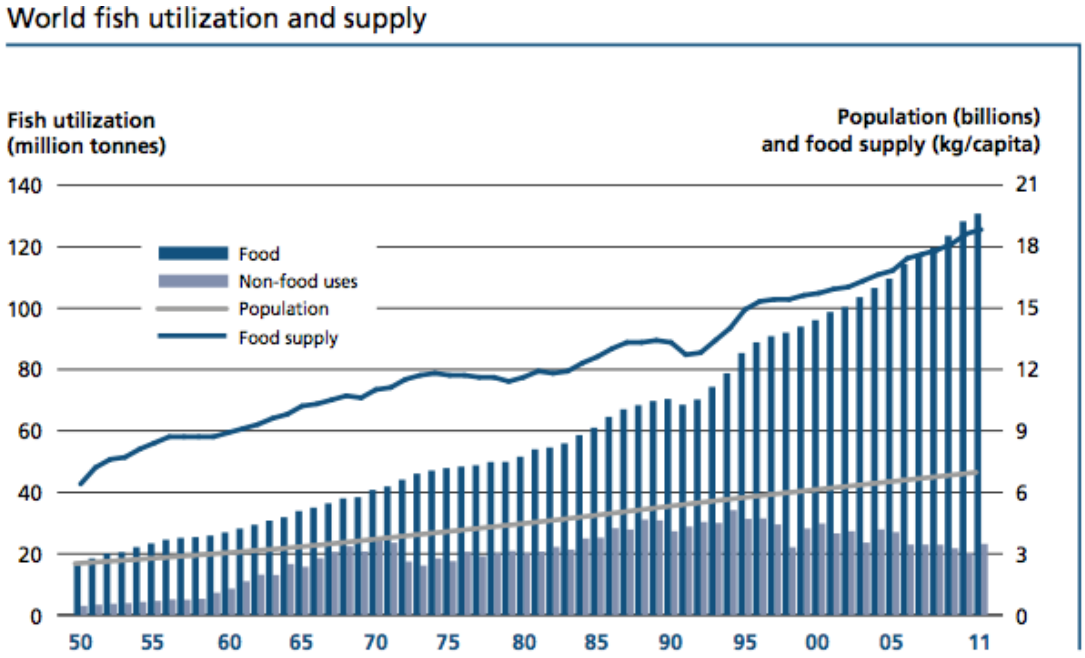

Figure2: World fish utilization and supply

Overall global capture fisheries production continues to remain stable at about 90 million tonnes although there have been some marked changes in catch trends by country, fishing area and species.

The Northwest Pacific is still by far the most productive fishing area. Catch peaks in the Northwest Atlantic, Northeast Atlantic and Northeast Pacific temperate fishing areas were reached many years ago, and total production had declined continuously from the early and mid-2000s, but in 2010 this trend was reversed in all three areas.

Total global capture production in inland waters has increased dramatically since the mid-2000s with reported and estimated total production at 11,2 million tonnes in 2010, an increase of 30 % since

2004. Despite this growth, it may be that capture production in inland waters is seriously underestimated in some regions. In the last three decades (1980–2010), world food fish production of aquaculture has expanded by almost 12 times, at an average annual rate of 8,8 %. Global aquaculture production has continued to grow, albeit more slowly than in the 1980s and 1990s.

World aquaculture production attained another all-time high in 2010, at 60 million tonnes (excluding aquatic plants and non-food products), with an estimated total value of US$ 119 billion. In 2010, global production of farmed food fish was 59,9 million tonnes, up by 7,5 % from 55,7 million tonnes in 2009 (32,4 million tonnes in 2000). Farmed food fish include finfishes, crustaceans, molluscs, amphibians (frogs), aquatic reptiles (except crocodiles) and other aquatic animals (such as sea cucumbers, sea urchins, sea squirts and jellyfishes) (Figure 3). Freshwater fishes dominate global aquaculture production (56,4 %, 33,7 million tonnes), followed by molluscs (23,6 %, 14,2 million tonnes), crustaceans (9,6 %, 5,7 million tonnes), diadromous fishes (6,0 %, 3,6 million tonnes), marine fishes (3,1 %, 1,8 million tonnes) and other aquatic animals (1,4 %, 814300 tonnes). While feed is generally perceived to be a major constraint to aquaculture development, one-third of all farmed food fish production (20 million tonnes) is currently

achieved without artificial feeding, as is the case for bivalves and filter-feeding carps (Figure 3).

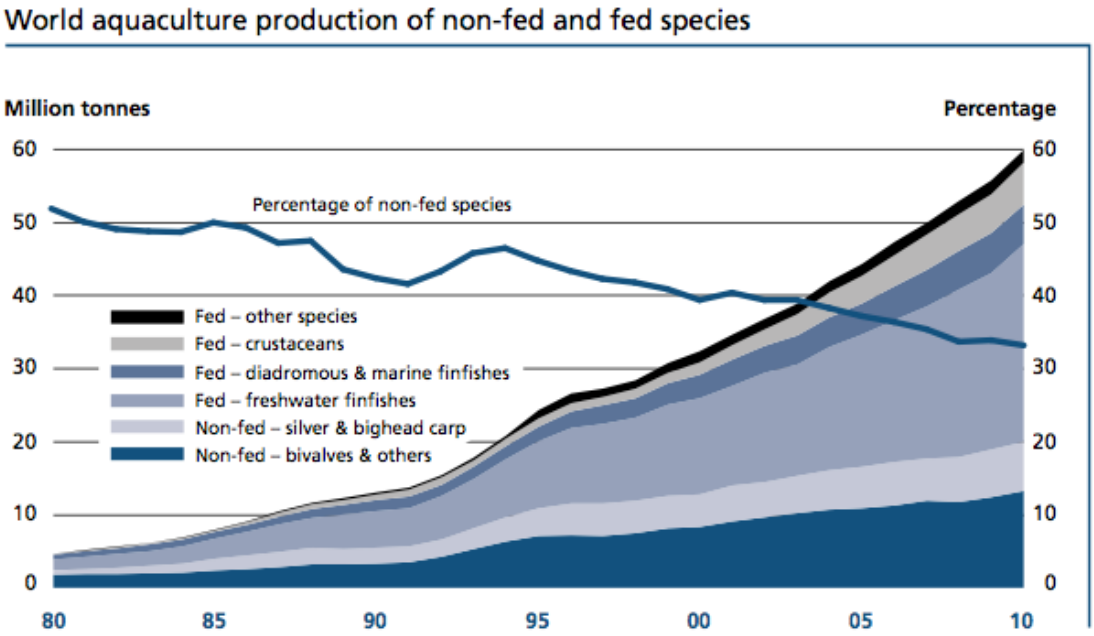

Figure 3: World aquaculture production of no-fed and fed species

Fisheries and aquaculture provided livelihoods and income for an estimated 54,8 million people engaged in the primary sector of fish production in 2010, of whom an estimated 7 million were occasional fishers and fish farmers. The world's marine fisheries increased markedly from 16,8 million tonnes in 1950 to a peak of 86,4 million tonnes in 1996, and then declined before stabilizing at about 80 million tonnes. Global recorded production was 77,4 million tonnes in 2010. The Northwest Pacific had the highest production with 20,9 million tonnes

(27 % of the global marine catch) in 2010, followed by the Western Central Pacific with 11,7 million tonnes (15 %), the Northeast Atlantic with 8,7 million tonnes (11 %), and the Southeast Pacific, with a total catch of 7,8 million tonnes (10 %). About 29,9 percent of stocks are overexploited, producing lower yields than their biological and ecological potential and in need of strict management plans to restore their full and sustainable productivity.

Most of the stocks of the top ten species, which account in total for about 30 % of world marine capture fisheries production, are fully exploited and, therefore, have no potential for increases in production, while some stocks are overexploited and increases in their production may be possible if effective rebuilding plans are put in place. The two main stocks of anchoveta in the Southeast Pacific, Alaska pollock in the North Pacific and blue whiting in the Atlantic are fully exploited. Atlantic herring stocks are fully exploited in both the Northeast and Northwest Atlantic. Japanese anchovy in the Northwest Pacific and Chilean jack mackerel in the Southeast Pacific are considered to be overexploited. Chub mackerel stocks are fully exploited in the Eastern Pacific and the Northwest Pacific. The largehead hairtail was estimated in 2009 to be overexploited in the main fishing area in the Northwest Pacific. Among the seven principal tuna species, one-third were estimated to be

overexploited, 37,5 % were fully exploited, and 29 % non-fully exploited in 2009.

The overall situation when summarized by FAO statistical areas shows three main patterns in catch trends:

- Areas that have demonstrated oscillations in total catch are the Eastern Central Atlantic (Area 34), Northeast Pacific (Area 67), Eastern Central Pacific (Area 77), Southwest Atlantic (Area 41), Southeast Pacific (Area 87), and Northwest Pacific (Area 61). These areas have provided about 52 % of the world's total marine catch on average in the last five years. Several of these areas include upwelling regions that are characterized by high natural variability.

- Areas that have demonstrated a decreasing trend in catch since reaching a peak at some time in the past. This group has contributed 20 % of global marine catch on average in the last five years, and includes the Northeast Atlantic (Area 27), Northwest Atlantic (Area 21), Western Central Atlantic (Area 31), Mediterranean and Black Sea (Area 37), Southwest Pacific (Area 81), and Southeast Atlantic (Area 47). It should be noted that lower catches in some cases reflect fisheries management

measures that are precautionary or aim at rebuilding stocks, and this situation should, therefore, not necessarily be interpreted as negative.

- Areas that have shown continuously increasing trends in catch since 1950 and includes the Western Central Pacific (Area 71), Eastern (Area 57) and Western (Area 51) Indian Ocean. They have together contributed 28 % of the total marine catch on average over the last five years.

However, in some regions, there is still high uncertainty about the actual catches owing to the poor quality of statistical reporting systems in coastal countries. In spite of the worrisome global situation of marine capture fisheries, good progress is being made in reducing exploitation rates and restoring overexploited fish stocks and marine ecosystems through effective management actions in some areas. In the United States of America, 67 % of all stocks are now being sustainably harvested, while only 17 % are still overexploited.

Concerning utilization of the world's fish production, 40,5 % (60,2 million tonnes) was marketed in live, fresh or chilled forms, 45,9 % (68,1 million tonnes) was processed in frozen, cured or otherwise prepared forms for direct human consumption, and 13.6 % destined for non-food uses in 2010 (Figure 4).

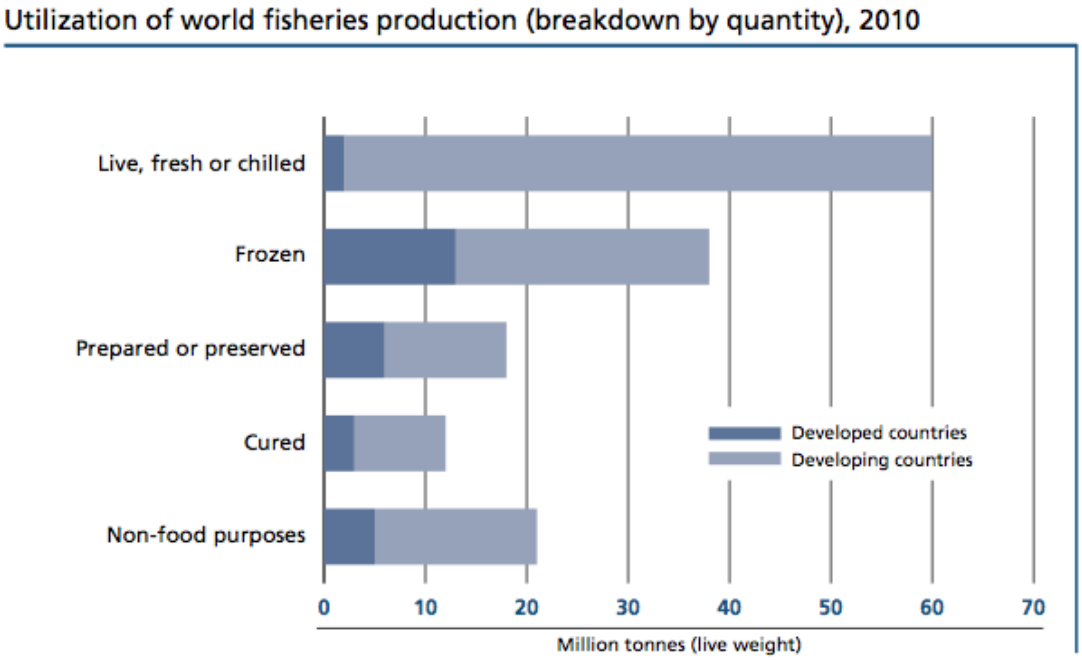

Figure 4: Utilization of world fisheries production (breakdown by quality), 2010

Since the early 1990s, there has been an increasing trend in the proportion of fisheries production used for direct human consumption rather than for other purposes. Whereas in the 1980s about 68 % of the fish produced was destined for human consumption, this share increased to more than 86 % in 2010, equalling 128,3 million tonnes. In 2010, 20,2 million tonnes was destined to non-food purposes, of which 75 % (15 million tonnes) was reduced to fishmeal and fish oil; the remaining 5,1 million tonnes was largely utilized as fish for ornamental purposes, for culture (fingerlings, fry, etc.), for bait, for pharmaceutical uses as well as for direct feeding in aquaculture, for livestock and for fur animals. Of the fish destined for direct human consumption, the most important product

form was live, fresh or chilled fish, with a share of 46,9 % in 2010, followed by frozen fish (29,3 %), prepared or preserved fish (14,0 %) and cured fish (9,8 %). Freezing represents the main method of processing fish for human consumption: the proportion of frozen fish grew from 33,2 % of total production for human consumption in 1970 to reach a record high of 52,1 % in 2010 (Figure 5).

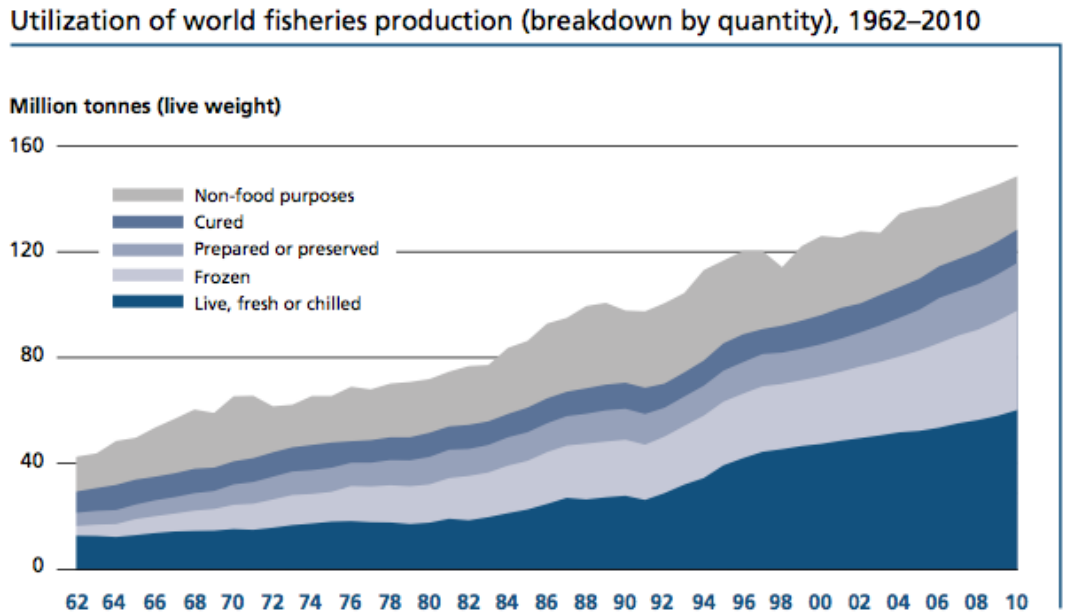

Figure 5: Utilization of world fisheries production (breakdown by quality), 1962 – 2010

The share of prepared and preserved forms remained rather stable during the same period, and it was 26,9 % in 2010. Developing countries have experienced a growth in the share of frozen products (24,1 % of the total fish for human consumption in 2010, up from 18,9 % in 2000) and of

prepared or preserved forms (11,0 % in 2010, compared with 7,8 % in 2000). Owing to deficiencies in infrastructure and processing facilities, together with well-established consumer habits, fish in developing countries is commercialized mainly in live or fresh form (representing 56,0 % of fish destined for human consumption in 2010) soon after landing or harvesting. Cured forms (dried, smoked or fermented) still remain a traditional method to retail and consume fish in developing countries, although their share in total fish for human consumption is declining (10,9 % in 2000 compared with 8,9 % in 2010).

Technological development in food processing and packaging is progressing rapidly. Processing is becoming more intensive, geographically concentrated, vertically integrated and linked with global supply chains. These changes reflect the increasing globalization of the fisheries value chain, with large retailers controlling the growth of international distribution channels. Fish and fishery products continue to be among the most traded food commodities worldwide, accounting for about 10 % of total agricultural exports and 1 % of world merchandise trade in value terms. The share of total fishery production exported in the form of various food and feed items increased from 25 % in 1976 to about 38 % (57 million tonnes) in 2010 (Figure 6).

Trade plays a major role in the fishery industry as a creator of employment, food supplier, income generator, and contributor to economic growth and development. For many countries and for numerous coastal, riverine, insular and inland regions, fishery exports are essential to the economy. For example, in 2010 they accounted for more than half of the total value of traded commodities in Greenland, Seychelles, Faeroe Islands and Vanuatu. Since late 2011 and early 2012, the world economy has entered a difficult phase characterized by significant downside risks and fragility, and key markets for fisheries trade have slowed sharply. Among the factors that might influence the sustainability and growth of fishery trade are the evolution of production and transportation costs and the prices of fishery products and alternative commodities, including meat and feeds. In the last few decades, the growth in aquaculture production has contributed significantly to increased consumption and commercialization of species that were once primarily wild-caught, with a consequent price decrease, particularly in the 1990s and early 2000, with average unit values of aquaculture production and trade declining in real terms. Subsequently, owing to increased costs and continuous high demand, prices have started to rise again.

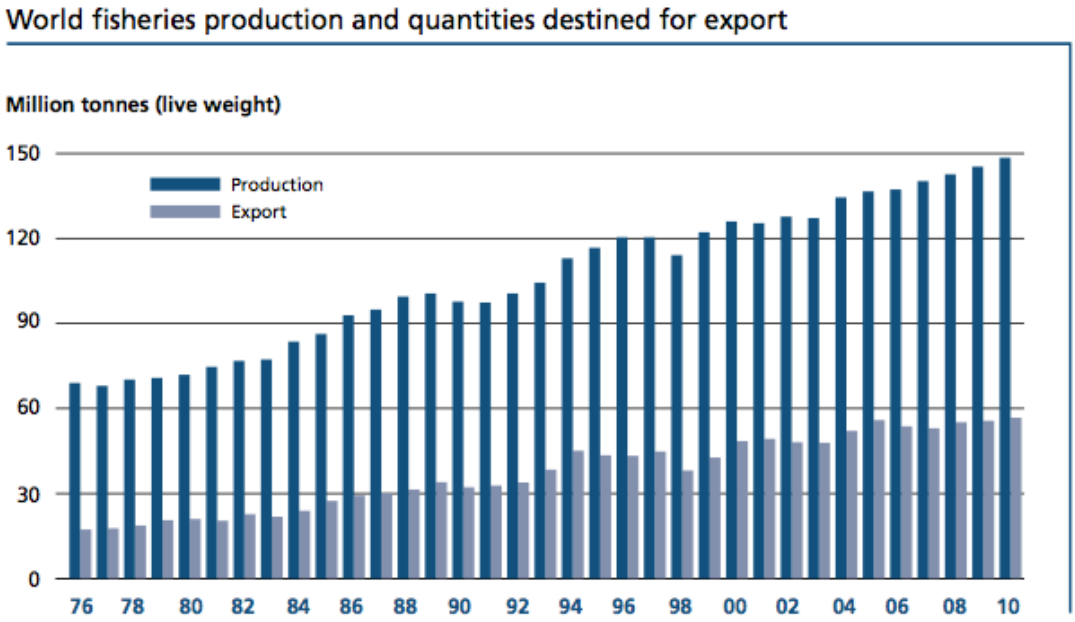

Figure 6: World fisheries production and quantities destined for export

Trade in fish and fishery products is characterized by a wide range of product types and participants. In 2010, 197 countries reported exports of fish and fishery products. The role of fishery trade varies among countries and is important for many economies, in particular for developing nations.

The fishery market is very dynamic and it is changing rapidly. It is becoming much more complex and stratified, with greater diversification among species and product forms. High-value species such as shrimp, prawns, salmon, tuna, groundfish, flatfish, seabass and seabream are highly traded, in particular towards more prosperous markets. Low-value species such as small pelagics are also traded in large quantities, mainly

being exported to feed low-income consumers in developing countries. In the last two decades, aquaculture has contributed to a growing share of the international trade in fishery commodities, with species such as shrimp, prawns, salmon, molluscs, tilapia, catfish (including *Pangasius*), seabass and seabream. Aquaculture is expanding in all continents in terms of new areas and species, as well as intensifying and diversifying the product range in species and product forms to respond to consumer needs. Many of the species that have registered the highest export growth rates in the last few years are produced by aquaculture. However, it is difficult to determine the extent of this trade because the classification used internationally to record trade statistics for fish does not distinguish between products of wild and farmed origin. Hence, the exact breakdown between products of capture fisheries and aquaculture in international trade is open to interpretation.

Owing to the high perishability of fish and fishery products, 90 % of trade in fish and fishery products in quantity terms (live weight equivalent) consists of processed products (i.e. excluding live and fresh whole fish). Fish are increasingly traded as frozen food (39 % of the total quantity in 2010, compared with 25 % in 1980). In the last four decades, prepared and preserved fish have nearly doubled their share in total quantity, going from 9 % in 1980 to 16 %in 2010. Notwithstanding their

perishability, trade in live, fresh and chilled fish represented 10 % of world fish trade in 2010, up from 7 % in 1980, reflecting improved logistics and increased demand for unprocessed fish. Trade in live fish also includes ornamental fish, which is high in value terms but almost negligible in terms of quantity traded. In 2010, 71 % of the quantity of fish and fishery products exported consisted of products destined for human consumption.

1.1 Fish consumption

Fish and fishery products represent a valuable source of nutrients of fundamental importance for diversified and healthy diets. With a few exceptions for selected species, fish is usually low in saturated fats, carbohydrates and cholesterol. Fish provides not only high-value protein, but also a wide range of essential micronutrients, including various vitamins (D, A and B), minerals (including calcium, iodine, zinc, iron and selenium) and polyunsaturated omega-3 fatty acids (docosahexaenoic acid and eicosapentaenoic acid). While average fish consumption pro capite may be low, even small quantities of fish can have a significant positive nutritional impact by providing essential amino acids, fats and micronutrients that are scarce in vegetable-based diets. There is evidence of beneficial effects of fish consumption in relation to coronary heart disease, stroke, age-related macular degeneration and mental health. There is also convincing evidence of benefits in terms of growth and development, in particular for women and children during gestation and infancy for optimal brain development of children.

On average, fish provides only about 33 calories pro capite per day. However, it can exceed 150 calories pro capite per day in countries where there is a lack of alternative protein food and where a preference

for fish has been developed and maintained (e.g. Iceland, Japan and several small island States). The dietary contribution of fish is more significant in terms of animal proteins, as a portion of 150 g of fish provides about 50–60 percent of the daily protein requirements for an adult (Figure 7).

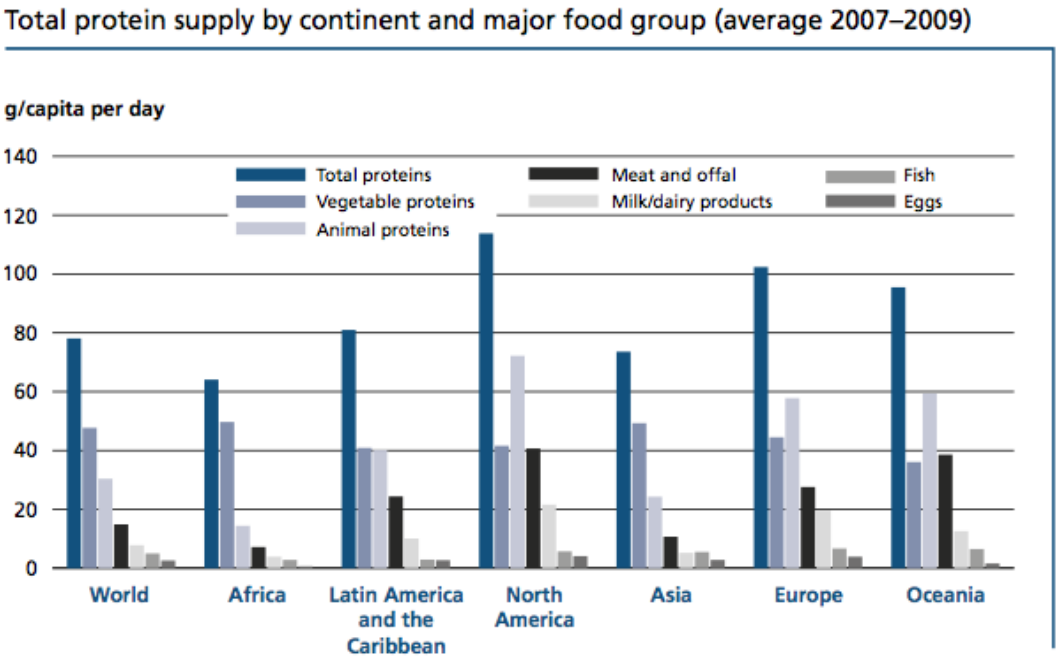

Figure 7: Total protein supply by continent and major foorìd group (average 2007 – 2009)

Fish proteins can represent a crucial component in some densely populated countries where total protein intake levels may be low. In fact, many populations, more those in developing countries than developed ones, depend on fish as part of their daily diet. For them, fish and fishery products often represent an affordable source of animal protein that may

not only be cheaper than other animal protein sources, but preferred and part of local and traditional recipes. For example, fish contributes to, or exceeds, 50 % of total animal protein intake in some small island developing States, as well as in Bangladesh, Cambodia, Ghana, the Gambia, Indonesia, Sierra Leone and Sri Lanka. In 2009, fish accounted for 16,6 % of the global population's intake of animal protein and 6,5 % of all protein consumed (Figure 8).

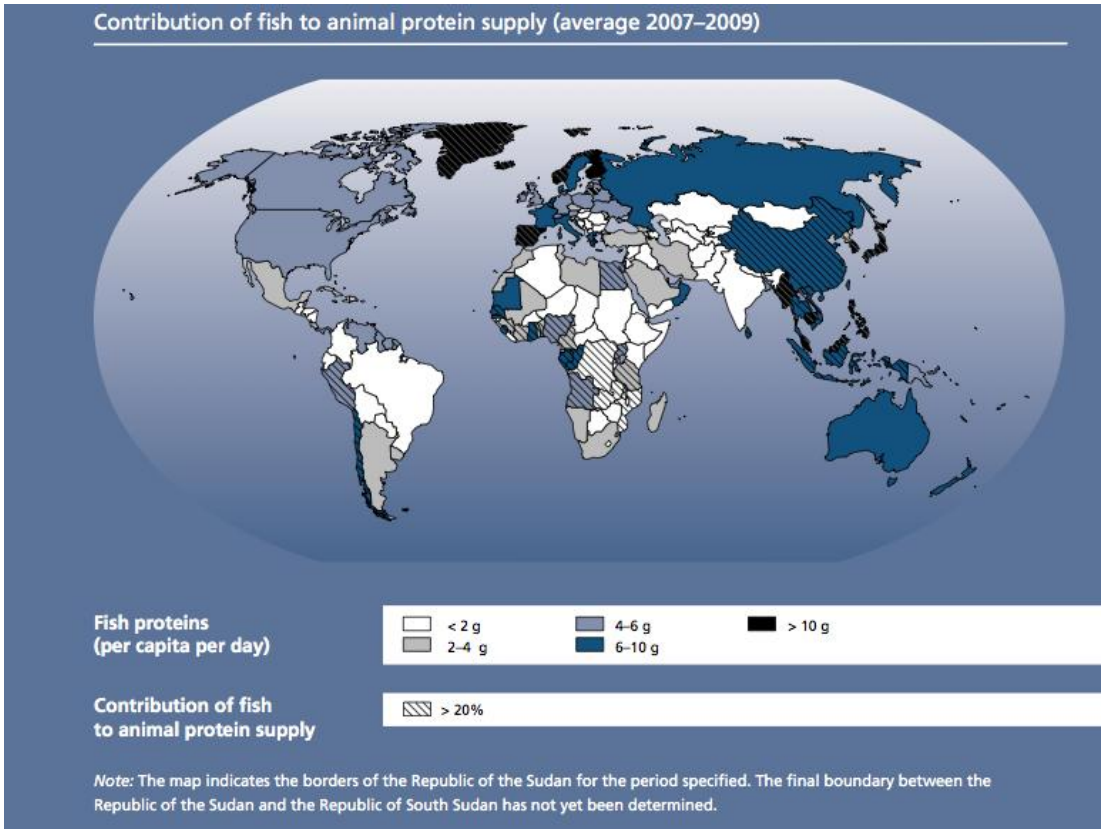

Figure 8: Contribution of fish to animal protein supply (average 2007 – 2009)

Globally, fish provides about 3 billion people with almost 20 % of their

average intake pro capite of animal protein, and 4.3 billion people with about 15 % of such protein (Figure 9).

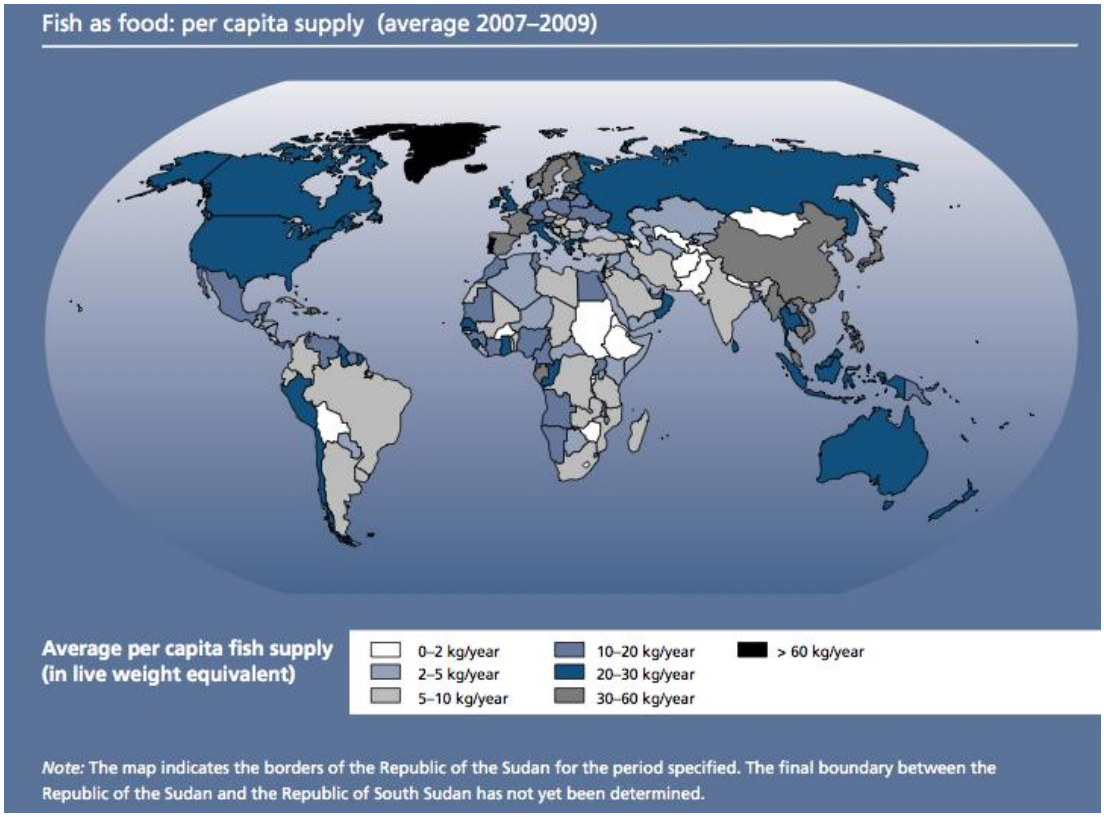

Figure 9: Fish as food: pro capite supply (average 2007 – 2009)

Differences in fish consumption exist between the more-developed and the less-developed countries. Although annual pro capite consumption of fishery products has grown steadily in developing regions (from 5,2 kg in 1961 to 17,0 kg in 2009) and in low-income food-deficit country (from 4,9 kg in 1961 to 10,1 kg in 2009), it is still considerably lower than that of more developed regions, even though the gap is narrowing. Annual consumption of demersal and pelagic fish species has stabilized

at about 3 kg and 3,4 kg pro capite, respectively. Demersal fish continue to be among the main species favoured by consumers in Northern Europe and in North America (8,6 kg and 7,0 kg pro capite per year, respectively, in 2009), whereas cephalopods are mainly preferred by Mediterranean and East Asian countries. Of the 18,4 kg of fish pro capite available for consumption in 2009, about 74 % came from finfish. Shellfish supplied 26 % (or about 4,5 kg pro capite, subdivided into 1,7 kg of crustaceans, 0,5 kg of cephalopods and 2,3 kg of other molluscs).

The global growth in fish consumption mirrors trends in food consumption in general. Food consumption pro capite has also been growing in the last few decades. With the exception of the periods of the food and economic crises, the global food market, including the fish market, has experienced unprecedented expansion and a change in global dietary patterns, becoming more homogeneous and globalized. This change has been the result of several factors, including rising living standards, population growth, rapid urbanization and opportunities for trade and transformations in food distribution. A combination of these factors has led to growing demand for proteic food products, in particular meat, fish, milk, eggs as well as vegetables in the diet, with a reduction in the share of staples such as roots and tubers.

1.2 Food safety

Today, food safety remains a major concern facing the seafood industry and it is a critical component in ensuring food and nutrition security worldwide. The production and consumption of safe food are central to any society and they have a wide range of economic, social and, in many cases, environmental consequences. In 1994, FAO published Assurance of Seafood Quality in response to the growing need for guidance on the subject from Members. A decade later, in 2004, FAO published an expanded and revised technical paper Assessment and Management of Seafood Safety and Quality that addressed new developments, especially with regard to food safety and the adoption, internationally, of the Hazard Analysis and Critical Control Point (HACCP) system and risk analysis concepts.

In the 1980s, food trade expanded dramatically with more food products crossing national and continental borders. Exports from developing countries increased. At the same time, several food scares, caused by bacterial (e.g. *Salmonella* and *Listeria*) and chemical (e.g. mycotoxins) contamination meant that food safety was an issue of major public concern. This concern was exacerbated during the 1990s by "*mad cow disease*" and the "*dioxin crisis*", and these food safety problems forced

regulators to rethink food safety strategies, integrating the various components of the value chain and introducing traceability requirements. Expansion of the food industry and food distribution systems across borders and continents required the development of quality assurance systems to support business-to-business contractual agreements and verification of conformity of food supplies with the specifications. At the same time, the development of bilateral, regional and multilateral trade agreements brought about changes in national and supranational food control systems to harmonize requirements and procedures.

Food-borne illnesses continue to be a major public health problem worldwide. It is estimated that up to 30 % of the population in industrialized countries are affected annually, and the situation in developing countries could be worse, although less-developed data systems means quantification is difficult.

The public health significance of seafood-borne illnesses depends on the probability of illness (number of cases) and the severity of 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.

Risk analysis consists of three separate but integrated parts:

- risk assessment;
- risk management;
- risk communication.

The management and control of food-borne diseases is carried out by several groups of people. First, it involves technical experts assessing the risk, i.e. examining epidemiological, microbiological and technological data about the hazard and the food. Risk managers at the government level decide what level of risk society will tolerate, while balancing other considerations, e.g. the cost of risk management measures and their effect on the affordability and utility of foods. Risk managers in both industry and government are then required to implement procedures to minimize the risk. In the current international food safety management environment, the tolerable level of hazard at the point of consumption is expressed as “food safety objectives”.

Risk communication is an integral part of risk analysis and provides timely, relevant and accurate information about the risk of eating food to industry, consumers and public bodies alike. Perception of risk has both technical and emotional dimensions, and risk communication should address both these aspects. Often, non-technical information provided by media, consumer groups or industry captures the attention of the general

public exposed to the risk. Risk communication should address the concerns of the public and not dismiss these as irrational.

Food control includes all activities carried out to ensure the safety and quality of food. Every stage from initial production to processing, storage, marketing and consumption must be included in a food safety and quality programme. The overall goal is to provide a systematic approach to all control and inspection activities through a managed programme based on proper scientific principles and appropriate risk assessment, leading to careful targeting of inspection and control resources. Furthermore, the risk assessment must be carefully documented, including any constraints that may have affected the quality of the risk estimated, transparent and fully available to independent assessors. Sufficient financial and personnel resources must be made available. However, it must be emphasized that no management system can offer zero risk in terms of consumer health protection.

Along fish value chain there are lots of concern regarding public health as microbial contaminations, presence of chemical residues, presence of parasites, presence of pharmaceutical substances etc..

Aim of this work was to propose an approach to antibiotic resistance and parasites risk assessment in fish production value chains.

CHAPTER 2. OCCURRENCE OF ANTIBIOTIC RESISTANCE IN BACTERIA ISOLATED FROM SEAWATER FISH AND OTHER MARINE ORGANISMS CAUGHT IN CAMPANIA REGION

2.1 Introduction

Pollution from pharmaceuticals in the aquatic environment is now recognized as an environmental concern in many countries. This has led to the creation of an extensive area of research, including:

- their chemical identification and quantification;
- elucidation of transformation pathways when present in wastewater treatment plants or in environmental matrices;
- assessment of their potential biological effects;
- development and application of advanced treatment processes for their removal and/or mineralization.

Pharmaceuticals are a unique category of pollutants, because of their special characteristics, and their behavior and fate cannot be simulated with other chemical organic contaminants. Over the last decade the scientific community has embraced research in this specific field and the outcome has been remarkable.

There has been an increasing concern in recent years about the occurrence, fate, and adverse effects of pharmaceutical residues in the aquatic environment. Some of the most widely and frequently drug classes employed, for example antibiotics, are used in quantities similar to those of pesticides and in some countries drugs could be sold without the requirement of a prescription (Fatta-Kassinos et al., 2011). Antibiotics are defined as naturally occurring, semi-synthetic and synthetic compounds with antimicrobial activity that can be applied parentally, orally or topically. They are probably the most successful family of drugs so far developed for improving human health. Besides this fundamental application, antibiotics have also been used for preventing and treating animals and plants infections as well as for promoting growth in animal farming (McManus et al., 2002; Smith et al., 2002; Singer et al., 2003; Cabello, 2006). All these applications made antibiotics to be released in large amounts in natural ecosystems.

