UNIVERSITÀ DEGLI STUDI DI

MODENA E REGGIO EMILIA

RESEARCH DOCTORATE IN FOOD AND AGRICULTURAL SCIENCE, TECHNOLOGY AND BIOTECHNOLOGY

Cycle XXXIII

ANISAKIS SPP. AS A POSSIBLE RISK FOR MEDITERRANEAN FISH CONSUMERS: OPTIMIZATION OF INSPECTION AND BIOMOLECULAR PROCEDURES

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ABSTRACT

My PhD thesis aimed to investigate the possible risks to Mediterranean consumers related to the presence of *Anisakis* spp. in fish and fish products of the FAO Zone 37 and adopt prevention procedures based on inspection and molecular techniques. The PhD activity was divided into 3 work packages. In the first work package, I analysed fish samples belonging to 5 species from markets of Sicily (Southern Italy) for the detection of *Anisakis* spp. nematodes by visual inspection and a modified chloro-peptic digestion, and their identification by molecular methods. The preliminary analysis of the fish samples showed the presence of 8172 larvae. All the fish species examined showed different prevalence of infestation, with a maximum of 100% in *Lepidopus caudatus* and a minimum of 2.7% in *Sardina pilchardus*. About 81% of the larvae examined by PCR-RFLP (Restriction Fragment Length Polymorphism) analysis belonged to *Anisakis pegreffii* species. The seasonal infestation trend of *Anisakis* was evaluated in all the fish samples examined. The results of the seasonal infestation trend showed a marked connection with the ecological aspects of the fish species examined. The results of this work package could be useful to plan a seasonal fishing strategy aimed at reducing the health risks of consumers related to *Anisakis.*

In the second work package, I assessed the possible presence of *Anisakis* spp. larvae in aquaculture fish samples. In particular, I examined 151 *Dicentrarchus labrax L.* samples from farms and fish markets of Sicily (Southern Italy) for *Anisakis* larvae detection. All the samples were examined by visual inspection and modified chloro-peptic digestion. Two nematode larvae were found in the viscera of only one *Dicentrarchus labrax L.* sample from a farm located in Greece (FAO 37.3), giving a total prevalence of infestation of 0.7%. The larvae were molecularly identified as *Anisakis pegreffii*. This is the first report on the presence of *Anisakis* parasites in farmed *Dicentrarchus labrax* of Mediterranean Sea, suggesting that the risk of exposure to *Anisakis* spp. in farmed fish remains very low.

In the third work package, I optimized and validated a commercial system based on Loop-Mediated Isothermal Amplification (LAMP) technique for the sensitive and rapid detection of *Anisakis* spp. DNA in processed fish products. Processed fish samples experimentally infected with *Anisakis* spp. larvae and DNA were used for specificity and sensitivity tests. The validation of the LAMP assay gave positive amplification for all the processed fish samples artificially contaminated with *Anisakis* spp., giving sensitivity values equal to 100%. The specificity tests provided no amplification for other Anisakidae and Raphidascarididae genera and uninfected samples. The limit of detection (LOD) of the LAMP assay proposed was 10^2 times lower than the real-time PCR method used for comparison purposes. To the best of my knowledge, this is the first report regarding the application of the LAMP assay for the detection of *Anisakis* spp. in processed fish products. The LAMP assay optimized and validated could be a reliable and convenient tool for the rapid detection of *Anisakis* DNA in processed fish products, useful in preventing the possible presence of *Anisakis*-related allergens and safeguarding the health of sensitive consumers.

SINTESI

La mia tesi di dottorato ha avuto lo scopo di indagare sul possibile rischio per i consumatori del Mediterraneo relativo alla presenza di larve di *Anisakis* spp. in prodotti della pesca della zona FAO 37, ed adottare procedure di prevenzione basate tecniche ispettive e lo sviluppo di metodiche molecolari. L'attività di dottorato è stata suddivisa in 3 work packages.
Durante il primo work package ho analizzato campioni di pesce appartenenti a 5 specie provenienti da mercati ittici della Sicilia per la rilevazione, mediante esami ispettivi (ispezione visiva e digestione cloro-peptica modificata) di nematodi appartenenti al genere *Anisakis* e la loro successiva identificazione mediante metodi molecolari. Le analisi ispettive preliminari hanno rivelato la presenza di 8172 larve di *Anisakis* spp. I campioni di pesce esaminati hanno mostrato differenti valori di prevalenza di infestazione, con un massimo del 100% nei campioni di *Lepidopus caudatus* ed un minimo del 2,7% nei campioni di *Sardina pilchardus.* Circa l'81% delle larve esaminate attraverso metodo PCR-RFLP (Restriction Fragment Length Polymorphism) appartenevano alla specie *Anisakis pegreffii.* Successivamente, è stato analizzato il trend stagionale di infestazione di *Anisakis* nei campioni esaminati. I risultati hanno mostrato una marcata relazione con gli aspetti ecologici delle specie di pesce esaminati. I dati ottenuti in questo *work package* si rivelano utili per una possibile pianificazione stagionale dell'attività di pesca al fine di ridurre possibili rischi ai consumatori legati alla presenza di *Anisakis* spp.
Nel secondo *work package*, ho valutato la possibile presenza di larve di *Anisakis* spp. i campioni di pesci d'acquacoltura. In particolare ho analizzato 151 campioni di spigola (*Dicentrarchus labrax L.*), provenienti da mercati ittici della Sicilia, per la ricerca di larve di *Anisakis* spp. I campioni sono stati analizzati attraverso ispezione visiva e digestione cloro-peptica modificata. Le indagini hanno rilevato la presenza di due larve di *Anisakis* spp. nei visceri di un solo campione di spigola proveniente da acquacoltura greca (FAO 37.3), determinando una prevalenza di infestazione totale del 0,7%. Le due larve sono state identificate molecolarmente come *Anisakis pegreffii*. I risultati ottenuti rivelano in primo report sulla presenza di parassiti *Anisakis* spp. in pesci di allevamento del Mediterraneo, confermando comunque un basso rischio d'esposizione ad *Anisakis* per questi prodotti.
Nel *work package* finale ho ottimizzato e validato un sistema commerciale basato sul metodo LAMP
(Loop-Mediated Isothermal Amplification) per la determinazione rapida e sensibile della presenza di DNA di *Anisakis* spp. in prodotti della pesca trasformati. Sono stati utilizzati campioni infestati sperimentalmente da DNA e larve di Anisakidae e Raphidascarididae per testare la specificità e sensibilità del metodo. Il processo di validazione ha determinato una positività per tutti i campioni infestati con *Anisakis* spp., mostrando valori di sensibilità pari al 100%. Il test di specificità non ha determinato alcun segnale di amplificazione per altri parassiti Anisakidi e Raphidascarididi e per campioni non infestati artificialmente. Il limite di rivelabilità del metodo (LOD) ottenuto si è rivelato 100 volte inferiore del metodo di Real-Time PCR usato come confronto. I risultati ottenuti si riferiscono al primo studio sull'applicazione del metodo LAMP per la ricerca di *Anisakis* spp. nei prodotti della pesca trasformati. Il metodo ottimizzato e validato si rivela un economico ed affidabile strumento di autocontrollo nel prevenire la possibile presenza di allergeni *Anisakis*-correlati nei prodotti della pesca trasformati, utile alla salvaguardia dei consumatori sensibili.

1. INTRODUCTION

In recent years, the public and scientific attention has been particularly focused on the research of parasites during the production and marketing of fish products.

The fish products occupy an important position in the international trade, as their world demand is constantly increasing. For this reason, it is important to guarantee the safety of these products in order to protect the consumers. Nowadays, European consumers are increasingly protected not only by the increase and accuracy of the controls implemented but also by the continuous research works in various research centers, in order to broaden the knowledge on parasites belonging to the marine environment.

In particular, the parasites that we refer to are those belonging to the Anisakidae family. The marine roundworms, belonging to this large family, are among the most studied parasites since the presence of some species has immediate repercussions on human health and causes considerable economic damages. The economic damage, in fact, is significant due to the enormous quantities of fish seized every year (Urquhart et al., 2002).

Specifically, this PhD thesis is focused on the nematodes belonging to the genus *Anisakis* since, in addition to their high zoonotic potential, these parasites are characterized by a high number of hosts involved in their biological cycle. Indeed, *Anisakis* spp. is one of the most common parasites that can be found in marine organisms all over the world, with the characteristic of infesting the viscera and musculature of many species of teleost fish (Cipriani et al., 2016; Mattiucci et al., 2004; Nieuwenhuizen and Lopata, 2013 ; Setyobudi et al., 2011). Abollo et al. (2001) stated that *Anisakis* is a parasite with a wide preference for hosts, in fact it is found in over 200 species of fish, 25 species of cephalopods and in over 50 species of mammals around the world.

From recent studies (Mo et al., 2014) it was possible to ascertain the presence of these parasites in farmed fish. Furthermore, the larvae of *Anisakis* spp. are gaining an increasing attention in the feed sector since the allergens of *Anisakis* spp., possibly present in fishmeal-based feed, can cause allergic reactions in humans despite the larva being no longer viable (Armentia et al ., 2006; Fæste et al., 2015). In fact, it has been shown how these allergens can transfer from feed to the final product as they are resistant to the temperatures adopted for cooking and to the digestive process of the fish host.

The following paragraphs will illustrate the risks related to the presence of anisakid parasites in the fishery products most consumed in the Mediterranean, highlighting their geographical distribution. Furthermore, given the few studies carried out in the sector, the presence of parasites of *Anisakis* spp. in aquaculture fish taking a further look at the breeding sector of euryhaline species.

1.1 Systematic classification of the Anisakidae family

The Anisakidae family includes several genera of nematodes that carry out their life cycle in the marine environment. Anisakids have a cosmopolitan

distribution and a life cycle involving invertebrates, fish and marine mammals (Gaglio et al., 2018; Llarena-Reino et al., 2012).

Over the past fifty years, the systematic classification of this family has been the subject of controversial debates. In fact, for some authors the Anisakidae family included other subfamilies, while for others it was a separate family, separate from the Raphidascarididae family (Pereira and Luque, 2017).

In 1974, Gerhard Hartwich proposed a first classification scheme for the members of the Anisakidae family based, on the characteristics of the excretory system and the digestive tract. According to this author, three subfamilies can be distinguished: Anisakinae, Geoziinae and Raphidascaridinae (Lymbery and Cheah, 2007; Mattiucci and Nascetti, 2008).

In 1991, Hans Peter Fagerholm, using a series of studies, proposed a new classification by dividing the superfamily Ascaridoidea into two families: Anisakidae and Raphidascarididae. The Anisakidae family was made up of the Anisakinae subfamily to which the genera *Anisakis* and *Pseudoterranova* belonged and the Contracaecinae subfamily which included the genera *Contracaecum, Galeiceps* and *Phocascaris* (Fagerholm, 1991; Lymbery and Cheah, 2007) (**Table 1**). Instead, several genera belonged to the Raphidascarididae family, including *Hysterothylacium* and *Raphidascaris*.

	Hartwich, 1974	Fagerholm, 1991	
Family	Anisakidae	Anisakidae	Raphidascarididae
Subfamily	Anisakinae	Anisakinae	
	Geoziinae		
	Raphidascaridinae	Contracaecinae	

Table 1 – Anisakidae classification schemes proposed by Hartwich, 1974 and Fagerholm, 1991.

The advent of molecular methods has made the possibility to highlight the different genetic structure in ascarids (Mattiucci and Nascetti, 2008). Consequently, such molecular methods have provided a significant contribution to the understanding of the systematics and biology of these nematodes (Costa et al., 2013; Li et al., 2018).