The full extent and consequences of the presence of these compounds in the environment are still largely unknown. These compounds have been detected in a wide variety of environmental water samples including sewage flows, surface and groundwater, with concentrations generally ranging from traces to ppb levels.

It is now known that some pharmaceuticals can persist in the environment and, either via the food chain or via drinking water, can make their way back to humans. It is also accepted that some of these compounds are beginning to be associated with adverse developmental effects in aquatic organisms at environmentally relevant concentrations, that are usually believed to be infinitesimal and harmless (Khetan and Collins., 2007). When pharmaceuticals are regarded as pollutants, their environmental fate and biological potency can be predicted or assessed on the basis of their special physicochemical characteristics.

There are three risks deriving from immoderate appliance of antibiotics resulting in environmental contamination with original substances or derivatives:

- An indirect impact on health via resistant micro-organisms;
- A direct organic damage;
- Some influences on the biotic environment.

The main interest regarding the use of antibiotics in human and animal treatment is the development of resistant bacteria strains representing a health risk to humans and animals; the application of veterinary antibiotics to food animals is supposed to enhance the selection for strains resistant to antibiotics used in human medicine. Transmission of these strains might be performed via direct contact with animals or via

the food-chain. As antibiotic resistance protects antibiotic-producing organisms from their own products, and other originally susceptible organisms from competitive attack, it is as ancient as antibiotics.

Not only direct therapeutic use of antibiotics, but also indirect contact might enhance the resistance of bacteria, not taking into account the bacteria's origins: resistance genes have been isolated from human, animal and environmental sources (Levy, 1997; Van den Bogaard and Stobberingh, 1999; Angulo et al., 2004). Recently, multiresistant bacteria have been isolated from hospitals all over the world, representing a serious therapeutic problem in human medicine (Lee et al., 2001; Morris and Masterton, 2002). Increasing resistance to fluorochinolones and third generation cephalosporins has been noted in food-borne pathogens *Campylobacter* species (spp.) and *Salmonella spp*., particularly. In *Salmonella spp.* and *Escherichia coli*, multi-drug resistance is a worrying possibility, as well.

Thus, even if the main risk of provoking resistance was related to the clinical application of antibiotics and the abundance of certain pathogens in a restricted area such as a hospital or stables, the influence of bacteria located elsewhere cannot be denied. Resistance can be transferred to environmental bacteria. This is supported by several studies showing

resistance patterns in bacteria isolated from soils (Onan and La Para, 2003; Sengeløv et al., 2003).

Once in the environment, antibiotic efficiency depends on the physical–chemical properties and a variety of other environmental factors such as prevailing climatic conditions, soil types etc.

The effects of sub-inhibitory concentrations against non-marine aquatic bacteria are mainly unknown, but the impact of various antibiotics remaining active against bacteria living in wastewater has been documented (Kummerer, 2003). Resistant and multi-resistant bacteria have been detected in wastewater and sewage treatment plants, possibly entering the food chain directly via sewage sludge used as fertiliser or wastewater serving for irrigation (Guardabassi et al., 1998; Witte, 1998; Feuerpfeil et al., 1999; Kummerer, 2003). Antibiotic effects on organisms living in the aquatic environment such as algae and daphnids (*Daphnia magna*) have been reported at concentrations between 5 and 100 mg/l (Holten-Lutzøft et al., 1999; Wollen- berger et al., 2000).

Under test conditions in aquatic systems, most of the examined antibiotic compounds have been persistent, while only few have been partially biodegraded (Al-Ahmad et al., 1999; Kummerer et al., 2000).

Antimicrobials released into the environment can enhance the formation of single, cross- and even multiple resistance in pathogens, commensal

and environmental bacteria (Wegener et al., 1996; Al-Ahmad et al., 1999). Additionally, there is a gap of reliable studies on the relationship between antibiotic residues and the occurrence of resistant bacteria. Even if a general link between antibiotic use and percentage of resistant strains is assumed (Nwosu, 2001), it is unclear at which threshold concentrations a shift towards an increase in resistant bacteria is to be expected.

Drugs administered to humans and animals are excreted with urine or faeces (Forth et al., 1996) and attend the sewage treatment plant (Stumpf et al., 1996); successively if substances are hydrophilic or are metabolized to a more hydrophilic form of the parent lipophilic drug, will pass the waste water treatment plant and end up in the receiving waters where they may be are present at very low concentrations; it is important noted that several substances could stimulate a response in humans and animals also at low doses with a very specific target (Jobling et al., 2006). A recent study showed that a mixture of drugs at the concentrations actually found in the aquatic environment of some Italian areas is able to exert toxic effects on the proliferation of human and zebra fish (*Danio rerio*) cells cultures (Pomati et al., 2006).

World wide there is a remarkable concern about the increased prevalence of antibiotic resistance: the growing alarm related to the spreading of the

resistance of antibiotics considered of first choice in the treatment of specific human infections suggests measures for antimicrobial resistance surveillance of bacteria circulating in humans, animals and food products. Aim of this study was to evaluate the presence of residues of antimicrobial substances and to assess the antimicrobial resistance in bacteria species isolated from wild caught seawater fish and fishery products caught in Tyrrenian sea along the coast of Campania region (southern Italy).

2.2 Methods

2.2.1 Sampling

Samples were collected in a defined area of the gulf of Salerno (Campania Region, Southern Italy) with the support of the mobile station of the Fish Research Laboratory of the *Department of Veterinary Medicine and Animal Production, University of Naples "Federico II".* The sampling area was chosen because it is close to sewers conveying hospital wastewater.

The research concerned 56 samples (Figure 10): 33 fish (7 species), 13 cephalopods (2 species) and 10 gasteropods (1 specie) present in the sampling zone during sampling period; fish species, collected at a depth of 5-7 meters and at a distance of about 50 meters from the coast, were: red scorpionfish (*Scorpaena scrofa*, 6 samples), giant goby (*Gobius cobitis,* 7 samples), atlantic horse mackerel (*Trachurus trachurus,* 4 samples), brown meagre (*Sciaena umbra,* 3 samples), white seabream (*Diplodus sargus,* 6 samples), fathead mullet (*Mugil cephalus,* 5 samples), green wrasse (*Labrus viridis,* 2 samples), common octopus (*Octopus vulgaris,* 7 samples), european cuttlefish (*Sepia officinalis,* 6 samples) and red-mouthed rock shell (*Thais haemastoma,* 10 samples) respectively. Samples after capture were immediately transported on ice

to the lab of the *Department of Veterinary Medicine and Animal Production, University of Naples "Federico II"*. An aliquot was subjected to microbiological analysis and the other was frozen at -80 °C until analyzed.

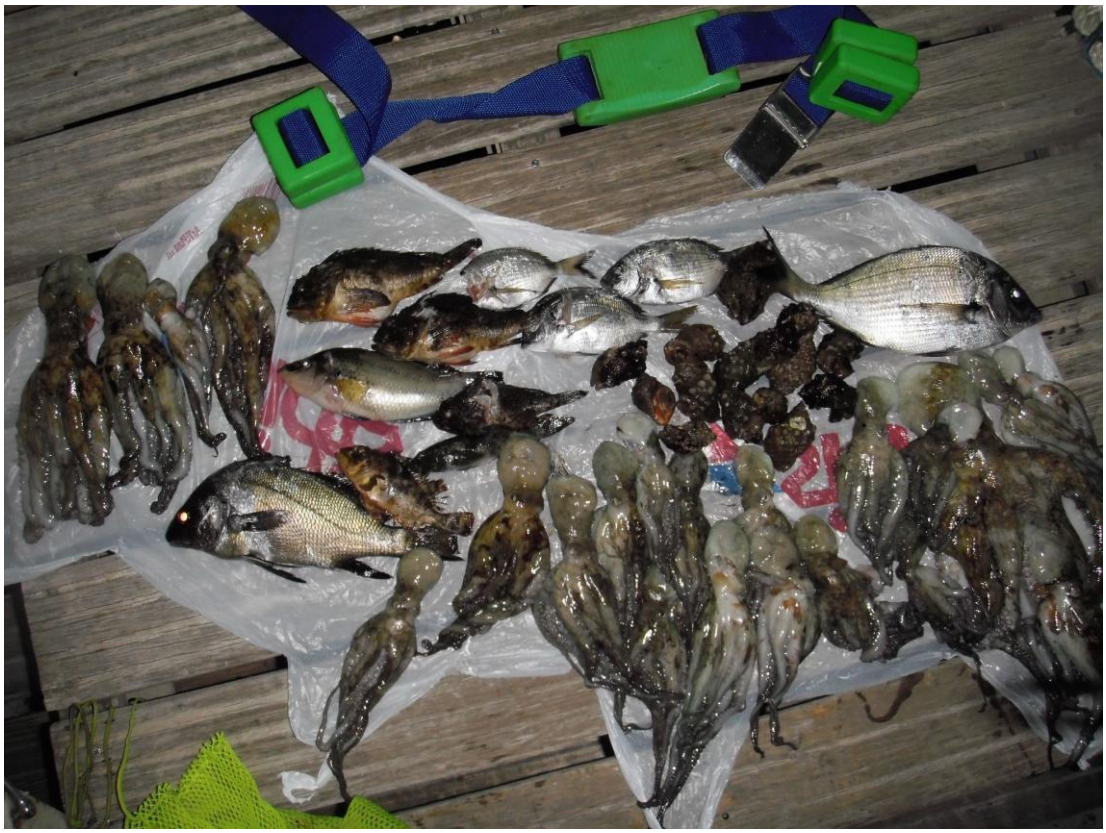

Figure 10: Samples

2.2.2 Microbial analysis and antibiogram

All samples were analyzed for the presence of microbial species of the genus *"Vibrio"* according to recognized ISO methods. Briefly all samples were scrubbed and analytical portions (25 g) were aseptically removed and collected in a sterile bag with 225 ml of alkaline saline

peptone water (ASPW) (Figure 11). According to ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 indications for fresh products, the samples were homogenized using a stomacher (PBI International, Milan, Italy) at 11000 rev min^{-1} for 3 min and incubated at 37 °C and 42 °C for 6 h. A further enrichment was performed employing 1 ml of the first enrichment and 9 ml of ASPW. This broth culture was incubated at 37 °C and 42 °C for 18 h. The enrichment cultures from incubation were plated onto thiosulphate-citrate-bile salt sucrose (TCBS) (Oxoid, Hampshire, UK) agar and incubated at 37 °C for 24 h. Typical colonies were transferred into Nutrient Agar plates (Oxoid, Hampshire, UK) added to 5 g/l NaCl to bring it to a final concentration of 1% and incubated at 37 °C for 24 h according to ISO/TS method.

Figure 11: Microbial analisys

After incubation at 37 °C for 24 h, the isolates were subjected to the Gram stain, the oxidase test using Oxidase Sticks (Oxoid, Hampshire, UK), Triple-Sugar-Iron (TSI) (Oxoid, Hampshire, UK) and biochemical identification with API 20E (bioMérieux, Marcy l'Étoile, France) (Figure 12) according to Di Pinto et al. (2008). The identification profiles were obtained by the APIweb software (bioMérieux, Marcy l'Étoile, France) according to to the instructions of the manufacturer.

Figure 12: ApiTest

The strains isolated were subjected to the antibiotic resistance test using standard methods. Antibiotic susceptibility was determined by the agar diffusion method according to French national guidelines. Bacterial suspensions prepared in sterile 0,85% saline matching an optical density of 0.5 McFarland standard corresponding to 10^8 cfu/ml and diluted 1:100 in physiological saline were inoculated by lawn onto Muller-Hinton agar (Difco, Le Pont de Claix, France). Each antibiotic test was run in duplicate on freshly prepared agar plates. After incubation for 24 h at 37

°C, organisms were classified as sensitive (S), intermediate (I) or resistant (R) according to the inhibition zone diameter (29). The antibiotics tested were Teicoplanin (TEC), Cephalexin (CN), Penicillin (P), Oxacillin (OX), Amoxicillin/Clavulanic Acid (AMC), Cefotaxime (CTX), Vancomycin (VA), Sulfamethoxazole (SXT), Rifampicin (RD), Cefoxitin (FOX), Plaritromicin (PRL), Ciprofloxacin (CIP), Chloramphenicol ©, Tobramicin (TOB), Tetracycline (TE), Tigecycline (TGC), Linezolid (LZD) and Fosfomycin (FOS).

2.2.3 Detection of residues of antibiotics

Analysis were performed on fish muscle. Each sample analyzed consisted of a pool of fish and fishery products grouped by species (i.e. sample of red scorpionfish consisted of a pool of six red scorpionfish). The detection of antibiotics residues was carried out using the kit "*Premi® Test*" (*Biopharm, Darmstadt, Germany*), as a screening method, according manufacturer's instructions. The kit is based on the growth inhibition of *Bacillus stearothermophilus*, a microorganism sensible to the residues of different antibiotics. This test is able to detect residues of β-lactam antibiotics, cephalosporins, macrolides, tetracyclines, sulphonamides, aminoglycosides, quinolones, amphenicols and polypeptides. The principle on which is based the test is the following: a

standard number of spores is embedded in an agar medium with selected nutrients (Figure 13).

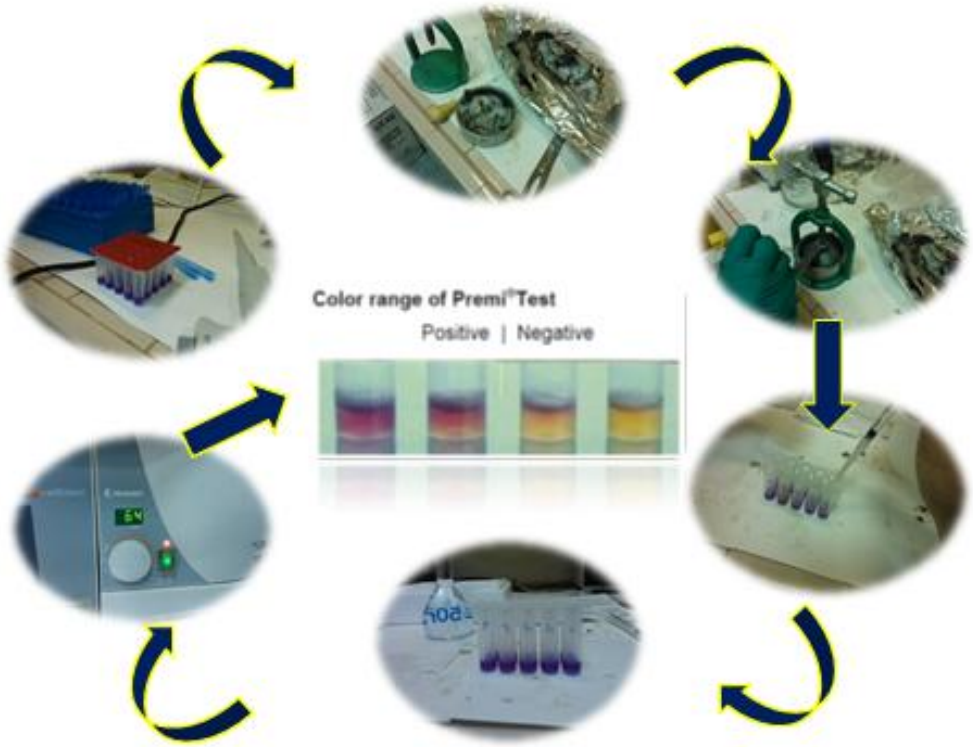

Figure 13: Premi ® Test

When *Premi ® Test* is heated to 64 °C, spores can germinate. If antimicrobial substances are absent, spores germinated producing hydrogen and a clear color change from purple to yellow occurs. When anti-microbial compounds are present above limit of detection, spores will not be able to germinate and there will be no colour change.

Samples positive to *Premi ® Test* were analyzed by the mean of HPLC-DAD method suggested by Fernandez-Torres et al. (2011) for the following compounds (97-99,9 % purity, Sigma-Aldrich - USA):

Sulfadiazine (SDI), Trimetroprim (TMP), Oxytetracycline (OXT), C and SXT. HPLC-UV method proposed by De Jesùs Valle et al. (2008), was used for VA detection. Antibiotic selection was made considering drugs commonly used in farms in the Campania region. All reagents used were of analytical grade. Measurements were made with a Jasco (Mary's Court, Easton, MD, USA) liquid chromatograph equipped with UV and diode array (DAD) detector, an injector with a loop of 50 µL, a quaternary pump, a vacuum degasser an a thermostated column compartment. For first method the separation of the analyzed compounds was conducted by means of a Phenomenex C_{18} (150 mm × 4.6 mm I.D., particle size 5 µm) analytical column with a C_{18} (4 mm × 4 mm, particle size 5 µm) guard-column. The mobile phase consisted of a mixture of 0,1% (v/v) formic acid in water pH 2,6 (phase A) and acetonitrile (phase B). A gradient eluition program at 1 mL/m-1 flow rate was used. After a step of 8 min with 99 % (phase A) a linear elution gradient to 65 % in 25 ~~m~~in was performed. The column effluent was monitored by DAD detector in the range of 200-400 nm. The sample extraction was conducted as follows: After homogenization of sample (2 g of lyophilized tissue + 5 of deionized water) 50 µL of Proteinase-K solution was added; to the mixture, centrifuged for 2,5 hours, 100 µL of formic acid was then added. Finally the samples was treated three times with 5

mL of dichloromethane and the extracts were evaporated under nitrogen. 50 µL of the residue, reconstituted with 1 mL of deionized water, were injected.

For the vancomycin detection method , chromatographic separation was carried out by the means of a Nucleosil 120 C18 5 µm column (length, 15 cm; inner diameter, 0.4 cm) using a mixture of 50 mM $NH_4H_2PO_4$ (pH 4)–acetonitrile (92:8, v/v) as the mobile phase at a flow rate 1 mL/min and a column temperature of 40 °C with UV detection at 220 nm. Regarding extraction procedure briefly: a mixture of 500 µg of sample with 20 µL of 60% perchloric acid was vortexed for 30 s, followed by centrifugation at 10,900 rpm, after the supernatant was collected and an aliquot of 50 µL was injected into the chromatographic system.

2.2.4 Statistical analysis

The χ^2 test performed with the Epi-Info statistical program (version 6.0; Centers for Diseases Control and Prevention, Atlanta, GA, USA) was used to test the effect of the antibiotics on the bacteria growth and to assess the effect type (resistance or sensibility) of each molecule among the different isolated microbial strain.

2.3 Results and discussion

2.3.1 Microbial analysis and antibiogram

The microbial species isolated were *Vibrio alginolyticus (Va), Vibrio parahaemolyticus (Vp), Shewanella putrefaciens (Sp)* and *Acromonas spp. (Ac)* (Table 2); *Vp* is a bacteria naturally present in marine and estuarine aquatic environments and is part of the natural flora of fish and bivalve mollusks. According to the findings of Yamakazi et al. (1996) in our study the prevalence of *Vp* in coastal fish species was higher than those caught away from the coast. There are no known information that might clarify the abundance of *Vp* in certain fishing area; however it is still likely that some fishing areas could be an excellent ecosystem so that the microorganism can express his replicative capacity and result in higher concentrations than other sites. *Va,* isolated from *Sepia officinalis* and *Trachurus trachurus* samples, is frequently detected from fin fish, shellfish, seawater, and sediment (Gjerde and Boe, 1981). It has not been not widely recognized as a fish pathogen. *Sp* a saprophytic microorganism common in marine environments is one of the major causes of spoilage of fish and fishery products (Jergensen and Huss, 1989);

Microbial species	Samples									
	S. officinalis	*M. cephalus*	*T. trachurus*	*S. scrofa*	*O. vulgaris*	*L. viridid*	*G. cobitis*	*S. umbra*	*D. sargus*	*T. haemastoma*
V. cholerae	-	-	-	-	-	-	-	-	-	-
V. parahaemolyticus	+	-	+	+	-	-	-	-	+	-
V. vulnificus	-	-	-	-	-	-	-	-	-	-
V. fluvialis	-	-	-	-	-	-	-	-	-	-
V. mimicus	-	-	-	-	-	-	-	-	-	-
Other spp.	*Va*	*Va*	*Va*	*Va*	*Va*	*Va*	-	*Va*	*Va*	-
Other generes	*Sp*	-	-	*Sp; Ac*	*Sp*	-	*Sp*	-	-	*Sp*

Table 2: Microbial species isolated in seawater fish and fishery products: Vibrio parahaemolyticus (Vp) was isolated in Sepia officinalis, Trachurus trachurus, Scorpaena scrofa and Diplodus sargus; Vibrio alginolyticus (Va) was isolated in all samples except in Gobius cobitis and Thais haemastoma; Shewanella putrefaciens (Sp) was isolated in cuttlefish, Scorpaena scrofa, Octopus vulgaris, Gobius cobitis and Thais haemastoma; Acromonas spp. (Ac) was isolated only in Scorpaena scrofa.

Water bacteria might be indigenous to aquatic environments, or exogenous, transiently and occasionally present in the water as a result of shedding from animal, vegetal, or soil surfaces.

Moreover several pollutants in seawater might exert selective activities, as well as ecological damage in water environment, resulting in antibiotic resistance: Baquero et al. (2008) noted that resistance profiles of aquatic pseudomonads depend on the species composition, but also from the site in which they were isolated, being more antibiotic-resistant along shorelines and in sheltered bays than in the open water, indicating the influence of nonaquatic organisms or pollutants.

The frequency of antibiotic resistance among microbial strains isolated was shown in table 3 and table 4. Although only 7 species of fish and 3 species of fishery products were studied to determine the incidence of antibiotic resistance, all the strains isolated were resistant to one or more of the antibiotics tested; the frequency of resistance varied from 16,6% to 50% in different samples; 69,45% of the microbial strains isolates showed resistance to more than 4 molecules tested. *Va* showed antimicrobial resistance against 9 antibiotics, *Vp* against 4 antibiotics, *Sp* against 6 antibiotics and *Ac* against 4 antibiotics tested. Accordinto to Martinez (2003), more than 90% of bacterial strains originated in

seawater are resistant to more than one antibiotic. Multiple antibiotic resistance has been reported in a wide range of human pathogenic or opportunistic bacteria such as *Campylobacter spp*. (Randall et al., 2003), *Klebsiella pneumoniae* (Cameiro et al., 2003), *Salmonella sp.* (Randall et al., 2004), *Pseudomonas aeruginosa* (Ziha-Zari et al., 1999), *E. coli* (Miranda et al., 2008) and also in fish pathogens (Schmidt et al., 2000). In all bacterial strains, resistance against TEC and VA, drugs belonging to the class of glycopeptides having similar mechanisms of action on bacterial cell wall synthesis, and against P and OX, drugs belonging to the class of β-lactam antibiotics, was observed. Spectra of activity of TEC and VA are limited to Gram–positive bacteria including methicillin–resistant strains of *S. aureus* and *S. epidermidis* and for this reason the resistant Gram–negative bacteria isolates could be not sensitive to mechanism of action of these molecules. VA has a shorter half–life than TEC and requires multiple dosing to maintain adequate serum levels. In contrast, the pharmacokinetics of TEC allow for once–daily dosing and it is a drug associated with a lower incidence of nephrotoxicity or ototoxicity. For these reasons TEC is more cost–effective and its role in hospitals is likely to increase. Resistance to VA could be related not only to the use of VA in human medicine but also to a cross-resistance due to the use of *Avoparcin*, a glycopeptides utilized

to improve performance in poultry flocks (Bager et al., 1997), which are present in the area near to sampling zone. Resistance against P and OX could be related to the large use of β-lactam antibiotics in human and veterinary medicine. The sensitivity against C detected in all bacterial strains coupled with the absence of C residues in fish sampled, confirm the limited administration to humans and the compliance concerning the proihibition for use in food producing animals. However, according Kerry et al. (1996), it is important to underline that resistance phenomena are not systematically correlated with the presence of the corresponding drugs.

Antibiotic resistance profiles among bacterial strains isolates (% resistant strains) were presented in table 5. As a whole, all culturable bacteria were significantly ($P < 0,001$) affected by the presence of the tested antibiotic molecules. In particular, for seven molecules (FOS, FOX, AMC, PRL, TOB, TE and LZD) for the four different bacteria strains isolated a statistically significant ($P < 0,05$) effect (resistance or sensibility) was observed. The other molecules didn't show statistically significant effect ($P > 0,05$) among the different isolated microbial strains; four of them showed very high resistance (mean values: VA: 100 %, OX: 98,9 %, TEC: 97,7%, P: 85,7%) particularly.

Samples	Antibiotics tested																	
	TEC	***CN***	***P***	***OX***	***AMC***	***CTX***	***VA***	***SXT***	***RD***	***FOX***	***PRL***	***CIP***	***C***	***TOB***	***TE***	***TGC***	***LZD***	***FOS***
Va* isolated from *S. umbra	R	S	R	R	R	S	R	I	S	I	R	S	S	I	S	I	S	I
Va* isolated from *S. umbra	R	I	R	R	R	S	R	S	S	I	S	S	S	I	S	S	S	S
Va* isolated from *S. officinalis	R	R	R	R	R	R	R	I	I	R	R	S	S	I	S	I	S	S
Va* isolated from *S. officinalis	S	S	R	R	R	S	R	S	S	I	R	S	S	I	R	S	S	S
Va* isolated from *S. officinalis	R	S	R	S	S	S	R	S	S	I	S	S	S	S	S	S	S	S
Va* isolated from *T. trachurus	R	I	R	R	R	S	R	I	S	I	I	S	S	I	S	S	S	S
Va* isolated from *T. trachurus	R	I	R	R	R	R	R	R	I	I	R	S	S	S	S	S	S	S
Va* isolated from *T. trachurus	R	I	R	R	R	R	R	I	I	I	R	S	I	I	S	S	S	S
Va* isolated from *T. trachurus	R	S	R	R	R	I	R	I	I	I	R	S	S	I	S	S	S	I
Va* isolated from *T. trachurus	R	I	R	R	I	S	R	S	S	I	S	S	S	I	S	S	S	S
Va* isolated from *O. vulgaris	S	S	R	R	R	S	R	S	S	I	R	S	S	I	R	S	S	S
Va* isolated from *O. vulgaris	R	I	R	R	S	S	R	S	S	I	R	S	S	I	S	S	S	S
Va* isolated from *O. vulgaris	R	I	R	R	S	S	R	S	S	I	R	S	S	I	S	S	S	S
Va* isolated from *M. cephalus	R	S	R	R	R	S	R	I	S	S	S	S	S	I	S	S	S	S
Va* isolated from *M. cephalus	R	I	R	R	S	S	R	S	S	I	R	S	S	I	S	S	S	S
Va* isolated from *M. cephalus	R	I	R	R	R	S	R	S	S	I	S	S	S	I	S	S	S	S
Va* isolated from *M. cephalus	R	S	R	R	R	S	R	I	S	I	R	S	S	I	S	I	S	I
Va* isolated from *L. viridis	R	I	R	R	I	S	R	S	S	I	S	S	S	I	S	S	S	S
Va* isolated from *L. viridis	R	S	R	R	R	S	R	I	S	S	S	S	S	I	S	S	S	S
Va* isolated from *D. sargus	R	I	R	R	R	S	R	I	S	I	I	S	S	I	S	S	S	S
Va* isolated from *D. sargus	R	I	R	R	R	S	R	I	S	I	I	S	S	I	S	S	S	S
Va* isolated from *D. sargus	R	I	R	R	R	S	R	I	S	I	I	S	S	I	S	S	S	S

Table 3: Frequency of antibiotic resistance among the bacteria isolated: Teicoplanin (TEC), Cephalexin (CN), Penicillin (P), Oxacillin (OX), Amoxicillin/Clavulanic Acid (AMC), Cefotaxime (CTX), Vancomycin (VA), Sulfamethoxazole (SXT), Rifampicin (RD), Cefoxitin (FOX), Plaritromicin (PRL), Ciprofloxacin (CIP), Chloramphenicol (C), Tobramicin (TOB), Tetracycline (TE), Tigecycline (TGC), Linezolid (LZD) Fosfomycin (FOS). Microbial strains were classified as sensitive (S), intermediate (I) or resistant (R); all Vibrio alginolyticus (Va) isolated from different samples are resistant to P and VA and are sensitive to C.

Samples	Antibiotics tested																	
	TEC	*CN*	*P*	*OX*	*AMC*	*CTX*	*VA*	*SXT*	*RD*	*FOX*	*PRL*	*CIP*	*C*	*TOB*	*TE*	*TGC*	*LZD*	*FOS*
Ac* isolated from *S. scrofa	R	S	R	R	I	S	R	S	S	I	S	S	S	S	S	S	S	S
Sp* isolated from *S. officinalis	R	S	S	R	S	S	R	S	S	I	S	S	S	I	S	S	S	R
Sp* isolated from *S. officinalis	R	S	S	R	S	S	R	S	S	I	S	S	S	S	S	S	I	R
Sp* isolated from *G cobitis	R	S	R	R	R	I	R	I	S	R	S	S	S	I	I	S	I	S
Sp* isolated from *T. haemastoma	R	S	R	R	R	I	R	I	S	R	S	S	S	I	I	S	I	S
Sp* isolated from *O. vulgaris	R	S	S	R	S	S	R	S	S	I	S	S	S	I	S	S	S	R
Sp* isolated from *S. scrofa	R	S	I	R	S	S	R	S	S	S	S	S	S	S	S	S	S	R
Sp* isolated from *S. scrofa	R	S	R	R	R	I	R	I	S	R	S	S	S	I	I	S	I	S
Vp* isolated from *S. officinalis	R	S	R	R	S	S	R	I	I	S	S	S	S	I	S	S	S	S
Vp* isolated from *T. trachurus	R	I	R	R	S	S	R	S	S	S	S	S	S	I	S	I	S	S
Vp* isolated from *S. scrofa	R	I	R	R	R	I	R	I	S	R	S	S	S	I	S	S	S	S
Vp* isolated from *S. scrofa	R	I	R	R	S	S	R	S	S	R	S	S	S	I	S	I	S	S
Vp* isolated from *D. sargus	R	S	R	R	S	S	R	I	I	S	S	S	S	I	S	S	S	S
Vp* isolated from *D. sargus	R	I	R	R	R	I	R	I	S	R	R	S	S	I	S	S	S	S

Table 4: Frequency of antibiotic resistance among the bacteria isolated: Teicoplanin (TEC), Cephalexin (CN), Penicillin (P), Oxacillin (OX), Amoxicillin/Clavulanic Acid (AMC), Cefotaxime (CTX), Vancomycin (VA), Sulfamethoxazole (SXT), Rifampicin (RD), Cefoxitin (FOX), Plaritromicin (PRL), Ciprofloxacin (CIP), Chloramphenicol (C), Tobramicin (TOB), Tetracycline (TE), Tigecycline (TGC), Linezolid (LZD) Fosfomycin (FOS). Microbial strains were classified as sensitive (S), intermediate (I) or resistant (R); all microbial strains isolated from different samples are resistant to TEC and VA and are sensitive to C.

Microbial strains (n)	Antibiotics tested																	
	TEC	***CN***	***P***	***OX***	***AMC***	***CTX***	***VA***	***SXT***	***RD***	***FOX***	***PRL***	***CIP***	***C***	***TOB***	***TE***	***TGC***	***LZD***	***FOS***
V. alginolyticus (22)	90,9	4,5	100	95,5	72,7	13,6	100	4,5	0	4,5	50	0	0	0	9,1	0	0	0
V. parahaemolyticus (6)	100	0	100	100	33	0	100	0	0	50	17	0	0	0	0	0	0	0
S. putrefaciens (7)	100	0	42,9	100	43	0	100	0	0	43	0	0	0	0	0	0	0	57
Acromonas spp.(1)	100	0	100	100	0	0	100	0	0	0	0	0	0	0	0	0	0	0
Mean of all isolated microbial strains	97,7	1,1	85,7	98,9	37,2	3,4	100	1,1	0	24,4	16,7	0	0	0	2,3	0	0	14,3

Table 5: Antibiotic resistance profiles among bacterial strains isolates (% resistant strains). Teicoplanin (TEC), Cephalexin (CN), Penicillin (P), Oxacillin (OX), Amoxicillin/Clavulanic Acid (AMC), Cefotaxime (CTX), Vancomycin (VA), Sulfamethoxazole (SXT), Rifampicin (RD), Cefoxitin (FOX), Plaritromicin (PRL), Ciprofloxacin (CIP), Chloramphenicol (C), Tobramicin (TOB), Tetracycline (TE), Tigecycline (TGC), Linezolid (LZD) Fosfomycin (FOS). All strains are resistant to VA and sensitive to CIP, C, TOB, TGC and LZD.

2.3.2 Detection of residues of antibiotics

Residues of antibacterial substances were detected in common octopus, european cuttlefish and red-mouthed rock shell (Figure 14).

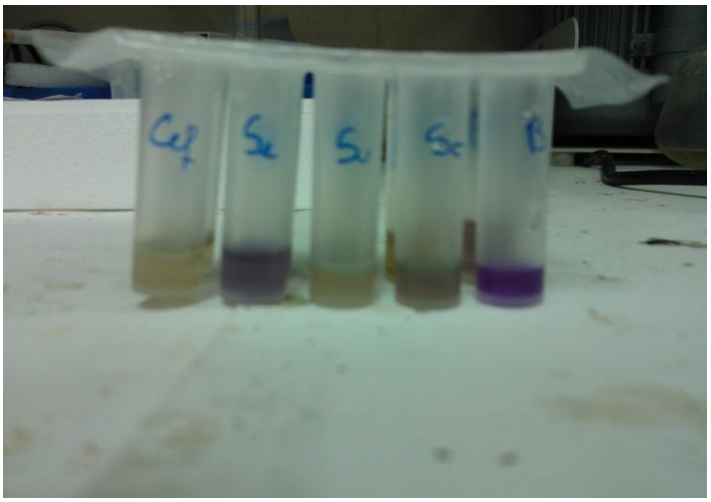

Figure 14: Premi ® Test results

At the confirmatory analysis only two of the examined drugs were detected. Oxytetracycline and Sulfamethoxazole were quantified with 3.62 µg/kg and 0.48 µg/kg respectively. Levels detect ed were in compliance with LMR established by UE Reg. 37/2010. The presence of antibiotics might be due to the increased possibility of accumulation in fishery products that, for a period of their life cycle, remain for a long time in the same fishing area. In our study, the sampling area is within a stretch of coastline that spans the mouth of one river and several streams that cross a lot of livestock and agricultural fields.

Studies on residues of pharmacologically active molecules have shown elimination rates in the aquatic environment after the depuration treatment generally between 60 and 90%, for a wide variety of polar drugs (Ternes, 1998). The elimination of drugs in common sewage treatment plants is often incomplete and recent works show the presence of antimicrobial residues in river waters (Iglesias et al.,2012). Polar antibiotics cannot be eliminated effectively as much of the process of elimination is based on absorption on activated sludge and so ultimately on hydrophobic interactions. Another route of elimination of drugs in the aquatic waste water is related to the dispersion of manure "contaminated" on the fields as fertilizer through runoff into streams of wastewater and those used for irrigation. It was showed that sulfa drugs, such as sulfadimethoxine, are sufficiently stable in the manure as to maintain a significant residual activity up when the manure is used for fertilizing (Migliore et al., 1995; Boehm, 1996) and that some of the metabolites of antibiotics excreted may also be retransformed into the active drug progenitor; such as the glucuronide dicloramphenicol or the N-4-acetyl sulfamethazine converted into the manure in chloramphenicol and sulfamethazine respectively (Berger et al., 1986).

2.4 Conclusions

The risk of antibiotic resistance was considered significantly more serious than the risk associated with the presence of antibiotic residues in food (FAO/OIE/WHO, 1986). Results presented in this study provide evidence that seawater fishes collected in some area of Campania Region, especially in marine areas including mouths of streams, were contaminated by residues of antibiotic and antibiotic-resistant bacteria strains and that they might play an important role in the spread of antibiotic-resistance. The resistance of 97,7 % of isolated strains against TEC might suggest that the main sources of contamination were hospital discharges.

The spread of strains with antibiotic resistance from animal to animal does not meet the minimum barrier in the marine environment and resistance evolves as a consequence of promiscuous exchange and shuffling of genes, genetic platforms, and genetic vectors. The spread of strains with antibiotic resistance from animal to animal does not meet the minimum barrier in the marine environment and resistance evolves as a consequence of promiscuous exchange and shuffling of genes, genetic platforms, and genetic vectors. Future prediction and prevention of antibiotic resistance depends on the research investments in the

development of microbial source tracking as well as in the ecology, including water ecology, of antibiotic-resistant microorganisms.

CHAPTER 3. PARASITE RISK ASSESSMENT

For millennia a wide variety of products derived from marine and freshwater animals have been used for human consumption and to feed animals raised with the intention to be consumed by humans.

Parasitic diseases are those caused by eukaryotic organisms (both unicellular of multicellular). Human fishery product-borne parasitic diseases primarily include those caused by cestodes, trematodes and nematodes. These diseases are either caused by an infection following ingestion of viable parasites, or as an allergic (hypersensitivity) reaction against parasite antigens which occurs for nematodes of the family *Anisakidae*. While it was recognized the importance of meatborne parasitic zoonoses such as trichinellosis and cysticercosis, fishery product-borne parasitic disease like opisthorchiasis, intestinal trematodiasis, anisakiasis or diphyllobothriasis have received less attention despite the large numbers of human infections (Chai et al., 2005a).

In the past, these diseases were limited for the most part to populations living in low- and middle-income countries, but the situation is changing because of growing international markets, improved transport systems, and demographic changes (such as population movements). The World

Health Organization (1995) has estimated that the number of people currently infected with fish-borne trematodes exceeds 18 million, but worldwide the number of people at risk, including those in developed countries, is more than half a billion. The recognition of the public health significance of these zoonoses, their links to poverty and cultural traditions, to intensification of agriculture, to environmental degradation, and the lack of tools for control is increasing (World Health Organization, 1995, 2004). This is due also to the process by which priorities in national public health systems are developed, which is usually a competitive exercise, and in which the justification for devoting greater attention and resources to fish-borne parasitic zoonoses is generally handicapped by the lack of good data on health and economic impacts. Compared with other well-studied parasitic diseases, fish-borne parasitic zoonoses have been public health orphans in the world of research funding, due in no small measure to insufficient appreciation of a crucial fact that most of them exist as a complex of parasite species whose transmission is often dependent on well-entrenched human behaviors. Because the modes of human infection are so similar, collectively these zoonoses may in many locations have a much greater aggregate effect than some other better-known parasitic diseases. The difficulties of diagnosis, the complexities of human

cultural behaviors and the poor understanding of potential economic costs have made this field simultaneously daunting, scientifically obscure and, therefore, somewhat unattractive to investigators especially in developed countries. However, the challenge of developing a prevention and control strategy that accommodates strong cultural and agricultural traditions will test the imaginations and skills of researchers, an intellectual challenge that could provide the stimulation needed to build a more concerted international effort.

3.1 Anisakid nematodes and anisakiasis

Anisakiasis (anisakidosis) refers to infection of people with larval stages of ascaridoid nematodes belonging to the family *Anisakidae* (and possibly also *Raphidascarididae*). *Anisakis spp*. is a genus of nematode that causes human parasitic infection most commonly associated with consumption of raw fishery products. These worms, commonly called anisakids, utilize aquatic mammals, piscivorous birds, aquatic reptiles, or fish as definitive hosts, and aquatic invertebrates and fish as intermediate or paratenic hosts. Adult and larval anisakids often have major pathological effects in the alimentary tract and associated organs of their natural host species (reviewed by Smith, 1999). Humans become infected by consuming fish or cephalopod mollusks with larval anisakids in their flesh, viscera, or body cavity. Although mammalian hosts have been experimentally infected with worms from a number of species within the families *Anisakidae* and *Raphidascarididae*, human infections almost always involve *Anisakis simplex* and *Pseudoterranova decipiens*. A. simplex is about 2 cm long, easy to see in the viscera, but difficult to see in the fish musculature and in the belly flaps of white fish. Humans are accidental hosts in the life cycle of anisakid nematodes, and, although the parasites almost never develop further within the human alimentary tract, they may penetrate the tract and associated organs, with

severe pathological consequences and may produce a strong allergic reaction, often culminating in anaphylactic shock. Anisakiasis is therefore a serious zoonotic disease, and there has been a dramatic increase in its reported prevalence throughout the world in the last two decades.

3.2 Taxonomy and geographic distribution

Species within the superfamily *Ascaridoidea* are among the most thoroughly studied nematode parasites of vertebrates. Ascaridoids have been used extensively for studies of respiratory biochemistry, immunology, molecular genetics, and population genetics (Nadler and Hudspeth, 2000). However, the evolutionary taxonomy of the superfamily is very uncertain, largely because of the great variation of external features and life cycle patterns among different species (Fagerholm, 1991; Anderson, 1992).

Before the widespread use of cladistic analysis, most hypotheses of ascaridoid phylogeny were based on a few key morphological structures or life history features, such as the presence or absence of the ventriculus (Hsu, 1933), the structure of the secretory-excretory system (Hartwich, 1974), or male caudal morphology (Fagerholm, 1991). Differences in features used for phylogenetic reconstruction led to an array of contrasting interpretations and hypotheses of relationships, in turn leading to instability of ascaridoid classification, although the classification schemes of Hartwich (1974) and Fagerholm (1991) have been most commonly used (Table 6).

Authority	Family	Subfamily	Genera
Hartwich (1974)	Anisakidae	Anisakinae	*Anisakis, Phocanema* (= *Pseudoterranova*), *Terranova, Sulcascaris, Duplicaecum, Galeiceps, Contracaecum, Phosascaris*
		Geoziinae	*Goezia*
		Raphidascaridinae	*Raphidascaris, Raphidascaroides, Thynnascaris* (= *Hysterothylacium*), *Lappetascaris, Aliascaris, Heterotyphlum, Paranisakis, Paranisakiopsis*
Fagerholm (1991)	Anisakidae	Anisakinae	*Anisakis, Pseudoterranova, Terranova, Sulcascaris, Peritrachelius, Pulchrascaris, Paranisakiopsis*
		Contracaecinae	*Contracaecum, Galeiceps, Phosascaris*
	Raphidascarididae		*Raphidascaris, Raphidascaroides, Hysterothylacium, Lappetascaris, Heterotyphlum, Paranisakis, Goezia, Sprentascaris, Paraheterotyphlum*

Table 6: Contrasting classification scheme of Anisakid nematodes by Hartwich (1974) and Fagerholm (1991).

The anisakids, broadly defined, constitute those ascaridoids with an aquatic definitive host (fish, reptile, piscivorous bird, or mammal), whose transmission is dependent on water and usually involves aquatic invertebrate and fish intermediate or paratenic hosts (Anderson, 1992). Species identification in the Anisakidae has traditionally been complicated by a lack of distinguishing morphological characteristics, particularly in larval worms. Historically, therefore, only two major zoonotic species were recognized: the herring worm or whale worm *Anisakis simplex*, and the codworm or seal worm *Pseudoterranova decipiens*.

Recent molecular genetic studies, however, have shown that both of these morphospecies actually comprise a number of sibling species, genetically differentiated and often with distinct geographic ranges.

Three different species have been described within the *Anisakis simplex* complex (Mattiucci et al., 1997):

- *Anisakis simplex (sensu stricto)* is found in the north Atlantic Ocean between 30°N and the Arctic polar circle;
- *A. pegreffii* is distributed in southern oceans from 35°S to 55°S as well as in the Mediterranean Sea;
- *A. simplex* C is found in the northern Pacific and southern oceans below 30°N (Mattiucci et al., 1997).

Within each sibling species, there is very little genetic differentiation between populations located thousands of kilometres apart. This is thought to be caused by the homogenizing effects of gene flow, enhanced by the high mobility of fish hosts (Mattiucci et al., 1997). In addition to these three sibling species, four other species of Anisakis have been confirmed using genetic markers:

- *A. typica*, from the Atlantic Ocean, Indian Ocean, and Mediterranean Sea;
- *A. physeteris*, from the Atlantic and Mediterranean;
- *A. brevispiculata*, from the south east Atlantic;

- *A. zhiphidarum*, from the southeast Atlantic and Mediterranean (Mattiucci et al., 2005).

Six different species have been described within the Pseudoterranova decipiens species complex (Paggi et al., 1991, 2000; Mattiucci et al., 1998; George-Nascimento and Urrutia, 2000; McClelland, 2002).

- *Pseudoterranova decipiens (sensu stricto)* is found in the northeast Atlantic, in waters off northern Europe and Iceland, and in the northwest Atlantic, off eastern Canada;
- *Pseudoterranova krabbei* is found only in the northeast Atlantic, where it is sympatric with *P. decipiens (sensu stricto)*;
- *P. bulbosa* is confined to the Barents Sea in the northeast Atlantic, and is also found in the north Pacific, off Japan, where it is sympatric with *P. azarasi* (McClelland, 2002). Two species have been described from southern oceans;
- *P. decipiens E* in the Antarctic and *P. cattani* in the south Pacific, off Chile. In addition to these six sibling species, two other species of *Pseudoterranova* have been described, based on morphological criteria: *P. kogiae* and *P. ceticola* (Anderson, 1992).

Neither the species lists for *Anisakis* and *Pseudoterranova* nor the described geographic ranges of these species can be regarded in any sense as definitive. Further genetic studies will undoubtedly uncover

more species of anisakid nematodes and extend the geographic ranges of those species that have already been described.

3.3 Molecular systematic of Anisakis spp.

The inconsistency in morphological characters of Anisakis species impeded development of a credible scheme of their phylogeny. This prompted the need to classify these nematodes by genetic and/or biochemical methods. Thus, beginning in the late 1980s, researchers started to evaluate their taxonomy, and genetic differentiation and relationships between taxa of this genus (Nascetti et al., 1986; Mattiucci et al., 1986; Nadler et al., 1990, 1995).

Today, the existence of two main clades is clearly shown (Figure 15), by genetic studies, in the genus *Anisakis*: one including species showing the larval stage indicated as *Anisakis Type I* (sensu Berland, 1961), and a second sharing the larval morphology *Anisakis Type II* (sensu Berland, 1961). The first clade includes the species of *A. simplex complex* (i.e. *A. simplex (s.s.), A. pegreffii, A. simplex C*), *A. typica, A. ziphidarum* and *Anisakis sp.* The second includes the species *A. physeteris, A. brevispiculata* and *A. paggiae* (Mattiucci et al., 2005; Valentini et al., 2006). At the interspecific level, among the nine genetically characterized species of Anisakis, the highest genetic identity was observed between the three sibling species of the *A. simplex complex.*

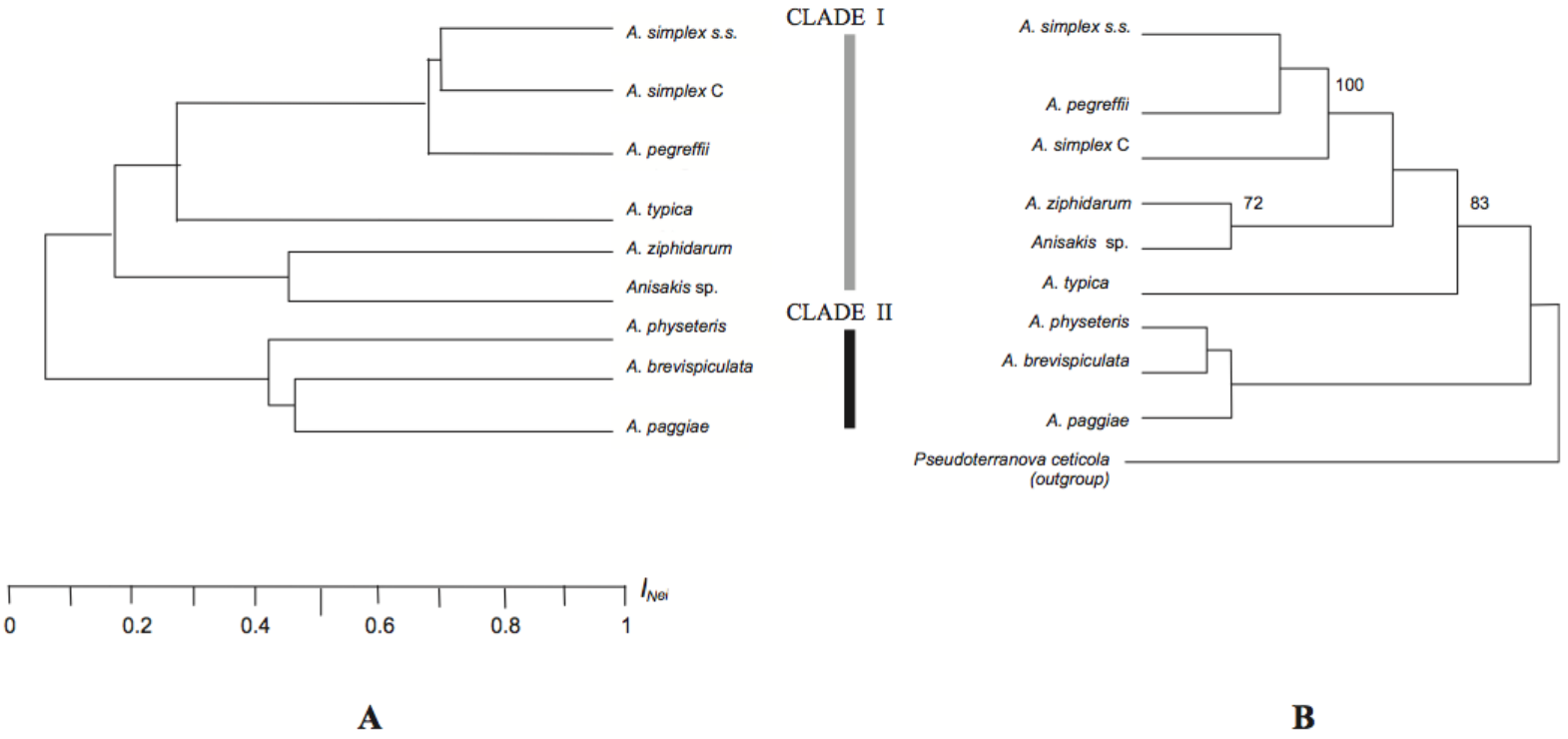

Figure 15: Genetic relationship among Anisakis spp.

3.4 Ecological data of Anisakis species included in Clade I

According to the genetic data, five species (*A. simplex sensu stricto, A. pegreffii, A. simplex* C, *A. typica, A. ziphidarum*) are included in this clade, and one new gene pool recently evidenced, and indicated as *Anisakis* sp. (Valentini *et al.*, 2006). A synopsis of ecological aspects of each species, including host preference and geographical aspects, is presented below.

3.4.1 The Anisakis simplex complex

Three species are so far included in the *A. simplex* complex: they are *A. simplex (s. s.), A. pegreffii, A. simplex* C. Their definitive hosts (cetaceans) and intermediate/ paratenic (squids and fish) identified today are listed in Tables 7 and 8.

- *A. simplex (s.s.)* (Nascetti *et al.*, 1986) is widespread between 35° N and the Arctic Polar Circle; it is present in both the western and eastern Atlantic and both western and eastern waters of the Pacific Ocean (Mattiucci *et al.*, 1997; 1998; Paggi *et al*., 1998b; Abollo *et al*., 2001) (Figure 16). The southern limit of this species in the north east Atlantic Ocean are the waters around the Gibraltar area. *A. simplex (s.s.)* is occasionally present also in the western part of the Mediterranean waters due to the migration of pelagic fish species in the far western Mediterranean Sea waters (Alboran Sea)

(Mattiucci *et al.*, 2004; and 2006) (Figure 16). *A. simplex (s. s.)* has been so far recorded in nine species of cetacean hosts. Four squid and 26 fish species were so far found harbouring larvae of this species along its geographical range. A sympatric area between *A. simplex (s. s.)* and *A. pegreffii* was identified along the Spanish and Portuguese Atlantic coast (Mattiucci *et al.*, 1997, 2004, 2006; Abollo *et al*., 2001) and in the Alboran Sea (Mattiucci *et al*., 2004, 2006). *A. simplex (s. s.)* also occurs with *A. simplex* C in the eastern Pacific Atlantic Ocean, where it has been identified in some definitive hosts along the East Pacific coast (Mattiucci *et al*., 1997, 1998; Paggi *et al*., 1998) (Tables 7, 8 and Figure 16). Although it has sympatric and syntopic occurrence in mixed infections at both larval and adult stages with other *Anisakis* species (Mattiucci *et al.*, 2004, 2005), reproductive isolation between *A. simplex (s .s.)* and both *A. pegreffii* and *A. simplex* C was proved by the lack of adult F1 hybrids, and/or backcross genotypes clearly demonstrated at nuclear level (Mattiucci *et al*., 1997, 2005).

- *A. pegreffii* (Nascetti *et al.*, 1986), previously indicated as *A. simplex* A (Nascetti *et al*., 1986), is the dominant species of the genus *Anisakis* in the Mediterranean Sea, being widespread in all

the fish species so far examined. Indeed, it is presently the most important aetiological agent of infection in pelagic and demersal fish of the Mediterranean waters. It appears also widely distributed in the Austral Region between 35° N and 55° S. In the Atlantic waters its upper limit of geographical range is represented by the Iberian coast of the NE Atlantic (Mattiucci *et al*., 1997, 2004; Abollo *et al*., 2001). It has been not reported so far from the west part of Atlantic Ocean (Figure 16). The genetic homogeneity between Mediterranean populations and those from Austral region seems to be maintained by the high levels of gene flow observed in this species, allowing the hypothesis of its wide occurrence also in other areas of the southern hemisphere. To date, it has been recorded as parasite at adult stage in three species of oceanic dolphins as definitive hosts, in 28 species of fish, and in two squids (Tables 7, 8). Among them, two definitive and 11 intermediate were found to be shared with *A. simplex (s. s.)*, in the contact area of the Iberian Atlantic coast waters (Figure 16). Whereas, two definitive and five intermediate hosts are shared by *A. pegreffii* and *A. simplex* C, in the austral region of New Zealand waters, the South African coast and the Southern Pacific Chilean coast (Table 8 and Figure 16).

- *A. simplex* C (Mattiucci *et al.,* 1997) shows, to date, a discontinuous range, including Pacific Canada, Chile, New Zealand waters, and the Atlantic South African coast. *A. simplex* C was identified so far at adult stage from three marine mammals and at larval stages it syntopically occurred with *A. pegreffii* in five fish species (Tables 7, 8 and Figure 16).
- • *A. typica* (Diesing, 1860) extends its range from 30° S to 35° N in warmer temperate and tropical waters (Table 9 and Figure 16) (Mattiucci *et al.,* 2002). In these areas it was found at adult stage in six dolphin species and at larval stages in 10 fish species. *A. typica* was recently identified also in the striped dolphin, *Stenella coeruleoalba* and in the European hake, *Merluccius merluccius* from the eastern Mediterranean Sea (Cyprus). Its presence in these waters could be the result of "lesseptian migration" (through the Suez Channel) (Mattiucci *et al.,* 2004) of its intermediate hosts from the Indian Ocean. Indeed, it is the only species to date responsible for the infections in fish species of these waters (Table 9 and Figure 16).
- *A. ziphidarum* (Paggi *et al*., 1998) was detected in the beaked whales, *Mesoplodon layardii* and *Ziphius cavirostris* from the South Atlantic Ocean (South Africa coast). Subsequently, it was

also recorded in the Mediterranean Sea, also parasite of *Z. cavirostris*. Since its first morphological description and genetic characterization, it has been recently genetically identified at adult stage also in other species of beaked whale, such as *M. mirus* and *M. grayi* in south Atlantic waters and in *Mesoplodon* sp. and *Ziphius cavirostris* from the Caribbean waters. Thus, its geographical range seems to be wide (Figure 16) and related to that of its definitive hosts. Scanty data are so far available concerning its infection in fish and/or squid. It is responsible for the low prevalence of infection in some fish species (Mattiucci *et al.*, 2004) that are reported in Table 9.

- *Anisakis* sp. (Valentini *et al.*, 2006) has been detected only at larval (L4) stage in the beaked whales *Mesoplodon mirus* and *M. grayi* from South African and New Zealand waters (Table 9 and Figure 16). This gene pool has been found reproductively isolated from the sympatric species *A. ziphidarum* occurring in the same hosts and geographic location. It is considered more closely related to *A. ziphidarum* rather than to the other species so far genetically characterized. Although evidenced only at adult stage, the third stage larva of this so far undescribed taxon shares the

morphotype Type I, and it was rarely identified in some fish species of North East Atlantic waters.

	A. simplex s.s.					*A. pegreffii*			*A. simplex C*	
LOCATION CODE	EA	IC	WA	EP	SA	CM	IC	SA	EP	SA
DEFINITIVE HOST SPECIES										
Cetaceans										
Neobalaenidae										
Caperea marginata	--	--	--	--	--	--	--	•	--	--
Balaenopteridae										
Balaenoptera acutorostrata	•	--	--	--	--	--	--	--	--	--
Monodontidae										
Delphinapterus leucas	--	--	•	--	--	--	--	--	--	--
Delphinidae										
Delphinus delphis	--	•	--	--	--	--	•	--	--	--
Globicephala melaena	--	•	--	--	•	--	--	--	--	•
Lagenorhynchus albirostris	•	--	--	--	--	--	--	--	--	--
Lissodelphis borealis	--	--	--	--	--	--	--	--	–	--
Orcinus orca	--	--	--	•	--	--	--	--	--	--
Pseudorca crassidens	--	--	--	•	--	--	--	--	•	--
Stenella coeruleoalba	--	•	--	--	--	--	--	--	--	--
Tursiops truncatus	--	--	--	--	--	•	--	•	--	--
Phocoenidae										
Phocoena phocoena	--	--	--	•	--	--	--	--	--	--

Table 7: Definitive host so far evidenced for the species of A. simplex complex (Codes: EA: North east Atlantic; IC: Iberian Atlantic coast; WA: Wst Atlantic; EP: North east Pacific; CM: Central Mediterranean Sea; SA: South Africa)

	A. simplex s.s.												*A. pegreffii*											*A. simplex* C		
LOCATION CODE	EA	SI	BS	IC	WA	EP	JA	BE	SA	MA	WM	AZ	CM	EM	WM	IC	EA	FA	SA	MA	NZ	AZ	BR	NZ	TA	SA
INTERMEDIATE HOST SPECIES																										
Cephalopods																										
Sepiidae																										
Sepia officinalis	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Ommastrephidae																										
Todaropsis eblanae	--	--	--	•	--	--	--	--	•	--	--	--	--	--	--	•	--	--	•	--	--	--	--	--	--	--
Ommastrephes sagittatus	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Ommastrephes angolensis	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Illex coindettii	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Fish																										
Pleuronectidae																										
Hippoglossus hippoglossus	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Scophthalmidae																										
Lepidorhombus boscii	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--
Bramidae																										
Brama brama	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Carangidae																										
Trachurus capensis	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Trachurus mediterraneus	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Trachurus picturatus	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	•	--	--	--	--
Trachurus trachurus	•	--	--	•	--	--	--	--	--	•	•	--	•	•	•	•	--	--	--	•	•	--	--	--	--	--
Emmelichthyidae																										
Emmelichthys nitidus nitidus	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Gempylidae																										
Thyrsites atun	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	•
Pinguipedidae																										
Parapercis colias	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	•	--	--
Scombridae																										
Scomber japonicus	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	•	--	--	--	--
Scomber scombrus	•	--	--	•	--	--	--	--	--	--	--	--	•	--	--	•	--	--	--	--	--	--	--	--	--	--
Thunnus thynnus	--	--	--	--	--	--	•	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	•	--	--	--
Sparidae																										
Spondyliosoma cantharus	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Trichiuridae																										
Lepidopus caudatus	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	•	--	--	--	--	--	--	--
Xiphiidae																										
Xiphias gladius	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Scorpaenidae																										
Scorpaena scrofa	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--
Sebastidae																										
Helicolenus dactylopterus	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	•	--	--	--	--	--	--	--
Triglidae																										
Eutrigla gurnardus	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Belonidae																										
Belone belone	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--

(continues)

(continued)

	A. simplex s.s.												*A. pegreffii*											*A. simplex C*		
LOCATION CODE	EA	SI	BS	IC	WA	EP	JA	BE	SA	MA	WM	AZ	CM	EM	WM	IC	EA	FA	SA	MA	NZ	AZ	BR	NZ	TA	SA
Scomberesocidae																										
Scomberesox saurus	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Gadidae																										
Boreogadus saida	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Gadus morhua	--	--	•	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Micromesistius poutassou	--	--	--	•	--	--	--	--	--	--	--	--	•	--	--	•	--	--	--	--	--	--	--	--	--	--
Theragra chalcogramma	--	--	--	--	--	•	•	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Trisopterus luscus	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Lotidae																										
Molva dypterygia	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Brosme brosme	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Merluccidae																										
Merluccius capensis	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Merluccius hubbsi	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--
Merluccius merluccius	•	--	--	•	--	--	--	--	--	•	--	--	•	•	•	•	•	--	--	•	--	--	--	--	--	--
Moridae																										
Pseudophycis bachus	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	•	--	--
Ophidiidae																										
Genypterus capensis	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Lophiidae																										
Lophius piscatorius	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Lophius vomerinus	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Trachichthyidae																										
Hoplostethus atlanticus	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--
Hoplostethus mediterraneus	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Salmonidae																										
Oncorhynchus gorbuscha	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Oncorhynchus keta	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Salmo salar	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Clupeidae																										
Clupea harengus	•	--	•	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Etrumeus whiteheadi	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Engraulidae																										
Engraulis encrasicolus	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Congridae																										
Conger conger	--	--	--	•	--	--	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--

Table 8: Intermediate host so far detected for the species of A. simplex complex (Codes: EA: North east Atlantic; BS: Baltic sea; WM: West Mediterranean; IC: Iberian Atlantic coast; WA: West Atlantic; EP: North east Pacific; JA: Japan sae; SI: Sakhalin islands; BE: Bering sea; MA: Mauritanian Coast; AZ: Azores Islands; FA:Falkland Islands; NZ: New Zealand; EM: East Mediterranean sea; BR: Brazil; TA: Tasmanian sea;EP: North east Pacific; CM: Central Mediterranean Sea; SA: South Africa)

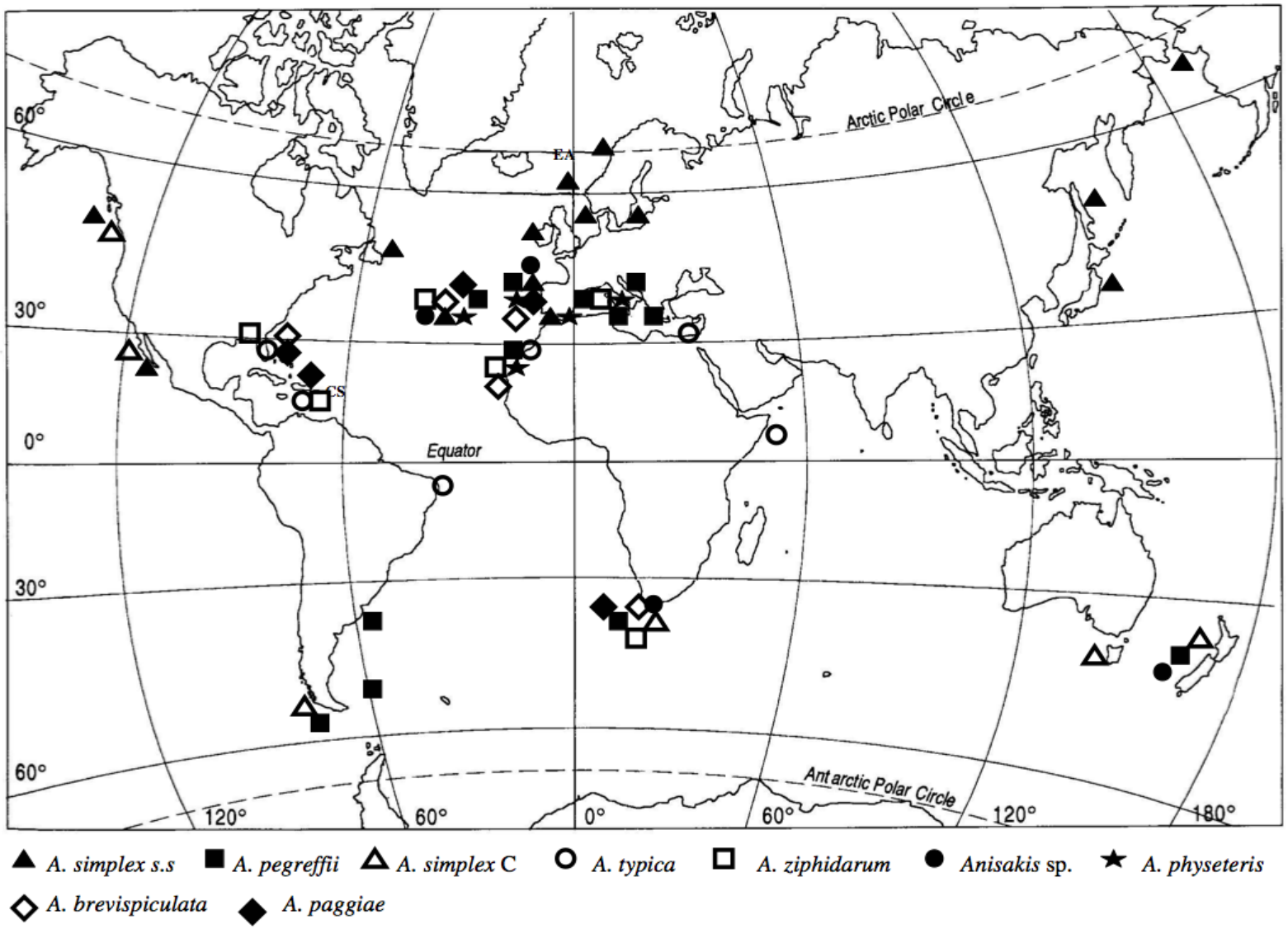

Figure 16: Geographical range of Anisakis spp genetically characterised

	A. typica							*A. ziphidarum*					*Anisakis* sp.			
LOCATION CODE	BR	AZ	SC	FL	CS	MA	EM	MA	AZ	CM	SA	FL	AZ	SA	NZ	IC
DEFINITIVE HOST SPECIES																
Cetaceans																
Ziphiidae																
Mesoplodon densirostris	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--
Mesoplodon europaeus	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--
Mesoplodon grayi	--	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--
Mesoplodon layardii	--	--	--	--	--	--	--	--	--	--	•	--	--	--	--	--
Mesoplodon mirus	--	--	--	--	--	--	--	--	--	--	--	--	--	•	•	--
Ziphius cavirostris	--	--	--	--	--	--	--	--	--	•	•	--	--	--	--	--
Delphinidae																
Globicephala macrorhynchus	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--
Sotalia fluviatilis	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Stenella attenuata	--	--	--	•	•	--	--	--	--	--	--	--	--	--	--	--
Stenella coeruleoalba	--	--	--	--	--	--	•	--	--	--	--	--	--	--	--	--
Steno bredanensis	--	--	--	--	•	--	--	--	--	--	--	--	--	--	--	--
Tursiops truncatus	--	--	--	•	•	--	--	--	--	--	--	--	--	--	--	--
INTERMEDIATE HOST SPECIES																
Fish																
Carangidae																
Trachurus picturatus	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Trachurus trachurus	--	--	--	--	--	--	•	--	--	--	--	--	--	--	•	--
Coryphaenidae																
Coryphaena hippurus	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Scombridae																
Auxis thazard	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Euthynnus affinis	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Sarda orientalis	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Scomber japonicus	--	•	--	--	--	--	--	--	•	--	--	--	--	--	--	--
Scomberomorus commerson	--	--	•	--	--	--	--	--	--	--	--	--	--	--	--	--
Thunnus thynnus	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Trichiuridae																
Aphanopus carbo	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--	--
Merluccidae																
Merluccius merluccius	--	--	--	--	--	•	•	•	--	--	--	--	--	--	--	•

Table 9: Definitive and intermediate host species so far detected for A. typical, A. ziphidarium and Anisakis sp. (Codes: IC: Iberian Atlantic coast; SC: Somali coast; MA: Mauritanian Coast; AZ: Azores Islands; NZ: New Zealand; EM: East Mediterranean sea; BR: Brazil; FL: Florida coastc; SA: South Africa; CS:Carribean sea)

3.5 Anisakis spp. included in Clade II

Three species of *Anisakis* share so far, at larval stage, the morphology known as Type II (*sensu* Berland, 1961). These species represent a complex of sibling species that could be genetically well recognised at both nuclear and mitochondrial level. They are *A. physeteris*, *A. brevispiculata* and *A. paggiae*.

- *A. physeteris* (Baylis, 1920) Its main definitive host is the sperm whale, *Physeter macrocephalus*; no adults genetically identified have been recorded in other cetacean hosts. Type II larvae of *A. physeteris* were genetically identified in very few host species and rarely occurring out of those examined during the study for *Anisakis* spp., thus suggesting that other intermediate hosts (mainly squid) are involved in the life-cycle of this parasite (Mattiucci *et al*., 2001, 2004).
- *A. brevispiculata* (Dollfus, 1966) was found genetically well distinct and reproductively isolated from the species *A. brevispiculata* (synonymised by Davey, 1971).
- *A. paggiae* (Mattiucci *et al*., 2005) was found as parasite, as adults, the pygmy sperm whale, *Kogia breviceps*, and of the dwarf sperm whale, *K. sima* (Table 10) from both Florida and the south African Atlantic coast. Scanty data are so far available to identify

the intermediate hosts in the life cycle of *A. paggiae* and *A. brevispiculata*. Very few larvae of Type II have been identified as belonging to these species in fish from Atlantic waters (Table 10), thus suggesting that other hosts, not yet detected, are involved in the life-cycles of these *Anisakis* species. Some morphological characters of diagnostic value available in male and female adult specimens were found to help in distinguishing *A. paggiae* from *A. physeteris* and *A. brevispiculata* (Mattiucci *et al*., 2005).

	A. physeteris						*A. brevispiculata*					*A. paggiae*			
LOCATION CODE	CM	MA	WM	IC	AZ	EM	SA	IC	FL	MA	AZ	SA	FL	IC	AZ
DEFINITIVE HOST SPECIES															
Cetaceans															
Physeteridae															
Physeter macrocephalus	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Kogiidae															
Kogia breviceps	--	--	--	--	--	--	•	•	•	--	--	•	•	--	--
Kogia sima	--	--	--	--	--	--	--	--	--	--	--	--	•	--	--
INTERMEDIATE HOST SPECIES															
Cephalopods															
Ommastrephidae															
Ommastrephes sagittatus	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Fish															
Trichiuridae															
Aphanopus carbo	--	--	--	--	•	--	--	--	--	--	•	--	--	--	•
Xiphiidae															
Xiphias gladius	•	--	--	•	--	--	--	--	--	--	--	--	--	--	--
Carangidae															
Trachurus trachurus	•	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Merluccidae															
Merluccius merluccius	•	•	•	•	--	•	--	--	--	•	--	--	--	•	--

Table 10: Definitive and intermediate host so far detected for A. physeteris, A. brevispiculata and A. paggie: (Codes: IC: Iberian Atlantic coast; SC: Somali coast; MA: Mauritanian Coast; AZ: Azores Islands; NZ: New Zealand; EM: East Mediterranean sea; BR: Brazil; FL: Florida coastc; SA: South Africa; CS:Carribean sea)

3.6 Reconciliation of genetics and morphology

The high genetic heterogeneity of the *Anisakis spp*. is supported by morphology of the species belonging to this genus as well, where two major clades can be delineated as follows:

- the ventriculus, at adult stage, is short, never sigmoid and broader than long in the species *A. physeteris, A. brevispiculata* and *A. paggiae* (Mattiucci *et al*., 2005), and longer than broad and often sigmoid in shape in the species included in clade 1;
- male spicules that are short, stout and of similar length can be observed in *A. physeteris*, *A. brevispiculata* and *A. paggiae* (Mattiucci *et al.,* 2005), long and often unequal (equal in *A. ziphidarum*, see Paggi *et al.,* 1998) in clade 1;
- type II larval morphology (*sensu* Berland, 1961) is characteristic of *A. physeteris, A. brevispiculata* and *A. paggiae* (Mattiucci *et al.*, 2001, 2004, 2005) (clade 2), whereas Type I morphology (*sensu* Berland, 1961) can be found in the species of the *A. simplex* complex, *A. typica*, *A. ziphidarum* and *Anisakis* sp. (clade 1).

The species of the *A. simplex complex* are so far morphologically indistinguishable at both adult and larval stage; consequently, only genetic and molecular methods can be used reliably to identify them at all the developmental stages. On the contrary, both *A. typica* and *A.*

ziphidarum are distinguishable at their male adult stage, but not so far at larval stage (Mattiucci *et al*., 2002; Paggi *et al*., 1998). A morphological key for the recognition of adult specimens of the species included in clade 2 (i.e. *A. physeteris, A. brevispiculata* and *A. paggiae*) was also given in Mattiucci *et al*., 2005.

3.7 Host parasite association and coevolution

The presence of two main clusters in the genus *Anisakis* is supported also by ecological data and specific host-parasite relationships. The sperm whales (i.e. *Physeteris catodon*, *Kogia breviceps* and *K. sima*) are hosts so far recorded for *A. physeteris, A. brevispiculata* and *A. paggiae* (Table 10) those worms included in the second clade (Figure 16). These hosts were never found parasitized by other species of *Anisakis spp*. The sole exception so far, is one individual of *P. macrocephalus* from the Mediterranean Sea, found harbouring three adult specimens of *A. pegreffii* out of the 320 specimens of *A. physeteris* genetically identified in syntopy. Oceanic dolphins in the *Delphinidae,* Arctic dolphins in the *Monodontidae,* and porpoises in the *Phocoenidae* are hosts of the species of the *A. simplex complex* and of *A. typica* (Mattiucci *et al*., 1997, 1998, 2002, 2005).

The beaked whales *Ziphius cavirostris, Mesoplodon layardii, M. mirus* and *M. grayi* are hosts of *A. ziphidarum* (Paggi *et al.*, 1998) and *Anisakis* sp., both partitioned into the second clade. Moreover, although some *Anisakis* spp., such as *A. ziphidarum* and *A. paggiae* and/or *A. brevispiculata* are found in the same sympatric areas, as the warm temperate tropical water basin, such as the Caribbean and Florida coasts of the Atlantic Ocean, however they were never identified in the same

cetacean species (Tables 9, 10, Figure 16) Phylogenetic relationships proposed elsewhere (Valentini *et al*., 2006) and reviewed here for species of genus *Anisakis* seem to align with that of their cetacean hosts (Milinkovitch, 1995; Nikaido *et al.,* 2001) (Figure 17).

The phylogeny of cetaceans proposed by Milinkovitch (1995) based on mtDNA (12S, 16S, and *cytb* partial sequences) and myoglobin sequences, and by Nikaido *et al*. (2001) based on retroposon analysis indicate a branching order of the cetacean lineages where the sperm whale and the pygmy sperm whales (*Physeteridae* and *Kogiidae*) represent basal taxa, followed by the beaked whales, and freshwater and marine dolphins as the most derived ones.

In accordance with that analysis, the branching order proposed for the *Anisakis* taxa showed that nematodes from the sperm whale and the pygmy sperm whales (*A. physeteris, A. brevispiculata* and *A. paggiae*) always occupy a basal lineage followed by those parasitizing the beaked whales (*A. ziphidarum* and *Anisakis* sp.) (Figure 17). The species from the "oceanic dolphins" (the definitive hosts of the *A. simplex* complex) consistently appear as the most derived ones, suggesting some level of parallelism or that co-evolutionary events could have accompanied the speciation of these endoparasitic nematodes and their definitive hosts (Figure 17).

Clearly, a broader dataset is needed to confirm co-speciation and/or host-switching events.