Recently, the classification most referred was based on the subdivision into two subfamilies: the Anisakinae subfamily which included genera such as *Anisakis*, *Pseudoterranova*, *Contracaecum*, *Phocascaris* and the Raphidascaridinae subfamily which included *Hysterothylacium*, *Raphidascaris* and other genera (Griglio et al. , 2012). However, several studies have been conducted for a couple of years, the results of which have led to consider the families Anisakidae and Raphidascarididae separate and to include, in the Anisakidae family, two subfamilies (Anisakinae and Contracaecinae) (Li et al., 2018; Pereira and Luque, 2017).

Therefore, the current classification of parasites belonging to the Anisakidae family is based on morphological and genetic characteristics (Li et al., 2018) and reflects that proposed by Fagerholm.

The systematic classification is shown below:

- Phylum NEMATODA
- Classe SECERNENTEA
- Ordine ASCARIDIDA
- Superfamiglia ASCARIDOIDEA
- Famiglia ANISAKIDAE
    - Sottofamiglia ANISAKINAE

        Genere *Anisakis*

Genere *Pseudoterranova*

- Sottofamiglia CONTRACAECINAE

Genere *Contracaecum*

Genere *Phocascaris*

- Famiglia RAPHIDASCARIDIDAE
  - Sottofamiglia RAPHIDASCARIDINAE

Genere *Hysterothylacium*

Genere *Raphidascaroides*

Genere *Raphidascaris*

1.2 Life cycle of the Anisakidae family

The biological cycle of anisakid nematodes involves different hosts and takes place in the marine environment through the succession of four stages of development (from L1 to L4) (**Figure 1**) (Buchmann and Mehrdana, 2016).

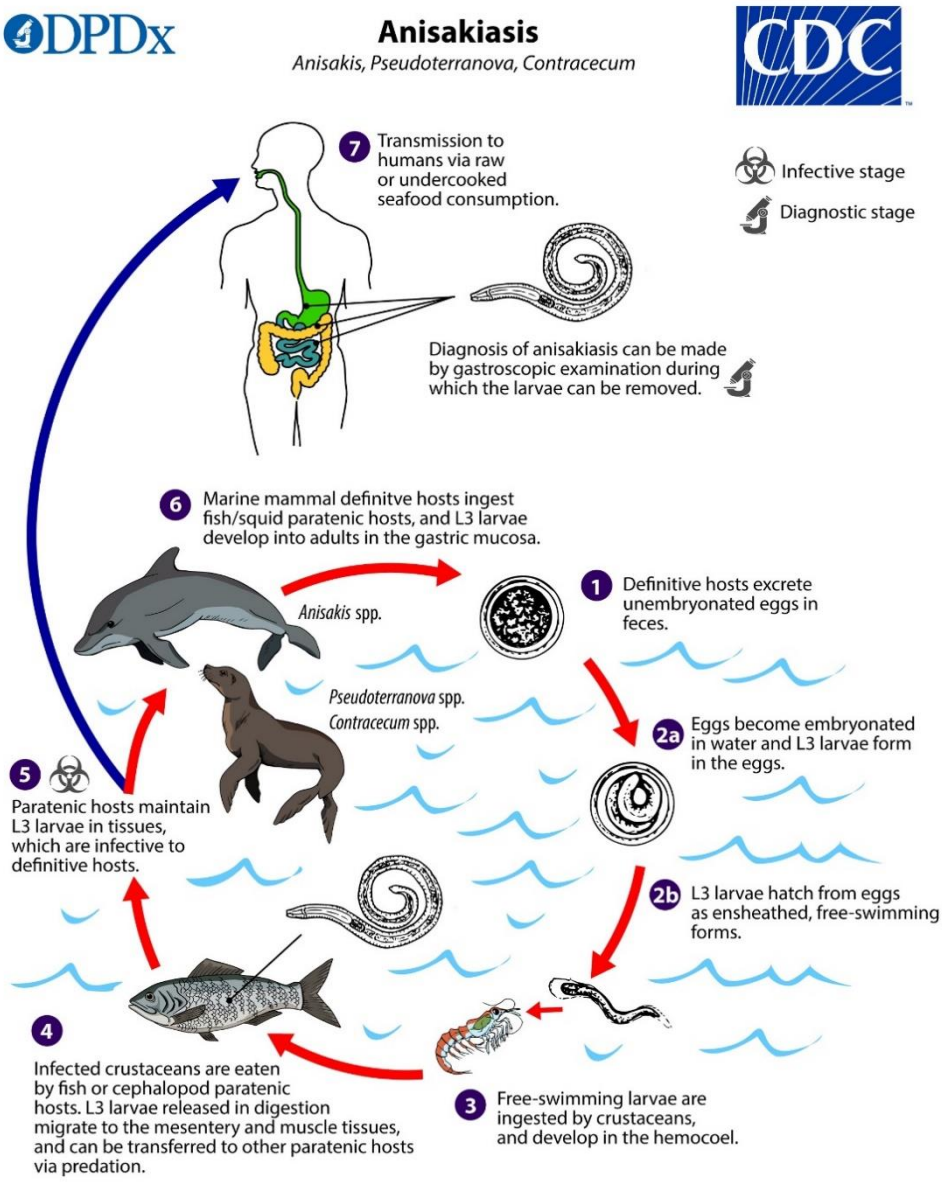


Figure 1. Schematic representation of Anisakidae family life cycle.

Marine mammals and piscivorous birds are the final hosts, while invertebrates, such as small crustaceans, and fish are the intermediate hosts. Specifically, fish act as main diffusion hosts (Bernardi, 2009; Chai et al., 2005; Cipriani et al., 2016; Griglio et al., 2012).

In particular, the definitive host varies in relation to the different parasite genera: the adult forms of *Anisakis* spp. are mainly found in the gastrointestinal tract of cetaceans (dolphins, porpoises and whales) (Griglio et al., 2012); those of *Pseudoterranova* spp. have pinnipeds (seals, sea lions and walruses) as final hosts (Chai et al., 2005; Griglio et al., 2012); the genus *Contracaecum* has ichthyophagous birds (cormorants, pelicans and herons) as definitive hosts and, to a lesser extent, pinnipeds (Buchmann and Mehrdana, 2016; Griglio et al., 2012);

finally, the genus *Hysterothylacium* has predatory teleosts as definitive hosts (Costa et al., 2018; González, 1998; Griglio et al., 2012).

It is important to note that it is possible to find parasite species belonging to the same genus in different intermediate hosts. For example, *A. simplex* s.s. (*sensu stricto*) is often found in demersal fish while *A. pegreffii* is frequently found in pelagic fish (Mattiucci et al., 1997). These differences appear to be related to the geographic distribution and dietary habits of the hosts than to the preferences of the parasites (Chai et al., 2005).

The cycle begins when the definitive host, through the faeces, releases the eggs produced by the adult parasites into the aquatic environment (Buchmann and Mehrdana, 2016), giving rise to the L1 larvae. These, once free in water, mature in the second stage L2 (Griglio et al., 2012). The cycle continues inside the first intermediate hosts as small crustaceans (amphipods and copepods), which ingest the larvae which in turn are preyed upon and ingested by fish and cephalopods (second intermediate or paratenic hosts) (Buchmann and Mehrdana, 2016; Chai et al., 2005). In these hosts, the parasite evolves to the third larval stage (L3) by penetrating the intestine and peritoneal cavity (Bernardi, 2009; Pravettoni et al., 2012). The larvae can reach a length of 1-4 cm and can migrate from the abdominal cavity, penetrating the internal organs (**Figure 2**) and become embedded in the muscle tissue of the fish (Bernardi, 2009; Griglio et al., 2012). In fish and cephalopods, visceral localization is clearly prevalent compared to muscle (Griglio et al., 2012).

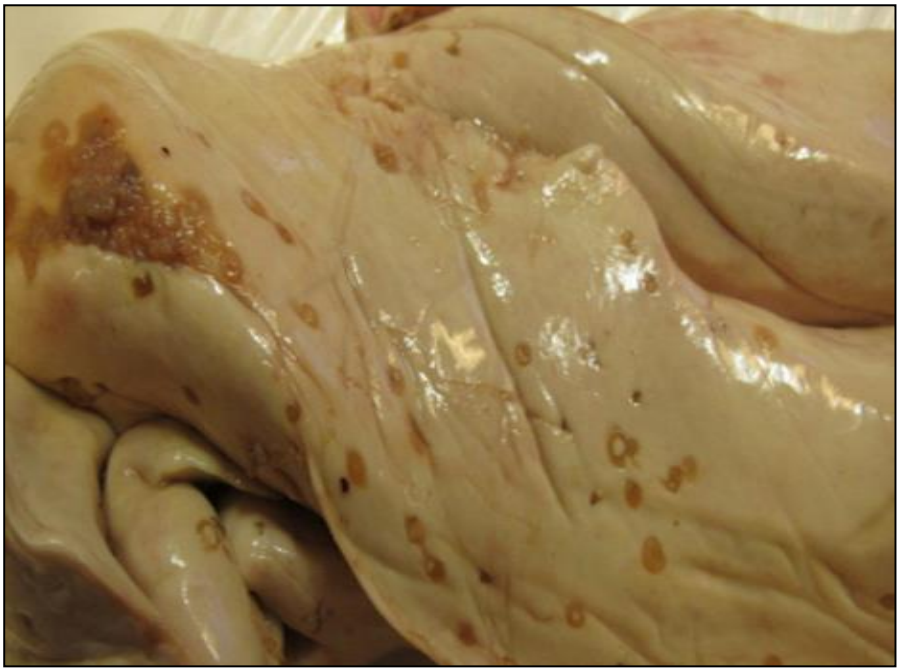


Figure 2 – Liver of *Gadus morhua* samples infested by encapsulated L3 *Anisakis* larvae (Buchmann e Mehrdana, 2016).

The main intermediate hosts that ingest these parasites, feeding on the infested crustaceans, are herrings (*Clupea harengus*), anchovies (*Engraulis encrasicolus*), sardines (*Sardina pilchardus*), mackerels (*Scomber scombrus*) and cod (*Gadus morhua*) (Abollo et al., 2001; McClelland and Martell, 2001). These intermediate fish are preyed upon by fish such as barracuda (*Sphyraena barracuda*), monkfish (*Lophius americanus*), conger eel (Conger conger) and silver scabbardfish (*Lepidopus caudatus*), which are paratenic hosts, i.e. hosts in which the parasite does not undergo to a development stage (Abollo et al., 2001; McClelland and Martell, 2001).

The parasites' life cycle ends when marine mammals or ichthyophagous birds feed on infested fish or cephalopods, representing the definitive hosts (Chai et al., 2005). In these animals, the larvae are localized in the stomach and intestines, maturing up to the fourth larval or pre-adult stage L4 and then develop up to the adult form (Griglio et al., 2012). This adult parasite lives in the stomach of the definitive host, where it reproduces.

The fertilized eggs are put back into the water through the feces of the hosts, starting a new cycle.

A very important aspect is related to the ease of *Anisakis* and *Pseudoterranova* parasites from one parathenic host to another, leading to the accumulation of a very high number of larvae, especially in adult fish and in large ones (Chai et al., 2005; Griglio et al., 2012). Furthermore, Adams et al. (1997) stated that demersal species tend to accumulate a greater number of nematode parasites during their life cycle.

Humans can accidentally be part of the biological cycle as a consequence of the consumption of fish or cephalopods infested with larvae (L3), in the muscle or in the viscera (Chai et al., 2005). The larvae do not complete their development in the accidental host but can penetrate the gastric or intestinal mucosa causing a series of symptoms and disorders (Ghittino, 1985; Nieuwenhuizen and Lopata, 2013).

1.3 Morphological characteristics of the parasites belonging to the Anisakidae family

Anisakid nematodes are filiform, cuticle-coated, circular-section parasites. The sexes are separate and have a complete digestive tract. The size and coloring are variable depending on the genus of the parasites. In general, the length varies from a few millimeters to a few centimeters (De Carneri, 2003; Mattiucci and Nascetti, 2008).