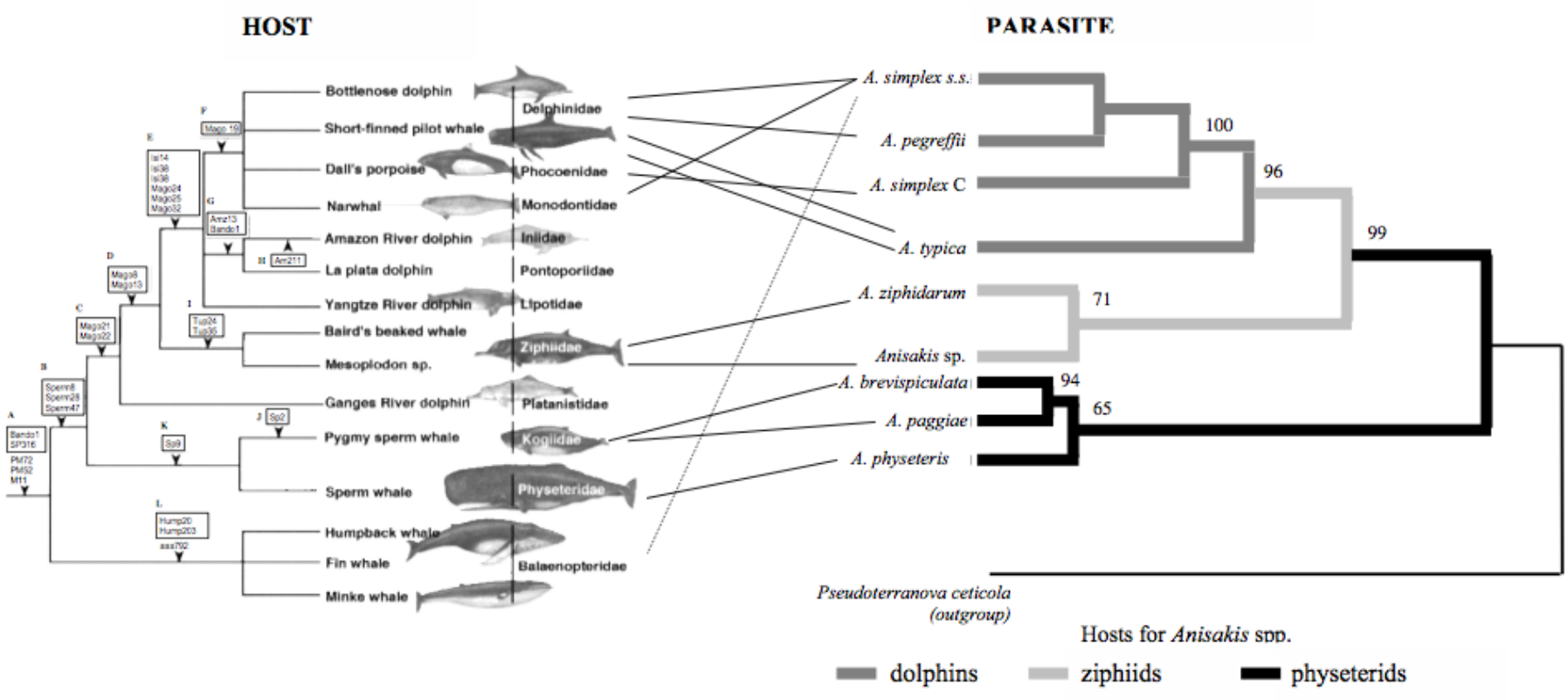

Figure 17: The pattern host and Anisakis parasite association

3.8 Life cycle

Anisakids typically utilise marine mammals or piscivorous birds as definitive hosts, with planktonic or benthic crustaceans acting as intermediate hosts and fish as main transport hosts. A wide range of fish species can carry larval anisakids including in the fish flesh thus representing the pathway for human infections. Adult *A. simplex* are found mainly in the gastrointestinal tract of cetaceans (dolphins, porpoises and baleen whales), while the adults of *Pseudoterranova spp*. and *Phocascaris spp*. live in pinnipeds (seals, sea lions and walrus), with the latter occurring only in the northern hemisphere including arctic waters. Some species of *Contracaecum* reach maturity in pinnipeds while others mature in fish-eating birds such as cormorans, pelicans and herons. However, the definitive host range of many anisakid species is still incompletely understood (Anderson, 1992). Additionally, there is some controversy whether or not any alternative transmission routes exist such as direct infection of fish by ingesting free-swimming larvae, or the transfer of larvae from crustaceans such as krill, to plankton-eating or omnivorous cetaceans, i.e. by skipping the fish transport host.

After final moulting, maturation and copulation, the female worms shed eggs within the definitive host's faeces, which embryonate and hatch in the water releasing free-swimming 3rd stage larvae (Koie et al., 1995).

The larvae are ingested by crustaceans such as decapods, copepods or amphipods in which they grow within the haemocoel. Fish and cephalopod molluscs (squids) become infected by eating planktonic or benthic crustaceans containing third stage larvae which bore through the wall of the digestive tract into the viscera and body cavity followed by host induced encapsulation (Anderson, 1992). When an infected fish is eaten by another fish, the encapsulated larvae become digested thus repeating the larval fish host cycle. This is important from an epidemiological and food safety perspective since the repeated transfer of larvae between fish within the natural food-chain may result in extensive accumulation, especially in large and older fish, sometimes harbouring hundreds or even thousands of encapsulated larvae (Smith and Wootten, 1978). However, the number of fish host cycles which individual larvae may carry through without loosing infectivity, has not yet been investigated. The definitive hosts become infected by eating fish or cephalopods containing the larvae. The generalised anisakid life cycle is shown in the Figure 18.

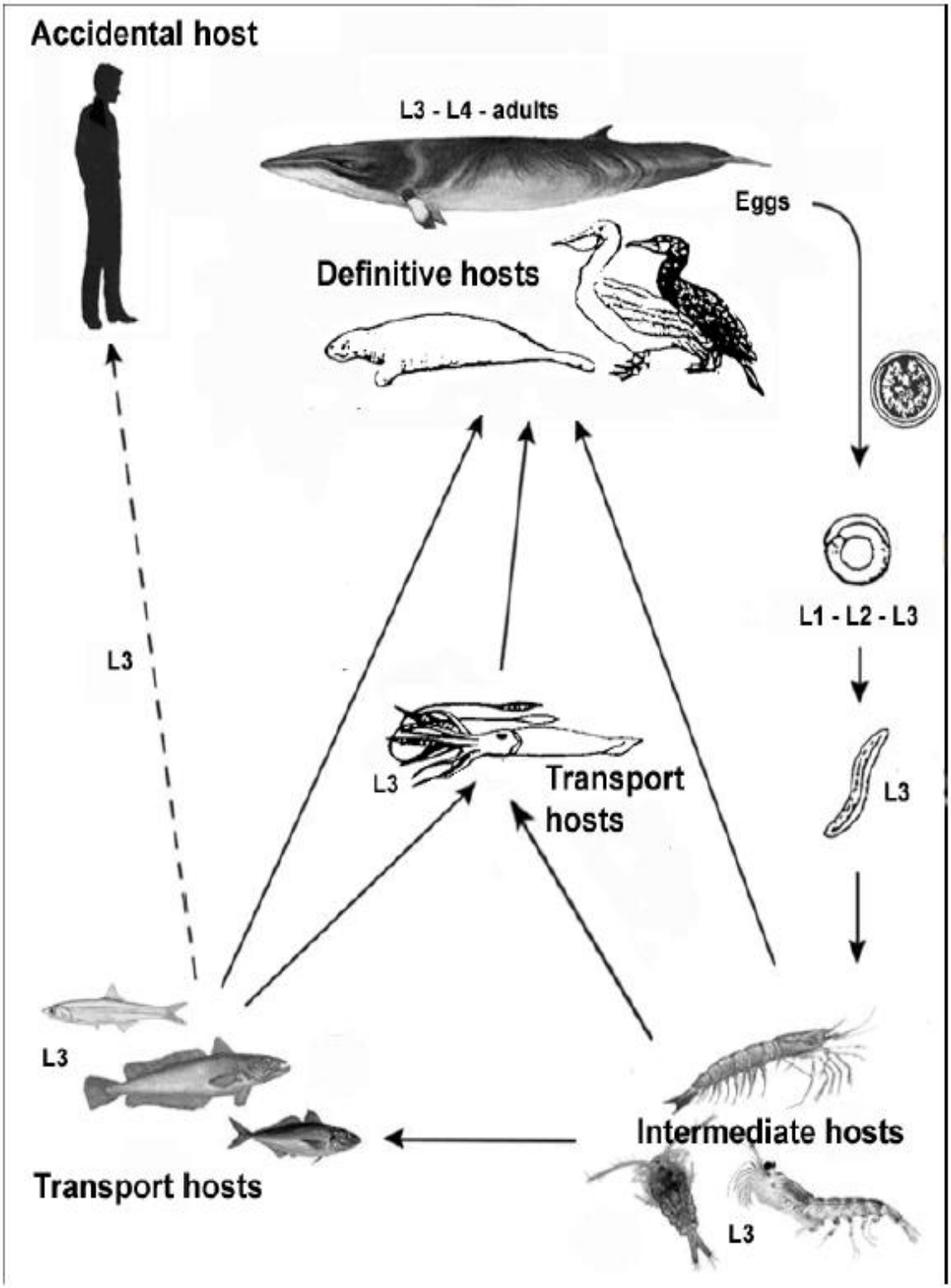

Figure 18: Life cycle of Anisakid parasites

3.9 Host range

The definitive and intermediate host ranges have not been completely described for any anisakid species. This is partly due to the uncertain species-level taxonomy and confusion over identification of different morphospecies, but also to low host specificity of the group.

3.9.1 Definitive Hosts

Adult worms in the *Anisakis simplex complex* appear to be associated principally with oceanic dolphins and porpoises, such as harbour porpoises (*Phocoena phocoena*), common dolphins (*Delphinus delphis*), bottle-nosed dolphins (*Tursiops truncatus*), and white-beaked dolphins (*Lagenorhynchus albirostrus*), although they are also frequently found in beluga whales (*Delphinapterus leucas*), and have been recorded from a wide range of other cetaceans, including minke whales (*Balaenoptera acutorostrata*), false killer whales (*Pseudorca crassidens*), and long-finned pilot whales (*Globicephala melaena*) (Greenwood and Taylor, 1978; Smith and Wootten, 1978; Pascoe, 1986; Wazura et al., 1986; Kuramochi et al., 1996; Ugland et al., 2004; Herreras et al., 2004; Mattiucci et al., 2005). They have also been recorded, although less frequently, from pinnipeds, such as harbour seals (*Phoca vitulina*), gray seals (*Halichoerus grypus*), subantarctic fur seals (*Arctocephalus*

tropicalis), and Stellar's sea lions (*Eumatopius jubatus*) (Smith and Wootten, 1978; Stroud and Dailey, 1978; Bester, 1989).

To our knowledge, adult worms in the *Pseudoterranova decipiens complex* have been found naturally only in pinnipeds. They seem to be associated most commonly with true seals (in the family *Phocidae*), particularly gray seals (*H. grypus*) and harbor seals (*P. vitulina*), although they have also been recorded from many other species, such as bearded seals (*Erignathus barbatus*), ribbon seals (*Phoca fasciata*), Weddell seals (*Leptonychotes weddelli*), and harp seals (*Phoca groenlandica*) (Stroud and Roffe, 1979; Paggi et al., 1991, 2000; Brattey and Stenson, 1993; Mattiucci et al., 1998; McClelland, 2002). Eared seals (*Otariidae*) appear to be infected less frequently, although adult worms have been reported in northern fur seals (*Callorhinus ursinus*), Stellar's sea lions (*E. jubatus*), Californian sea lions (*Zalophus californianus*), and South American sea lions (*Otario byronia*) (Keyes, 1965; Stroud and Roffe, 1979; George-Nascimento and Urrutia, 2000; McClelland, 2002).

Although there appear to be differences in host preference between different species in both the *Anisakis simplex* and *Pseudoterranova decipiens complexes*, different parasite species may be found in the same definitive host species, and sometimes in the same individual definitive

host (McClelland, 2002; Stobo et al., 2002; Mattiucci et al., 2005). The population structure of anisakid nematodes has been studied more frequently in intermediate fish hosts than in definitive hosts.

Prevalences and burdens of anisakids in definitive hosts vary widely with host species, geographic location, and season (McClelland, 2002; Herraras et al., 2004). As with all parasitic infections, adult worm abundances are positively skewed, with infection intensities of more than 20,000 worms per host being recorded (Ólafsdóttir, 2001). Intensity of infection is generally positively related to host age and size (McClelland, 2002).

3.9.2 Crustacean Intermediate Hosts

Larvae (L2 or L3) of *Anisakis* and *Pseudoterranova* have been reported from a wide range of crustaceans, including copepods, amphipods, isopods, euphausiids, and decapods, and occasionally from polychaete worms and mollusks (Smith and Wootten, 1978; McClelland, 2002). Euphausiids (krill) appear to be the most important hosts in the life cycles of Anisakis species, and copepods in the life cycles of *Pseudoterranova* species (McClelland, 2002; Smith and Snyder, 2005).

Prevalences and intensities of infection are usually low, with <1% of hosts being infected and rarely more than one worm per infected host (McClelland, 2002; Smith and Snyder, 2005).

3.9.3 Fish Intermediate Hosts

A very large number of fish and cephalopod species act as hosts for species of *Anisakis* and *Pseudoterranova*. For example, *Anisakis* larvae have been found in 200 fish species and 25 cephalopod species throughout the world (Abollo et al., 2001; Klimpel et al., 2004), while *Pseudoterranova* larvae have been reported from more than 75 fish species in the north Atlantic alone (McClelland et al., 1990; Desportes and McClelland, 2001). Primary fish hosts are planktivores, or predominantly planktivores, such as herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), blue whiting (*Micromesistius poutassou*), and juvenile plaice (*Hippoglossoides platessoides*), mackerel (*Scomber scombrus*), and cod (*Gadus morhua*), which acquire the parasite directly from crustacean invertebrate hosts (Abollo et al., 2001; McClelland and Martell, 2001). Secondary fish hosts are piscivores, such as blue shark (*Prionace glauca*), barracuda (*Sphyraena barracuda*), monkfish (*Lophius americanus*), and european conger (*Conger conger*), which usually acquire the parasite from infected planktivorous fish (Laffon-Leal et al., 2000; Abollo et al., 2001; McClelland and Martell, 2001).

Both prevalences and parasite burdens can be very large in fish hosts. They tend to increase with host age and size, and are usually greater in secondary than in primary hosts. For example, Costa et al. (2003) found

Anisakis larvae in 97% of black-scabbard fish (*Aphanopus carbo*) in waters of Portugal, with a mean intensity of 70 worms per fish, while over 80% of sculpins (*Myoxocephalus scorpius*) from Vega, Norway, were infected with *Pseudoterranova decipiens*, with a maximum intensity of 300 worms per fish (Jensen and Andersen, 1992). Prevalences and intensities of infection vary widely between fish hosts, both within and between anisakid species (e.g., Wharton et al., 1999; Abollo et al., 2001; Álvarez et al., 2002; Costa et al., 2003). These differences appear to be related more to geographic distribution, feeding habit, and growth rate of hosts than to behavioural or physiological host preferences of the parasites (Konishi and Sakurai, 2002; McClelland, 2002). *A. simplex* and *Pseudoterranova spp*. occur most often in benthic or demersal fish, while *A. pegreffi* is found more frequently in pelagic fish (Abollo et al., 2001; Anderson, 1992; Mattiucci et al., 1997; Paggi et al., 1991). These differences appear to be more related to geographic distribution and the feeding habits of hosts rather than to behavioural or physiological host preferences of the parasites.

In an individual fish, the majority of anisakid larvae are typically encapsulated as flat tight spirals, measuring 4 to 5 mm in cross section, as well as on or within visceral organs, mesenteries and peritoneum. However, a smaller number of larvae can migrate from the abdominal

cavity resulting in the presence of worms in the fish musculature, which may be noticed by the final consumer and/or food safety authorities. Most of the flesh-invading larvae seem to reside in the belly flaps, some may, however, penetrate deeply into the dorsal musculature of their fish host.

An on-going investigation of the occurrence and spatial distribution of *A. simplex* third stage larvae in three commercially important pelagic fish species from the NE Atlantic has so far revealed significant differences as to various larval infection parameters between the fish hosts (Levsen and Midthun, 2007). Preliminary data suggest that the overall *A. simplex* prevalence in blue whiting is 100%, reaching 90% in the flesh. A significant difference in larval abundance in the flesh (per individual) was found between the smallest and the larger blue whiting, i.e. 7 ± 5 and 4 ±4 larvae per fish, respectively. The liver seems to be the most commonly infected organ carrying 69%, 50% and 43% of the total *A. simplex* burden in the smallest, medium sized and larger fish, respectively. There was also a significant decrease in larval abundance in the liver with increasing fish size. In Atlantic mackerel, the overall prevalence was 97%, while 70%, 57% and 24% of the smallest (< 300 g), medium sized (300-500 g) and larger fish (> 500g) carried *A. simplex* in the flesh, respectively. The mean abundance in the flesh was 2 ± 3

larvae per fish in the smallest size group. In herring, both prevalence and abundance increased with body size. The abundance in the flesh was low, reaching a maximum of 0,5 ± 2 larvae per fish in the largest size group (150-300g). The most prominent infection site in both mackerel and herring was the pylorus area including the posterior stomach blind-sack, carrying between 57% and 81% of all larvae. The findings suggest that the *A. simplex* infection pattern in pelagic fish is related to specific life history, e.g. the feeding habits and age of the host species, and, probably, host specific immunological characteristics. Additionally, the visceral organ topography seems to be important. For example, the relatively larger liver in small blue whiting probably "entraps" most of the larvae immediately after their emergence in the visceral cavity. These findings further suggest that the larvae encapsulation site is not dependent on the availability of nutrients, e.g. in the liver, but rather on the immunological capacity of each individual host to control parasite development and migration. In younger cod (<30 cm), more than 40% of the worm burden was found in the flesh, whereas in older cod (>30 cm) less than 12% of worms occurred in the flesh. In whiting, the worm burden in the flesh was higher in older fish (>40%) and lower (3%) in younger, smaller fish. A study of the larvae of *Pseudoterranova decipiens (sensu stricto)* in a number of fish species in eastern Canadian

waters found that worms were almost totally confined to the flesh of young, demersal fish, but became increasingly prevalent in the body cavity and surrounding musculature of older, benthic fish (McClelland et al., 1990). On the other hand, the larvae of *P. bulbosa* are usually confined to the surface of the liver in plaice (*H. platessoides*) from the Barents Sea (Bristow and Berland, 1992). Smith (1984) suggested that an understanding of microhabitat preference in anisakid larvae required the sites of L3 penetration from the lumen of the alimentary tract into the body cavity to be studied in terms of distribution, arrangement, and connection of organs of different fish species at different ages. Such studies are hampered by the fact that the mechanisms of larval penetration within hosts are still unknown.

Although there are large differences in the prevalence and abundance of larval anisakids between species of demersal fish, there is no evidence of physiological host specificity. Rather, as described earlier for pelagic fish, the pattern of infection between and within fish species is driven by features such as feeding habits and habitat utilisation. Thus, cod and monkfish probably acquire much of their parasite burden via larvae from other fish hosts (Petrie, 2009). A number of authors have demonstrated how the larvae of *A. simplex* and *P. decipiens* are able to transfer between fish hosts, and in the case of *A. simplex* between multiple hosts

(Smith, 1974). Other demersal species, such as whiting, which do not feed directly on the seabed, do not acquire *Pseudoterranova*, but acquire *A. simplex* probably through feeding of invertebrate hosts such as euphausiids. As with some pelagic species such as herring, many demersal fish show an increase in prevalence and abundance of larval anisakids with age and size (Wootten and Waddell, 1977) and this may reflect an increase in the ingestion of larvae with prey and the longevity of larvae which are known to be able to survive for a minimum of 60 weeks in fish (Smith, 1984). However, the ability of individual larvae to establish and survive in some fish species such as Atlantic mackerel seems to be governed, at least in part, by the host's immune system (Levsen and Midthun, 2007). Thus, the apparent trade off between the need to cope with the infection and the ability of individual larvae to escape the host's immune response may result in a negative non-linear relationship between fish host age and parasite abundance.

A positive relationship between body size or age and larval nematode prevalence and/or abundance has been demonstrated in several commercially important fish species from different areas of the North Atlantic, including cod and herring (Banning and Becker, 1978; Bussmann and Ehrlich, 1979; Davey, 1972; Levsen and Midthun, 2007; McGladdery, 1986; Platt, 1975; Smith, 1984; Smith and Wootten, 1978;

Valero and Martín-Sánchez, 2000). However, additional information on the possible relationships between fish body size and larval abundance in the flesh (or other edible parts of fish such as the liver and roe of cod) appears to be scarce. Except for the above mentioned study on Atlantic mackerel which revealed higher *A. simplex* abundances in the flesh of smaller mackerel (Levsen and Lunestad, 2010; Levsen and Midthun, 2007) recently was found that in Norwegian spring spawning (NSS) herring, fish size and larval abundance in the flesh were positively correlated only in the largest size group (> 400g). Thus, even if data on the overall larval prevalence or abundance in fish species from given areas was available, it is not possible to reliably predict the presence or absence, or level of infection of *A. simplex* larvae in the flesh of the specific wild fish host species.

Indeed, the findings show that samples of NSS herring with high larval abundance in or on the organs of the visceral cavity, may be free of larvae in the fish flesh, or vice versa (Levsen and Lunestad, 2010). In demersal fish there appears to be differences in *A. simplex* distribution between host species.

In whiting for example, Wootten and Waddell (1977) found that significantly more larvae were present in the muscle in some samples, whereas in cod and other demersal species relatively few parasites are

present in the flesh. *P. decipiens* appears to show trophism for the musculature in that the largest proportion of the total worm burden is found in this site in many species, especially of demersal fish (McClelland, 2002a). Within the musculature of cod and monkfish, a higher proportion is found in the hypaxial muscles (belly flaps) than in the epaxial muscles (fillets) (Petrie, 2009; Wootten and Waddell, 1977; Young, 1972). The reasons for the difference in distribution are not known.

3.9.4 Accidental Hosts

Accidental hosts become infected by eating intermediate hosts (typically fish or cephalopods) that contain larval anisakids. The anisakid larvae do not complete development in the accidental host, but may penetrate the alimentary tract and invade associated organs, causing a range of pathological effects. Humans are, of course, the accidental hosts of most interest to us, but invasive anisakid larvae have also been reported from other fish-eating mammals, such as sea otters (*Enhydra lutris*) and brown bear (*Ursus arctos*) (Rausch, 1953; Davey, 1971; Jefferies et al., 1990), and experimental infections have been established in a wide range of laboratory mammals, including rats, mice, guinea pigs, rabbits, dogs, and cats (Smith, 1999). Fish-eating birds act as natural definitive hosts for a range of anisakid species in the genus *Contracaecum*, but a number of

species, including fulmars (Fulmarus glacialis), have also been reported as accidental hosts of *Anisakis* and *Pseudoterranova* (Riley, 1972; Smith, 1999).

3.9.5 Distribution of parasites in fish body, pre and post mortem

Some studies have found that larval nematodes migrate from the visceral organs to the muscle after the death of the fish host, and that this migration may be enhanced by the cold storage or processing of ungutted fish (Van Thiel et al., 1960; Smith and Wootten, 1975; Hauck, 1977; Smith, 1984; Abollo et al., 2001). Van Thiel (Van Thiel, 1962) suggested that this occurred in herring and subsequently other authors (Smith and Wootten, 1975) reported a significant increase in the proportion of *A. simplex* larvae in the muscle of herring after fish were kept in ice for up to 48 hours after capture. These observations suggest that encapsulation of larvae from the viscera is followed by migration into the muscle. Hauck (Hauck, 1977) also found a significant increase in numbers of *A. simplex* larvae in the muscle of cold smoked Pacific herring with time after capture. It was hypothesized that this apparent migration was due to post-mortem changes in the decomposing viscera and/or the exposure of larvae to the cold smoking temperatures and brining salinities.

Other studies, however, have not been able to demonstrate postmortem migration of larvae (Cattan and Carvajal, 1984; Roepstorff et al., 1993). Differences between fish species may also be important. Smith (1984) reported that storage of ungutted herring (*C. harengus*) and mackerel (*S. scombrus*) on ice (3–5 °C) resulted in postmortem migration of *Anisakis simplex (sensu lato)* larvae into the flesh, but no significant migration was seen in blue whiting (*Micromesistius poutassou*), whiting (*Merlangius merlangus*), and walleye pollock (*T. chalcogramma*). On the basis of these results, Smith (1984) suggested that larval migration was related to the location of lipid deposits, with mackerel and herring being fatty species with higher lipid storage in the flesh. Roepstorff et al. (1993), however, found no migration of *Anisakis* larvae into the flesh of herring when fish were maintained on ice, in chilled sea water, or in 10 ºC sea water and examined with pepsin-HCl digestion; there were no increases in larval numbers in the flesh even though after 5 days the viscera had disintegrated completely and many larvae had migrated out of the fish via anal or gill openings. It is probable that the postmortem migration behavior of anisakids is affected by a complex of parasite, host, and external environmental variables.

The reasons for the discrepancy between different studies are unknown. Karl (Karl et al., 2002) found no evidence of post-mortem migration of

A. simplex larvae into the muscle of haddock, saithe and ocean perch after capture. A similar lack of any evidence for post-mortem migration of *A. simplex* larvae was reported in Chilean hake (Cattan and Carvajal, 1984).

In pelagic fish, for example North Sea and NSS herring, the largest proportion of flesh residing *A. simplex* larvae were found in the belly flaps and no significant difference between the left and right flesh side was found (Karl et al., 2002; Levsen and Lunestad, 2010).

It is therefore not clear when, under what conditions and in which fish specie post-mortem migration of *A. simplex* larvae occurs.

3.10 Detection methodologies in fishery products

Fish can be examined for the presence of parasites by a variety of methods including visual inspection, slicing, candling, pressing, digestion, by Polymerase Chain Reaction (PCR) and by multilocus enzyme electrophoretic analyses (MAE). (Lopez and Pardo, 2010; Mossali et al., 2009). Visual inspection of fillets will reveal worms embedded near the surface which can be removed easily with a knife during processing. Worms embedded deep in the flesh however are not immediately obvious, but some can be detected by candling, that is shining a bright light through the fillet. The simplest kind of candling table is a box about 50 cm square with a ground glass or perspex top about 6 mm thick. The inside of the box is white, and is lit by two fluorescent tubes giving a white uncoloured, light. To use the box, the fillet is laid down on the illuminated top; worms show up as dark shadows in the flesh, and can be removed with forceps or a knife. Light from above the box should be restricted. The box is unsuitable in bright sunlight and although an experienced operator can handle up to 300 fillets an hour, user fatigue can result in reduced detection rates (Wootten and Cann, 2001). In commercial practice, visual inspection and candling is effective at detecting *Pseudoterranova* in thin skinless fillets of white fish, particularly cod; however the method does not work well

on thick fillets with the skin on. Candling is less effective in detecting *A. simplex*. Time can be saved by candling a sample of fillets from a batch of suspect fish to determine the level of infestation which can be used to establish whether the whole batch needs to be candled, and whether the batch is more suitable for other purposes.

A comparison of the efficacy of detection methods showed that visual inspection and candling of fillets detected only approximately 50% of the numbers of parasites detected by combining both candling and destructive slicing. Candling was demonstrated to be effective in fillets of up to 2.5 cm in thickness, after which effective detection could not be achieved. In contrast, when applying successively more accurate detection methods i.e. candling, artificial digestion and UV illumination, Levsen (Levsen et al., 2005) showed that only 7 to 10% of the *A. simplex* larvae in the fillets of herring, mackerel and blue whiting were detected by candling. However, visual inspection and candling appear to be sufficiently effective in detecting worms in the belly flaps recovering at least 75% of the larvae present. In herring and mackerel it was found that there was no significant difference in the numbers of *A. simplex* recovered by digestion and pressing (Petrie, 2009). The pressing method is widely used for systematic detection of nematode larvae in the flesh of fish in specific surveys.

This method utilises the fluorescence of frozen *A. simplex* larvae (Pippy, 1970) and is based on visual inspection of flattened/pressed and deep-frozen fish fillets or viscera under UV-light (Karl and Leinemann, 1993). Prior to the pressing process, each fish is gutted, manually filleted and deskinned before placing the visceral organs and both left and right flesh side (fillets incl. belly flaps) into clear plastic bags. The fillets are then pressed to 1–2 mm thickness in a hydraulic press. The bags containing the flattened fillets or viscera are then deep-frozen (≤ -18 °C) for at least 12 h prior to visual inspection under a 366 nm UV-light source. Any *A. simplex* larvae present appear as more or less brightly fluorescent spots in the samples. Additionally, the method allows the approximate determination of the larval infection site in the fillets, i.e. whether they are situated in the dorsal (upper) or ventral (lower) portion of the fish flesh (Levsen and Lunestad, 2010). The digestion method involves the use of a pepsin/hydrochloric acid solution to free anisakid larvae from muscle or other tissues (Jackson et al., 1981; Smith and Wootten, 1975). Pepsin is added to a 0.85 NaCl solution to a concentration of 10 mg/l. Pieces of fish for examination are then placed in a suitable glass container and pepsin solution added. The pH of the solution is then adjusted to pH 2 with concentrated hydrochloric acid and the solution incubated overnight at 38 ºC. The solution is then sieved (1.5 x 1 mm

mesh) and the contents of the sieve examined for larval nematodes. Live parasites survive the process unharmed and are easily detected. Dead worms, e.g. from frozen material, are also recovered using this method. The method recovers virtually all anisakid nematodes although it is time consuming and thus used for specific surveys rather than mass screening. DNA differential diagnosis is considered very useful for the definitive identification of clinically obtained worms. Several methods for identification of anisakid species such as PCR-based restriction fragment length polymorphism (PCR–RFLP) and sequencing of rRNA gene or mitochondrial DNA have been developed. By using multilocus enzyme electrophoresis (Nascetti et al., 1986; Mattiucci et al., 1997) and most recently a new set of rDNA markers based on the polymorphisms obtained by RFLP (D'Amelio et al., 2000), the species *Anisakis simplex* (commonly known as herringworm or whaleworm) has revealed as a complex of at least three sibling species (*A. pegreffii, A. simplex sensu stricto* and *A. simplex C*). They are characterised by marked differences in their genetic structure as well as in ecological traits, such as geographic distributions and host preferences (Mattiucci et al., 1997). Data may be obtained by performing PCR ofintergenic transcribed spacer (ITS), and mitochondrial cytochrome c-oxidase subunit 2 (mtDNA cox 2).

A real-time polymerase chain reaction (PCR) method has been developed for the identification of *A. simplex* in seafood products (Lopez and Pardo, 2010) combined with an optimized DNA extraction procedure. The method is highly specific and sensitive with a detection limit of 40 ppm parasite in 25g of sample, and may be used for fresh and processed material. This method is likely to be most suitable for testing of batches of fish products in specific surveys rather than screening of industrial fish.

3.11 Allergy caused by parasits in fishery products

The human host defence system is comprised of numerous cellular and protein components that interact in a highly complex manner in order to preserve self and to neutralize or destroy non-self.

However, this system sometimes induces an overreaction by a specific defence mechanism responding inappropriately to environmental encounters. Allergy is a clear example of this over-reaction that occurs in certain individuals responding inappropriately to non-self molecules termed allergens. Allergens are antigens that provoke a specific allergic reaction, and antigens are generally defined as moities that provoke immune responses.

Allergy can occur as Type I immune hypersensitivity, also known as immediate hypersensitivity, and is mediated by antibody of the immunoglobulin E type (IgE). Allergic reactions of this kind can range from mild to severe, of which the most serious (anaphylaxis) can be rapidly fatal if not treated promptly. Although IgE antibody mediates Type I allergic reactions, people having IgE antibody to a particular protein will not necessarily suffer an allergic response to it. Allergens triggering the Type I hypersensitivity are generally proteins. Another medically important immunological hypersensitivity reaction is a Type IV hypersensitivity, also known as delayed or contact sensitivity which

is not antibody mediated but is caused by activation of T-lymphocytes at affected sites. Allergens triggering the Type IV hypersensitivity are generally low molecular weight haptenes.

Parasites, especially helminths, produce in infected hosts a Th2 biased immunologic response with production of specific IgE against the parasite. This has to be taken into account when analysing studies on IgE production in relationship with parasite contact. This is especially important, as several studies claim the allergenicity of parasites by finding specific IgE production measured in serum or evidenced by a positive skin prick tests. The production of specific IgE is not to be mistaken as allergy.

A. simplex are, so far, the only fishery-product associated parasite causing clinical allergic responses. The principal clinical allergic responses due to *A. simplex* are:

- Gastro-allergic anisakiasis, in which allergic symptoms are additional symptoms in an acute gastric parasitism after eating raw or undercooked fishery products containing live larvae;
- Allergy to *A. simplex*, resulting from contamination of fishery products with allergens with no necessity for live parasite to elicit the allergic reaction.

Additional allergic responses occur and are:

• Chronic urticaria associated with *A. simplex* sensitization, whose mechanism is not yet clarified;

• Eosinophilic gastroenteritis, rheumatological and dermatological symptoms as well as occupational rhino-conjunctivitis and asthma.

The allergy to *A. simplex* and gastro-allergic anisakiasis is commonly recognised in some regions in Spain (Audicana and Kennedy, 2008); however these are rarely or have never been reported in other parts of Europe. It is not known if this is dues to lack awareness and rare application of diagnostic tests, or to true differences in the incidence of the disease. The remaining allergic diseases associated with *A. simplex* sensitization are very rare.

No other fishery product associated parasites have been clearly implicated with allergic reactions, although studies claim allergenicity of parasite extracts based on positive skin prick tests or specific serum IgE, these are not necessarily associated with clinical allergy.

3.12 Sensitization and exposure to A. simplex

There is general agreement that, in most instances, an infection is required to initiate allergic sensitivity to Anisakids. This hypothesis is difficult to establish with certainty, since cases of anisakiasis may go unrecognised or are not medically investigated. The possibility that sensitisation can occur via exposure to antigen alone, in the absence of a live infection, cannot, however, be excluded; there is experimental evidence indicating that nematode materials can generate allergic type (Th2) immune responses without infection (Hewitson et al., 2009), although only a few cases of such autoallergenicity have been reported (Audicana et al., 2002; Cuende et al., 1998; Vidaček, 2009).

Once sensitisation has occurred, response to nematode allergens can be highly aggressive and generate severe allergic disease (Audicana and Kennedy, 2008). Through direct clinical observation, some authors have shown that an infection can provoke a concurrent *A. simplex* allergic episode in a sensitised individual, and claim this is the principal mechanism for disease (Daschner et al., 1998; Daschner et al., 2000b). However, others consider that allergic episodes can not only be elicited by infection as described above, but also by exposure to allergen remaining in food fish treated so that no viable larvae remain (Audicana et al., 1997; Audicana et al., 2002; Vidaček, 2009). The relative

epidemiological impact for each route of provoking an allergic episode is unknown, however there is general agreement that consumption of fishery products containing viable *A. simplex* larvae presents a greater risk for allergy and initiation of the *A. simplex*-allergic state than consumption of fishery products containing non-viable parasites. Consequently, prevention of sensitisation to *A. simplex* should focus on the prevention of infection.

3.13 Urticaria and anaphylaxis due to A. simplex allergy from food

Acute urticaria and angioedema affects 20% of the population at some time in their lives, particularly in young adults (Kaplan, 1992), and although usually self-limiting and not life threatening, the condition is nevertheless unpleasant as a result of the intense itching, inability to sleep and even disfigurement when angioedema is present. Angioedema is associated with urticaria in 30% of cases, and is potentially life threatening because of the risk of oedema of the glottis. Anaphylaxis is a rapid onset and dangerous syndrome characterized by urticaria, angioedema, severe respiratory and gastrointestinal symptoms, collapse and shock. The first signs of an allergic reaction usually appear within 60–120 minutes after ingestion of infected fish but can take up to six hours (Audicana et al., 2002).

In the Basque Country of northern Spain, *A. simplex* is now considered to be the main factor associated with urticaria and angioedema in adults following fish and shellfish consumption and is responsible for 8% of acute urticaria and angioedema cases (Pozo, 1998) and for 27 % of anaphylactic episodes (Audicana, 2002). This constitutes a similar or even higher prevalence compared with other sources of ingested allergens in adult population (fruits, nuts, shellfish and fish flesh). Although the majority of cases were not life threatening, more than 50%

of the patients required emergency treatment and there was a single near fatal case of respiratory arrest (Audicana, 2000).

As the awareness about anisakiasis and *Anisakis* as a food allergen increased (SCVPH, 1998), sporadic reports from France (Petithory, 2007), Italy (Foti et al., 2002), Portugal (Falcao et al., 2008), and in regions of Spain other than Basque Country (Añíbarro, 2007; Moreno-Ancillo et al., 1997), demonstrated that this allergy occurred elsewhere in Europe and was not confined to the Basque Country and Japan, where more IgE sensitization to *A. simplex* than to fish proteins has been diagnosed (Kimura et al., 1999). However, Italian and Portuguese cases were similar to those of the Basque Country because they involve true allergy following exposure to cooked fish, whereas reports from other parts of Spain (Madrid and its surrounding provinces) describe gastroallergic cases caused by consumption of raw or undercooked fish (Daschner et al., 2000b). General risk factors for serious or fatal food reactions for allergens in foods include: young age, multi-sensitivity, presence of uncontrolled asthma, previous serious food reactions, and eating out of the home. Disease severity will be exacerbated by the lack of immediate availability of epinephrine.

Atopic allergic diseases are familiar and have a genetic basis. In contrast, patients allergic to *A. simplex* have a lack of previous atopic dermatitis,

asthma, or rhinitis and are generally aged 40–50 years (Audicana et al., 2002). In an Italian study, atopic subjects had a lower risk of *Anisakis* allergy than non-atopic subjects and *A. simplex* allergy was associated with consumption of uncooked seafood (especially anchovies and squid) and an increased risk with age (Foti et al., 2006). In a study of food induced anaphylaxis, logistic regression analysis revealed that age and specific IgE level were the unique risk factors associated with *A. simplex* allergy (Audicana, 2002). In many cases, the patients did not suspect allergy to *A. simplex* or to fishery products because of previous tolerance but attributed allergic disease to analgesic and/or antibiotic treatment. Other characteristics from the clinical histories of patients include the patients and their general practitioners linked the symptoms with medications (although such causes were later rejected and the episodes occurred at night (Audicana et al., 2002). A genetic predisposition to *A. simplex* allergy and the presence of HLA class II alleles has been shown (Sanchez-Velasco et al., 2000). This association, together with the different habits of fish consumption, could explain some differences in allergy rates because the HLA inheritance is a good marker of genetic differences.

3.14 Gastro-allergic anisakiasis (GAA)

As described above, urticaria was initially described as a systemic symptom accompanying human gastric anisakiasis in Japan and was estimated to be present in about 10 % of acute parasitic episodes (Asaishi et al., 1980). Reports from Spain in the late 1990's also described urticaria as the most common allergic reaction, with frequent abdominal symptoms of gastric anisakiasis. This clinical presentation was designated as gastro-allergic anisakiasis (Daschner et al., 1998; Daschner et al., 2000a).

Gastro-allergic anisakiasis is defined as an acute IgE-mediated generalized reaction (urticariaangioedema- anaphylaxis) after the intake of *A. simplex* infected fish, where the live larva induces the symptoms during penetration of the gastric mucosa (Daschner et al., 2000a). Allergic symptoms are typically accompanied by gastric/abdominal symptoms, but these often remain mild or even absent (Alonso-Gomez et al., 2004; Daschner et al., 1998).

As soon as acute allergic symptoms were recognised as associated with *A. simplex* a series of publications appeared describing a high frequency of urticaria or anaphylaxis in previously "idiopathic" reactions. These reports came initially from different regions of Spain, but progressively reports of cases of allergy related to *A. simplex* from other,

predominantly European, countries. In parallel, a series of reports of *A. simplex* gastric or intestinal parasitism without allergic symptoms were also published. It is difficult to evaluate whether the high number of reported cases is due to a real increase of *A. simplex* and associated disorders or a heightened awareness due to the reports in the scientific literature.

The rise in GAA can, at least in part be accounted for by awareness and knowledge of allergic disease presentation. This is due to the peculiar clinical characteristics of GAA, which is difficult to suspect if the attending physician is not aware of this condition. The main clue for a suspicion of an allergic reaction being possibly caused by *A. simplex* is a careful history in the emergency room. The patient's history has to include the question of intake of raw or undercooked fish in the 24 hour period before the onset of urticaria, angioedema or anaphylaxis. True food allergy with an immediate hypersensitivity reaction is usually straightforward to diagnose since the allergic symptoms begin almost immediately after the suspected food agent has been consumed. However, GAA can begin up to 24 hours after the intake of the parasitized fish and this characteristic seems to be the reason for a high proportion of under diagnosed anisakiasis prior to more widespread recognition of GAA. This entity has been described as a host response to

an acute parasitism and should clearly be differentiated from the IgE-mediated acute allergy caused by non-viable *A. simplex* material, which would thus reflect a true food-allergy (Daschner et al., 2002).

GAA is clinically composed by two types of reactions: the first being the local reaction of the gastric mucosa followed by the generalized allergic reaction. The gastric reaction reflects the previously described gastric parasitism where the third stage *A. simplex* larva penetrates the gastric mucosa by action of their enzymatic peptidases and produces the epigastric pain, nausea and vomiting. Early Japanese reports proposed this local reaction to be an IgE-mediated allergic reaction (Kasuya and Koga, 1992). GAA has been described as a simultaneous primary and secondary immunologic reaction as all immunoglobulin isotypes, including specific IgM are present from the first day of parasitism. With the exception of IgM, all other antibody isotypes (IgE, IgA, IgG, IgG4) show significant elevation after one month, with production of IgE antibodies against additional antigens as demonstrated by serial immunoblotting studies (Daschner et al., 2002). This immunologic polyclonal stimulation is produced by an active live larva, even if it is removed after some hours either by gastroscopic extraction or spontaneously as occurs in the majority of cases. Serial determination of immunoglobulins has gained importance in the diagnosis of allergic

reactions for distinguishing between a food-allergy like reaction and that induced by a viable larva (Daschner et al., 1999). It has been proposed that the generalized allergic reaction reflects a similar local reaction which is a protective host reaction against invasion in order to prevent a further penetration of the larva and produces the different forms of chronic reaction (Daschner et al., 2005). There is a clear negative association between cases of GAA and the chronic forms of intestinal anisakiasis, where no allergic reactions are described (Asaishi et al., 1980; González Quijada et al., 2005).

The first allergic symptoms can appear as late as 24 hours or longer after the intake of the viable parasite in a meal containing fishery-products, because it is not the direct contact of the *A. simplex* surface allergens leading to the allergic reaction but the excretory-secretory (E/S) products released by the larva during gastric penetration. Major *A. simplex* allergens like Ani s 1 and Ani s 7 have been identified in the excretory compartment which bears enzymatic activity allowing penetration of mucosal tissue (Anadon et al., 2009; Gomez-Aguado et al., 2003). Oral challenge tests have been performed with non-viable larvae in patients with previous GAA and patients have not developed any reaction (Daschner et al., 1999; Sastre et al., 2000). Even proteins that remain stable after heat treatment or pepsin digestion have not produced any

allergic reaction in such tests, when excretory-secretory proteins were administered orally, supporting the hypothesis that only viable larvae are capable of inducing allergic reactions in these patients (Baeza et al., 2004). Systemic allergic reactions vary between urticaria/ angioedema and anaphylactic shock, as in other IgE-mediated acute allergic reactions. Some patients with predominantly digestive symptoms display an erythematous reaction in the head, neck and superior thoracic region, sometimes without pruritus. This reaction is not necessarily IgE-mediated and could be due to other non-specific mechanisms, e.g. a vasomotor reaction of the nervous system. The latency of symptoms between fish intake and the allergic reaction can range between minutes to more than 24 hours. Abdominal symptoms appear over the same time period, but with a tendency to a shorter time interval. Some patients have diarrhoea, which could be due to a nervous reaction by the digestive tract or as a distant reaction in the course of anaphylaxis. It has been shown, that GAA can also be diagnosed in patients without abdominal symptoms (Alonso-Gomez et al., 2004).

GAA is an acute reaction with a mainly rapid resolution. Allergic symptoms rarely remain for longer than 24 hours, whereas the remission of abdominal symptoms depends on the extent of the local gastric tissue damage. Risk factor for GAA, have a clear association with the

consumption of raw and minimally processed fish and fishery products (Pozio, 2008; van Thiel and van Houten, 1966). This is also true for allergic reactions as well as for non-allergic gastric or intestinal anisakiasis. For GAA there is an association between increasing risk and age, this is probably a confounding factor associated with fish and fishery product eating habits: previous episodes of atopy are not a risk factor (Falcao et al., 2008). However, patients with allergic symptoms due to *A. simplex* sensitization, displaying no difference in the overall atopy status, but have a higher frequency of allergy against house dust mites and a diminished frequency of allergy against other aeroallergens, mainly pollen (Daschner, 2008). In one study an association of hypersensitivity to *A. simplex* with HLA class II DRB1*1502-DQB1*0601 haplotype has been described and it has been suggested that, the DRB1*1502 being the uncommon Thai allele in Spain region, the pressure of this eating habit selected for an association with this haplotype in two areas as far apart as Japan and Spain (Sanchez-Velasco et al., 2000).

3.15 A. simplex sensitization associated chronic urticaria (CU)

Chronic urticaria is a disabling disease with an important impact on the quality of life. Some studies discuss a possible association between chronic urticaria or pruritus and sensitization to *A. simplex* (Daschner et al., 2005; Gracia-Bara et al., 2001). The possible pathological mechanism has not been studied, but it has been postulated that *A. simplex* antigens following consumption of fish could be responsible for the perpetuation of hives and has opened a debate of a diet without *A. simplex* antigens (Audicana and Kennedy, 2008; Daschner et al., 2005), i.e. any fish, in at least a subgroup of patients. In patients with CU in endemic areas, the frequency of *A. simplex* sensitization is much higher than in the general population. This fact led to investigations into a possible causal relationship between *A. simplex* sensitization and CU. With the help of the measurement of specific IgG4 against A. simplex, CU patients were assigned to two groups with a different outcome after a two-month diet without any fish. Patients with detectable specific IgG4 were more prone to a remission of urticarial symptoms than those without detectable specific IgG4 or without sensitization to *A. simplex* (Daschner et al., 2005). However it has been reported that patients with GAA mount an immunological response by all immunoglobulin isotypes, including IgG4. CU patients with high specific IgG4 levels are

likewisesupposed to have suffered parasitism by this nematode. Thus, it has been proposed that, contrary to patients with GAA, some patients react with a chronic urticarial reaction after contact with A. simplex. If this is correct, a diet excluding non-viable *A. simplex* larvae after the diagnosis of GAA does not lead to new allergic episodes, even after several years, and is not contradicted by the fact that some patients begin a chronic urticarial reaction after an episode of parasitic contact.

In this disease, it is possible that live larvae of *A. simplex* as well as non-viable larvae or related antigens are involved in the pathogenesis of a chronic urticarial reaction although the mechanism for disease has yet to be elucidated.

3.16 Diagnosis

3.16.1 Diagnosis of Anisakis allergy

The diagnosis of allergy to *A. simplex* is based on the following criteria:

- a compatible history, such as urticaria, angioedema or anaphylaxis following fishery-product consumption,
- positive skinprick test,
- specific-IgE against *A. simplex* (radioimmunoassay) with values >0.7 kU/l,
- a lack of reaction to proteins from the host fishery products and/or other possible cross-reacting antigens such as crustaceans, snails, cockroach, dust mites and insects.

Other foods and allergens should also be screened in such cases (Audicana and Kennedy, 2008). IgE immunoblotting with sera from allergic patients appears to be specific to *A. simplex* because cross-reactivity was not observed in sera from African patients with other parasitosis (Del Pozo et al., 1996). However, when sera from other control populations (with no seafood consumption and with multiple parasitic diseases) were tested, the specific IgE against *A. simplex* antigens were detected (Moneo et al., 2000). Interestingly, the *A. simplex* antigens recognized by the monoclonal antibody (mAb) UA3 were also

detected in all patients with unambiguous *A. simplex* allergy (Lorenzo et al., 1999a). It is hypothesised that the antigen recognized by this mAb could provide a tool for specific diagnosis of *Anisakis* allergy.

Currently, the best confirmation of food allergy is a double-blind challenge-test against a placebo (Audicana et al., 2002; Audicana and Kennedy, 2008), although this may be precluded on ethical considerations. Some authors report surprising results since oral *A. simplex* challenge tests were negative whereas conjunctival tests were positive. These studies could be biased by patients' recruitment (gastro-allergic versus. allergic patients) and the antigen challenge since lyophilized or extracted larvae may not be representative of the natural allergen exposure (Sastre et al., 2000).

Somatic and E/S recombinant allergen proteins production can help in the diagnostic techniques (prick tests and in vitro tests) and even in the development of allergen vaccines (immunotherapy) in the future.

It is common to find IgE against *A. simplex* in subjects who do not react allergically to this parasite. The identification of specific IgE against the parasite is not a reliable indicator of allergy, and specific IgE has been detected in 25% of otherwise healthy controls (Del Pozo et al., 1997). Possible explanations for the existence of IgE against *A. simplex* without clinical manifestations are: crossreactivity with other nematodes because

of presence of a panallergen, such as tropomyosin; crossreactivity with carbohydrates or phosphorylcholine and cross-reactivity with glycans present in glycoproteins of other nematodes; the presence of biotinyl-enzymes that can stimulate the production of IgE in some patients; or a prior episode of anisakiasis which has not been diagnosed (Audicana and Kennedy, 2008).

3.16.2 Diagnosis of gastro-allergic anisakiasis as an acute parasitism

Initial experience with gastroscopic evaluation of GAA was useful for diagnosis and extraction of the nematode, however subsequent clinical experience later showed that gastroscopic intervention is only rarely required because of spontaneous resolution (Alonso-Gomez et al., 2004). Clinical history is fundamental: the intake of raw or undercooked fishery products in the 24 hours previous to the onset of an acute allergic reaction is the main criterion leading to initial clinical suspicion. This probability is heightened if abdominal symptoms like epigastric pain, nausea or vomiting precede or accompany the allergic reaction. A correct clinical history and further allergic evaluation can provide a strong indication of the initial diagnosis. The skin prick test (SPT) with an extract of *A. simplex* is highly sensitive. Specificity depends on the prevalence of sensitization in the population. Cross-reactivity with other nematodes or arthropods has been described to give false positive results.

Although the presence of a positive skin prick test or a specific serum-IgE against *A. simplex* is attributed to a previous acute *A. simplex* infection, the reactivity does not necessarily appear in the clinical context of the actual allergic reaction (Daschner et al., 2005). Thus other diagnostic methods are being studied. A serial follow up of specific (and total) IgE is useful, as the polyclonal stimulation induced by the viable larva produces elevated levels after one month (with respect to the levels at day 0) with decreasing values after 6 months (Daschner et al., 1999).

Other diagnostic methods are still experimental and not commercially available. The most promising is major allergen Ani s 7 which is a component of the excretory-secretory fraction of *A. simplex* and the only allergen recognized by 100% of infected patients (Anadon et al., 2009). No antibody against a single allergen has been identified, that discriminates within the group of parasitized patients with and without allergic symptoms. Even patients with a history of intestinal anisakiasis show specific IgE against *A. simplex* or against single allergens like Ani s 1 (Caballero and Moneo, 2002).

3.16.3 Diagnosis of Anisakis sensitization associated chronic urticaria

In patients with chronic urticaria who are sensitized against *A. simplex* (detectable specific IgE in serum or by skin prick test) and display detectable serum specific IgG4, the chronic urticaria is likely to be

induced by parasite proteins. The response to a temporary diet excluding fishery products can simultaneously be of diagnostic and therapeutic value. Otherwise, most patients that respond with clinical improvement to this temporary diet (Daschner et al., 2005) tolerate fishery products afterwards. If no clinical response is observed after the temporary diet the interpretation will mainly be that chronic urticaria and *A. simplex* sensitization are independent features.

With respect to other serum isotypes, specific IgG4 is produced in all patients with GAA (Daschner et al., 2002). Its presence in patients with chronic urticaria sensitized against *A. simplex* has been attributed to a previous contact with viable *A. simplex* but it is presently not clear if its absence in *A. simplex* associated chronic urticaria is due to either a longer time interval from the last parasitic episode, a different immunologic response after parasitism, or a marker of an allergic reaction to nonviable *A. simplex* proteins (Daschner et al., 2005).

Given the complexity of the parasites risk assessment, the aim of this section was the epidemiological evaluation of the presence of *Anisakis* in different fish species of commercial interest and the subsequent application of a monitoring tool for the evaluation of parasite infection in fish lots assessing parasites impact on marketed fish.

CHAPTER 4. GENETIC IDENTIFICATION AND DISTRIBUTION OF THE LARVAL PARASITES *ANISAKIS PEGREFFII* AND *ANISAKIS SIMPLEX (S. S.)* IN FISH TISSUES OF *MERLUCCIUS MERLUCCIUS* FROM TYRRHENIAN SEA AND SPANISH ATLANTIC COAST: IMPLICATIONS FOR FOOD SAFETY

4.1 Introduction

European hake *Merluccius merluccius* (L.) is one of the most important and widely distributed fish species, occurring both in Western European (Casey and Pereiro, 1995) and Mediterranean Sea fisheries (Oliver and Massutì, 1995; Ardizzone and Corsi, 1997). The existence of different stocks within the species in European Atlantic waters has been suggested on the basis of a multi-methodological approach: the North-East Atlantic and the Mediterranean Sea populations of *M. merluccius* can be actually considered as separate stocks (Inada, 1981; Alheit and Pitcher, 1995; Roldan et al., 1998; Lundy et al., 1999; Abaunza et al., 2001; Cimmaruta et al., 2005, Milano et al., 2014; Mattiucci et al., 2014a). Atlantic and Mediterranean populations of European hake show remarkable differences in demographic and life history traits such as growth rate, size at maturity, recruitment patterns and spawning season (Froese and Pauly 2013, and references there in).

The species is fished commercially throughout its range, and it is the most important demersal species fished in Western Europe. It commands a high price on the European fish markets where it is selling mostly as fresh fish in both Italian and Spanish markets.

The species of the genus *Anisakis* include nematode parasites of marine organisms, with crustaceans which act as first intermediate hosts, fishes and squid as intermediate/paratenic hosts, and mainly cetaceans as definitive ones (Mattiucci and Nascetti, 2006; 2008). Larval stages of the parasites *Anisakis* spp. are commonly infecting viscera and musculature of many species of teleost fish (Mattiucci and Nascetti, 2008). Actually, nine species of the genus *Anisakis* were genetically detected and characterised (Mattiucci et al., 2009; 2014b). The third stage larvae of these species, recovered from the fish hosts, show the morphotype indicated as *Anisakis* Type I or Type II (*sensu* Berland, 1961), but they cannot be identified to their species level based on traditional morphological analysis. Among the genetic methodologies used for the species identificaton of *Anisakis* spp. larvae, the allozyme markers, as based on several diagnostic loci among the species of *Anisakis* so far genetically detected, are able to recognise the species at any of their life-history stages. In addition, among the other molecular markers used for *Anisakis* detection (Mattiucci et al., 2014b), the high substitution rate of

the mtDNA *cox2* sequences found so far in *Anisakis* spp. allows their correct identification.

To date different species of *Anisakis* are known to occur commonly in *M. merluccius*, the two most common parasites of hakes from Mediterranean and NE Atlantic waters are *A. pegreffii* and *A. simplex* (s. s.), respectively (Mattiucci *et al.*, 2004; Valero et al., 2006). Statistical significant differences observed in the distribution patterns of *Anisakis* spp. larvae, genetically identified, have been used also as biomarkers in the stocks characterization of the fish species in European waters (Mattiucci et al., 2004; 2014a). However, up to now, no detailed information has been given regarding the infection levels by different species of *Anisakis* in the viscera and in the flesh of the fish host, *Merluccius merluccius* in the Mediterranean Sea and NE Atlantic waters. Besides the ecological aspects related to the differential distribution of *Anisakis* spp. larvae detected in this fish host, data on the distribution of larval *Anisakis* spp. in the edible parts of the fish with respect to the viscera of the same hosts species, are important due to the larvae's potentially consumer health hazardous property (Thiel et al., 1960). Indeed, larval stages of the genus *Anisakis* are the etiological agents of human anisakiasis. Over the last 30 years, there has been a marked

increase in the reported cases of human anisakiasis throughout the world, likely due, among others, to:

i) the application of diagnostic techniques, including the molecular identification of the parasites removed by gastroendoscopy (D'Amelio et al., 1999; Fumarola et al., 2009; Umehara et al., 2007; Mattiucci et al., 2013), and in granulomas surgically removed (Mattiucci et al, 2011);

ii) the increasing global demand for seafood;

iii) a growing preference for raw or lightly cooked food, especially in many Western countries, with increased risk of parasite exposure (EFSA, 2010).

So far, among the nine species of *Anisakis* genetically detected (Mattiucci et al., 2009), the two species *A. simplex* (*(s. s.)*) and *A. pegreffii* are recognised as zoonotic species of human anisakiasis (Umehara et al., 2007; D'Amelio et al., 1999; Mattiucci et al., 2011, 2013).

Out of the consumer health implications, anisakid nematodes have also a considerable quality reducing effect as well due to their most unappealing appearance in fish intended for consumption (Karl and Levsen, 2011).

The aim of the present work was to obtain data regarding the occurrence, in general, and the distribution in the flesh, in particular, of the species of the genus *Anisakis* in the European hake, *Merluccius merluccius*, captured in two fishing grounds of Mediterranean and NE Atlantic waters, in order to provide some epidemiological data contributing to the risk assessment analysis of this zoonotic parasite in sea food.

4.2 Methods

4.2.1 Fish sampling and parasitological survey

A total of 130 specimens of European hake, *Merluccius merluccius* (Figure 19) were sampled between November 2012 and May 2013 (Table 11). In particular, 65 were fished by a commercial fishing net in the area FAO 27 (western Iberian Sea, ICES division IXa) of the NE Atlantic Ocean, while 65 specimens were fished in the area FAO 37.1.2 (central Tyrrhenian Sea) of the Mediterranean Sea (Table 11). Fishes were weighted to the nearest 0.1g and measured (total length) to nearest 0.1 cm. The mean weight and mean length of the European hakes were respectively 329,985g ± 90,98 and 35,95 cm ± 3,75 for the 65 specimens of FAO 27 area, while 286,06 g ± 110,25 and 33,86 cm ± 4,07 for the 65 fish of FAO 37.1.2 area (Table 11).

	n	***Mean lenght (Range)***	***Mean weight (Range)***	***N***	***N allozyme (% of tot)***	***N mtDNA cox2***
Fao 27 IXa Atlantic coast of Spain	65	35.9 (29.0 – 45.0)	329.2 (160.8 – 497.0)	2836	1244 (44%)	45
Fao 37.1.2 Tyrrhenian Sea	65	33.9 (26.3 – 47.0)	286.0 (120.1 – 631.0)	154	154 (100%)	23

Table 11: Total number (n) of Merluccius merluccius examined from the two different fishing grounds, with values of mean length (expressed in grams), mean weight (expressed in millimeters), reported with the number (N) of larvae of Anisakis collected, number (N allozyme) of larvae identified by allozymes, number (N mtDNA cox2) of larvae sequenced at mtDNA cox2 gene.

Traditional parasitological survey was carried out for the detection of larval *Anisakis* spp..

Figure 19: Merluccius merluccius

Visceral cavity, digestive tract, liver, gonads and mesenteries of each fresh individual fish were first examined under a dissecting microscope for a standard parasites inspection.

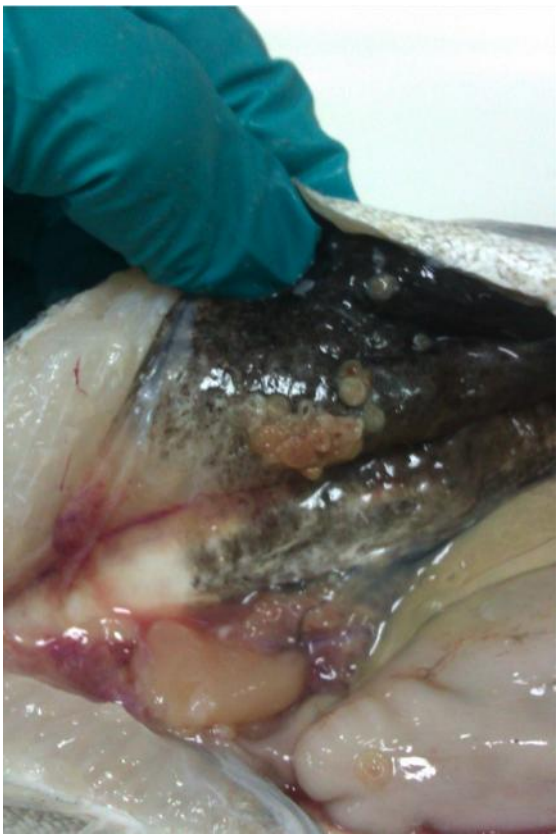

Figure 20: Anisakids in visceral cavity

Afterward, all the viscera were digested in pepsin solution, according to the procedures as reported in Llarena-Reino et al., (2013a), in order to recover eventual undetected parasites. The fish musculature was separated into the hypaxial (ventral) and epaxial (dorsal) regions, following the horizontal septum; afterward, each part was digested separately in pepsin solution, according to Llarena-Reino et al., 2013. Digestion was carried out in an acid solution (pH = 1,5) with HCl at 0,063 M. Assay uses liquid pepsin at concentration of 0,5% and a ratio 1:10 sample weight/solution volume was used. Digestion was performed at incubation temperature of 37 °C, for 30’ (Figure 21).

Figure 21: Pepsin digestion

All the nematodes obtained from the parasitological inspection and pepsin digestion were washed in physiological saline, counted, and stored at -50 °C until their genetic/molecular identification.

4.2.2 Multilocus allozyme electrophoresis (MAE)

The genetic identification of the larval specimens of *Anisakis* spp. collected during the parasitological survey (Table 11) was first undertaken using multilocus allozyme electrophoresis (MAE) on the frozen samples. A total of 1310 larval specimens of *Anisakis* spp. were genetically identified using multilocus allozyme electrophoresis (MAE). Standard horizontal starch gel electrophoresis was performed at those enzyme loci, which have been proven to be diagnostic between the three sibling species (Mattiucci et al., 1997, 2009; Mattiucci and Nascetti, 2006). These are: adenylate kinase (*Adk-2*, EC 2.7.4.3), leucine-alanine peptidase (*PepC-1*, *PepC-2*, EC 3.4.11), sup eroxide dismutase (*Sod-1*, EC 1.15.1.1), leucine-amino peptidase (*Lap-2*, EC 3.4.11.1), and leucine-leucine peptidase (*Pep B*, EC 3.4.11). Their staining procedures are those reported in detail by Mattiucci et al. (1997). Isozymes were numbered in order of decreasing mobility from the most anodal one. Allozymes were identified by numbers indicating their mobility (in mm, standardized conditions) relative to the most common allele, designated as *100*, found in the reference population (i.e. *Anisakis pegreffii* from the

Mediterranean Sea). The statistical significance of departures from the Hardy-Weinberg equilibrium was estimated using the $\chi 2$ test. Genetic analysis was performed using BIOSYS-2 software (Swofford and Selander, 1997).

4.2.3 DNA extraction, amplification and sequencing of the mtDNA cox2 gene

A certain number of the *Anisakis* spp. larvae first identified by allozymes, (i.e. 23 specimens from the Tyrrhenian Sea, and 45 from the Atlantic Spanish coast) (Table 1) were sequenced at mtDNA *cox2* gene. The total DNA was extracted from 2 mg of tissue from each single nematode by using the cetyltrithylammonium bromide method (CTAB) (Valentini et al., 2006). The mitochondrial cytochrome *c* oxidase subunit II (*cox2)* gene was amplified using the primers 211F (5'-TTT TCT AGT TAT ATA GAT TGR TTY AT-3') and 210R (5'-CAC CAA CTC TTA AAA TTA TC-3') (Nadler and Hudspeth, 2000) spanning the mtDNA nucleotide position 10,639-11,248, as defined in *Ascaris suum* [GenBank X54253]. The PCR (polymerase chain reaction) was carried out using the following conditions: 94 °C for 3 min (initial denaturation), followed by 34 cycles at 94 °C for 30 sec (denaturation), 46 °C for 60 sec (annealing), 72 °C for 90 sec

(extension), followed by post amplification at 72 °C for 10 min (Valentini et al., 2006).

The sequences obtained at the mtDNA *cox2* for the larval specimens of *Anisakis* spp. analyzed in the present study, were compared with those already obtained for the same gene in the species *A. pegreffii* and *A. simplex s.s* and with respect to the other species of the genus *Anisakis.* Therefore, the following mtDNA *cox2* sequences of the species of the genus *Anisakis,* retrievable from GenBank, were used for the identification of those larval specimens, previously identified by allozymes: *A. simplex (s. s.)* (DQ116426), *A. pegreffii* (DQ116428), *A. berlandi* (KC809999), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. nascettii* (FJ685642), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433), *A. paggiae* (DQ116434). Phylogenetic trees were rooted using the ascarids *Ascaris suum* and *Toxocara canis* as outgroups.

4.2.4 Statistical analysis of the epidemiological data

The parasitic infection levels of the infestation by *Anisakis spp*. larvae in the fish species were calculated at the parameters of prevalence (P, %), abundance (A) following Bush et al. (1997) and Rosza et al. (2000), using the Software Quantitative Parasitology QPweb, implemented for the web (Reiczigel and Rozsa, 2005). The statistical significance of the

differences observed in the prevalence (P) and abundance (A) values of the infestation by larvae of the two different species were assessed by the Fisher's exact test and Bootstrap t-test, respectively, using the Software Quantitative Parasitology QPweb (Reiczigel and Rozsa, 2005). Differences were considered significant when $P < 0.05$.

Differences in the average abundances of *A. pegreffii* between localities and between *Anisakis simplex (s. s.)* and *A. pegreffii* among tissues (i.e. viscera and flesh) for each locality (i.e. Tyrrhenian Sea *versus* Atlantic coast of Spain) were evaluated by means of a Kruskall-Wallis one-way ANOVA.

Finally, the relationship between the observed abundance values by *Anisakis* spp. larvae infecting in the viscera and flesh of European hake sampled in the Tyrrhenian Sea *versus* those found in the fish from the Spanish Atlantic coast, was measured by means of a linear regression analysis.

An overall MANOVA test of multivariate regression was performed to evaluate the possible dependence between the size and weight of European hake (*Merluccius merluccius*) with the parasitic burden of *Anisakis* spp. larvae sampled in the viscera and flesh, respectively. Raw data were fourth-root transformed and 'zero-adjusted' by adding 1 as a 'dummy variable' to all cells (Clarke and Warwick, 2001), due to the

high number of non-infected individuals. The significance of the regression was given by the Rao's F statistic computed from the Wilks lambda with one-tailed F-test.

The phylogenetic analysis on the sequences data sets obtained at Anisakis spp. larval specimens examined was carried out by Maximum Parsimony (MP) by using PAUP* (Swofford, 2003). MP analysis was performed using the heuristic search with tree-bisection-reconnection (TBR) branch-swapping algorithm; the reliabilities of the phylogenetic relationships were evaluated using nonparametric bootstrap analysis on 1000 pseudoreplicates (Felsenstein, 1985). Bootstrap values ≥70 were considered well supported (Hillis and Bull, 1993).

4.3 Results

4.3.1 Genetic identification of Anisakis spp. larvae using MAE and sequences analysis of mtDNA cox2 gene

A total of 2990 larval specimens of *A. simplex* (*s. l.*) were collected from *Merluccius merluccius* captured in the two different fishing grounds (FAO27 IXa and FAO37.1.2) (Table 12). 1310 larvae, out of those collected, were identified by allozymes electrophoresis (MAE) (Table 12). According to the alleles observed at the diagnostic loci, i.e. *Sod-1* ^{100}, *Adk-2*^{100}, *PepB*^{100}, *PepC-1*^{100}and *PepC2*^{100}, 476 specimens were assigned to the species *A. pegreffii*; whereas, according to the diagnostic alleles *Sod-1*^{105}, *Adk-2*^{105}, *PepB*^{70}, *PepC-1*^{90}and *PepC2*^{96}, as indicated in Mattiucci et al. (1997), 814 larvae corresponded to the species *A. simplex (s. s.)* (Table 12). In particular, 154 larvae were identified as *A. pegreffii* in *M. merluccius* samples from the Tyrrhenian Sea and 322 from the hakes fished in the Atlantic coast of Spain. While, the species *A. simplex (s. s.)* was identified (N=814) only in the fish specimens fished in the Atlantic coast of Spain. 20 larval specimens collected in hakes from the FAO27 area showed an heterozygote genotype at all the diagnostic allozyme loci between *A. pegreffii* and *A. simplex (s. s.)*, likely representing F1 hybrid genotypes. No hybrid genotypes were observed in larval *Anisakis* collected from the Tyrrhenian Sea hake samples.

	Viscera						***Ventral flesh***						***Dorsal flesh***						***Total***					
	P (%)		P^1	A		P^2	P (%)		P^1	A		P^2	P (%)		P^1	A		P^2	P (%)		P^1	A		P^2
	A. simplex (s. s.)	*A. pegreffii*		*A. simplex* (s. s.)	*A. pegreffii*		*A. simplex* (s. s.)	*A. pegreffii*		*A. simplex* (s. s.)	*A. pegreffii*		*A. simplex* (s. s.)	*A. pegreffii*		*A. simplex* (s. s.).	*A. pegreffii*		*A. simplex* (s. s.)	*A. pegreffii*		*A. simplex* (s. s.)	*A. pegreffii*	
Fao 27	98.5	93.8	ns	20.15 ± 29.31	11.46 ± 16.64	*	78.5	53.8	***	8.17 ± 15.78	1.41 ± 2.42	*	38.5	10.8	***	1.68 ± 8.08	0.29 ± 1.51	ns	98.5	93.8	ns	30.00 ± 44.71	13.17 ± 18.63	*
Fao 37.1.2	-	70.8	-	-	2.15 ± 3.04	-	-	9.2	-	-	0.14 ± 0.50	-	-	7.7	-	-	0.08 ± 0.27	-	-	73.8	-	-	2.37 ± 3.10	-
P^3	-	***		-	-		-	***		-	-		-	ns		-	-		-	***		-	-	
P^4	-	-		-	*		-	-		-	*		-	-		-	ns		-	-		-	*	

Table 12: Prevalence (P) and abundance (A) infection levels by Anisakis simplex (s. s.) and A. pegreffii larvae identified in Merluccius merluccius from the two sampling areas and according to their site of infection in the fish host.

P^1= probability level of the statistical significance of the comparison of prevalence value by Fisher's exact test
P^2= probability level of the statistical significance of the Bootstrap 2-sample t-test for the comparison of mean abundance value
P^3= probability level of the statistical significance of the comparison of prevalences by Fisher's exact test
P^4= probability level of the statistical significance of the Bootstrap 2-sample t-test for comparison of mean abundance values
**** $p < 0.001$, * $p < 0.05$, ns = not significative*

In addition, some of the same specimens of *A. pegreffii* and *A. simplex* (s. s.) previously identified by allozymes (MAE), were sequenced at the mitochondrial *cox2* gene (mtDNA *cox2*).

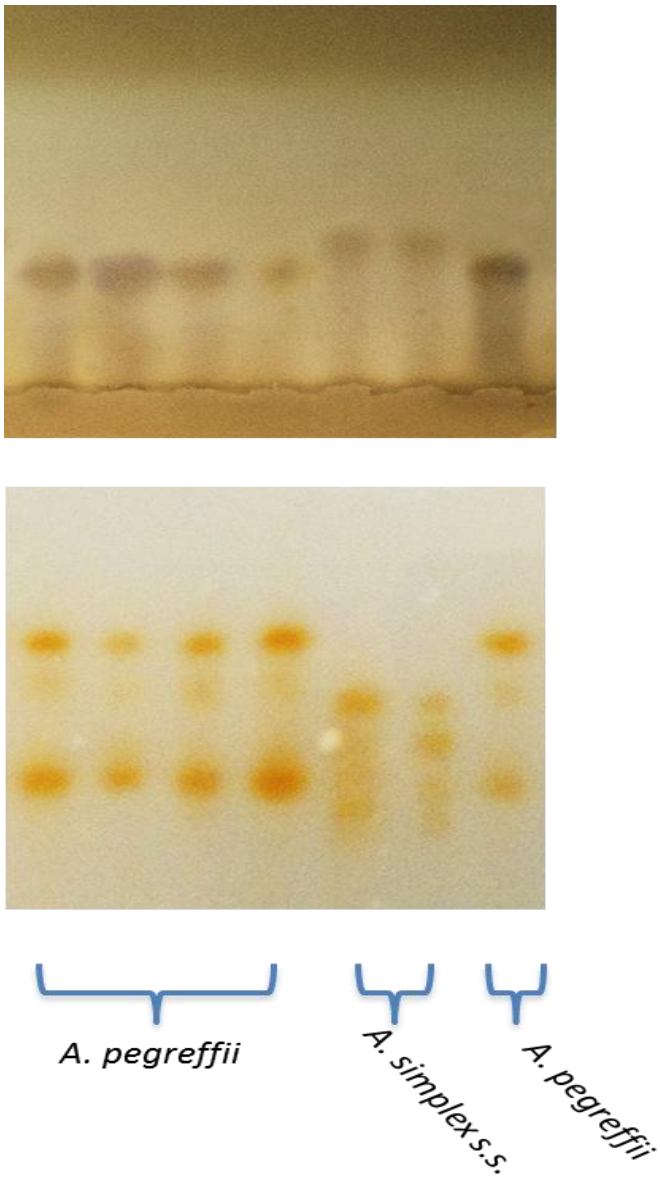

Figure 22: Multilocus allozymes electrophoresis

The 23 specimens of *Anisakis* spp. collected from hakes of the Tyrrhenian and 31 from the hakes from Atlantic coast of Spain matched

99% or 100% the sequence at the mtDNA *cox2* gene of *A. pegreffii* previously deposited in GenBank (Mattiucci et al., 2014b); Analogously, 14 specimens of *Anisakis* spp. from the hakes fished from the FAO27 matched 99% or 100% the sequence deposited in GenBank for the species *A. simplex (s. s.)* at the gene mtDNA *cox2* (Mattiucci et al., 2014b).

The strict consensus of the Maximum Parsimony tree, inferred from the mtDNA cox2 sequences datasets depicted all the specimens of *A. pegreffii* (*n*=54) as forming a unique phylogenetic lineage including the sequence deposited in GenBank, well distinct from the other phylogenetic lineages formed by different species of the *A. simplex s.l.* complex (Figure 23). On the other hand, the specimens of *A. simplex (s. s.)* from hakes of FAO27 (n=14) clustered in the same clade at the MP analysis, representing a distinct phylogenetic lineage from the other species of *Anisakis* considered (Figure 23).

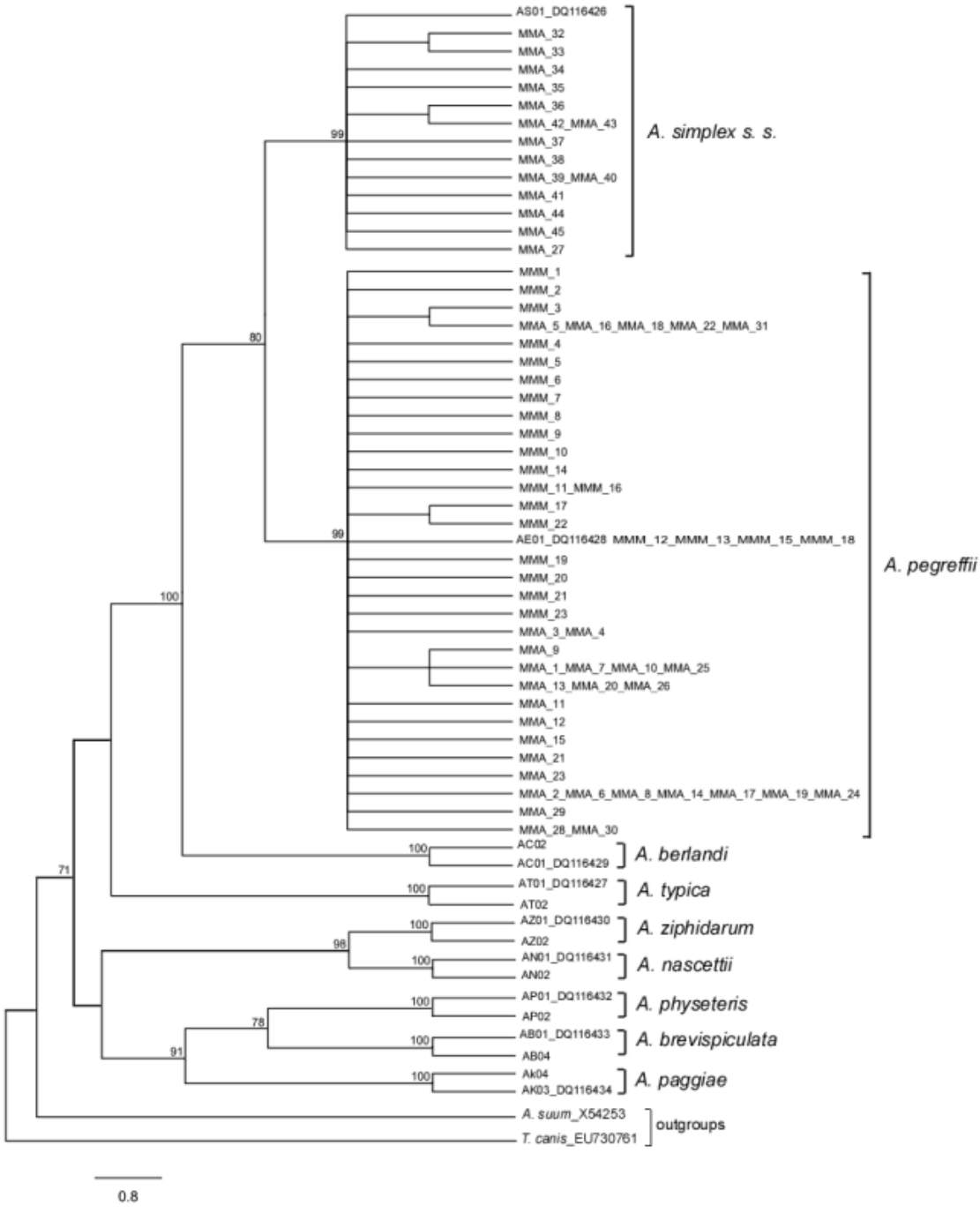

Figure 23: Anisakis phylogenetic lineage

This finding confirmed the results achieved by allozymes, regarding the identification to the specific level of those specimens as belonging to the species *A. simplex* (s. s.) or to *A. pegreffii* (Table 11).