The different morphological characteristics of these nematodes are not sufficient for the identification of species, especially in larval forms (Griglio et al., 2012). Therefore, accurate and precise molecular methods based on the use of sequence analysis of mitochondrial DNA

(mtDNA) and ribosomal DNA (rDNA) have been performed to correctly identify the species belonging to the different genera (Abollo et al., 2003; Cavallero et al. ., 2012; Costa et al., 2018; Zhu et al., 2000).

Given the recent systematic reformulation of the genus *Hysterothylacium* and given their possible zoonotic potential affirmed by recent studies (Yagi et al., 1996), this genus will not be morphologically described. **Figure 3** shows the cephalic ends of the three different genera.

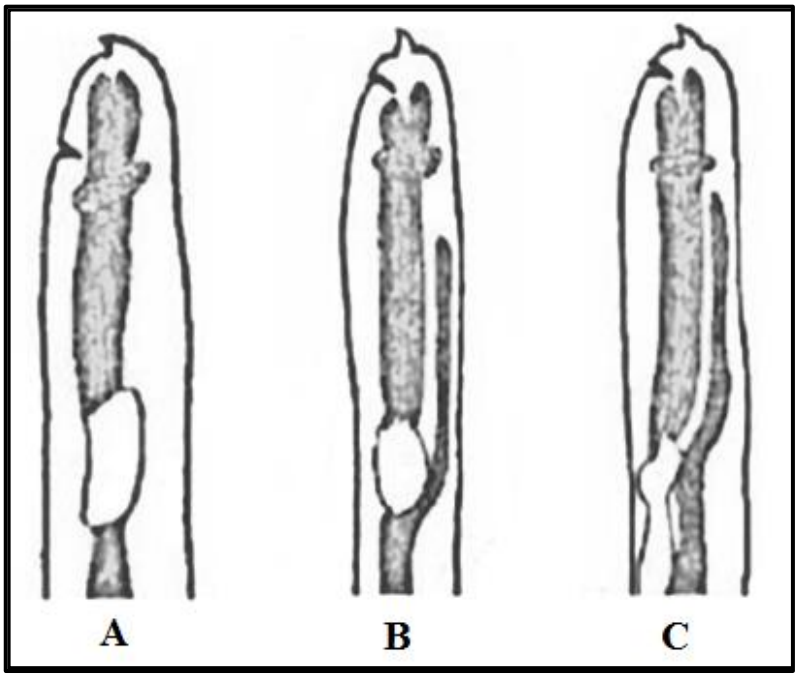


Figure 3. Cephalic extremities in comparison: **A.** *Anisakis* spp.; **B.** *Pseudoterranova* spp.; **C.** *Contracaecum* spp.

1.3.1 Anisakis genre

The larval forms of this nematode have a length that varies between 1 and 3 cm and a diameter of 0.44-0.54 mm, consequently they are easily visible in fish products. They have a color that varies from white to yellowish, are thin and tend to roll up on themselves (Fig. 4) (C.Re.N.A.; Ghittino, 1985; Sakanari and McKerrow, 1989).

The main morphological characters are located in the anterior end (excretory pore, terebrant tooth), in the caudal end (mucron or conical structure) and in the gastrointestinal part (ventricle).

Parasites of the genus *Anisakis* do not have an esophageal appendage and intestinal cecum. To view all the morphological elements, macroscopic examination is not sufficient

but the use of an optical microscope is necessary (**Figure 4**). It is clear that the specific diagnosis must rely on further molecular investigations (Buchmann and Mehrdana, 2016) as the *Anisakis* species are characterized by marked peculiarities in their genetic structure and ecological traits, differentiating themselves for the geographical distribution and for the host preferences (Chai et al., 2005; D'Amelio et al., 2010; Farjallah et al., 2008).

Figure 4. *Anisakis simplex* s.l. L3 larvae (Buchmann e Mehrdana, 2016).

Compared to the larval forms, the adults are distinguished by their larger size, reaching up to 5 cm of length. As for the morphological characters, the cephalic extremity of the adults does not present the tooth because there is the appearance of the lips, while at the caudal extremity there is the disappearance of the mucron and the manifestation of sexual characteristics, such as the eggs in the females and the copulatory spicules in males.

On the basis of the morphological characteristics of the cephalic extremity, the appearance of the ventricle and the caudal extremity, visible under an optical microscope, the third stage *Anisakis* larvae differs into two morphotypes (I and II) (Berland, 1961) (**Figure 5**). The subdivision of the genus *Anisakis* into two clades was confirmed by molecular genetics techniques that involve the use of nuclear and mitochondrial markers (Griglio et al., 2012).

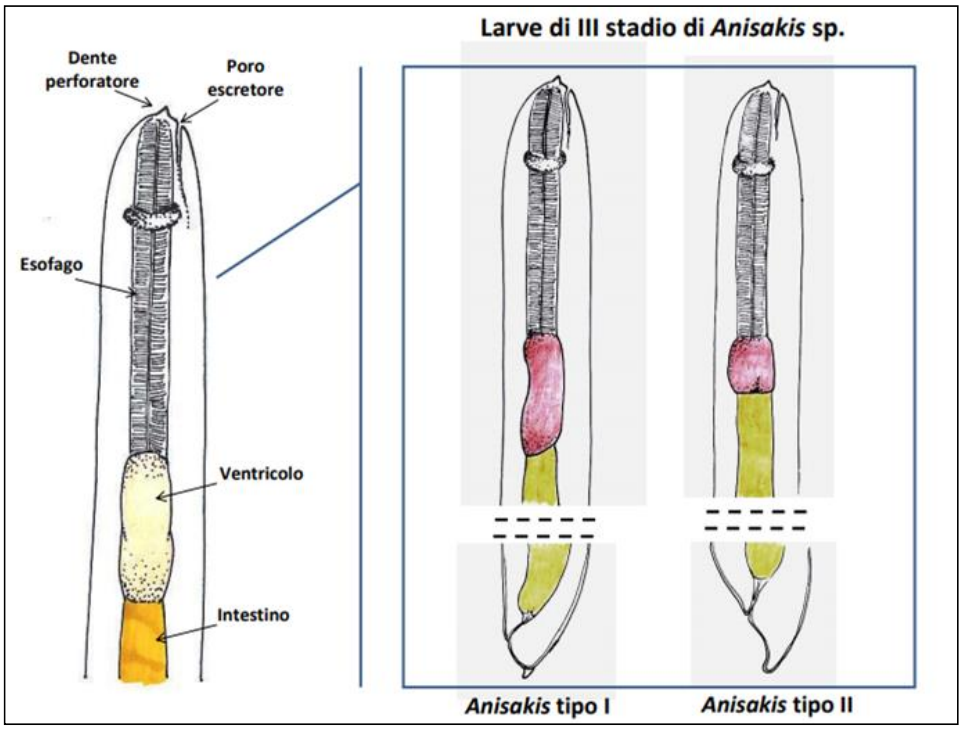


Figure 5. Representation of morphological characteristic of *Anisakis* spp. L3 larvae.

The larvae of morphotype I have a boring tooth in the cephalic end, a cylindrical ventricle and a mucron in the caudal end (**Figure 6**).

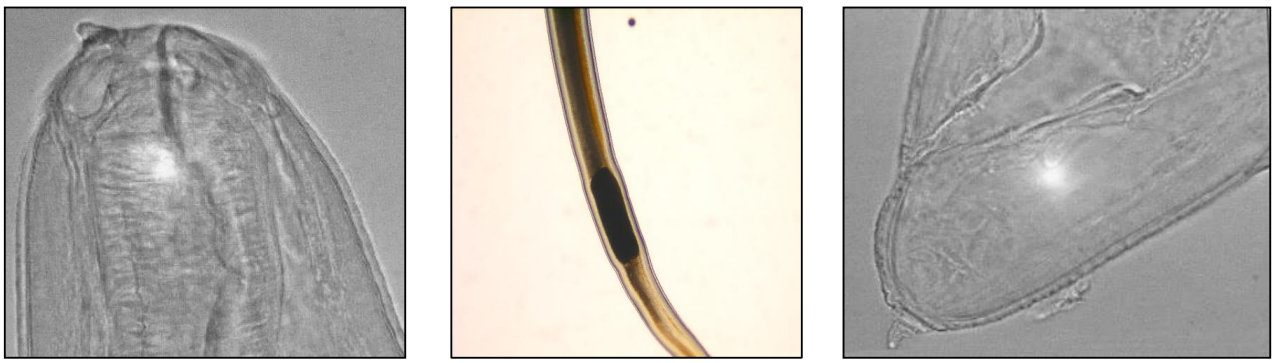


Figure 6. From left to the right: cephalic end with boring tooth; cylindrical ventricle; caudal end with mucron.

The morphotype I is composed by the following species:

- *A. simplex s.s.* (*sensu stricto*);
- *A. pegreffii*;
- *A. berlandi* (ex *A. simplex* C);
- *A. ziphidarum*;
- *A. typica*.

The *A. simplex s.s.*, *A. pegreffii* e *A. berlandi* species belong to the complex *Anisakis simplex s.l.* (*sensu lato*) (Griglio *et al.*, 2012).

The species belonging to morphotype II have a terebrant tooth in the cephalic end, a spherical ventricle and a conical caudal termination (**Figure 7**).

Figure 7. From left to the right: cephalic end with terebrant tooth; spherical ventricle; conical caudal termination.

The morphotype II comprises the following species:

- *A. physeteris*;
- *A. brevispiculata*;
- *A. paggiae*.

1.3.2 Pseudoterranova genre

The larvae of the genus *Pseudoterranova* are easily visible as they have a red-brown color, a length between 2.5 and 4 cm and a diameter between 0.3 and 2 mm. Like the Anisakis larvae, they roll up on themselves but less compactly (**Figure 8**) (Sakanari and McKerrow, 1989).

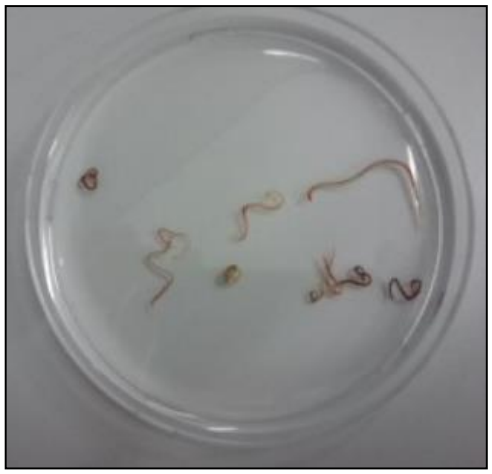


Figure 8. *Pseudoterranova spp.* larvae.

As in the genus *Anisakis*, they do not have the esophageal appendix and the excretory pore is positioned in a similar way. On the contrary, the morphological differences consist in the presence of the intestinal cecum, a shorter ventricle (**Figure 9**) and a small spine in the posterior extremity (Sakanari and McKerrow, 1989).

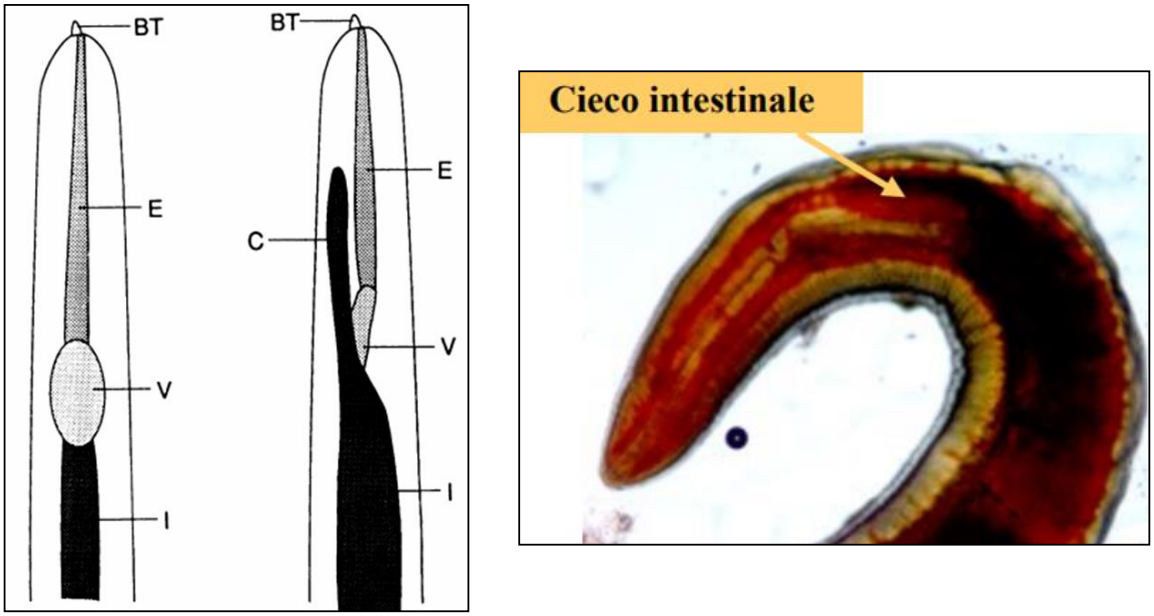


Figure 9. Cephalic extremity of *Anisakis* spp. (left) and *Pseudoterranova* spp. (right). BT=boring tooth; E=esophagus; V=ventricle; C= caecum; I=intestine (Sakanari e McKerrow, 1989); cephalic extremity and intestinal caecum.

This genus comprises *Pseudoterranova decipiens* s.l. (sensu lato) which includes four main twin species, with genetic and morphological differences and a widespread distribution in the Atlantic and Pacific Oceans: *P. krabbei, P. decipiens ss, P. bulbosa* and *P. azarasi* (Griglio et al., 2012; Mattiucci and Nascetti, 2008).

1.3.3 Contracaecum genre

The larvae of this genus have two very tapered ends, assuming the characteristic shape called "hook". They have a greenish-white color and an average length of 1 cm (C.Re.N.A.) (**Figure 10**). Compared to the genus *Anisakis* and *Pseudoterranova*, they have smaller dimensions (Mattiucci and Nascetti, 2008).

Figure 10. *Contracaecum* spp. larva.

By light microscopy, distinctive features are observed such as the presence of the intestinal cecum and the esophageal appendage, the excretory pore in the cephalic extremity (as for the two genera previously described) and finally the absence of spines or mucrons in the caudal extremity (**Figure 11**).

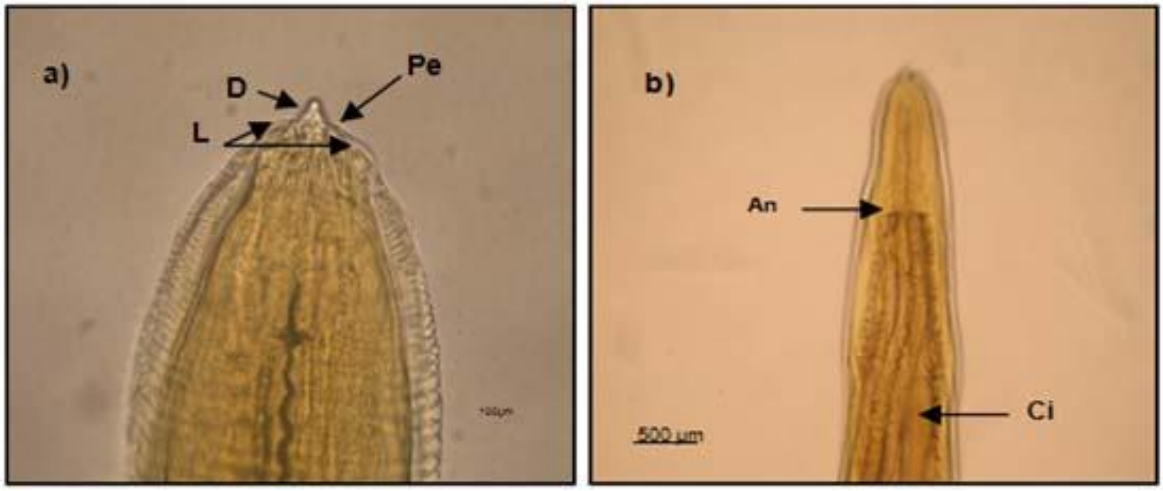


Figure 11. Microscope picture of cephalic extremities of *Contracoecum* spp. Larva. boring tooth, D; excretory pore, Pe; ventrolateral lips, L b). nervous ring, An; intestinal caecum, Ci.

This genus includes *Contracaecum osculatum* s.l. (sensu lato) to which belongs five twin species that are genetically different with different geographical distributions. The first three species (*C. osculatum A, C. osculatum B* and *C. osculatum ss*) were found in the Boreal-Arctic region (Nascetti et al., 1993), while the other two (*C. osculatum D* and *C. osculatum E*) were discovered in the Antarctic region (Orecchia et al., 1994).

Contracaecum ogmorhini s.l. also belongs to the genus *Contracaecum*. which includes two twin species*: C. ogmorhini s.s.* and *C. margolisi*, discovered respectively in the Boreal region and in the Austral region (Mattiucci et al., 2003). Three other species apart are: *C. osculatum baicalensis, C. radiatum* and *C. mirounga*.

1.1. Geographic distribution of the *Anisakis* genre

As initially mentioned, the attention will be paid to the genus *Anisakis* as it is the target parasite of the present study (**Figure 12**).

According to an opinion of the European Food Safety Authority (EFSA), no fishing area can be considered free from the presence of *Anisakis* spp. Therefore, fish and fish products can be at risk of infestation due to the variation in the distribution of these parasites. Therefore, their presence in caught fish products is not linked to alteration phenomena but represents an essential and natural phenomenon linked to the ecosystem (EFSA, 2010; Griglio et al., 2012).

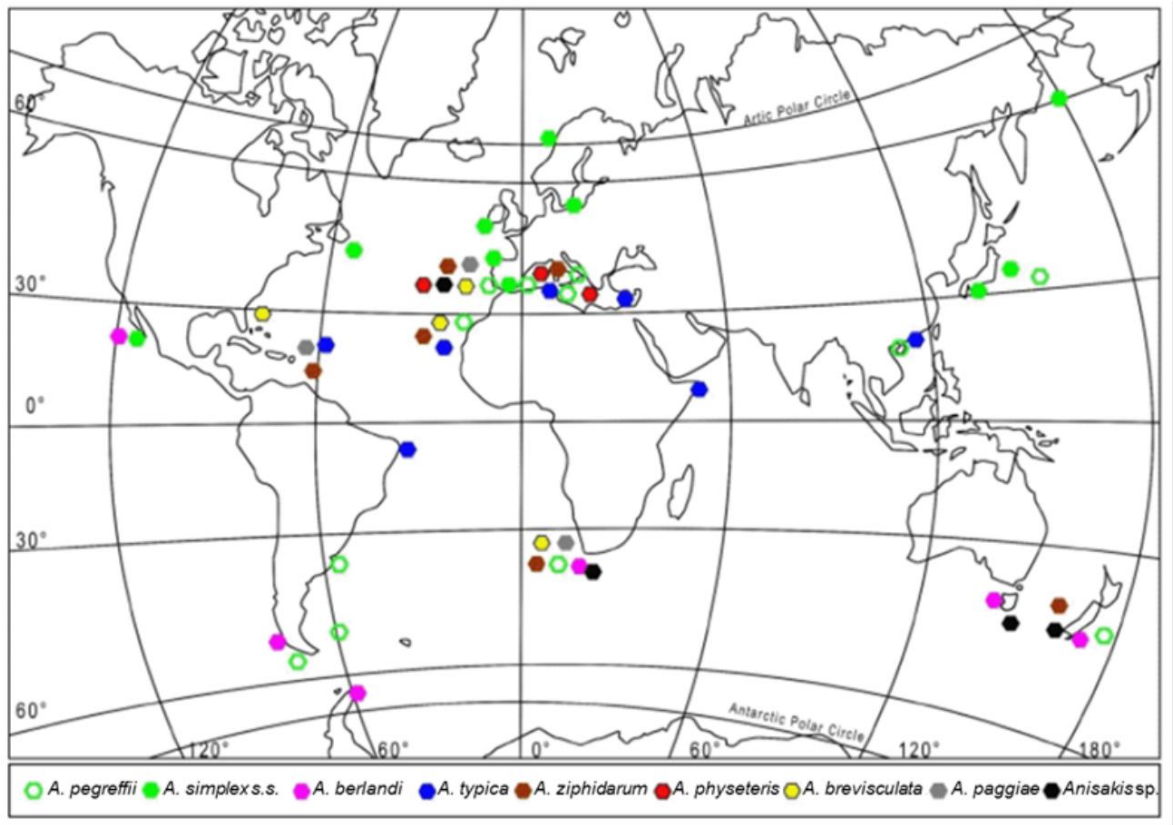


Figure 12. Geographic distribution of *Anisakis* spp. Genetically characterized (Mattiucci e Nascetti, 2006).

A. simplex s.s. is known for having a cosmopolitan distribution and is mostly present in the western and eastern Atlantic and in the Pacific (Mattiucci et al., 1997; Mattiucci and Nascetti, 2008). It is also the main species of the North Atlantic part of the Strait of Gibraltar (Mattiucci et al., 2004). It is occasionally widespread in the western waters of the Mediterranean due to the migration of pelagic fish from the Atlantic to the Mediterranean (Costa et al., 2016; Farjallah et al., 2008; Mattiucci and Nascetti, 2008).

A. pegreffii is the most widespread species in the Mediterranean Sea with a high prevalence of infestation in various fish species (Ferrantelli et al., 2015; Mattiucci et al., 2004; Mattiucci and Nascetti, 2006). The high abundance of this species in the Mediterranean Sea can be explained by the presence of various species of dolphins, such as *Tursiops truncatus*, which is one of the main definitive hosts of *A. pegreffii* (Farjallah et al., 2008). The data obtained in the study conducted by Cavallero et al. (2012) confirm that the *A. pegreffii* species is the most widespread in fish off the Italian coast.

The *A. berlandi* species, formerly known as *A. simplex C*, has a discontinuous distribution including the waters of Chile, Canada and New Zealand as well as the South African Atlantic coast (D'Amelio et al., 2012; Mattiucci and Nascetti, 2006).

As for *A. ziphidarum*, it is a species that has been found in the southern Atlantic and in the Mediterranean Sea (Mattiucci and Nascetti, 2006).

The *A. typica* species is generally found in temperate waters (D'Amelio et al., 2012; Mattiucci and Nascetti, 2006) and it has been found in the Indian Ocean and in the Mediterranean Sea (Farjallah et al., 2008; Griglio et al., 2012).

Another species widespread in the Mediterranean is *A. physeteris* (Farjallah et al., 2008), also found in the Atlantic Ocean (Mattiucci and Nascetti, 2006). Finally, *A. brevispiculata* and *A. paggiae* are widespread in the temperate waters of the Atlantic Ocean, from the Gulf of Mexico to the coasts of the Iberian Peninsula (D'Amelio et al., 2012).

Some species of the genus *Anisakis* are very adapted to the oceanographic characteristics of the Mediterranean waters as the larval forms prefer a temperature range between 34 and 37° C. Therefore, they cannot change when temperatures are significantly lower (10-11°C) (Smith and Wootten, 1978).

1.4 Infestation in the Mediterranean ichthyofauna

The presence of nematodes in the coelomic cavity and in the musculature of fish and cephalopods has been known since the 1950s.

The prevalence of infestation in fish and cephalopods varies considerably from less than 1% to almost 100% depending on the fishing area and fish species as well as their biology and eating habits. In addition, the prevalence of infestation is related to the weight and age of the fish, presenting higher prevalence values in adult and larger fish (Pozio, 2013).