4.3.2 Parasitic infection data by A. pegreffii and A. simplex (s. s.)

Regarding the fish sampled from the FAO 27 Atlantic waters, only one *M. merluccius* out of the 65 examined, resulted uninfested by *Anisakis* spp. larvae; 2 hakes presented a single infection by *A. simplex (s. s.)*, while only 1 specimen showed the single infection by *A. pegreffii* larvae. The remaining 61 fish of the 65 examined reported a mixed infection by both *A. simplex (s. s.)* and *A. pegreffii*. Further, 53 fish specimens resulted positive to *Anisakis* infestation in fillets; 35 of these showed also a mixed infection with both *Anisakis* species. Only 2 of the total 111 fish (1,8%) infested by *Anisakis* harboured larvae in the flesh whereas the viscera resulted apparently uninfected.

As concerning the *M. merluccius* fished from the Tyrrhenian Sea, 47 fish showed a single infection by *A. pegreffii;* its occurrence in the fish fillets was observed in 8 out of the 65 examined.

The parasitic infection levels observed at the parameters of prevalence (P) and abundance (A) of the infection by *Anisakis simplex (s. s.)* and *A. pegreffii* larvae recovered from *M. merluccius* captured from the two different fishing grounds, are reported in Table 12. Because *A. simplex*

(s. s.) was not found in any of sampled individuals in the Mediterranean Sea, the statistical analysis within and between locations followed exclusively the parasitic burden of *A. pegreffii*.

In the hakes fished from the FAO 27 fishing ground, *A. simplex (s. s.)* and *A. pegreffii* co-infected the same fish individual fish host, both in viscera and in the fillets (Table 12). However, while no significant difference (*p*= 0.36) in the prevalence (P) values was recorded between the two *Anisakis* species found in the viscera of the host (P= 98.5% by *A. simplex (s. s.)* and P= 93.8% by *A. pegreffii*), the abundance level of the infection by *A. simplex* (s. s.) resulted significantly higher than that observed by *A. pegreffii* (A= 20.51 and 11.46, respectively, p= 0.045). In particular, concerning the infestation by the two *Anisakis* species in the flesh of the hakes, the prevalence (P) of *A. simplex (s. s.)* resulted statistically significant higher than *A. pegreffii* in both ventral and dorsal flesh, being respectively P= 78.5% Vs P= 53.8% (p= 0.0051) and P= 38.5% Vs P= 10.8% (p= 0.0004). *A. simplex* (s. s.) resulted significantly more abundant in the ventral flesh, the hypaxial, (with a value of A= 8.17 for *A. simplex* (s. s.) and A= 1.41 for *A. pegreffii*, p= 0.0026) (Table 12, and Figure 23). While, no statistical difference (*p*=0.411) was recorded in the abundance values of the two species in the dorsal flesh of *M. merluccius* (Table 12).

Concerning the occurrence of *A. pegreffii* in the examined *M. merluccius*, it resulted present in fish from both the sampling areas. The average amount of parasitic burden of *A. pegreffii* (F = 3.885, *p* = 0.001) differed significantly in fish from the Atlantic waters and fish from the Tyrrhenian Sea.

Its infection levels in terms of prevalence and abundance were statistically significant higher in the fish from the FAO27 fishing ground, rather than in the fish species from the Tyrrhenian Sea (Table 12). *A. pegreffii* larvae were in fact significantly higher in the viscera of *M. merluccius* from FAO27 area (P= 93.8, A= 11.46) than from fish from FAO 37.1.2 fishing grounds (P=70.8, A= 2.15) (Prevalence *p*= 0.001 and Abundance *p*= 0.002) (Table 12). Concerning the presence of this *Anisakis* species in the ventral flesh of the fish, statistically significant higher levels of prevalence and abundance was recorded in FAO 27 area compared to the FAO 37.1.2 (respectively, P= 53.8% *Vs* P=9.2% *p*= 0.0001; A= 1.41 *Vs* A= 0.14 *p*= 0.06) (Table 12, and Figure 23). The small infection by *A. pegreffii (n* =5*)* of the dorsal fillet of the fish of the FAO 37.1.2 did not evidence any statistically significant differences from the analogous location in the fish collected from the Atlantic fishing area.

In addition, European hakes sampled from FAO 27 Atlantic area showed significant differences in abundance among tissues of both *A. simplex (s.*

s.) and *A. pegreffii* (One-way ANOVA, $F_{2,192} > 40$ and $p < 0.0001$ in both infections). The same trend has been observed in the average abundance of the infection by *A. pegreffii* among different hosts tissues in the fish sampled in the Tyrrhenian Sea (One-way ANOVA, $F_{2,192} = 53.56$, $p < 0.0001$).

Concerning the relationship among the parasitic burden by *Anisakis* spp. and fish size, the overall Manova test showed a significantly positive correlation between body length of the hakes and *Anisakis* levels of infection (p = 1,71E-06), whereas no correlation was found between infection levels and weight of the fish in either locality. Multivariate regression analysis showed the relative dependence between the size of European hake and the parasitic burden of *Anisakis* (Overall MANOVA, $F = 4.912$, $p < 0.0001$) was mainly related to the host length (Wilks' lambda = 0.64, $p < 0.0001$). The abundance of *Anisakis* (*A. simplex* (s. s.) *plus A. pegreffii*) in the viscera of the Atlantic fish specimens reached a higher degree of correlation with respect to host size (cumulative r^2 = 0.977), in comparison to the abundance values observed in the flesh (cumulative r^2 = 0.692); the last was mostly due to the infection by *A. simplex (*s. s.) (r^2 = 0.522, F = 33.88, $p < 0.001$). A relation between the burden of *A. simplex (s. s.)* in the viscera and flesh of Atlantic fishes was found (r = 0.912) and the same relation, with a lower significance (r =

0.586), occurred for *A. pegreffii*. Instead, the same relation did not occur for *A. pegreffii* in different tissues of fishes sampled in the Mediterranean (r = 0.018).

4.4 Discussion

In the present parasitological survey, two parasite species of *Anisakis* were identified as larval stages in the European hake from the two different fishing grounds: they are *A. simplex (s. s.)* and *A. pegreffii*. They occurred in simpatry and sintopy in the fish caught from the Atlantic FAO 27 IXa area, while only *A. pegreffii* was detected in fish sampled from the Tyrrhenian Sea waters. The occurrence of F1 hybrid genotypes in this sympatric area of the two species was also documented on the basis of the same genetic markers (allozymes) in this fish species (Mattiucci et al., 2004).

Both *Anisakis* species occurred in viscera and flesh of the fish species. However, significant differences were found in the infection levels both in terms of geographic origin of the fish species (fishing grounds), and of infection site by the two species of *Anisakis*.

Indeed, for instance, the overall parasitic burden by *A. pegreffii* reported in the FAO 37.1.2 (Tyrrhenian Sea) resulted significantly lower compared to the level of the same parasite species identified in *M. merluccius* from the FAO 27 IXa Atlantic area (Table 12, Figure 24).

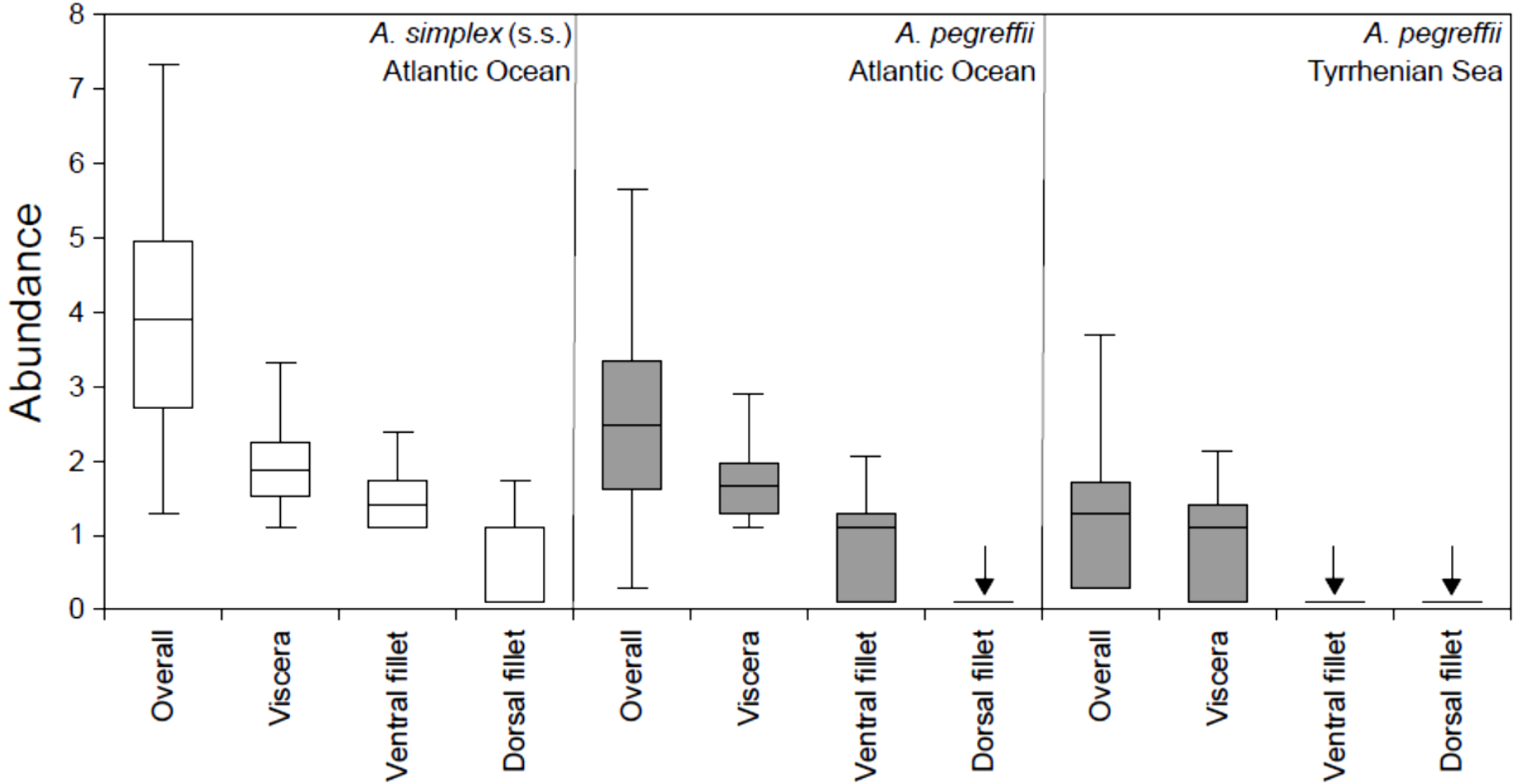

Figure 24: Anisakis abundance

While, *A. simplex (s. s.)* showed significant higher values with respect to the species *A. pegreffii*, both in the visceral and organs of the fish species as well as in its flesh, with respect to those observed for the same location in the fish host by *A. pegreffii,* co-infecting the same individual fish host (Table 12 and Figure 23). The higher infection levels shown by *A. simplex (s. s.)* with respect to *A. pegreffii* in the present study are generally in agreement with those previously reported by the two parasite species infecting European hake in those fishing grounds (Mattiucci et al., 2004), despite the fact that Mattiucci et al., (2004) did not report the infection levels in the fish muscle.

On the other hand, the assumption that geographic fishing ground of the fish species represents a parameter affecting the parasitic infection levels by different species of *Anisakis* and their populations, has been documented by previous Authors (Suzuki et al., 2010; Valero et al. 2006; Mattiucci et al., 2014a). Such differences which have been found in the present study in the fish species *M. merluccius*, concerning the infection levels by *A. pegreffii* from two fishing grounds, could be related to the fact that the Atlantic coast of Spain is an area inhabited by several cetacean species, such as "oceanic dolphins" and baleen whales, which are suitable definitive hosts of this species in Atlantic Ocean waters (Mattiucci and Nascetti, 2008). While, in the Mediterranean Sea a lower

level of infection by *Anisakis* has been related also to the higher level of habitat disturbance, which affects the population density of the suitable intermediate and definitive hosts directly involved in the life-cycle of this parasite species (Mattiucci and Nascetti, 2008).

The infection levels by different species of anisakid nematodes in the flesh of the fish represents a crucial data from a food safety perspective, being the fillets the part used for commercial purpose.

This study represents the first record reporting the occurrence of different species of *Anisakis* in the fish muscle of *M. merluccius*. Indeed, previous data about the infection levels by *Anisakis simplex (s. s.)* were those produced by Herreras et al., (2000) in Argentinean hake, *Merluccius hubbsi*, and by Valero et al., (2006). However, in those studies, no genetic identification of the *Anisakis* larvae collected was carried out, and no co-infection by different species was detected.

The larval spatial distribution of the two *Anisakis* species detected in the flesh of *M. merluccius*, with their relative frequencies, are reported in Figure 25. In the area of simpatry, the hypaxial (ventral) region of the European hake hosted the majority of larvae (531) of *A. simplex (s. s.)*, and (92) of *A. pegreffii*, compared to the epaxial (dorsal) one (109 of *A. simplex (s. s.)*, 19 of *A. pegreffii*). These findings are in accordance with previous data from different fish host species (Levsen and Lunestad

2010; Kark and Levsen, 2011; Levsen and Karl 2014, Llarena et al., 2013), and confirm that most of the *Anisakis* spp. larvae penetrating the fish flesh located in its ventral area, surrounding the visceral organs. However, a statistically significant lower relative proportion was recorded in the infection in flesh by *A. pegreffii* with respect to the species *A. simplex (s. s.)*.

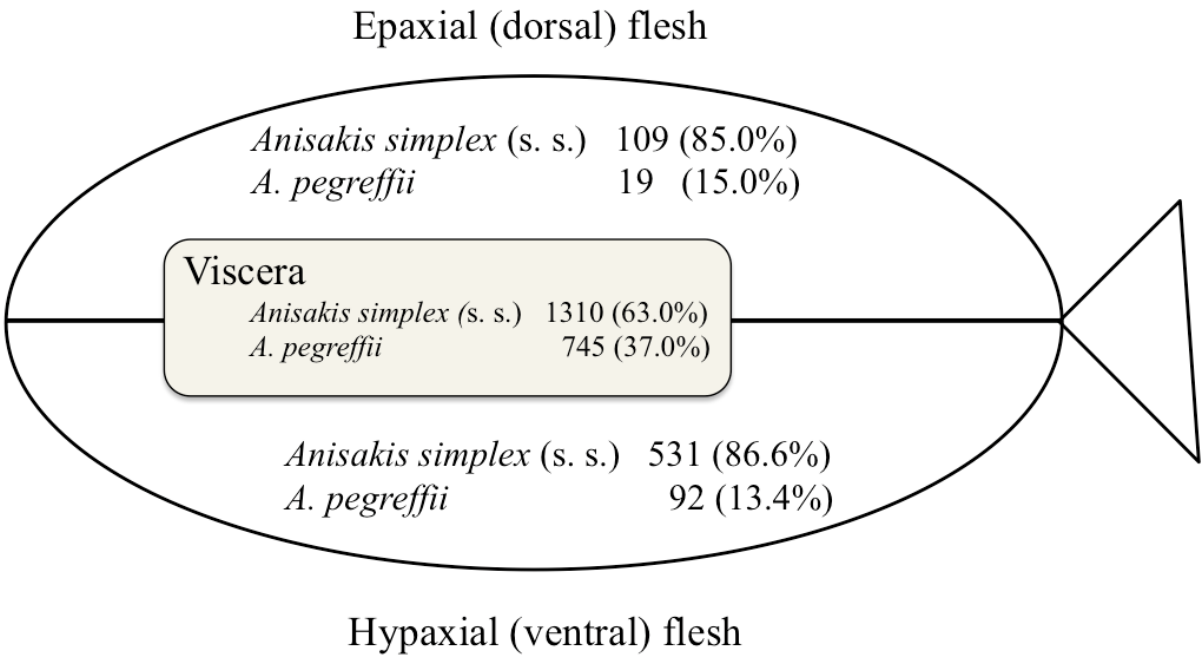

Figure 25: Anisakis spatial distribution

Indeed, *A. simplex (s. s.)* (640) outnumbers (almost 6 times higher) the average number (111) of *A. pegreffii* in the fish fillets. Similar findings were previously observed in the fish species *Scomber japonicus* from the Pacific coast of Japan, co-infected by *A. simplex (s. s.)* and *A. pegreffii*,

where the average number of *A. simplex (s. s.)* per fish was 12 times higher than that for the species *A. pegreffii* (Suzuki et al., 2010). Moreover, the same Authors have also found that the penetration rate in agar of *A. simplex (s. s.)* was higher than that of *A. pegreffii*. The Authors concluded that human anisakiasis reported in Japan (Umehara et al., 2007) is mainly caused by *A. simplex (s. s.)* because it penetrates the muscle of the fish species at higher rate with respect to *A. pegreffii* (Suzuki et al., 2010). Similar observations has been obtained by the experimental infection of the rainbow trout and olive flounder with larvae of the two species, i.e. *A. simplex (s. s.)* and *A. pegreffii*; indeed, the *A. simplex (s. s.)* was found to have migrated to the fish muscle, while *A. pegreffii* larvae remained in the fish body cavity (Quiazon et al., 2011). Analogously, Abattouy et al., (2011) found in the fish flesh only the 5,5% of the total larvae of *A. pegreffii* identified in *Scomber japonicus* caught in the North of Morocco Mediterranean waters.

Being *M. merluccius* the most important demersal species fished in western Europe, the study on the presence and localization of *Anisakis (s. l.)* species in this fish host offers a crucial food safety instrument to evaluate the risk assessment associated with the presence of this parasite. Therefore, in terms of food safety it is a priority to better understand the infection rate of different *Anisakis* species in the fish muscle with respect

to the proportion in the body cavity of the fish, assuming that viscera and organs in the body cavity, together with parasites, are generally removed during fish processing. Data so far acquired seems to indicate that the two species *A. pegreffii* and *A. simplex (s. s.)* differ in their site of infection among host fish species, with *A. pegreffii* showing a lower ability to invade the fish muscle. It has been also observed, in experimental infection, that at high temperature (>25 °C) the *Anisakis* larvae are likely able to migrate to the flesh of the fish, however, again, less ability was found in the species *A. pegreffii* (Quiazon, et al., 2011). These findings need to be investigated also in other fish species. On the other hand, it has been hypothesized that the ability of *Anisakis* spp. larvae to migrate in the fish muscle could be related to differences in the muscle tissue of various fish species, such as the fatty acid content (Smith, 1983).

Furthermore, our study seems to suggest that while *A. simplex (s. s.)* showed a significant positive correlation between the proportion of larvae in visceral and in the fish muscle, in the case of the infection by *A. pegreffii* this trend was not statistical significant, with always a lower percentage of larvae migrating in the flesh, despite a high quantity of larvae found in the body cavity and viscera of *M. merluccius* examined. This result is consistent with similar observation reported by Kark and

Levsen (2011) in grey gurnards. Indeed these Authors reported in grey gurnards "*...a significantly positive relationship between both abundance and intensity of the larvae in the flesh and the viscera (r= 0.64 and r= 0.59, respectively), i.e. the number of larvae in the flesh tend to increase with increasing infection level in the viscera...*" (Karl and Levsen, 2011). Only the 1,8% of the fish positive to *Anisakis* showed an infestation in the flesh and no larvae in the viscera. The Authors underlined that fish which "*...appear to be free of nematodes in the viscera may still carry A. simplex larvae in the flesh, i.e. the larval infection level in the viscera cannot be used as reliable indicator for the approximate Anisakis burden in the flesh...*" (Karl and Levsen, 2011).

Thus we underline the importance to carry out a parasitological survey concerning the detection of larval anisakid nematodes in fish which includes not only the inspection in the viscera, but, even more important, the evaluation of the parasitic burden in the fish fillets, which represents the quote of risk to human health. In this respect, the correct genetic/molecular identification of the anisakid nematodes involved in the fish infection represents the base for the epidemiological survey in order to identify the zoonotic species involved.

Attain detailed information of the distribution of different *Anisakis* species in flesh of fish could draw useful guidelines for fishing industry,

for the inspection and for the detection of the anisakid larvae in the commercial edible part of the host fish. Recent results showed how candling, visual inspection, and related standard parasite inspection, represent not a satisfying method of detection of *Anisakis* larvae in the flesh of various fish hosts species (Levsen et al, 2005; Llarena et al., 2013a). Pepsin digestion resulted a valuable method for the detection of anisakid nematodes in fish flesh: when applied to fresh fillets the digestion keeps parasites alive and easy to detect, and to be identified by genetic/molecular methodologies; it also allows recovery of dead worms from frozen material. The only limits of pepsin digestion method are that is time consuming, difficult to adopt in commercial mass screening of huge amount of fish (EFSA, 2010).

The relative low prevalence of the species *A. pegreffii* so far observed with respect to *A. simplex (s. s.)* in the fish muscle is of health interest, and poses the basis to carry out further epidemiological data on this anisakid parasite in other fish species of the Mediterranean Sea, which must include an accurate inspection of fish fillets.

CHAPTER 5. RISK-BASED SURVEILLANCE FOR ANISAKIDS IN FROZEN FISH PRODUCTS FROM THE ATLANTIC

5.1 Introduction

Anisakid nematodes are present worldwide in the gut and flesh of many marine fish and cephalopod species, which act as trophic bridges assuring and widening the parasite life cycle. Differences in host range depending on nematodes species have been found. These differences appear to be related more to geographic distribution and to feeding habits of hosts rather than the parasite's preferences towards certain host behaviour and physiology, with no evidence of host specificity. A relative prevalence and/or abundance of larval nematode and a positive relationship between body size/age of fish in several commercially important fish species from different Atlantic areas have been demonstrated (Banning and Becker, 1978; Bussmann and Ehrlich, 1979; Davey, 1972; Levsen and Midthun, 2007; McGladdery, 1986; Platt, 1975; Smith, 1984; Smith and Wootten, 1978; Valero and Martín-Sánchez, 2000).

The aim of this work is to provide data for improving the epidemiological understanding of anisakids in commercial fish from

different Atlantic FAO fishing areas and to apply a monitoring tool for the evaluation of parasite infection in fish lots with the goal to assess parasites impact on marketed fish.

5.2 Methods

5.2.1 Sampling

A total of 771 fish samples belonging to several commercial frozen lots (33 different species) from 5 different FAO fishing areas were examined (Table 13). After thawing every sample was measured, weighed and eviscerated. Then, heads and tails were removed (Figure 26) and the remaining musculature was separated into the hypaxial (ventral) and epaxial (dorsal) regions following the horizontal septum (Figure 27, 28, 29).

Figure 26: Remiving head and tail

Figure 27: Filleting

Figure 29: Dorsal and ventral fillets

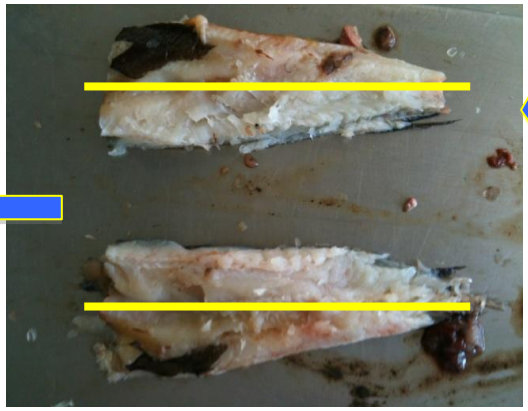

Figure 28: Filleting

Host	*Individuals (N)*	*FAO area*
Alepocephalus bairdii	25	FAO 27
Aphanopus carbo	10	FAO 27
Atlantoraja castelanui	10	FAO 41
Caelorinchus fasciatus	25	FAO 41
Clupea harengus	25	FAO 27
Conger conger	25	FAO 27
Dicologlossa cuneata	25	FAO 34
Galeoides decadactylus	25	FAO 34
Genypterus blacoides	25	FAO 41
Glyptocephalus cynoglossus	25	FAO 21
Illex argentinus	10	FAO 47
Lepidopus caudatus	9	FAO 47
Macruronus magellanicus	28	FAO 41
Macrurus berglax	25	FAO 21
Mallotus villosus	25	FAO 21
Melanogrammus aeglefinus	25	FAO 27
Merluccius capensis	25	FAO 47
Merluccius hubbsi	25	FAO 41
Merluccius paradoxus	34	FAO 47
Merluccius polli	30	FAO 47
Micromesistius australis	17	FAO 41
Molva dypterygia	25	FAO 27
Patagonotothen ramsayi	25	FAO 41
Regalecus glesne	11	FAO 27
Reinharditius hippoglossoides	25	FAO 21
Salilota australis	25	FAO 41
Serranus cabrilla	25	FAO 47
Todaropsis angolensis	10	FAO 47
Trachurus trachurus	22	FAO 47
Trachurus trecae	50	FAO 34
Trachyrhynchus scabrus	25	FAO 27
Urophycis chus	30	FAO 21
Zeus faber	25	FAO 27

Table 13: Host sample

5.2.2 Artificial peptic digestion

The whole fish muscle (hypaxial and epaxial regions separately) of each individual was digested in an ACM-11806 Magnetic Stirrer Multiplate in pepsin solution (according to Llarena-Reino et al., 2013a). Digestion was performed at incubation temperature of 37 °C, in an acid solution (pH =

1,5) with HCl at 0,063 M. Liquid pepsin at concentration of 0,5% and a ratio 1:20 sample weight/solution volume was used. The digestion solution was filtrated through a sieve and the rests of digestion and nematodes were inspected under stereomicroscope and under UV light (figure 30). All anisakids (Figure 31) were placed in a petri dish and then in individual eppendorf with ethanol 70% for further molecular diagnosis.

Figure 30: Anisakids under UV-light

Figure 31: Anisakids

5.2.3 Molecular analysis

All anisakid larvae were identified at the genus level by microscopic examination of diagnostic characters. For specific parasite diagnosis, some larvae from each fish species were collected and then genetically

identified as follows: DNA extractions were performed employing the commercial kit NucleoSpin®Tissue kit (Macherey-Nagel) following manufacturer recommended protocols.

Figure 32: DNA extraction kit *Figure 33: DNA amplification*

DNA quality and quantity was checked in a spectrophotometer Nanodrop® ND-1000 (Nanodrop technologies, Inc).

The entire ITS (ITS1, 5.8S rDNA gene and ITS2) was amplified using the forward primer NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') and the reverse primer NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') (Figure 33).

PCR reactions were carried out in a total volume of 25 µl containing 100 ng of genomic DNA, 10 µM of each primer, 2.5 µl of 10x buffer, 0,5 µl of dNTPs and 5 U/µl of Taq DNA polymerase (Roche). PCR cycling

parameters included denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 75 s, and a final extension at 72 °C for 7 min. PCR products were purified for sequencing using ExoSAP-IT © following manufacturer recommended protocols.

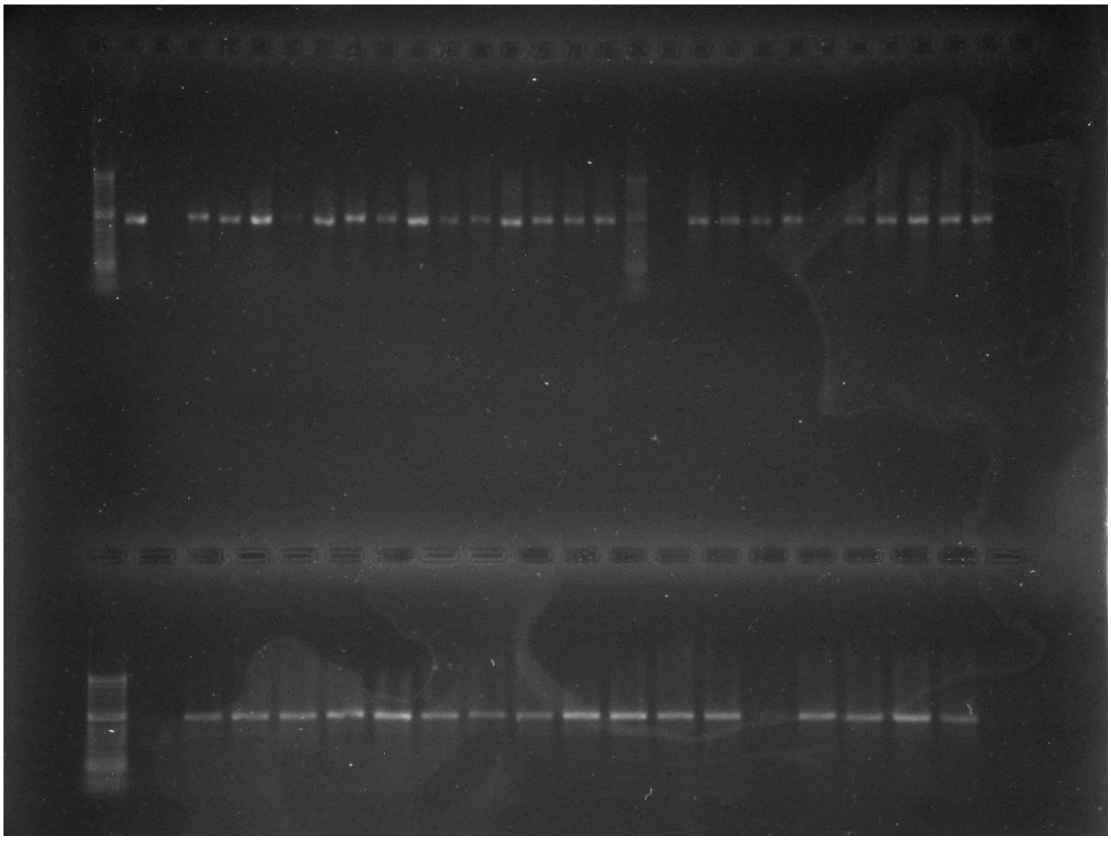

Figure 34: DNA electrophoresis

Sequencing was performed by Secugen (Madrid, Spain) and the chromatograms were analysed using the programme ChromasPro version 1.41 Technelysium Pty LtdA. All sequences were searched for

similarity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Center for Biotechnology Information (USA) (Figure 35).

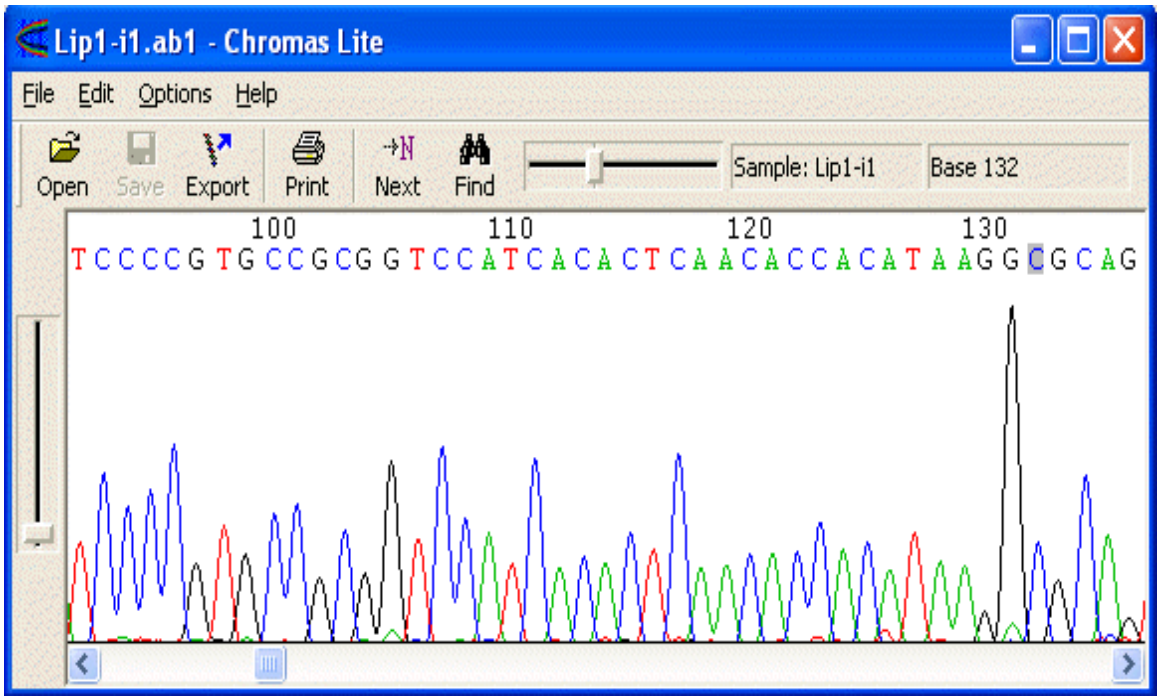

Figure 35: Blast chromatogram

5.2.4 Risk categorisation

Demographic infection values for each fish lot was established according to Bush et al (1997). A risk ranking tool based on a scenario tree modeling developed by Llarena et al. (2013b) was used as a novel inspection approach for risk categorisation of wild fish stocks of commercial interest. The scoring system, namely SADE (Site of

infection, Assurance of quality, Demography, Epidemiology), presents a categorization of parasite infection. This tool is presented in a highly visual and rapid-reference format. Fish lots are grouped according to four homogeneous categories (indices or ‘‘bins’’ of disease importance, namely S, A, D, and E), which are further divided with some accommodation into subcategories (denoted by numerals). The objective of SADE is the score of fish lots. By summing the numerical values assigned to each batch along the four categories, the SADE system adopts a 10-point scale. Each company must determine the level of score that sets off the implementation of measures to ensure food safety and quality of processed batches. The highest score indicates parasite-free fish lots. The lowest scores refer to serious weaknesses in the fish evaluated; that means a fish lot that should be reprocessed to guarantee its visual quality and/or safety attributes.

- *Site of infection* (the S category assesses the anatomic exposure of fish flesh recorded at inspection).
    - S0: disseminated (spread throughout the whole flesh)
    - S1: located in the epaxial zone
    - S2: located in the hypaxial zone, including the visceral body cavity
    - S3: parasite-free

- *Assurance of quality*: macroscopic pathological-unaesthetic commercial findings (the A category shows whether there are manufacturing and/or visual parasite problems reported at line or on site in contaminated fish lots).
    - A0: both topics included in A1 (pathological changes and parasite motility)
    - A1: gross pathological changes in infected tissues (undesirable components such as nodules in bellyflaps, melanized capsules in fillets, milky flesh, hemorrhages in the vent areas (e.g., Beck et al., 2008) or commercial reject due to a live parasite, mostly associated with parasite motility in fresh fish (e.g., Pascual et al., 2010)
    - A2: neither pathological nor commercial problems
- *Demography of infection* (the D category assesses the quantity of infection recorded at inspection, upon adapted and combined criteria based on CODEX STAN 165 [1989], CODEX STAN 190 [1995], CX/FFP 08/29/7, and on Wooten and Cann [2001]).
    - D0: density > 5 parasites/kg
    - D1: density 2–5 parasites/kg
    - D2: density < 2 parasites/kg

- *Epidemiological relevance of the species* (the E category describes the risk of the hazard after parasite species diagnoses, based on EFSA opinion and previous clinical evidences, already cited).
    - E0: zoonotic species of parasite (or its metabolites) associated with gastrointestinal diseases, other documented allergies, and/or clinical manifestations
    - E3: species of parasite with no published evidencebased data demonstrating human health affection. The importance of this point in terms of food security leads to assigning it a value of 3 points

5.2.5 Flow diagram: An easy tool to use the scoring system

Based on the SADE scoring system and following an HACCP schema, the flow diagram herein proposed was subsequently generated to standardize epidemiological stages provided by fish-inspection results. Figure 36 illustrates this flow diagram as a user-friendly tool that can be easily implemented and controlled by the technicians and followed by fish workers.