Recent studies highlighted the continuous increase in the prevalence of infestation of these parasites (Griglio et al., 2012). According to Pozio (2013), this increase could be attributed to three factors: from the increase in the populations of marine mammals and therefore of potential definitive hosts following the growing protection of these animals; from the bad habit of throwing viscera into the sea by fishing vessels despite recent European restrictions to dispose the by-products ashore; by the growing attention focused on these zoonotic parasites. However, the reasons for this increase are uncertain and consequently it is necessary to conduct further studies in order to be aware of the causes of the variations in their prevalence (Griglio et al., 2012).

The fish species and the Mediterranean cephalopods most infested by the larvae of the genus *Anisakis* are the scabbardfish (*Lepidopus caudatus*), the horse mackerel (*Trachurus trachurus*), the mackerel (*Scomber scombrus*) and, to a lesser extent, the anchovies (*Engraulis encrasicolus*) and sardines (*Sardina pilchardus*).

Figure 13 shows the main Medirerranean species parasitized by Anisakidae larvae (Pozio 2005).

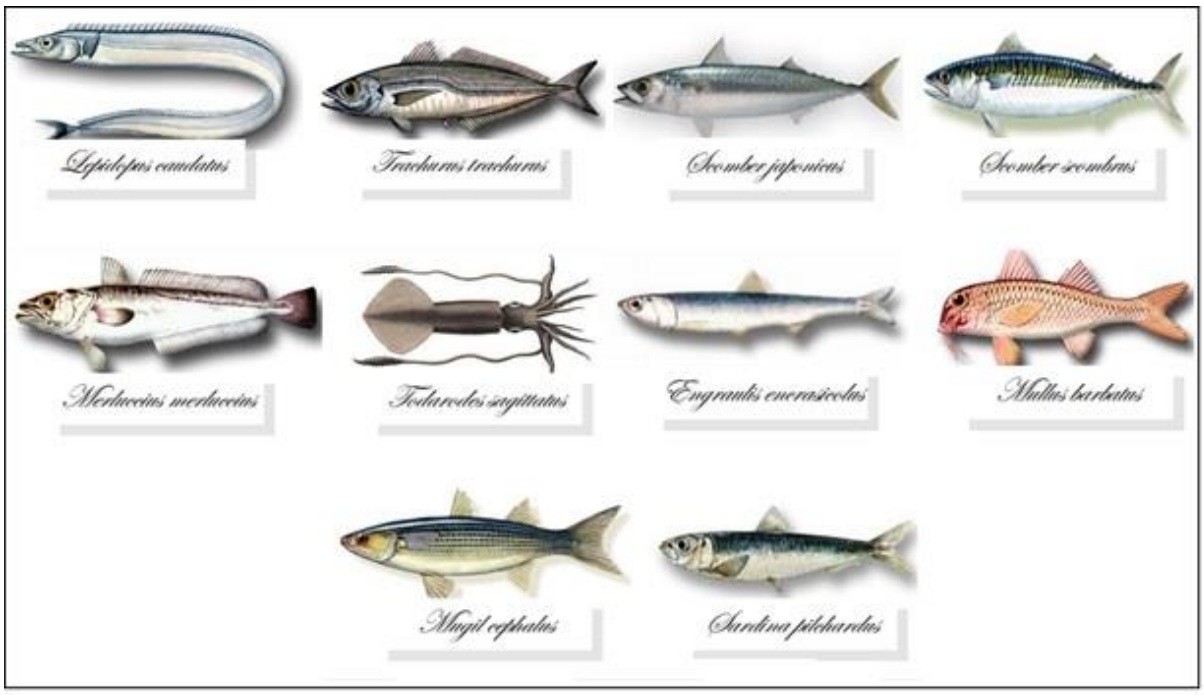


Figure 13. Mediterranean fish species infested by *Anisakis* spp.

Parasites belonging to the *Anisakis* genus are no longer related only to wild fish, but some studies have found their presence, albeit limited, also in aquaculture fish. It is assumed that this is mainly due to the increasing use of offshore farming systems, i.e. in the open sea, such as floating and submerged cages, by which crustaceans or small fish potentially infested with anisakid nematodes could pass (Mo et al., 2014).

Furthermore, a recent study (Fæste et al., 2015) has shown that fishmeal contained in aquaculture feed may be a cause of allergenic reactions related to *Anisakis* spp. since it derives from the viscera of fish that could be infested with anisakid larvae.

1.5 Anisakis and the aquaculture sector

Aquaculture is the farming of aquatic organisms such as fish, molluscs, crustaceans and algae in fresh, brackish and marine waters (Cataudella and Bronzi, 2001).

Aquaculture is a rapidly growing sector and assumes importance as it helps to satisfy, together with fishing, the growing demand for fish products by the world population. The increase in aquaculture activity has been favored by the rapid evolution of production techniques, particularly in the areas of feed and farming methods.

Furthermore, the high demand for fish products and the new needs proposed by the consumer, such as the demand for non-fatty fish, have contributed to the development of this sector (Cataudella and Bronzi, 2001; Cataudella and Spagnolo, 2011; FAO, 2018).

Over the years, the farming methods have undergone to a profound change. Initially, aquaculture was essentially represented by the fishing valleys, where the fish was farmed extensively. Subsequently, there was a gradual transition to intensive farming in ground tanks which were mostly integrated and replaced by farms with sea cages systems (Cataudella and Spagnolo, 2011) (**Figure 14**).

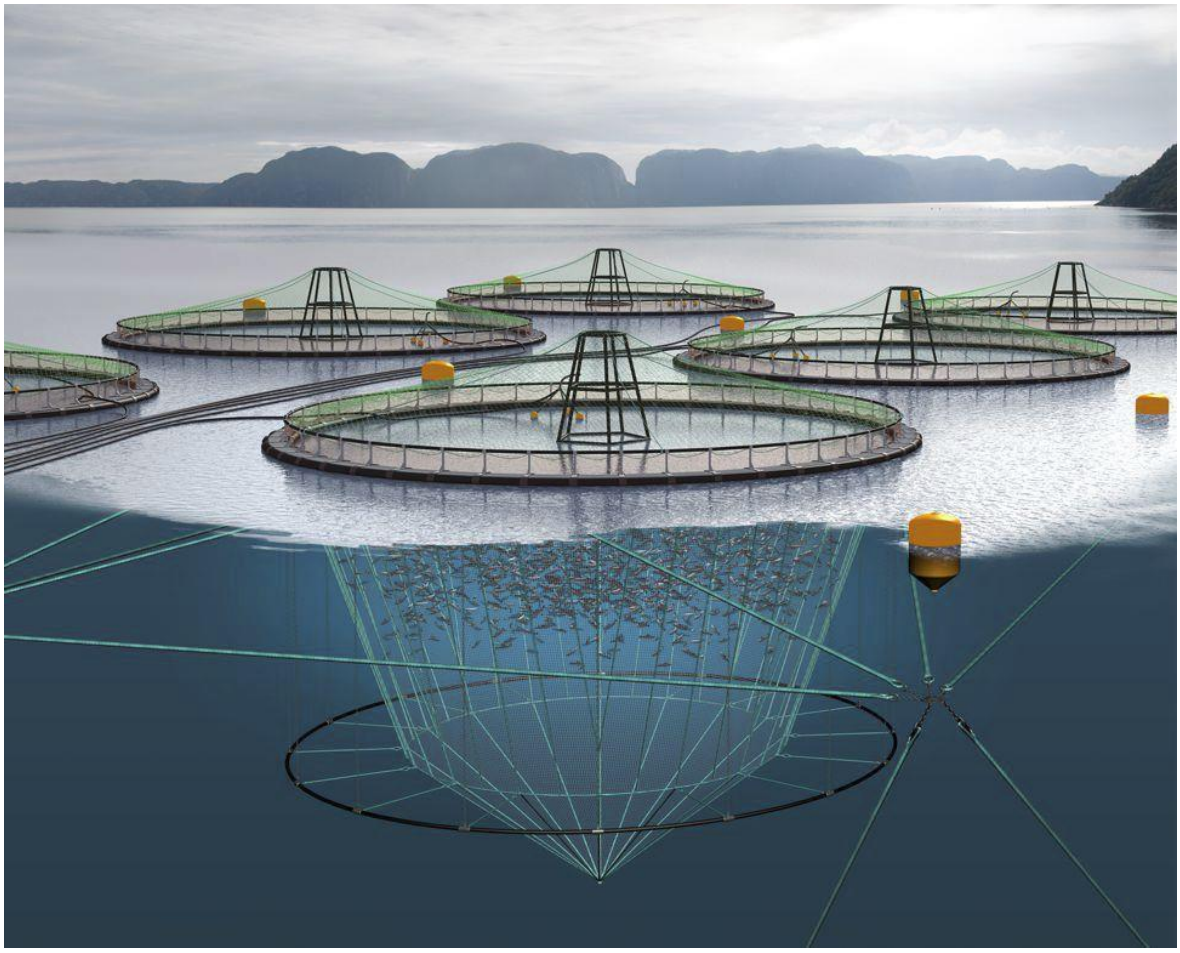


Figure 14. Floating cages for the farming of sea bass.

The aquaculture sector must also aim to have food safety and consumer protection among its main objectives. In particular, it is necessary to minimize the potential risks for consumers by ensuring the compliance with the regulations, including the absence of parasites (Cataudella and Bronzi, 2001).

In 2010, EFSA asserted that the risk of infestation with *Anisakis* spp. is negligible only for Atlantic salmon reared in floating cages or onshore tanks and fed with feed that does not contain live parasites since it considers sufficient scientific data demonstrating the negligible

risk of infestation by *Anisakis* spp. (EFSA, 2010). Furthermore, EFSA states that epidemiological data relating to the risk of *Anisakis* spp. are insufficient in other farmed fish species. Therefore, it recommends conducting new coordinated studies that can demonstrate the absence of *Anisakis* spp. larvae on the farm to get an exhaustive picture of the risk.

Even if the risk of encountering parasite larvae is practically nil, it is still advisable to subject the aquaculture product to a preventive freezing treatment if they are intended to be eaten raw or undercooked.

Mo et al. (2014) verified the presence of anisakid parasites in farmed Atlantic salmon, stating that the finding of these nematodes in farmed fish can be traced back to the ingestion of crustaceans or small infested fish introduced inside the breeding cages.

Currently, there are few studies focused on the prevalence of anisakid nematodes in caged fish of Mediterranean Sea such as sea bass (*Dicentrarchus labrax* L.) and sea bream (*Sparus aurata* L.).

The study by Peñalver et al. (2010) verified the absence of parasites in sea bass and sea bream samples from Iberian farms. However, Macrì et al. (2012) demonstrated the experimental susceptibility of sea bass and sea bream to parasites of the genus *Anisakis*.

During the aquaculture activity, a significant aspect takes on the feed sector. Particular attention is paid to fishmeal-based feeds as they are mainly composed of the viscera of marine fish commonly infested with *Anisakis* spp., so to raise 1 kg of fish it is necessary to use 5 kg of fish caught for feed production. Consequently, if these viscera were infested they could be the cause of allergic manifestations in humans.

Allergic reactions to *A. simplex* s.l. they can be caused by the consumption of dead larvae present in fish products or by the presence of allergens of these nematodes in feed (Fæste et al., 2015). Indeed, Audicana and Kennedy (2008) demonstrated the resistance of these allergens to both digestive enzymes and cooking. Furthermore, Mazzucco et al. (2012) reported cases of *A. simplex* allergy in operators handling fish-based feeds on a daily basis.

Fæste et al. (2015) showed that allergens can be transferred from feed to the final product causing various symptoms such as urticaria, angioedema, anaphylaxis and asthma. The detection of allergens in fish products could be due to their presence in the blood of fresh fish.

In addition to seafood, Armentia et al. (2006) found cases of positivity to allergens of *Anisakis* spp. in subjects with high sensitivity to *A. simplex* s.l. who had consumed chicken meat. This is because chicken feed usually contains a high percentage of fishmeal which could be contaminated by this parasite. Despite the high stress factors arising from the

processes of production of the feed and the subsequent cooking of chicken meat, the allergens of *Anisakis simplex*, transmitted by the feed, have at least partially maintained their biological activity causing the typical allergic symptoms.

Following these studies, which highlight the correlation between the feed sector and allergies caused by *Anisakis*, more attention is paid to the quality of feed production.

1.6 Anisakiasis

Until a few decades ago, Anisakiasis was relatively unknown. Today, health authorities are increasingly aware of the problems caused by these parasites and its response on human health and commercial value of fish products (Valero et al., 2006).

Anisakiasis is a gastrointestinal zoonotic disease caused by the ingestion of live third stage larvae of *Anisakis* spp. as consequence of the consumption of raw or undercooked fish products infested (Chai et al., 2005; Griglio et al., 2012; Setyobudi et al., 2011; Suzuki et al., 2010).

Only *A. pegreffii* and *A. simplex s.s.* have been reported as causative agents of human gastric, intestinal and gastro-allergic Anisakiasis (Guardone et al., 2018).

A. simplex s.l. is responsible for most of the Anisakiasis cases (Buchmann and Mehrdana, 2016); in particular, in the Mediterranean, *A. pegreffii* is the species most responsible for anisakiasis in humans. Parasites of this species can be found in many Mediterranean fish, belonging to different geographical distributions (Cipriani et al., 2016; Gaglio et al., 2018).

1.6.1 Epidemiology

The first case of Anisakiasis was described in 1876, in Greenland, by Rudolf Leuckart, (Griglio et al., 2012).

In Europe, the first case of Anisakiasis dates back to 1960, in the Netherlands, when the researcher P.H. Van Thiel reported the presence of the parasite inside the intestine of a patient following the ingestion of "cold" smoked herring (Griglio et al., 2012).

Since 1962, many studies have been extensively conducted on Anisakiasis as it has been diagnosed in thousands of patients worldwide (Griglio et al., 2012).

In Italy, the first case was found in Bari in 1996 (Griglio et al., 2012; Zanelli et al., 2017). Subsequently, several cases have been reported mainly in Southern Italy, where traditional preparations of salted sardines and marinated anchovies (**Figure 15**) are frequent (Piras et al., 2014). In fact, in all cases the patient declared the consumption of raw or marinated fish (Bernardi, 2009).

Figure 15. From left to right: salted sardines, marinated anchovies.

Of approximately 20000 cases diagnosed worldwide, more than 90% come from Japan and Europe (Holland, Germany, Spain, France and Italy), China, United States of America, Canada, New Zealand, Chile and Egypt (Audicana et al., 2002; Buchmann and Mehrdana, 2016; Chai et al., 2005; Nieuwenhuizen and Lopata, 2013). In Japan, about 2000 cases are diagnosed a year and the high prevalence of Anisakiasis is clearly related to the tradition of consuming raw fish (Audicana et al., 2002; Bernardi, 2009). According to Suzuki et al. (2010), almost all cases of Anisakiasis in Japan are caused by *Anisakis simplex* s.s.

In Europe, it is very complex to establish the exact incidence of the disease. However, it seems to be less than 20 cases per country per year (Guardone et al., 2018).

In Italy, the cases appear significantly underestimated and it is assumed that the incidence of human Anisakiasis is probably higher than what is reported, probably due to the difficult in diagnosis (Pampiglione et al., 2002; Zanelli et al., 2017). Consequently, conflicting estimates of the number of cases can be found (Guardone et al., 2018). In Italy, we assisted to an increase in the consumption of raw and/or undercooked fish, following the importation of products such as sushi and sashimi (**Figure 16**) (Bernardi, 2009; Costa et al., 2018). This phenomenon has led to a consequent growth, all over the world, of the cases of Anisakiasis in humans, becoming a great concern for human health (Setyobudi et al., 2011).

Figure 16. From left to right: Sushi; Sashimi.

However, the increase of Anisakiasis reports is also linked to the use of traditional treatment processes, such as marinating, which are unable to devitalize the larvae (Buchmann and Mehrdana, 2016).

Among the Mediterranean fish, anchovies (*Engraulis encrasicolus*) are the most consumed raw in Italy, because are consumed marinated in domestic ways. The consumption of raw marinated anchovies, not previously frozen at −20°C for 24 hours or at −18°C for 96 hours in domestic freezers, represents the main risk for Anisakiasis in the Italian population (Cipriani et al, 2016; Griglio et al., 2012; Guardone et al., 2018). In this regard, the studies conducted by Cavallero et al. (2012) and Cipriani et al. (2018) stated that several cases of Anisakiasis in Italy were caused by infested fish preparations such as marinated anchovies (**Figure 17**).

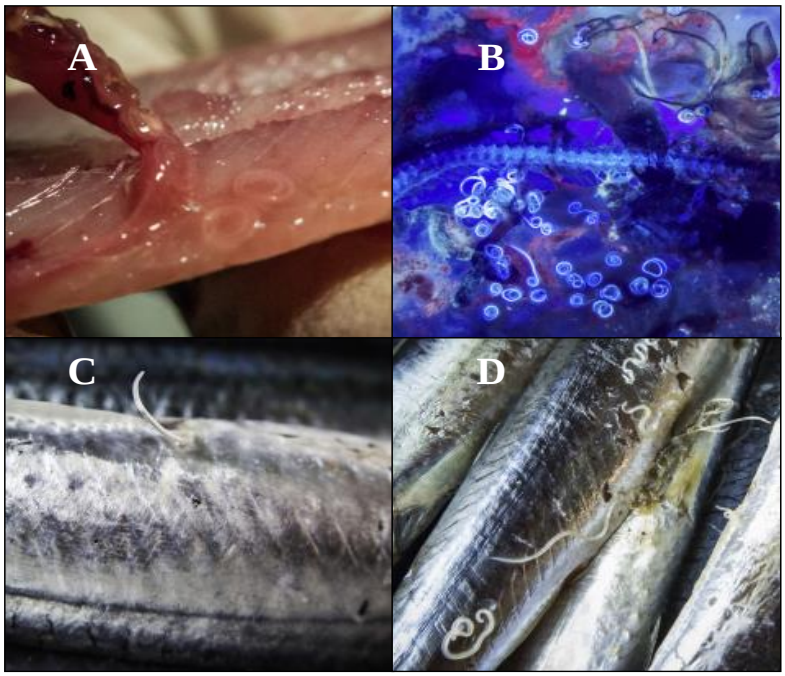


Figure 17. Localization of *A. pegreffii* larvae in *E. encrasicolus* **A.** intramuscular larvae; **B.** larvae in the viscera observed by UV light; **C and D** larvae located in the external part of the anchovy (Cipriani *et al.*, 2016).

Marinated anchovies are popular in other Mediterranean countries, in fact it is supposed to be the cause of most cases of Anisakiasis in Spain (Cavallero et al., 2012).

It is correct to specify that the significant increase in the prevalence of anisakiasis worldwide over the last 30 years is not only linked to a greater preference for raw and/or undercooked food, but also to two other main factors. First, new diagnostic techniques, in particular endoscopy, have been found to be essential for detecting the disease as before probably many cases of gastric Anisakiasis were not identified or were misdiagnosed (Cipriani et al., 2015; Pravettoni et al. , 2012). The other relevant factor concerns the increased controls on fish products and the application of new regulations in Europe (Chai et al., 2005; Oshima, 1987).

Following the continuous increase of raw fish consumption and the presence of parasites in fish products, EFSA published in 2010 the scientific opinion "Scientific opinion on risk assessment of parasites in fishery products" that recommends to Member States to conduct new epidemiological studies with the aim of gathering more information about Anisakiasis in humans (EFSA, 2010).

1.6.2 Clinical manifestations

Anisakiasis can present a non-invasive form, generally asymptomatic, in the event that the larvae remain in the gastrointestinal tract without penetrating the tissues. Infestation can only be diagnosed when the parasite is spontaneously expelled orally or with feces (Chai et al., 2005). In very rare cases, the esophageal form may occur, a phenomenon in which the larva after ingestion can return to the esophagus through gastric reflux. The symptoms encountered are dysphagia, burning and gastroesophageal reflux (Chung et al., 1999).

However, in invasive infestations, the larvae of *Anisakis* spp. stick to the gastrointestinal system with significant damage to the gastric or intestinal mucosa, such as haemorrhagic lesions, ascites, gastrointestinal perforations up to masses of granulomas (Pravettoni et al., 2012, **Figure 18**).

The anisakid larvae have the ability to be resistant to digestion processes; in fact, they are able to penetrate the gastric or intestinal wall on which they adhere through the terebrant tooth and the release of lytic enzymes after (proteases) even up to 6 days after the ingestion (Fig. 18). The proteolytic enzymes are secreted by the esophageal gland and other excretory glands located around the mouth and are capable of causing hemorrhagic lesions (Pravettoni et al., 2012). More rarely, the larvae can stick other organs or other sites such as the throat (Audicana et al., 2002; Chai et al., 2005).

Figure 18. Engraving of an *Anisakis* larva on the gastric mucosa (Sakanari, 1990).

Anisakiasis can be manifested in different ways, based on the location of the larvae and the histopathological lesions caused (Zhu et al., 1998). In particular, Anisakiasis can be divided in acute, chronic and ectopic forms. In addition to parasitic infestation, the presence of *Anisakis* larvae can also cause an allergic form (**Figure 19**).

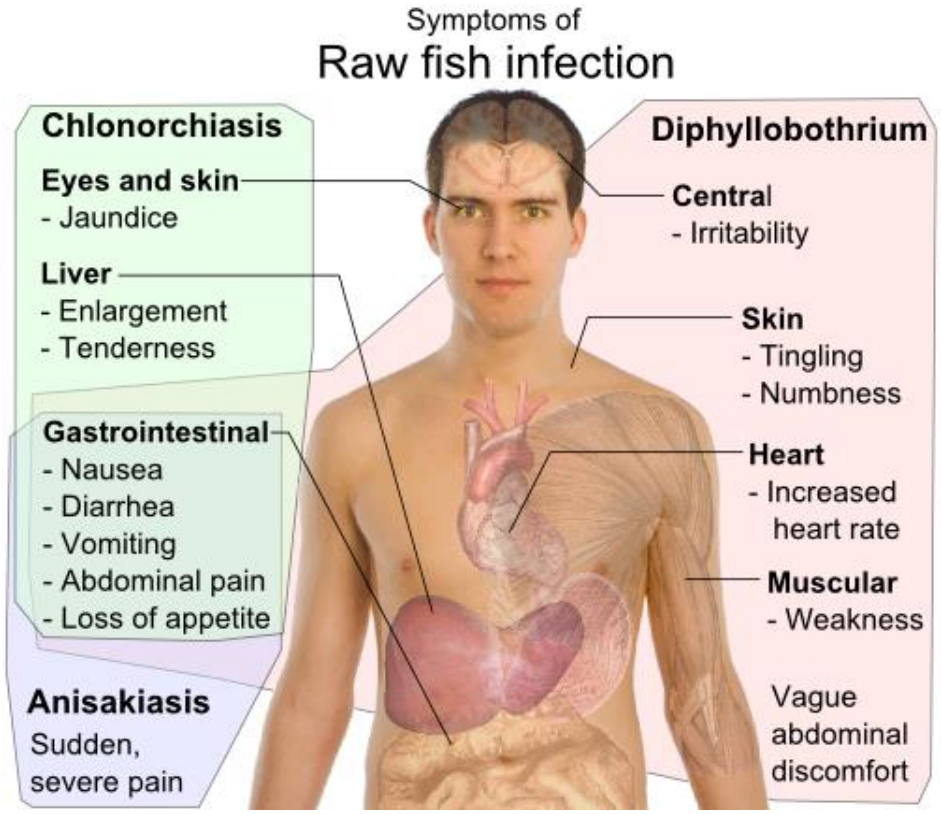


Figure 19. Symptomatology of Anisakiasis in humans.