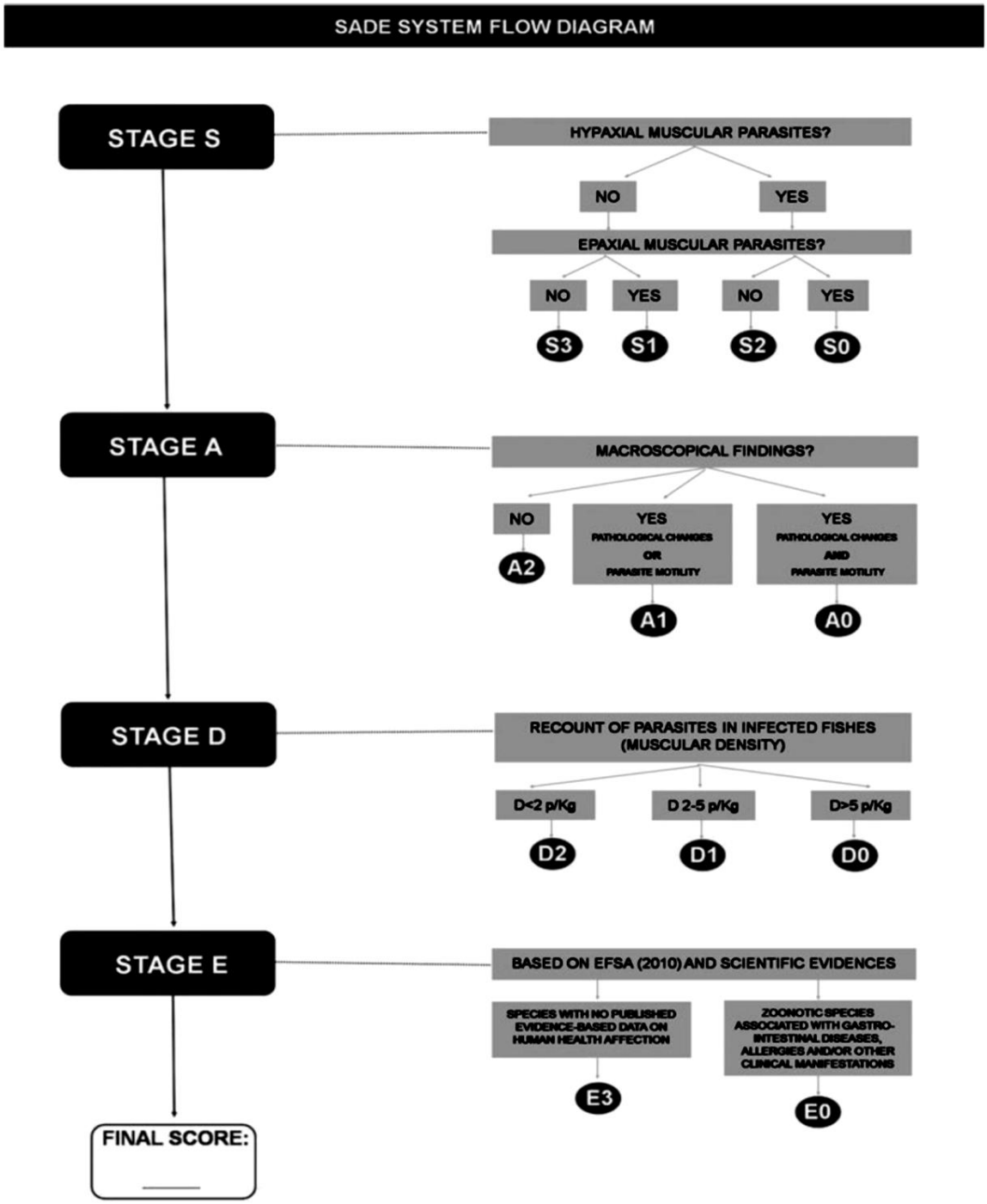

Figure 36: Flow diagram for risk categorization

5.3 Results

For each parasitized fish species the infection values were reported in Table 14. A total of 10897 anisakid larvae were found and collected in the flesh. The blue ling (*Molva dypterygia*), european conger (*Conger conger)* and Peter's Fish (*Zeus faber)* were the most highly infected species respectively. A total of 27,27 % of the species analyzed were anisakid-free: no larvae were detected in capelin (*Mallotus villosus),* torbay sole (*Glyptocephalus cynoglossus*),wedge sole (*Dicologlossa cuneata*), lesser african threadfin (*Galeoides decadactylus*), cunene horse mackerel (*Trachurus trecae*), tadpole codling (*Salilota australis*), spotback skate (*Atlantoraja castelnaui*) and comber (*Serranus cabrilla*). For every fish species, demography of infection showed higher values at the hypaxial region than in the epaxial (Figure 37). In fact, 50 % of the samples were parasite-free at the epaxial region and in all cases the epaxial infection took place simultaneously with hypaxial location.

Host	Parasites found/sequenced (N.)	PREVALENCE (% ± CI)			MEAN INTENSITY ± SD			MEAN ABUNDANCE ± SD		
		Epaxial	Hypaxial	Total	Epaxial	Hypaxial	Total	Epaxial	Hypaxial	Total
Alepocephalus bairdii	14/5	0	16 ± 0,143	16 ± 0,143	0	3,5 ± 2,38	3,5 ± 2,38	0	0,56±1,55	0,56 ±1,55
Aphanopus carbo	741/9	60 ± 0,30	100	100	1,33 ± 2,56	73,3 ± 28,46	74,1 ± 28,55	0,8 ± 2,29	73,3 ± 28,46	74,1 ±28,55
Atlantoraja castelnaui	0	0	0	0	0	0	0	0	0	0
Caelorinchus fasciatus	3/3	4 ± 0,07	8 ± 0,10	12 ± 0,12	1	1	1	0,04 ± 0,2	0,08 ±0,27	0,12± 0,33
Clupea harengus	13/12	0	32 ± 0,18	32 ± 0,18	0	1,625 ± 0,74	1,625 ± 0,74	0	0,52 ± 0,87	0,52 ± 0,87
Conger conger	2879/12	64 ± 0,18	100	100	2,5 ± 2,12	113,56 ± 96,34	115,16 ± 96,77	1,6 ±2,08	113,56 ±96,34	115,16 ± 96,77
Dicologlossa cuneata	0	0	0	0	0	0	0	0	0	0
Galeoides decadactylus	0	0	0	0	0	0	0	0	0	0
Genypterus blacoides	83/11	28 ± 0,17	64 ± 0,18	80 ± 0,15	1,57 ± 0,78	4,5 ± 4,93	4,15 ± 4,76	0,44 ±0,82	2,88 ±4,48	3,32 ±4,56
Glyptocephalus cynoglossus	0	0	0	0	0	0	0	0	0	0
Illex argentinus	2/2		10 ±0,18			2			0,2 ± 0,63	
Lepidopus caudatus	44/6	0	100	1	0	4,88 ± 3,18	4,88 ± 3,18	0	4,88 ± 3,18	4,88 ± 3,18
Macruronus magellanicus	3/2	3,57 ± 0,06	3,57 ± 0,06	7,14 ± 0,1	2	1	1,5±0,7	0,071 ± 0,377	0,035 ± 0,188	0,107 ±0,41
Macrurus berglax	314/13	0	84 ± 0,14	84 ± 0,14	0	14,95 ± 16,92	14,95 ± 16,92	0	12,56 ± 16,42	12,56 ± 16,42
Mallotus villosus	0	0	0	0	0	0	0	0	0	0
Melanogrammus aeglefinus	7/5	0	12 ± 0,12	12 ± 0,12	0	2,33 ± 0,57	2,33 ± 0,57	0	0,28 ± 0,79	0,28 ± 0,79
Merluccius capensis	6/6	4 ± 0,07	16 ± 0,14	20 ± 0,15	1	1,25 ± 0,5	1,2 ± 0,5	0,04 ±0,2	0,2 ±0,5	0,24 ± 0,52
Merluccius hubbsi	216/14	36 ± 0,18	84 ± 0,14	84 ± 0,14	3,55 ± 6,57	8,66 ± 8,1	10,19 ± 8,79	1,32 ± 4,2	7,28±8,08	8,56 ±8,9
Merluccius paradoxus	15/14	2,94 ± 0,05	23,52 ± 0,14	23,52 ± 0,14	1	1,75 ± 0,88	1,875 ± 0,99	0,029 ± 0,17	0,411 ±0,85	0,441 ± 0,92
Merluccius polli	3/3	0	10 ± 0,1	10 ± 0,1	0	1	1	0	0,1 ± 0,3	0,1 ± 0,3
Micromesistius australis	61/12	11,76 ± 0,15	70,58 ± 0,21	70,58 ± 0,21	1	4,91 ±3,47	5,08 ± 3,34	0,11 ±0,33	3,47 ±3,69	3,58 ±3,65
Molva dypterygia	5113/8	36	100	100	2,22 ± 1,48	203,72 ± 90,78	204,52 ± 91,14	0,8 ±1,38	203,72 ± 90,78	204,52 ± 91,14
Patagonotothen ramsayi	7/0	0	16 ± 0,14	16 ± 0,14	0	1,75 ±1,5	1,75 ±1,5	0	0,28 ±0,84	0,28 ±0,84
Regalecus glesne	4/3	0	36 ± 0,28	36 ± 0,28	0	1,33 ± 0,57	1,33 ± 0,57	0	0,36 ± 0,67	0,36 ± 0,67
Reinharditius hippoglossoides	125/14	0	44 ± 0,194	44 ± 0,194	0	8,92 ± 14,2	8,92 ± 14,2	0	5 ± 11,39	5 ± 11,39
Salilota australis	0	0	0	0	0	0	0	0	0	0
Serranus cabrilla	0	0	0	0	0	0	0	0	0	0
Todaropsis angolensis	3/3		10 ±0,18			3			0,3 ± 0,94	
Trachurus trachurus	68/ 12	0	45,45 ± 0,2	45,45 ± 0,2	0	6,8 ± 14,54	6,8 ± 14,54	0	3,09 ± 10,13	3,09 ± 10,13
Trachurus trecae	0	0	0	0	0	0	0	0	0	0
Trachyrhynchus scabrus	18/6	0	32 ± 0,18	32 ± 0,18	0	2,25 ± 1,83	2,25 ± 1,83	0	0,72 ±1,45	0,72 ±1,45
Urophycis Chus	31/7	10 ± 0,1	10 ± 0,1	20 ± 0,14	1,33 ± 0,57	9 ±13,85	5,16 ± 9,72	0,133 ± 0,43	0,9 ±4,55	1,033 ±4,52
Zeus faber	1124/12	72 ± 0,17	100	100	2,05 ± 1,35	43,48 ± 32,11	44,96 ± 32,66	1,48 ±1,47	43,48± 32,11	44,96 ± 32,66

Table 14: Infection values

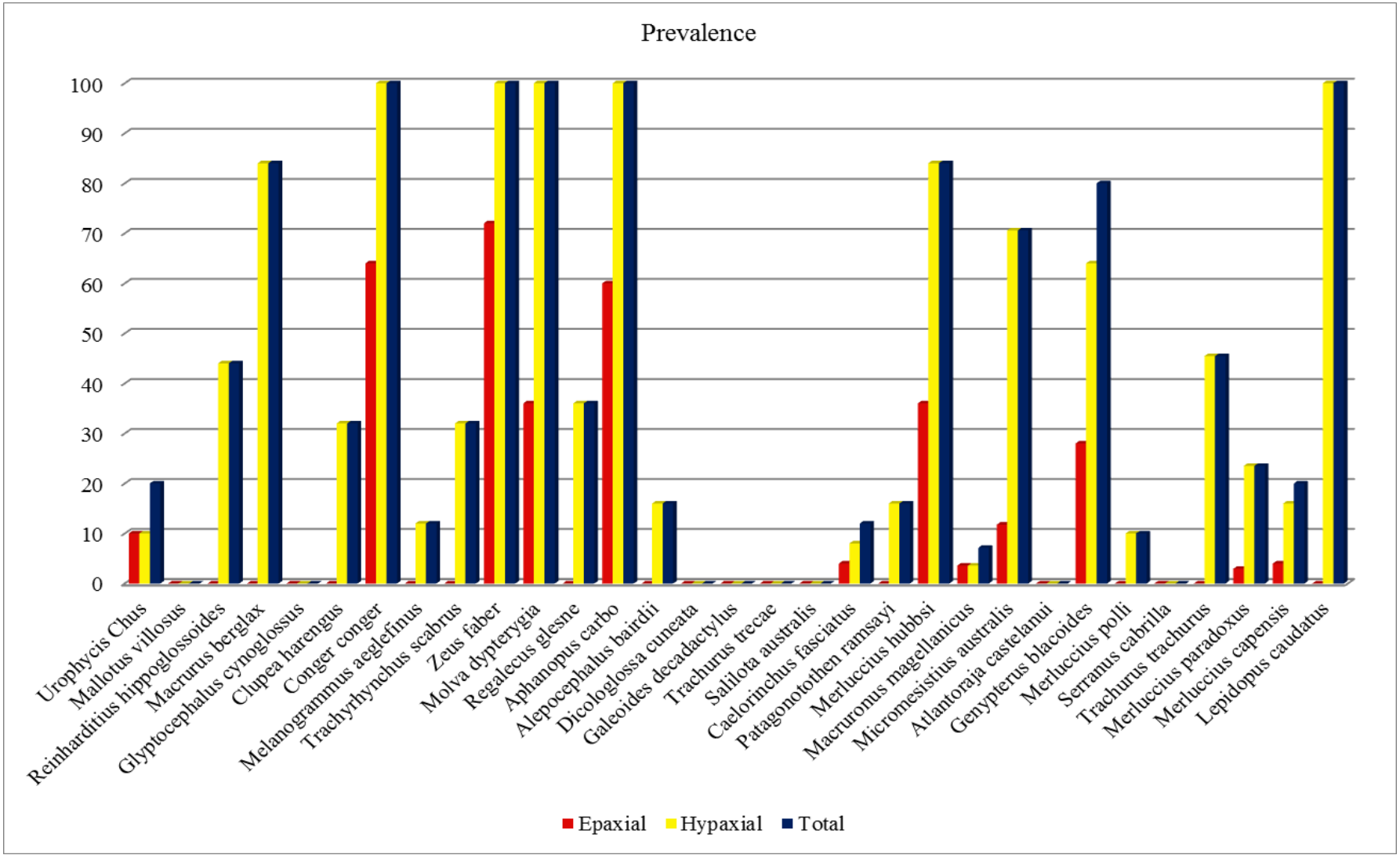

Figure 37: Parasites prevalence

A total of 194 anisakid larvae, previously identified to the genus level, were used for species identification by PCR-sequencing (Figure 38).

All the anisakid larvae detected in fish from FAO area 21 were identified as *A. simplex (s.s.)*; In FAO area 27 89,85% of parasites were identified as *A. simplex (s.s.)*, 7,24 % as *Pseudoterranova* spp. and 2,89% as *A. pegreffii*. In FAO area 41, up to 65,9 % of parasites were identified as *A. pegreffii*, 18,18 % as *A. simplex C*, 9,09 % as *C. osculatum*, 2,27 % as *Pseudoterranova spp.*, 2,27 % as *H. aduncum* and 2,27 % as *A. simplex (s.s.)*; In FAO area 47, a 63,82% of parasites were identified as *A. pegreffii*, 25,53% as *A. typica* and 10,63% as *A. simplex C*. No parasites were found in fish species belonging from FAO area 34 (Figure 39).

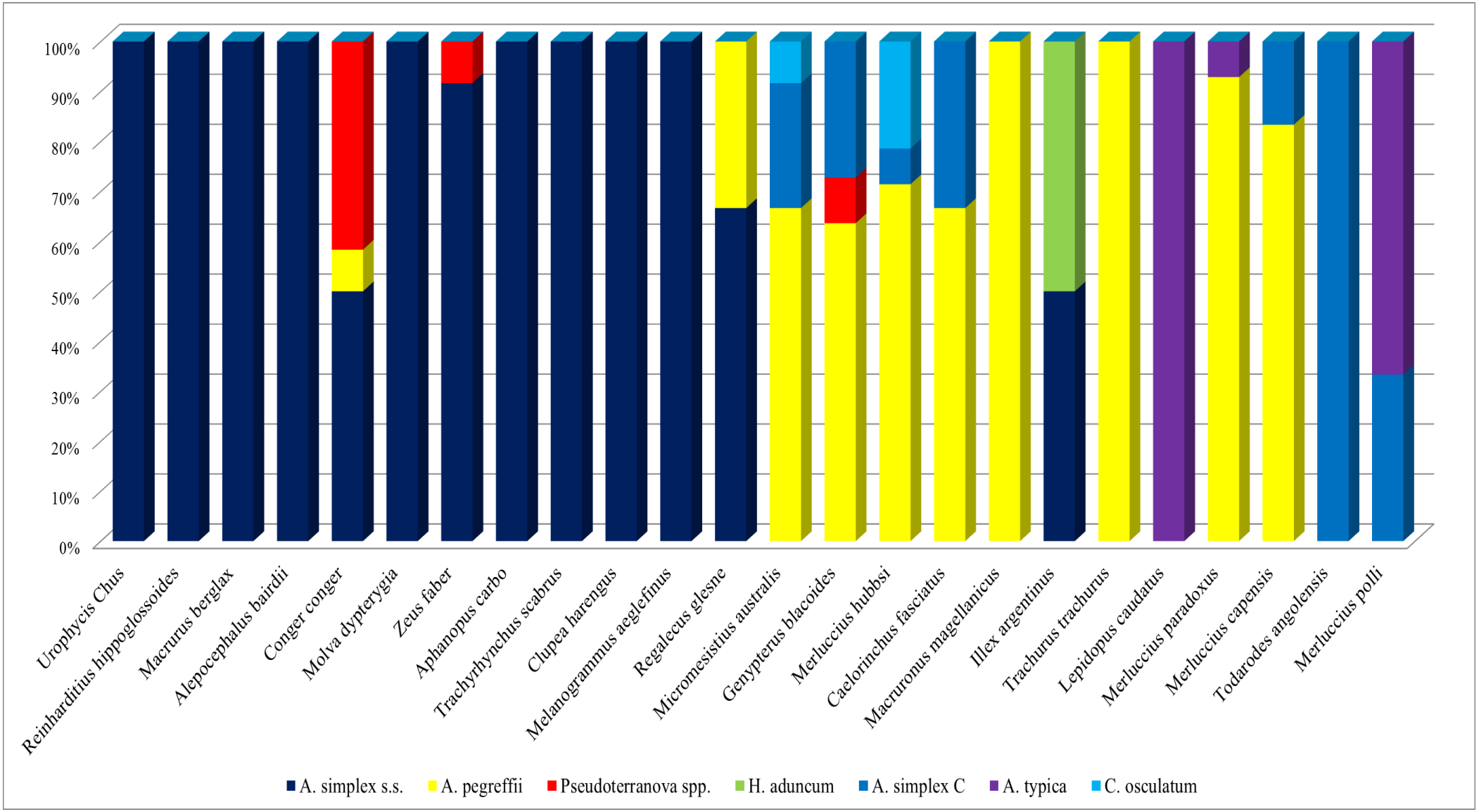

Figure 38: Parasites identification

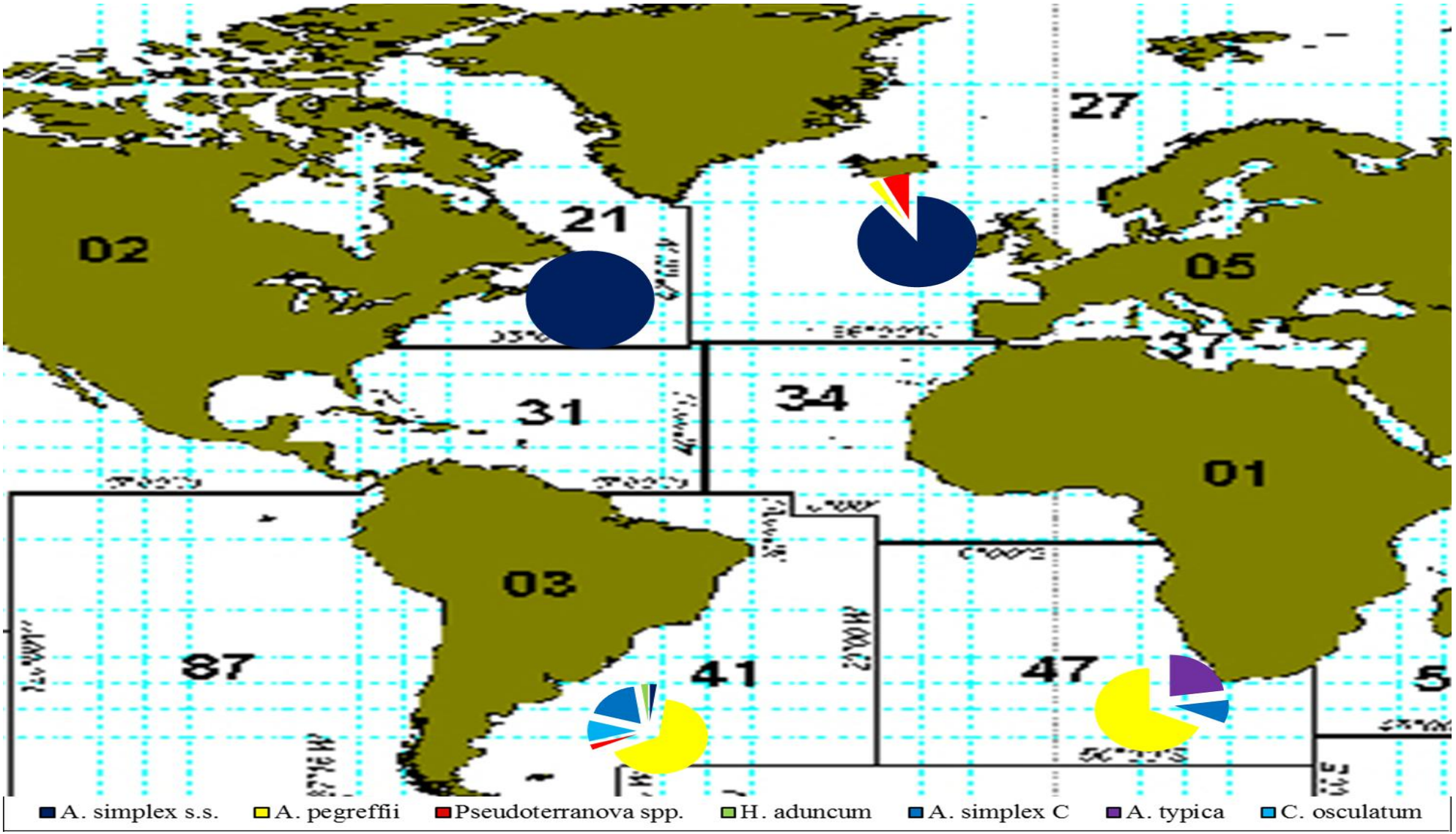

Figure 39: Parasites geographical distribution

The results showed mixed infection in 45,83 % of the infected fish analyzed. Table 15 reports the inspection data categorized by the SADE scoring system showing for each host species a “SADE Score” as results of the addition of the code points. In 24,25 % of cases, SADE score was 10, with no risk associated to those fish lots; On the other hand in 12,13 % of analyzed lots SADE score was 1, with a noticeable high risk because of a disseminated anisakid infection that could produce allergy for consumers and significant commercial rejection as a result of the unaesthetic appearance of a highly infected fish product.

Host	DENSITY (n. parasites/kg)			SADE CODE	SCORE
	Epaxial	Hypaxial	Total		
Alepocephalus bairdii	0	0,56	0,56	S2A2D2E0	6
Aphanopus carbo	1	91,625	92,625	S0A1D0E0	1
Atlantoraja castelanui	0	0	0	S3A2D2E3	10
Caelorinchus fasciatus	0,13	0,26	0,4	S0A2D2E0	4
Clupea harengus	0	1,73	1,73	S2A2D2E0	6
Conger conger	0,64	45,42	46,06	S0A1D0E0	1
Dicologlossa cuneata	0	0	0	S3A2D2E3	10
Galeoides decadactylus	0	0	0	S3A2D2E3	10
Genypterus blacoides	0,44	2,88	3,32	S0A2D1E0	3
Glyptocephalus cynoglossus	0	0	0	S3A2D2E3	10
Illex argentinus		0,44		D2	
Lepidopus caudatus	0	9,7	9,7	S2A2D0E0	4
Macruronus magellanicus	0,14	0,07	0,21	S0A2D2E0	4
Macrurus berglax	0	41,86	41,86	S2A2D0E0	4
Mallotus villosus	0	0	0	S3A2D2E3	10
Melanogrammus aeglefinus	0	0,8	0,8	S2A2D2E0	6
Merluccius capensis	0,22	1,14	1,37	S0A2D2E0	4
Merluccius hubbsi	0,73	4,06	4,8	S0A2D1E0	3
Merluccius paradoxus	0,16	2,35	2,52	S0A2D1E0	3
Merluccius polli	0	0,5	0,5	S2A2D2E0	6
Micromesistius australis	0,13	4,09	4,23	S0A2D1E0	3
Molva dypterygia	0,4	101,86	102,26	S0A1D0E0	1
Patagonotothen ramsayi	0	1,4	1,4	S2A2D2E0	6
Regalecus glesne	0	0,45	0,45	S2A2D2E0	6
Reinharditius hippoglossoides	0	7,14	7,14	S2A2D0E0	4
Salilota australis	0	0	0	S3A2D2E3	10
Serranus cabrilla	0	0	0	S3A2D2E3	10
Todarodes angolensis		1		D2	
Trachurus trachurus	0	7,72	7,72	S2A2D0E0	4
Trachurus trecae	0	0	0	S3A2D2E3	10
Trachyrhynchus scabrus	0	4,8	4,8	S2A2D1E0	7
Urophycis Chus	0,48	3,27	3,75	S0A2D1E0	3
Zeus faber	2,46	72,46	74,93	S0A1D0E0	1

Table 15: inspection data categorized by the SADE scoring system

5.4 Discussion

5.4.1 Epidemiology

Our study confirms the presence of anisakid species with public health concern (EFSA, 2010) in lots of fish species from different FAO areas. The high prevalence and remarkable mean intensity and mean abundance observed in this study support that *Anisakis* has the status of component parasite of many fish species and fishing areas; results confirm that *A. simplex (s. s.)* commonly occurs in various ecologically and economically important fish species such as herring, blue ling, argentine hake, european conger, Peter's fish and black scabbardfish. The blue ling and the european conger were the most highly infected species and this findings could be related to the feeding and predatory habits of these species which had as their main preys pelagic crustaceans and fish (such as roughhead grenadier), that, in this study, were also found infected by *Anisakis* larvae. As above mentioned, there was different geographical distribution of parasites that could reflect variations in the abundance of different hosts of *Anisakis* and *Pseudoterranova*.

A. simplex (s. s.) was never found in FAO area 47, while *Pseudoterranova spp.* was never found in FAO area 21 and FAO area 47. The life-cycles of *Anisakis* and *Pseudoterranova* are complex, involving many different potential hosts, and the factors governing the

geographical abundance of the life-cycles are not fully understood. The distribution of these parasites is further complicated by migrations of infected fish (Petrie, 2009).

Anisakis and *Pseudoterranova* are generally most abundant in European NE Atlantic waters that are traditionally some of the most productive fishing areas in Europe and the abundance of different hosts at all trophic levels presumably accounts for the overall abundance of the parasites.

A. pegreffii was never found in FAO area 21; Only 2 species showed mixed infection by *A. pegreffii* and *A. simplex (s. s.).*

5.4.2 Market considerations

Although the frozen condition assures no viable zoonotic parasite in the fish products analyzed, the risk of thermostable allergens in the edible part of fish for hypersensitized patients should be considered. Any-case, in some fish lots a high infection level could produce unaesthetic fish products lowering their commercial value. The standard quality for European fish production value chains, in agreement with the EU legislation, recognizes that any parasitized fish should be unfit for human consumption under a visual inspection scheme.

The implementation of this risk-based surveillance under the predictive SADE scheme makes it easier to categorise the risk of anisakids in the flesh of commercial fish. The scoring scheme which adopts a 10-point

scale help us to understand the severity of the risk and to early propose correction measures, within HACCP system, in those fish species with the lowest scores. This was the case in 29% of the fish lots (score 0-3) corresponding to *A. carbo, C. conger, M. dypterygia, Z. faber, G. blacoides, M. hubbsi, M. paradoxus, M. australis* and *U. chus*. In 22.5% of the fish lots the inspection was fair (score 4-5); finally in 48,5 % of the lots the score was good (score 6-7), very good (score 8-9) or excellent (score 10).

CHAPTER 6. TREATMENT FOR KILLING PARASITES IN FISHERY PRODUCTS

The critical control points for prevention of consumers' exposure to fishery-parasites are: the quality of the raw material, i.e. the catching or rearing of stock free from the parasites; the application of physicochemical treatments to fishery products to ensure killing of any parasites which may be present; or by the physical separation of parasite contaminated fishery products during processing. All three of these options are potential control measures for control of allergic diseases, and the second option (physical-chemical treatments to kill parasites) will also be effective at preventing infections. *A. simplex* allergens are highly resistant to heat and freezing (Falcao et al., 2008; Fernandez de Corres et al., 1996) therefore treatments which kill Anisakidae in fishery products may not protect the consumers against allergic hazards due to the ingestion: these aspects are not further considered in this chapter.

There is more information on the resistance to physical and chemical treatments by *A. simplex* than for other fishery parasites. The properties of *A. simplex* are likely to be similar to that of other multicellular parasites (although trematode metacercariae are considerably more heat

resistant), and information on other genera and species of organism will be given where available.

6.1 Assessing viability

Viability is here defined as the ability of individual anisakid larvae to survive various chemical and physical treatments or processing procedures. A viable larva is defined as that physically intact and motile, as demonstrated by spontaneous movements following stimulating by bending with forceps and a needle. It is not currently possible however to confirm that intact and motile larvae are capable of successfully infecting a human. It may be important in the future for the establishment of methods to assess whether viable larvae, as here defined, from different sources are capable to human infection.

6.2 Treatments defined by legislation

One of the first countries in Europe that applied freezing as preventive treatment for anisakiasis was the Netherlands in 1968, with the so called "Green Herring Laws" which stated that fresh herring should be frozen in such a manner as to reach a temperature of at least -20 °C within 12 hours and stored for a period of 24 hours prior to being released to the public. This resulted in a decrease of 40-50 human cases per year to less than 10 cases per year after the legislative action was implemented (Sakanari, 1995). In 1987 the EEC subsequently implemented legislation/recommendations for the similar freezing requirements (-20°C for 24h) as were implemented in the regulation of Netherlands (Eurofish-Report, 1987).

The EC Regulation 853/2004, modified by UE Regulation 1276/2011, states that fishery products to be consumed raw or almost raw and fishery products marinated and/or salted, if the processing is insufficient to destroy nematode larvae, requires freezing to a temperature of not more than –20 °C in all parts of the product for not less than 24 hours or −35 °C for not less than 15 h. The freezing treatment must be applied to either the raw or the finished product. The EC Regulation also states that food business operators need not carry out the freezing treatment if epidemiological data are available indicating that the fishing grounds of

origin do not present a health hazard with regard to the presence of parasites and the competent authority so authorises. Moreover, this regulation requires that a document from the manufacturer stating the type of process they have undergone must accompany treated fishery products when placed on the market, except when supplied to the final consumer (Chapter III, Section VIII, Annex III of Reg. 853/2004). Under the chapter V "Health standards for fishery products " in the same section, the Regulation also states that "food business operators must ensure that fishery products have been subjected to a visual examination for the purpose of detecting visible parasites before being placed on the market. They must not place fishery products that are obviously contaminated with parasites on the market for human consumption".

In the USA, the FDA (*Food and Drug Administration*) requires that all fish and shellfish intended for raw or semi-raw (e.g. marinated or partly cooked) consumption should be blast frozen to -35°C (-31°F) or below for 15 hours, or be completely frozen to -20°C (-4°F) or below for 7 days (FDA, 1998). The same freezing treatment is required in Canada (Weir, 2005). The temperature and time difference between the EU and US regulations reflects either the total storage time (FDA) or the time the product core achieves the critical temperature (AFSSA, 2007). These preventive measures have been adopted by the fish industry in Europe

and North America as part of their Hazard Analysis and Critical Control Points (HACCP) systems (Audicana and Kennedy, 2008).

The CODEX standard for salted Atlantic herring and salted sprat (CODEX, 2004) states that the viability of nematodes shall be examined after artificial digestion with magnetic stirring treatment. If living nematodes are detected, products must not be placed on the market for human consumption unless they are treated by freezing to -20ºC for not less than 24 h in all parts of the product, or adequate combination of salt content and storage time or by other processes with the equivalent effect.

6.3 Chemical treatment

Salting and marinating are the chemical treatments most commonly used to inactivate viable Anisakidae larvae.

6.3.1 Salting and Marinating

Anisakidae larvae are sensitive to salt only under certain conditions. It has been estimated that 28 days of storage in brine with 6.3% salt and 3.7% acetic acid in the aqueous phase of the fish was the maximum survival time for herring (Karl, 1995).

Marinating, is the process of soaking foods in a seasoned, often acidic, liquids with or without cooking. The aim of marinating is not only to inhibit the action of bacteria and enzymes, but also tenderize the connective tissue and change the taste, textural and structural properties of the raw material, resulting in a product with a characteristic flavour and an extended shelf life (Duyar, 2009).

The active ingredients of the marinade can include vinegar, lemon juice, wine, soy sauce, or brine. Early studies showed that *A. simplex* larvae are resistant to traditionally conditions of marinating and can survive 25 days in a mixture of salt and vinegar (Kuipers et al., 1960). Depending on the salt concentrations, the survival of larvae reaches 35 to 119 days (Karl, 1995). Thus the traditional marinating procedure for anchovies in vinegar has been one of the most important sources of human *A. simplex*

disease, both with and without allergic symptoms. Previous German and Danish procedures for marinating herring fillets require acetic acid concentrations of 5 to 7%, but require long treatment times to kill *A. simplex* larvae: 5 weeks by the German method with additional use of hydrogen peroxide, currently banned in several EU countries, and 6 weeks by the Danish method (Karl, 1995). Some preparations with short treatments for marinating are adopted due to economic

and organoleptic reasons, one recent study describes a marinating procedure for anchovies with the use of 10% acetic acid (vol/vol) plus 12% salt which guaranteed destruction of A. simplex larvae within 5 days (Sanchez-Monsalvez et al., 2005): the sensory characteristics of the product were shown to be satisfactory.

Arcangeli (1996) found that a marinade of vinegar (6% acetic acid) and 10% sodium chloride applied for 24 h to sardines, followed by the addition of sunflower seed oil and refrigeration for 13 days, inactivates all *A. simplex* larvae. The conditions for successful killing of *A. simplex* larvae (Table 16) in fishery products are reported.

Fish	Treatment	Parameters	Reference
Herring	salting	5% NaCl, > 17 weeks	(Karl, 1995)
		6-7% NaCl, 10-12 weeks[8]	
		8-9% NaCl, 6 weeks	AESAN, 2007
	dry salting	20 days	CEVPM, 2005
Anchovies	marinating	10% acetic acid plus 12% salt for a minimum of 5 days	Sanchez-Monsalvez, 2005
		2.4% of acetic acid and 6% of NaCl for 35 days	AESAN, 2007
		10% acetic acid, 12% NaCl for 5 days	Sanchez-Monsalvez et al., 2005
Sardines	marinating	6% acetic acid, 10% NaCl for 24h + 4°C for 13 days	(Arcangeli et al., 1996)
Herring	marinating	28 days in brine with 6.3% NaCl and 3.7% acetic acid	Karl, 1995
Sockeye salmon and canary rockfish	freezing	-35°C for 15h, followed by -18° for 24h	Deardorff and Throm, 1998
Arrowtooth flounder	freezing	-15°C for 96h; -20°C for 60h; -30°C for 20h; -40°C for 9h	(Adams et al., 2005)
In vitro larvae	freezing	L3 survived at -10°Cfor up to 4 h and at -5°C for 5 h. No survival at -15°C for few minutes	(Wharton and Aalders, 2002)
	heating	60°C for > 15 minutes	(Sanchez-Monsalvez et al., 2006)
	heating	>60°C (core temperature) for 1 min;	(Bier, 1976)
	heating	74° for 15 sec	Audicana and Kennedy, 2008
	heating	60°C for 10 min (3 cm thick fillet)	Wootten and Cann, 2001
	plant extract	[6]-shogaol at 62.5 µg/ml ; [6]-gingerol at 250 µg/ml	(Goto et al., 1990)
King salmon and Arrowtooth flounder	high pressure	414 MPa for 30-60 seconds	(Molina-Garcia and Sanz, 2002)
		276 MPa for 90-180 seconds	
		207 MPa for 180 seconds	
Herring	irradiation	6-10 kGy	(Van Mameren and Houwing, 1968)
Sea eel	irradiation	>1 kGy	Seo et al., 2006

Table 16: Condition for succesfull killing of A. simplex in fishery products

Considering the above presented data, many traditional marinating methods are not sufficient to kill *A. simplex* larvae. Chemical treatment, if not combined with freezing, must be optimised for each individual fish preparation, as survival *of A. simplex* larvae depends on various factors (fish size, fat content, and the active ingredients). Thus, studies on viability of larvae with marinating preparations for herring differ from those of anchovies. With respect to anchovies in vinegar sauce, the published data show effectiveness in killing *A. simplex* larvae if anchovies are treated with 10% acetic acid plus 12% salt for a minimum of 5 days.

According to the *Agencia Espanola de Seguridad Alimentaria* (AESAN, 2007), to kill A. simplex larvae, 35 days treatment together with 2.4% of acetic acid and 6% of NaCl are required. Thus, it can be concluded that vinegar and salt can reduce the hazard associated with A. simplex, but do not eliminate it nor do they reduce it to an acceptable level. Because of that, it is necessary to freeze products prior to marinating (for example Spanish escabechar).

6.3.2 Other chemical procedures

Vegetable products have been studied for their possible usefulness to kill *A. simplex* larvae under experimental conditions: shogaol and gingerol extracted from Zingiber officinale, as well as components of Perilla

leaves are able to kill *A. simplex* under specific conditions (Goto et al., 1990; Hierro et al., 2006; Hierro et al., 2004).

Other in vivo studies have been conducted about the activity against *A. simplex* larvae of chemical compounds, in particular monoterpenic derivatives obtained from different essential oils, such as alpha-pinene, beta-pinene, ocimene, myrcene, geranyl acetate, and cineole (Navarro et al., 2008). The most active compound was alpha-pinene. Further in vivo studies are required to investigate whether addition of these compounds to food could have a killing effects alone or in synergy with other compounds and treatments) on *A. simplex*. These in vitro studies should be extended in order to evaluate their usefulness in food processing.

6.4 Physical treatment

6.4.1 Freezing treatment

Factors affecting the efficacy of freezing for inactivating anisakid larvae include the temperature, time needed for reaching the final temperature in core fish tissues, maintenance time and fat contents of fish (AFSSA 2007).

One of the first study about effects of freezing on *A. simplex* larvae was conducted in 1953 (Gustafson, 1953), before the first legislative requirement about freezing fish for public health reasons (Green Herring Law) was adopted in the Netherland. In this initial study freezing and storage at -5 °C or -10 °C, even for several days, did not kill all the larvae. After 12 days at -10°C, 4% of larvae recovered were still alive. On storage at -17°C (internal temperature of -14°C), 5.5% of the larvae (33 out 600) were alive. After 24 hours and longer, the core temperatures had reached the freezer temperature and no live nematodes were recovered (Gustafson, 1953).

A. simplex larvae has been reported to survive freezing at -10°C for up to 4h which may be aided by the production of trehalose by the parasite which can act as a cryoprotectant (Wharton and Aalders, 2002). Monitoring of fish freezing in commercial blast freezers and under conditions which simulate those of a domestic freezer, indicate that it

may take several days for all parts of the fish to reach a temperature that will kill the larvae (for instance it was observed that 20 kg container of fish did not reach -35 °C until 28 hours;(Wharton and Aalders, 2002). This aspect, and the moderate freezing tolerance of larvae, emphasize the need for fish to be frozen at a sufficiently low temperature (at least -15 °C) for a sufficient time to ensure that fish are safe for consumption (Wharton and Aalders, 2002).