Despite the development of debilitating symptoms and immune hypersensitivity, the parasite is not suitable for living in humans as it is an accidental host. Therefore, the larvae do not complete their life cycle and therefore the infestation is transient (Audicana and Kennedy, 2008). **Table 2** shows the chronology of the events that occur during the infestation of the larva.

Time after ingestion	Infestation step
<1h	Adhesion to the mucosa
4 h to 7 days	Penetration into the mucosa and submucosa
7-14 days	Formation of granulomas
> 14 days	Death of the larva

Table 2. Synoptic scheme of Anisakiasis disease (Audicana e Kennedy, 2008).

Acute form

The acute form can be gastric or intestinal. The gastric form is the most found and is characterized by symptoms such as epigastric pain, nausea, vomiting or diarrhea, which occur approximately 4 to 12 hours after ingestion of the fish infested (Buchmann and Kania, 2012; Buchmann and Mehrdana, 2016; Griglio et al., 2012; Setyobudi et al., 2011; Zhu et al., 1998). The larva penetrates the gastric mucosa and the release of proteolytic enzymes cause erosive and/or hemorrhagic lesions. Generally, the most acute symptoms are alleviated within a few days, but if the gastric disease is not taken into account, chronic symptoms, such as those typical of ulcers, may be encountered (Pravettoni et al., 2012).

The intestinal form occurred after 12 hours - 7 days after the consumption of fish manifesting colic-like pains, nausea, vomiting or diarrhea (Griglio et al., 2012). Infested individuals can develop ascites, i.e. the accumulation of fluid in the peritoneal cavity (Pravettoni et al., 2012).

Chronic form

The chronic form occurs about 7-14 days after the ingestion; once have penetrated the wall of the gastrointestinal mucosa, the larvae induce the formation of secondary lesions such as abscesses or, more frequently, granulomas with eosinophilic infiltrate (Griglio et al., 2012 ; Pravettoni et al., 2012).

Symptoms are nonspecific such as chronic diarrhea, melaena, colicky pains and organ perforation symptoms. The clinical picture can also worsen if intestinal obstruction phenomena are triggered or the parasite calcification occurs after about 6 months.

However, paucisymptomatic forms with little pain or completely asymptomatic forms may also be present (Griglio et al., 2012).

Ectopic or extra-gastrointestinal form

The rare ectopic form occurs when the larva, after having penetrated the gastrointestinal mucosa, migrate to extra-gastrointestinal sites or organs, such as the peritoneal cavity, liver, pancreas, lung, ovary, peritesticular tissues, esophagus and tongue.

Once penetrated into one of these sites, an abscess or more frequently a granuloma around the larva can be observed (Griglio et al., 2012).

In most cases, the clinical manifestations are not severe. However, symptoms of greater severity such as diffuse abdominal pain can occur after the larva has penetrated the intestinal wall (Audicana et al., 2003).

1.7 Allergic form and Anisakis-related allergy

In recent years it has been highlighted how the ingestion of fish infested with live *Anisakis* larvae can cause, following the infestation, a strong allergic reaction leading to the allergic Anisakiasis (Buchmann and Mehrdana, 2016). The latter must not be confused with the allergy caused by *Anisakis* spp. since it is triggered by the presence of post-mortem allergens of the parasite, regardless of whether live larvae are present in the food product.

About 11% of people affected by Anisakiasis have the gastro-allergic form caused by the antigens of the parasite and the enzymes produced to penetrate the gastric mucosa. Clinical symptoms encountered are swelling, hives, airway obstruction, asthma and anaphylactic shock, sometimes accompanied by gastrointestinal symptoms. Transient and itchy bumps and edematous areas can be found (Chai et al., 2005; Griglio et al., 2012; Nieuwenhuizen and Lopata, 2013; Valero et al., 2006). The most severe clinical form is anaphylaxis and it occurs mainly in Mediterranean and Asian countries (Pravettoni et al., 2012).

Several studies have associated the onset of urticaria and allergic reactions to gastrointestinal infestation caused by nematodes belonging to *A. simplex*, identifying these parasites as the etiological agents of IgE-mediated allergic reactions (Audicana et al., 2002; Audicana and Kennedy, 2008).

A study conducted by Mattiucci et al. (2013) reported cases of gastro-allergic reactions associated with *Anisakis pegreffii* infestation in Italy. Eight patients experienced symptoms such as acute gastric pain and nausea, which appeared following the consumption of raw fish. In particular, 3 patients experienced acute allergic reactions.

The immune response is triggered by the contact with some antigens of the live larva and are divided into three groups. The first group is characterized by excretory/secretory allergens and are proteases and protease-inhibitory enzymes, secreted during the penetration of the larva. The second group consists of somatic allergens that are present throughout the larva's body, while the third group is represented by cuticle allergens, secreted by the parasite to protect itself from stomach acid (Pravettoni et al., 2012).

Consequently, the subjects could be exposed to different allergens: during the penetration of the larva and its subsequent death (usually between 2 and 3 weeks) the patient is exposed to all the allergens of the larva; if the larva is eliminated intact through the gastrointestinal

tract, the patient is exposed only to excretory/secretory allergens. Finally, if the larva ingested is dead, the subjects are mainly exposed to somatic and cuticular allergens and minimally to excretory/secretory allergens (Pravettoni et al., 2012).

Among the eleven allergens produced by *Anisakis simplex,* identified and characterized, Ani s 1 is the one of greatest interest. It belongs to excretory/secretory allergens and has a molecular mass of 24 kDa. Furthermore, it is devoid of any significant homology with other known allergens, therefore it is highly specific for allergic patients, of which 85% develop IgE with this protein (Mattiucci et al., 2013; Pravettoni et al., 2012).

When the subject comes into contact with allergens during the infestation of the live larva, the immune system begins to produce type 2 helper T lymphocytes, which through specific interleukins, mediate the production of class E immunoglobulins (IgE). IgE are the classic antibodies produced following the allergic response and are responsible for the symptoms described above (Pravettoni et al., 2012). Once the B lymphocytes produce the IgE, they bind to mast cells which are immune cells rich in granules. Subsequently, after the allergens binding on the IgE present on the surface of the mast cells, their degranulation follows with the release of vasoactive molecules (histamine, serotonin, heparin, leukotrienes and prostaglandins) which will induce an inflammatory reaction (**Figure 20**).

The risk of allergy is higher for fish products containing live *Anisakis* larvae than for those containing non-viable larvae. However, there have been some cases where the consumption of cooked or canned seafood has led to specific allergic reactions (Nieuwenhuizen and Lopata, 2013). In fact, allergenic capacity is partly maintained after cooking. Different studies have demonstrated the temperature resistance of *Anisakis* allergens (Audicana et al., 2002) and the resistance to pepsin, a digestive enzyme (Nieuwenhuizen and Lopata, 2013), suggesting that food ingestion contaminated with *Anisakis* larvae could sensitize or induce clinical symptoms even if the food is cooked.

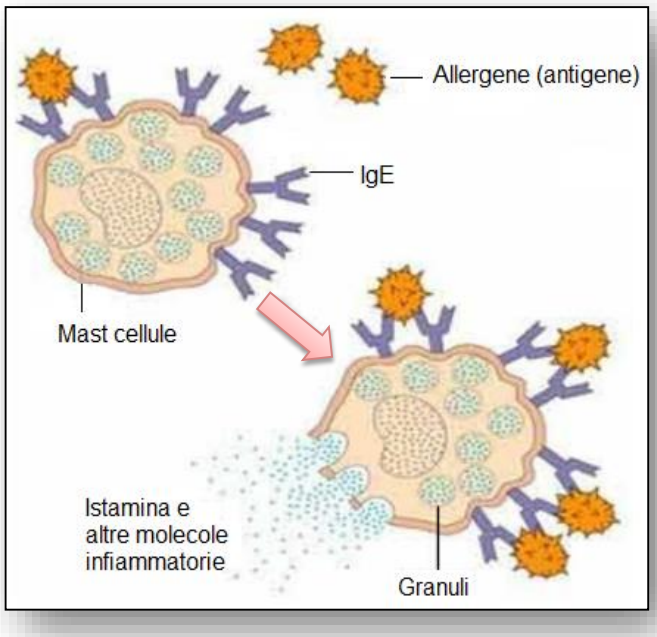


Figure 20. Representation of the IgE-mediated allergic reaction.

The subjects who most frequently suffer *Anisakis* allergy are patients with chronic urticaria or fish sector operators who handle fresh fish products or fish meal every day (Mazzucco et al., 2012).

1.8 Diagnosis

The diagnosis is not always simple and immediate as the symptoms and clinical picture are comparable to other pathologies such as appendicitis, acute abdomen, gastric cancer or Crohn's disease (EFSA, 2010).

The study conducted by Sakanari and McKerrow (1989) states that, out of 92 cases of Anisakiasis in Japan, over 60% of cases have been misdiagnosed.

Often, due to clinical symptoms, gastric anisakiasis is misdiagnosed as a peptic ulcer or stomach cancer, whereas the intestinal form is misdiagnosed as appendicitis or peritonitis (Chai et al., 2005; Pozio, 2013).

The use of endoscopy, immediately after the onset of gastric symptoms, led to the observation of live larvae that penetrate the mucosa (Audicana et al., 2002). Endoscopic examination can often be used to provide a definitive diagnosis for gastric Anisakiasis (**Figure 21**), while the clinical diagnosis of intestinal anisakiasis is difficult as it requires careful examination of clinical symptoms and patient history (Chai et al., 2005; Zhu et al., 1998).

Larvae can be found for up to 6 days after consuming seafood, but if endoscopy is delayed, in the worst cases, the larva can pass through the mucosa (causing ectopic disease) preventing the visualization (Pravettoni et al., 2012).

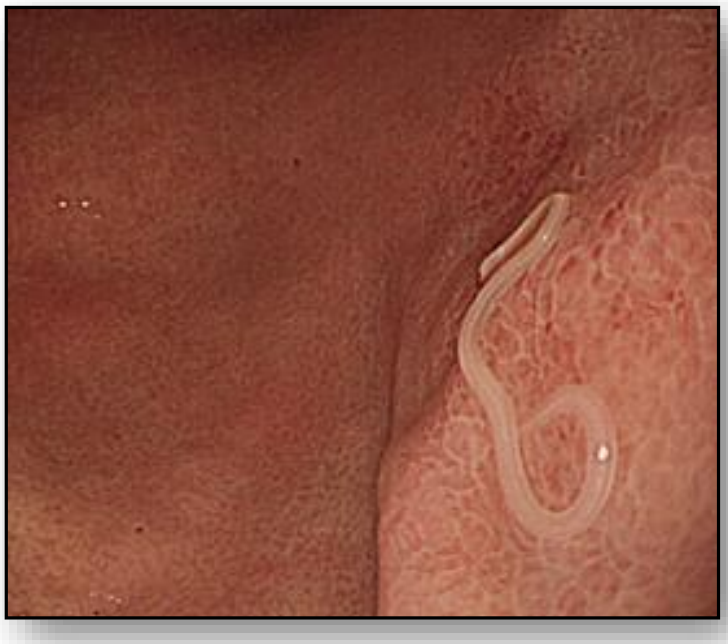


Figure 21. Endoscopic visualization of *Anisakis* spp. larva in the gastric mucosa (Hosoe *et al.*, 2014).

In addition to endoscopic examination, the clinical diagnosis can be made with immunoassays, such as skin-prick tests (**Figure 22**), complement fixation, immunoblotting, indirect immunofluorescence, immunodiffusion, immunoelectrophoresis, radioallergoabsorption test (RAST) and ELISA (Audicana et al., 2002; Desowitz et al., 1985).