A study was conducted on fish fillets at -35° C for 15 hours to determine the effects of commercial blast-freezing on the viability of third-stage larvae of *A. simplex* encapsulated in the muscle and viscera of sockeye salmon (*Oncorhynchus nerka*) and canary rockfish (*Sebastes pinniger*). The frozen fish were subsequently stored at -18°C, and samples taken after 1, 24, 48, and 72 hours of storage.

Four live but comatose larvae were found out of 1671 larvae recovered after blast freezing and 1 hour storage at -18 °C, but no viable larvae were recovered from fish stored for the longer periods (Deardorff and Throm, 1988).

The EU Hygiene Regulation (Reg. 853/2004) requires that frozen fishery products must be kept at a temperature of not more than -18°C in all parts of the product. Some studies demonstrate that there is a direct correlation between time and temperature in order to kill *A. simplex*

larvae (Adams et al, 2005) and that 100% of A. simplex larvae in fish muscle are killed at a temperature of -15°C for 96h, so this minimum period should be recommended for the storage at -18°C, to ensure successful parasite killing.

For cestode larvae, the *Diphyllobothrium plerocercoid* is inactivated if the fish is kept in household freeze at -18 ºC for at least one day (Salminen, 1970).

Freezing of fish at -10 °C (5 days) will kill Clonorchis and Opisthorchis metacercariae (World Health Organization, 1979). It should be noted that 1-star and 2-stars freezers can reach temperature of -6°C and -12 °C respectively, and only 3-star and 4-star domestic freezers may operate at a temperature of -18 °C or less, thus the fish need to be frozen in all parts of the products for a time longer than 24 hours to ensure that nematode parasites are inactivated (Wharton and Aalders, 2002).

It is to be expected that the lethality of freezing to nematode larvae will be a function of temperature and dwell time. Even -5°C shows some lethality, but the results of the Gustafson experiments suggests the critical temperature to ensure a high proportion of nematodes are killed within several hours (> 24 hours) is at least -17 °C. It is also apparent from the results of the investigations reported that freezing of itself may not kill all larvae. A very small proportion of larvae may survive, but

they are moribund, that is, the larvae do not show spontaneous movement, but will move when stimulated. It is not known, but has been suggested that such moribund larvae are not be capable of infecting humans.

However, assuming motile larvae are infectious provides a greater margin of safety for example in HACCP analyses. Therefore freezing should be followed by a period of storage in the frozen state to ensure complete elimination of the infectious hazard.

6.4.2 Heat treatment

Studies showed that a core temperature of 60 °C for 1 minute is sufficient to kill any larva present in the fishery product (Bier, 1976). However, reaching such a core temperature depends on the product thickness and composition. It has been estimated that a 3 cm thick fillet should be heated at 60°C for 10 minutes to ensure all larvae are destroyed (Wootten and Cann, 2001).

Heating temperatures of ≥ 60 °C for at least 1 min when cooking or smoking fish (Bier, 1976), or heating temperatures up to ≥ 74 °C for at least 15 seconds when microwave cooking (Adams et al., 1999) of fish to be eaten raw have been recommended to kill the parasites and prevent infections (Audicana and Kennedy, 2008).

For *Diphyllobothrium spp*., plerocercoids do not survive temperatures above 56ºC. Thus, the infection risk is eliminated if the fish is fried, boiled, or adequately smoked (Salminen, 1970).

Thus freezing and cooking remain the reference processes guaranteeing the destruction of larvae, under well defined conditions. It should nevertheless be recalled that these treatments may not inactivate allergens. Treatments which provide an equivalent level of protection as freezing at a temperature of not more than -20ºC for not less than 24 hours in all parts of the product for the killing of *A. simplex* larvae include:

- Freezing at -35ºC for 15 hours or at -15°C for at least 96 hours;
- Cooking at > 60ºC for at least 1 minute (core temperature)

6.4.3 High hydrostatic pressure

High hydrostatic pressure has been demonstrated to be an effective technique for treating food to reduce the number of pathogenic microorganisms and to extend shelf life (Knorr, 1999). A pressure of 200 MPa for 10 minutes at 0-15 °C kills *A. simplex* larvae, as well as pressures down to 140 MPa when the treatment time is increased up to 60 minutes. In addition, cycles of compression and decompression applied for a specific time were found to be more effective at killing larvae than a single pressure treatment for a similar time (Molina-Garcia

and Sanz, 2002). It should be noted that such long treatment times would be impractical for the food industry. A pilot study was performed to determine the effect of high hydrostatic pressure on the viability of *A. simplex* larvae in raw fillets of king salmon and arrowtooth flounder, and to evaluate the effects of the treatment on the colour and texture of the fillets. Different pressure and time combinations were required to kill 100% of the larvae, and were as follows: 414 MPa for 30-60 seconds, 276 for 90-180 seconds, and 207 MPa for 180 seconds. For 100% killing, however, a significant increase in the whiteness of the flesh was observed: this effect on the colour and appearance of the fillet may limit its application to the processing of fish for raw consumption (Dong et al., 2003). However, pressure treatment could be applicable to processed fish, e.g. marinated and cold-smoked fish, where the tissues are already substantially modified. In these processes, the pressure needed to kill parasites could be lower when combined with other treatments (Molina-Garcia and Sanz, 2002). In a recent study, the application of a pressure of 300 MPa for 5 minutes has resulted in the inactivation of *A. simplex* larvae in the tissues of mackerel (*Scomber scombrus*), and a similar procedure has been suggested for the treatment of other fatty fish such as sardines and anchovies (Brutti et al., 2009). These experimental studies

should be extended in order to evaluate their usefulness in food processing.

6.4.4 Drying

No specific data was found in the literature on the efficacy of drying for inactivating parasitic larvae in fishery products, thus drying cannot be considered an effective treatment for that purpose.

6.4.5 Irradiation

In 1986, the Scientific Committee for Foods (SCF, 1986) concluded that fish and shellfish could be irradiated at doses up to 3 kGy (overall average irradiation dose), as those values were considered to be acceptable from a public health standpoint. Irradiation has been applied to fresh, frozen as well as dried fish, fishery products, and shellfish.

Irradiation doses that kill *A. simplex* larvae in salted herring were reported to be higher than 6–10 kGy (Loaharanu, 1997a; Van Mameren and Houwing, 1968). Similarly, another study found *A. simplex* larvae to be highly resistant to irradiation doses of 2 kGy or 10 kGy (FAO/IAEA 1992). Another recent study based on an in vivo experiment in rats demonstrates that *A. simplex* third-stage larvae in the sea eel (*Anago anago*) are not inactivated up to 1 kGy (Seo et al., 2006). Irradiation is therefore not effective in inactivating *A. simplex* larvae, since they

appear to be highly resistant to the irradiation doses which are normally recommended.

For liver flukes, investigations in Thailand demonstrated that low dose irradiation of freshwater fish can prevent infectivity of metacercariae of *O. viverrini* when such fish are prepared in local dishes made from raw or semi processed fish (Bhaibulaya, 1985). At 0.5 kGy, the metacercariae could not develop in hamsters and caused no infection in their livers. The effective inactivation of *Opisthorchis* metacercariae through irradiation has also been recently reported (Naz'mov et al., 2001), although high doses were used (12.5-25 kGy), much above the recommended levels. These experimental studies should be extended in order to evaluate their usefulness in food processing.

6.4.6 Low voltage current

A treatment to inactivate *A. simplex* larvae based on the application of electrical discharge through the fish has been patented in Spain in 2005 (ES 2 213 486 B1). The fish, either a single large fish (e.g. tuna) or pools of small fish (sardines, anchovies), are placed in an electrolyte bath. This is claimed to inactivate the larvae and leave the organoleptic properties as unaltered. Nevertheless adequate studies to prove the effectiveness of this method are not currently available. These experimental studies

should be extended in order to evaluate their usefulness in food processing.

6.4.7 Smoking treatment

Smoking techniques can be categorised into hot smoking and cold smoking. Hot smoking exposes foods to smoke and heat in a controlled environment; products are subjected to temperatures > 60 °C (average reference parameters: 70 °C - 80 °C for 3-8 hours approximately). *A. simplex* larvae are unable to withstand such conditions (FDA/CFSAN, 2001; Sainclivier, 1985). Cold smoking can be used as a flavour enhancer for example to salmon or scallops, and smokehouse temperatures for this process are maintained below 38 °C: the process lasts from a few hours to a several days. During cold smoking, temperatures are insufficiently high for killing parasite larvae (Khalil, 1969; Szostakowska et al., 2005), thus the products must undergo an initial inactivation treatment.

Gardiner reported that neither cold smoking for 12 h at 25,6 °C nor refrigeration for 27 days killed *A. simplex* larvae in salmon (Gardiner, 1990). This analysis indicated that fresh salmon and cold-smoked salmon had 1-3 and 1-5 *A. simplex* viable larvae per 200 g of fish, respectively. A similar result was found in whole Pacific herring (*Clupea harengus pallasi*), where *A. simplex* larval remained viability after

brining and smoking at an average temperature of 19 °C for 24 h was 100% and 87.5%, respectively (Hauck, 1977). Thus during hot smoking, products are treated at > 60 °C for some hours, and A. simplex larvae are unable to withstand such conditions. During cold smoking, instead, the temperature are too low (< 38 °C) in order to kill the parasitic larvae.

In summary, many traditional marinating and cold smoking methods are not sufficient to kill *A. simplex* larvae. Such treatment, if not combined with freezing, must be optimised for each individual fishery-product preparation, as survival of *A. simplex* larvae depends on various factors (fish size, fat content, and the active ingredients). Freezing raw fishery products prior to smoking remains the most effective way to ensure that viable parasites are killed in cold-smoked products to be consumed by the public. There is insufficient information to show that alternative treatments, including high hydrostatic pressure, drying, irradiation, and low voltage currents, are effective at killing anisakid larvae under conditions that preserve the products' organoleptic qualities.

CHAPTER 7. SURVIVAL OF *ANISAKIDS* LARVAE IN SALTED ANCHOVIES (*ENGRAULIS ENCRASICOLUS*) PREPARED ACCORDING TO TRADITIONAL PROCEDURE

7.1 Introduction

Anisakids have been found in parasitological studies of fish of the genus *Engraulis* (Rello et al., 2009, Sánchez-Monsalvez et al, 2005, Henning, 1974, Solas et al., 2009, Mladineoa et al., 2012, Song et al., 1995). These nematodes have also been detected as parasites of the European anchovy, *E. encrasicolus* belonging to the *Engraulidae* family (Cuéllar et al., 1991; De la Torre Molina et al., 2000; Osanz, 2001). Since the European anchovy represents one of the most attractive Mediterranean fisheries product (Solas et al., 2009) traditionally consumed without thermal preparation that has been already related to *Anisakis* zoonosis in men (López Peñas et al., 2000; Foti et al., 2002; AAITO-IFIACI Anisakis Consortium, 2011), it is necessary to include anchovies into more firm risk assessment frames. Most Italian cases of anisakidosis have been ascribed to Salted anchovies, a traditional heavy-salted and ripened food product, is another highly popular dish in Italy and in other Mediterranean countries prepared from fish of the *E. encrasicolus* species. Sodium chloride is an essential ingredient in the ripening

anchovy process, contributing not only to the flavour and texture of this type of product but also to the microbiological stability (Pleps et al., 2006). The salt penetration rate depends on thickness of muscle, temperature, freshness of fish and fat content (Clucas, 1982). The finished product, that contains approximately between 14 and 15 % of NaCl shows a tender consistency and a specific pleasant aroma and taste as a result of the enzymatic activity on the fish flesh (Fisinger et al., 1982) and contain an high nutritional value due to high content in polyunsaturated fatty acids.

Aim of study was to evaluate the survival of *anisakids* larvae, naturally present in fish, in salted anchovies prepared according to traditional procedure, at different stages of ripening process.

7.2 Methods

7.2.1 Sampling

Two different batches (A and B) of ripened salted anchovies were prepared using two different fish lots. Anchovies (9-15 cm - 150 kg, Figure 40) were caught in Adriatic sea along coast of Abruzzo region (Central Italy) on March and July 2013 for trial A and B respectively; they were held in ice and stored in refrigerated boxes. Temperature was monitored by the means of data-logger and didn't exceed +3 °C.

Figure 40: Anchovies

In approximately 5% of anchovies (500 anchovies, 8 Kg) demographic infection values were established according to Bush et al. (1997).

Nematodes (Figure 41) were isolated from fish trough visual inspection and by peptic digestion according to Llarena-Reino et al. (2013a). All

samples were transferred into beakers containing pepsin solution, placed on a ACM-11806 magnetic stirrer and set the heating plate at 37 °C; when the tissue was thoroughly digested, the solution was poured into a sieve. *Anisakidae* larvae were detected on the sieve, collected, counted and washed with 0.9% NaCl solution, and preserved in 70% ethanol for further identification; they then were examined under the stereomicroscope with transmitted light for their morphological identification according to Berland (1961), Smith and Wooten (1984), Peter and Maillard (1988), and Peter and Cabaret (1995).

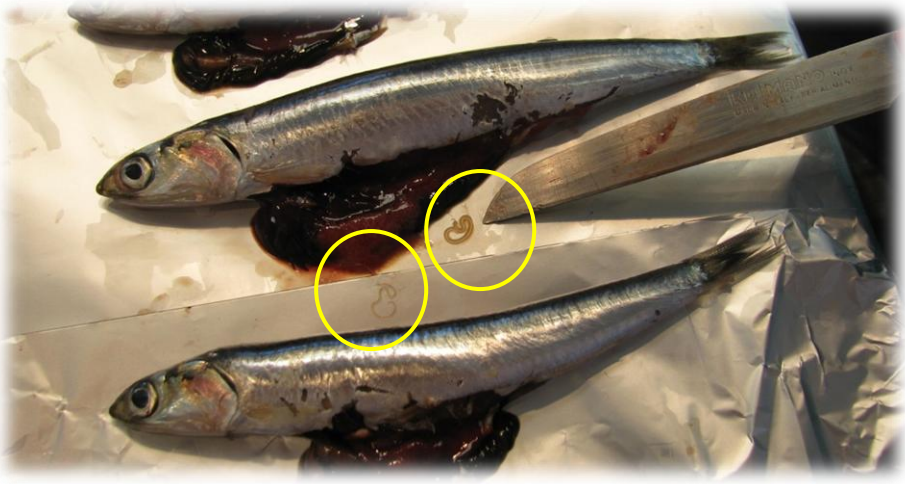

Figure 41: Anisakids in E. encrasicolus

The remaining amount of anchovies (about 142 Kg) were used to produce salted anchovies at processing factory according to the following traditional method.

7.2.2 Salting process

Intact fishes (not beheaded or gutted) were mixed with salt (100 kg NaCl; ratio fishes:salt = 3:2, Figure 42) according to the usual techniques used by the company.

Figure 42: First step of salting process

In order to avoid loss of parasites, intact anchovies, not eviscerated, were used.

The product was then placed in a 20 liter plastic drums and each drum was singly pressed with a weight of 25 kg. All the drums were kept in a room at a controlled temperature of 25 °C.

The trials were carried out for 120 days, which is considered the time required to obtain commercial salted anchovies. Sampling (Figure 43)

were performed weekly for the first month at day 0, 7, 15, 23, 30 and then monthly: during each interval n. 65 salted anchovies (about 1 kg) were taken at various drums heights (surface, middle and bottom of the drum) and, after removal of parasites, subjected to chemical analysis.

7.2.3 Genomic DNA extraction and PCR-RFLP analysis

The genomic DNA (gDNA) was extracted from the worm using the "Tissue and Hair extraction kit" (Promega, Italy) combined with the "DNA IQ system kit" (Promega) and eluted in a final volume of 100 µl. The rDNA region of approximately 1Kb, comprising the ITS-1 region, 5.8S gene, ITS-2 region plus approximately 70 nucleotides of the 28S gene, was PCR amplified using the oligonucleotides NC5 (forward; 5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (reverse; 5'-TTAGTTTCTTTTCCTCCGCT-3') from 2 µl of the extracted gDNA and gDNA of *A. pegreffi* was used as control. The PCR reaction was performed according the protocol described in Zhu et al. (1998), using the Go-Taq master mix DNA polymerase (Promega) in a T-personal thermocycler (Whatman-Biometra, Germany). Aliquots (5–10 µl) of individual PCR products were digested with 10 units of either the restriction endonuclease HinfI or HhaI in a final volume of 20 µl at 37 ◦C for 16 h according to D'Amelio et al. (2000) and Abollo et al. (2003).

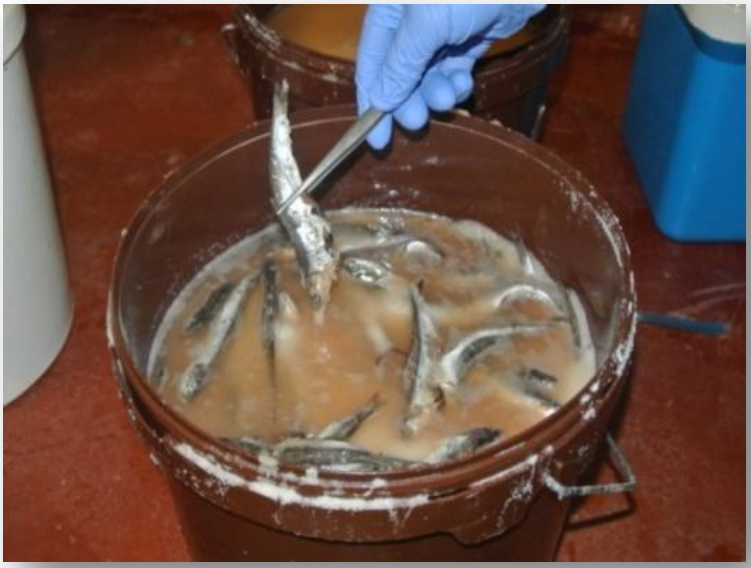

Figure 43:Sampling

7.2.4 Sensory quality

The evolution of the ripening process was assessed using the method developed by Fisinger et al. (1982) and modificated by Pérez-Villarreal et al. (1992). Sensory assessment of the salted anchovies fillets was carried out by a sensory panel of six trained panelists; six parameters were considered:

- flavour,
- flesh colour,
- odour,
- flesh texture,
- flesh adherence to backbone,
- saltiness perception.

The intensity of sensory properties was described through a descriptive scale with point range from 1 to 8 (1, raw fish; 6, optimum level of ripening; 8, spoiled). The scale was used to describe samples' overall acceptability. The fillets were placed in a plastic dish, held at room temperature for 30 minutes before served to the panellists. The panel evaluated the samples, coded with random three-digit numbers without any information on storage time. They were instructed to eat crackers and drink water between the product assessments. The average of the panelists judgment was taken as the score of the parameter.

7.2.5 Larvae viability

At each sampling intervall nematodes were carefully taken from fillets and viability was tested according to three procedures according to Leinemann and Karl, (1988) (A), Sommerville and Davey, (1976) (B) and to CODEX, (2004) (C):

A. Larvae were incubated at room temperature in 1% acetic acid for 24h and inspected microscopically for spontaneous movements and for movements stimulated by operator. Nematodes that didn't show spontaneous or stimulated movements were counted as dead.
B. Larvae were transferred into culturing medium (Medium 199) consisting of Earle's salts, L-glutamine and sodium bicarbonate and placed in thermostat for 24-48h at 37 °C with the aim to

simulate the conditions under which the parasite's life cycle proceeds.

C. Larvae were transferred into 0.5 % pepsin digestion solution and inspected visually for viability. Digestion conditions correspond to conditions found in the digestive tracts of mammals and guarantee the survival of nematodes.

When checking for viable nematodes in salted products, reanimation time of nematodes can last up to two hours and more.

7.2.6 Physicochemical analyses

Prior to analysis, whole fish were sliced and minced. The pH measurements was done with a digital pH-meter (Crison MicroTT 2022, Crison Instruments, Barcelona) by placing the electrode into the minced anchovies. The water activity (a_w) was measured (Aqualab 4TE – Decagon Devices Inc. USA). NaCl concentration was determined following the Volhard technique (Haouet et al., 2006). The TVB-N content of the sample was measured by the method of Conway's dish (Cobb et al., 1973). The TVB-N extract of the sample in 6% trichloroacetic acid (TCA, Sigma, St. Louis, MO, USA) was absorbed by boric acid and then titrated with 0,02 N HCl. All chemical tests were done in duplicate.

7.3 Results

7.3.1 Infection indexes

Infection indexes were reported in table 17.

	Average lenght (cm)	***Prevalence (% ± CI)***	***N. parasites***	***Mean abundance (± SD)***	***Mean intensity (± SD)***
Batch A	13,78	69,4 ± 0,04	1047	2,09 ± 1,975	3,01 ± 1,68
Batch B	13,96	65,6 ± 0,041	888	1,77 ± 1,93	2,76 ± 1,75

Table 17: Infection indexes

No difference between visual inspection and peptic digestion was noticed.

All anisakid larvae, isolated in each batch both in raw that in ripened samples, examined under the stereomicroscope for their morphological identification, belonged to *Anisakis* genus; a 5 % of *Anisakis* larvae (n. 100), previously identified at genus level, were used for species identification by PCR-sequencing.

All *anisakis* detected were identified as *A. pegreffii*.

7.3.2 Sensory Assessment

The behavior of sensorial characteristics was similar for both lots analysed; in small pelagic fish seasonal changes in fat content might affect the flavour and sensory characteristics of seafood products. In the first month all organoleptical parameters showed an increase and in the

following 3 month of ripening process all parameters were constant. In case of flesh adherence to backbone punctuation, at 30 days of ripening, both lots reached 5 points. Regarding to flavour and odour perception no bitter taste was perceived by panelists. The saltiness perception had a very high score at 23rd days of ripening until the end of the trial. With regard to textural properties the finished product showed a good panel acceptance. Sensory assessment during the whole ripening period were reported in figure 44.

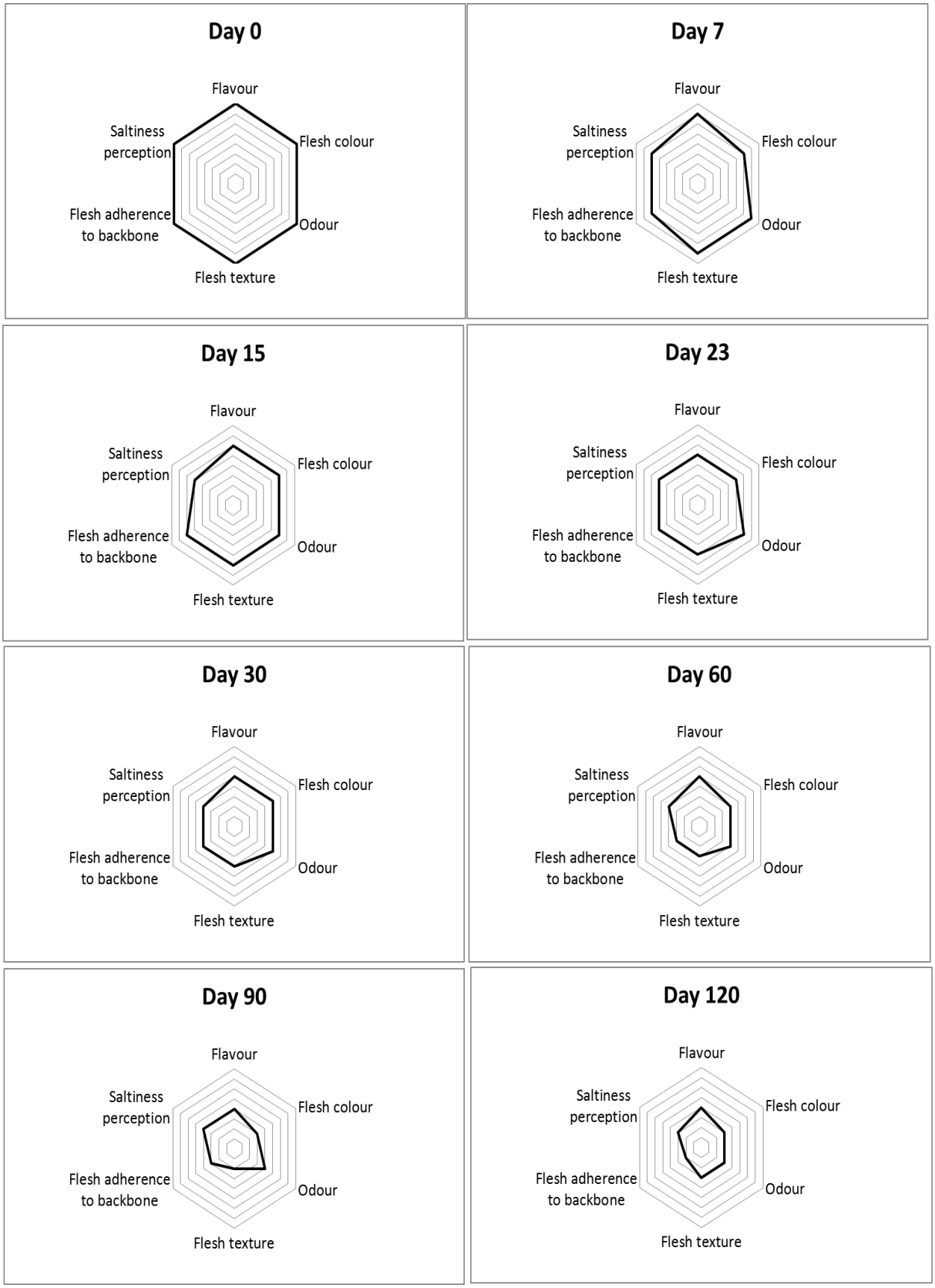

Figure 44: Evolution of sensory assessment

7.3.3 Physicochemical changes

The evolution of pH, $a_{w,}$ TVB-N (mg/100 g) and the NaCl (%) content was similar in the two batches (Table18, Figure 45, 46, 47, 48).

		Ripening time							
		0	**7**	**15**	**23**	**30**	**60**	**90**	**120**
Batch A	***a_w***	0,986	0,740	0,740	0,739	0,732	0,730	0,724	0,723
	pH	6,18	5,69	5,63	5,58	5,55	5,5	5,6	5,65
	NaCl	0,62	18,0	20,4	22,8	24,5	24,2	24,4	24,5
	TVB-N	10,93	12,76	20,3	27,8	31,1	37,2	45,7	50,1
Batch B	***a_w***	0,975	0,756	0,754	0,749	0,743	0,731	0,728	0,723
	pH	6,2	5,75	5,65	5,56	5,56	5,53	5,55	5,68
	NaCl	0,60	18,2	21,1	21,8	23,5	24,3	24,3	24,4
	TVB-N	12,4	13,5	20,3	25,7	33,6	38,6	44,6	53,2

Table 18: Evolution of pH, $a_{w,}$ TVB-N and the NaCl content in both batches during ripening process

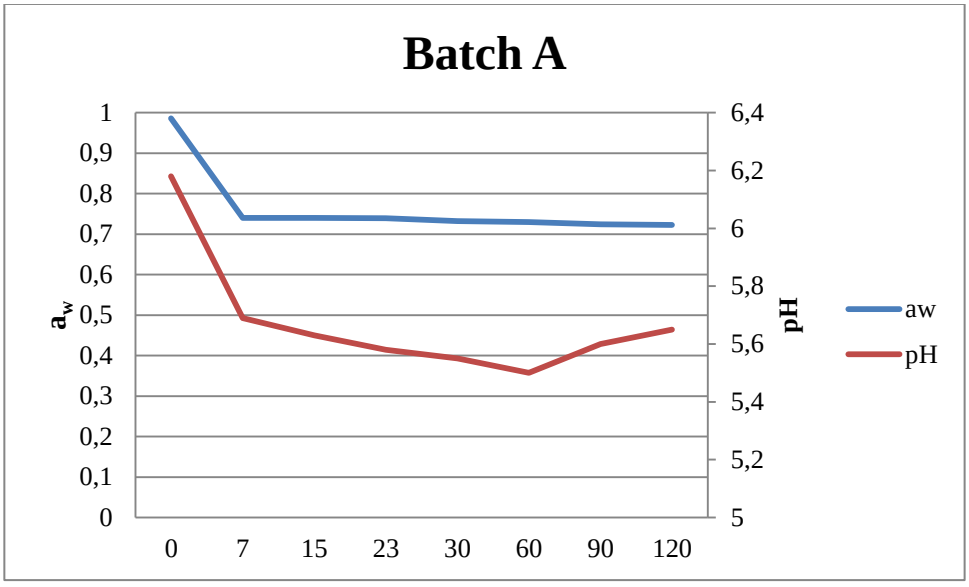

Figure 45: Evolution of pH and a_w for batch A during ripening process

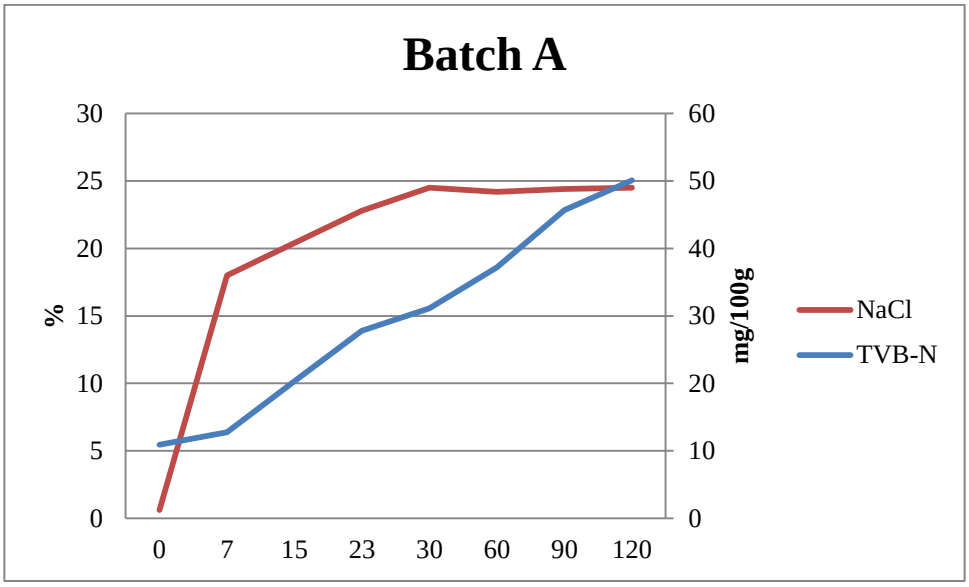

Figure 46: Evolution of TVB-N and the NaCl content for batch A during ripening process

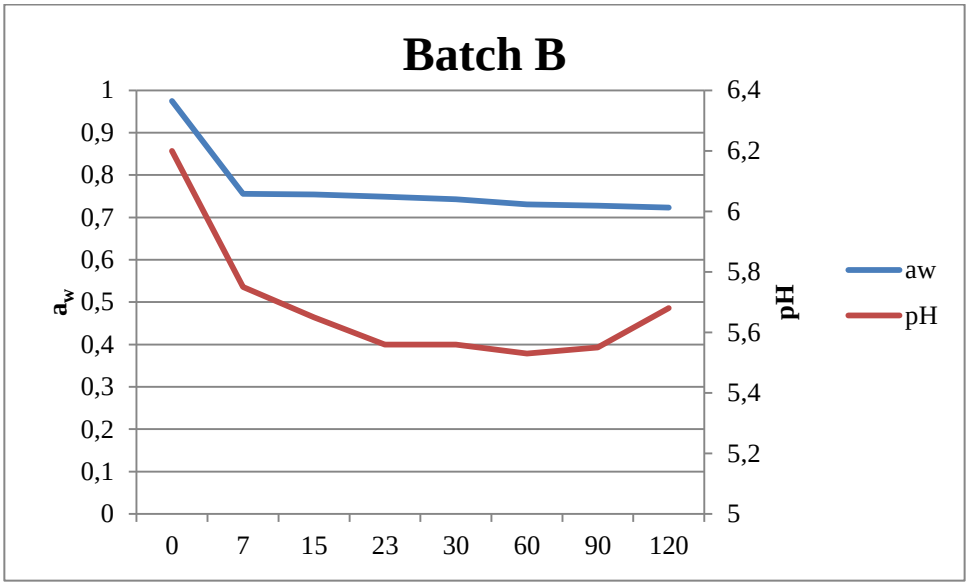

Figure 47: Evolution of pH and a_w for batch B during ripening process

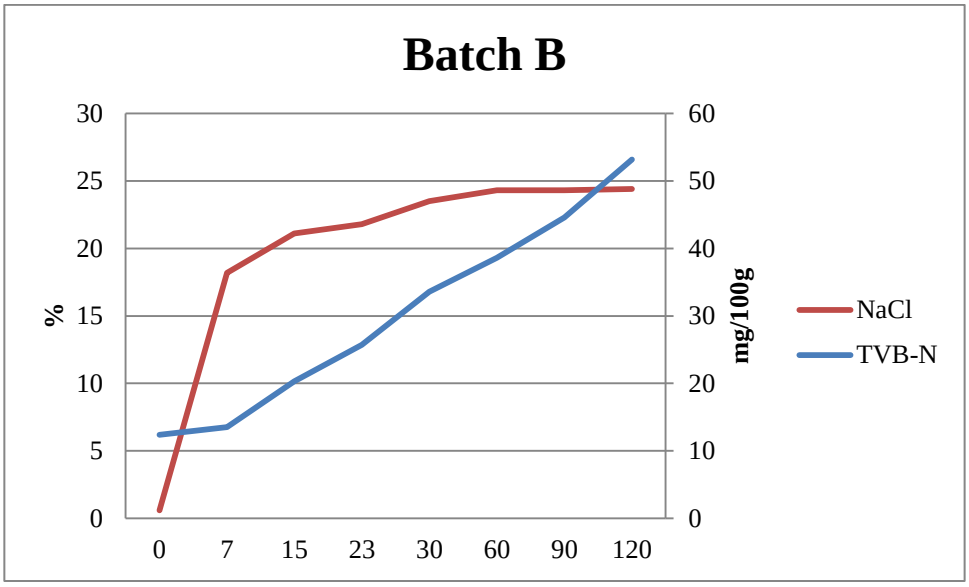

Figure 48: Evolution of TVB-N and the NaCl content for batch B during ripening process

After salting the chloride content (0,62%) increased rapidly in anchovies during the first week of salting up to 18 %. At the same time it can be observed a sharp decrease for a_w until values of 0,73. Salt content reached 24,5 % at the end of ripening process. A similar evolution has been reported by Srikar et al. (1993).

The pH of anchovy muscle decreased appreciably from 6,18 to 5,7, during the first week of ripening. Thereafter, the pH remained constant until the 90th day. A little increase in pH, observed during the last part of ripening, was related to the formation of volatile basic compounds (Yatsunami ea al. 1996). Moreover, according to Rodríguez-Jerez et al. (1993) salt concentration also could affects pH increase. Filsinger et al.

(1984) proposed the TVB-N determination as an objective index to monitor anchovy ripening.

TVB-N ranged from 10,93 to 53,2 mg/100 g; The increase in TVB-N values may be due to enzymatic and bacterial action, particularly to the growth of halophilic bacteria.

7.3.4 Larvae viability

All viable nematodes showed visible movements or spontaneous reactions when gently probed with dissecting needles at 0 and 7 ripening days.

Figure 49: Anisakid in salted samples

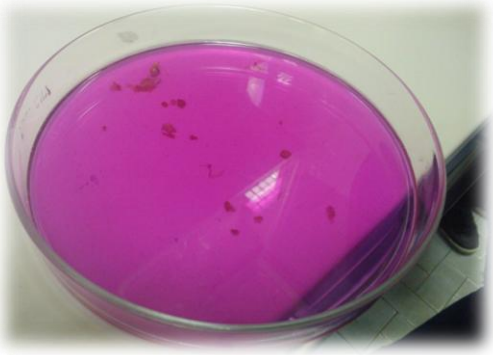

Figure 50: Viability evaluation

A single relaxation of coiled nematodes, which sometimes occurs, is not a clear sign of viability. Larvae not presenting spontaneous movements during viability test were counted as not viable. All larvae were not viable after 15 ripening days.

7.4 Discussion

Several authors have proposed a larval migration from viscera to musculature after the death of the host fish including fish of the genus *Engraulis*, such as *E. japonica* (Kino et al., 1993). Larval migration probably should produce a presence of larvae in fish muscle, increasing the risk of acquiring anisakiasis by the consumption of salted anchovies.

The EC Regulation 853/2004, modified by UE Regulation 1276/2011, states that fishery products to be consumed raw or almost raw and fishery products marinated and/or salted require freezing to a temperature of not more than –20 °C in all parts of the product for not less than 24 hours or −35 °C for not less than 15 h, if the processing is insufficient to destroy nematode larvae. A recent EFSA panel on risk assessment of parasites in fishery products stated that research on identification of alternative treatments for killing viable parasites in fishery products and on evaluation of their effectiveness compared to the freezing method described in the hygiene Regulations are needed. Study results showed that a salting period exceed 2 weeks is considered effective for the killing of parasites present in the product. It should however be stressed that the salting period appropriate to the achievement of the organoleptic characteristics of the product varies according to the company's procedures, but it is never less than 3

months. Companies only place on the market salted anchovies that elapse this time

It has been reported the ability of salt to inactivating nematodes; Grabda (1983) in a study on salted herring, stated that after 3 weeks of salting, with the salt content of the finished product between 12,2 to 14,6 %, there is no chance of infestations of humans by larvae present in the product.

In 2005 the "*Centre d'expérimentation et de valorization produits de la mer*" (CEVPM, 2005) has shown that under industrial production condition, a time of salting minimum of 21 days is necessary to allow the destruction of *A. simplex* larvae in salted herring fillets without freezing treatment.

Recently a report of the Scientific Committee of the "*Agencia Española de Seguridad Alimentaria y Nutrición*" (AESAN , 2007) regarding measures to reduce the risk associated with the presence of *Anisakis* stated that it is not necessary to freeze the product when:

- The concentration of salt in the fish reaches levels above 9% NaCl and remains unchanged for 6 weeks ;
- The concentration of salt in the fish reaches levels of NaCl between 10 and 20% and remains unchanged for four or five weeks (CODEX , 2004) ;

- The concentration of salt in the fish reaches levels of NaCl at least 20% and is maintained unchanged for 3 weeks (CODEX , 2004) ;

In our study, it was shown that, in salted anchovies, the dry salting process at mean salt concentration of 24% in all parts of the flesh at the end of the ripening period can be considered an effective method for devitalising anisakids larval forms present in the raw material and obtain a safe product for the consumer.

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