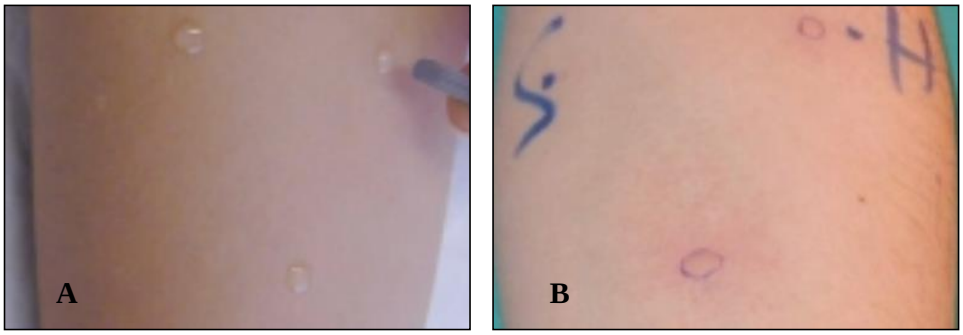


Figure 22. Positive *Skin-prick test* to *Anisakis simplex* allergens A. Placement of a saline solution (S in B) as negative control, 10 mg/ml of histamine (H in B) as positive control and 1 mg/ml of *Anisakis simplex* allergen. These liquids are introduced into the skin by puncture, B. The two red wheals indicate that the reaction is positive (Audicana et al., 2002).

However, the diagnosis by immunological tests can be difficult as cross-reactions with antigens related to other nematodes (eg *Ascaris* and *Toxocara*) can occur, detecting false positives (Audicana et al., 2002; Pozio, 2013).

1.1.1. Treatment

Actually, the only effective treatment for Anisakiasis is the endoscopic removal of live larvae by gastroscopy or colonoscopy (**Figure 23**) (Pravettoni et al., 2012).

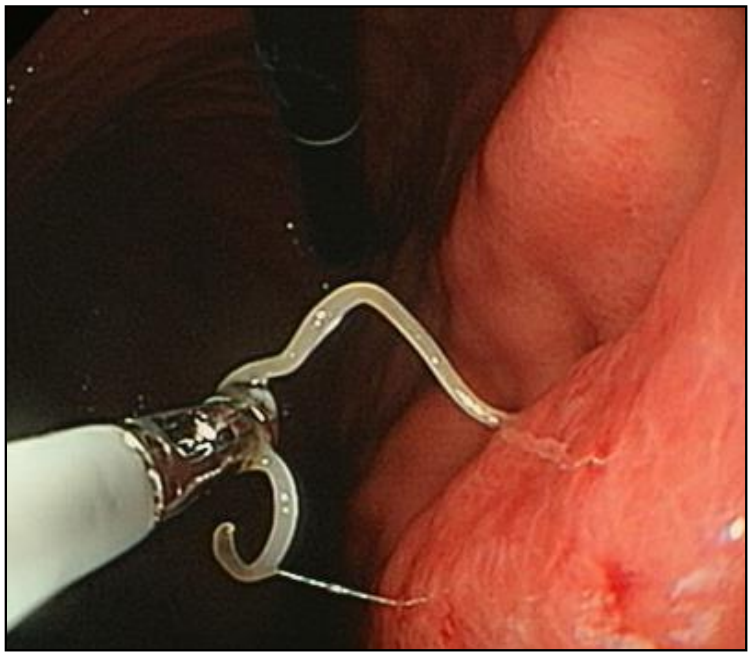


Figure 23. *Anisakis* larva removal using endoscopic forceps.

Sometimes, it is necessary a surgical removal of granulomas and the subsequent administration of corticosteroids to reduce inflammation (Pozio, 2013).

Currently, pharmacological treatments aimed at the elimination of parasites in vivo are being studied, such as anthelmintics (Thiabendazole, Albendazole and Ivermectin) which have been shown to be effective against these nematodes (Buchmann and Mehrdana, 2016; D'Amelio et al., 2012; Pozio, 2013).

1.9 Regulatory framework

The zoonosis caused by the ingestion of viable *Anisakis* larvae (L3) is increasingly subject to particular attention since it has significant repercussions in the field of food safety and product quality (Llarena-Reino et al., 2012).

In this regard, Regulation (EC) no. 853/2004, "which establishes specific hygiene rules for food of animal origin", regulates the production and marketing of fish products to ensure food safety. This regulation, in Annex III, section VIII, describes in detail all the structural requirements and hygienic conditions to be respected on ships during and after landing operations. In addition, the official controls to be carried out on land-based establishments are described to verify that the products meet the hygiene and temperature requirements in accordance with the law, as well as further checks on storage and transport conditions.

In Annex III, section VIII, chapter III, the regulation provides that fish products consumed raw, marinated, salted or subjected to treatments that do not guarantee the killing of the parasite "must be frozen at a temperature not exceeding −20° C in every part of the mass for at least 24 hours ". In addition, in Annex III, Section VIII, Chapter V, the regulation provides that before the placing on the market "food business operators must ensure that fishery products are subjected to a visual check for visible endoparasites" and " they must not place on the market for human consumption fishery products manifestly infested with parasites ".

Subsequently, the Regulation (EC) no. 2074/2005 gave more detailed indications on visual control. In particular, in Annex II, Section I, Chapter II, qualified personnel are recommended to carry out a visual inspection of the entire abdominal cavity (including liver and gonads) of a representative number of samples (Reg. (EC) No. 2074/2005).

Subsequently, Annex III of the Regulation (EC) no. 853/2004 is amended and extended by Regulation (EC) no. 1276/2011 "relating to the treatment for the killing of vital parasites in products in fishery products intended for human consumption". This regulation provides for other freezing time/temperature ratios, which consist in the possibility of freezing the fish, as well as at −20° C for at least 24 hours, also at −35 ° C for at least 15 hours (Reg. (EC) no. 1276/2011).

Finally, the Decree of 17 July 2013 of the Italian Ministry of Health established the mandatory information that must be made available to consumers regarding the "correct conditions of use of fresh fish and cephalopods". This information must be reported on a sign affixed in the places of sale and must be clearly visible and legible to the consumer (**Figure 24**) (Italian Ministerial Decree 17 July 2013). Again, the Italian Ministry of Health encourages the fish sector operators to carry out correct evisceration protocols of the fish

products in order to prevent Anisakis-related pathologies by reducing the possibility of migration of L3 larvae in the musculature (Ministry of Health; guidelines on hygiene of fishery products).

Figure 24. Sign to be exposed in the places of sale.

1.10 Prevention

The most effective tool to avoid Anisakiasis and *Anisakis*-related allergic forms still remains the prevention, with the aim not only of adopting a series of preventive measures but also of educating consumers on the real dangers associated with the consumption of raw fish. (Pravettoni et al., 2012).

To prevent the Anisakiasis it is essential to eliminate or inactive the larvae. This is possible with the implementation of various preventive measures such as immediate evisceration of the fish caught, freezing of the fish immediately after the capture and cooking of the fish product before consumption (Buchmann and Mehrdana, 2016; Chai et al., 2005 ; Valero et al., 2006). According to EFSA (2010), temperature treatments (cooking and freezing) are the most effective methods.

Regarding evisceration, this is carried out immediately after capture as the larvae are found mainly in the viscera and in some cases, after the death of the fish, they can migrate within the muscles (Chai et al., 2005). The larvae present in the muscle increase the probability of contracting the disease, therefore a prompt evisceration is necessary to reduce the possibility of migration of the larvae from the coelomic cavity to the muscle tissue. Some

fish, such as salmon, herring and Pacific halibut, appear to be more susceptible to post-mortem migration than other species (Adams et al., 1997).

The inactivation of the larvae can be achieved by cooking at temperatures of 60°C, up to the core of the product, for at least one minute. If the fish steak is 3 cm thick, it must be cooked at 60°C for at least 10 minutes in order to ensure that this temperature is reached at the core of the product (Chai et al., 2005; C.Re.NA; Sakanari and McKerrow, 1989).

All the fish products not intended for cooking or for treatments such as marinating, salting and smoking, must be frozen immediately after capture at −20°C for at least 24 hours. Similar treatment but with a different time/temperature ratio is that at −35 ° C for at least 15 hours (Audicana et al., 2002; Sakanari and McKerrow, 1989).

A series of studies have shown that vinegar and salt, used in marinating and salting procedures, do not guarantee the devitalization of the larvae (Buchmann and Mehrdana, 2016). Therefore, it is important to combine these treatments with the various freezing procedures, described above, in order to ensure the killing of the parasite.

In addition to these treatments, the current European Community Regulations require visual examination of fish with the elimination of heavily infested specimens and the extraction of visible larvae in less infested specimens (Audicana et al., 2002; Nieuwenhuizen and Lopata, 2013).

The purpose of the visual inspection is to detect any parasites with the unaided eye and predict their presence in the fish muscle in order to ensure that the contaminated fish does not pose a risk for consumers (Reg. (EC) No. 2074/2005; Llarena-Reino et al., 2012). However, Llarena-Reino et al. (2012) stated that visual inspection of the intestine is an inefficient method in predicting the presence of *Anisakis* larvae in fish muscle since it is unlikely that there is a frequent correlation between the number of parasites present in the abdominal cavity and those in the edible part of the fish. Furthermore, the accuracy of a visual inspection in a fishing industry largely depends on the training, experience and skills of the inspectors. Therefore, even today there are still some gaps to be filled using innovative prevention strategies, able to meet the needs of the European Food Safety Authority.

At present, the European authorities perform laborious and unreliable inspection method, such transillumination with UV (Jung-Soo et al. 2010) that cannot be applied for processed fish products such as anchovy paste, marinated anchovies, infant formulas etc. (Herrero et al. 2011). Furthermore, the current immunological methods for *Anisakis* allergy diagnosis give a high number of false positives due to the cross-reactivity with numerous panallergens (García-Palacios et al. 1996; Lluch-Bernal et al. 2002).

In this case, molecular biology methods are valuable tools in the detection of Anisakis spp. nematodes in processed seafood. Several studies showed that immunological and molecular methodologies yield comparable results concerning the detection of allergens in processed foods as sensitive and specific tools (Costa et al. 2014; Stephan et al. 2004).

2. PURPOSE OF THE THESIS

The presence of parasites belonging to the Anisakidae family has a significant impact in the fishing industry as it represents the main source of infestation in fish fauna. Furthermore, these parasites are no longer related only to wild fish but also can be present in farmed fish. Anisakid nematodes are capable of having immediate repercussions on human health and causing significant economic damages. Therefore, the purpose of my PhD thesis is to investigate the presence of nematode parasites belonging to the Anisakidae family in fishery and aquaculture products of the Mediterranean. In particular, with regard to fishery products, a survey on the seasonality of infestation was carried out in order to find possible preventive risk procedures based on fishing time. Inspection analysis and molecular methods were used for the detection and identification of these parasites in all the fish products examined. Regarding the inspection methods, chloropeptic digestion was used according to Reg. (EC) n. 2075/2005 by the use of a semi-automatic digestion system.

Finally, given the growing attention to allergological phenomena caused by the presence of *Anisakis* spp. larvae, it was decided to integrate the study described above with the analysis on the presence of these parasites in processed fishery products through the optimization and validation of a molecular method based on the Loop Mediated Isothermal Amplification (LAMP) technique.

The thesis activities were carried out in the laboratories of the C.Re.N.A. (National Reference Center for Anisakiasis), which is based at the Veterinary Health Institute of Sicily "A. Mirri".

The thesis was divided in e Work Packages (WP):

1. Assess the presence of Anisakidae in Mediterranean bluefish and evaluate their seasonal infestation trend;
2. Assess the presence of *Anisakis* spp. in Mediterranean farmed fish;
3. Carry out and validate a molecular method for the detection of *Anisakis* parasites in processed fish products.

3. MATERIALS AND METHODS

3.1 WORK PACKAGE 1

3.1.1 Sampling plan

The sampling was carried out within the monitoring plan "Research of Anisakidae larvae in fish products marketed in Sicily" The Monitoring Plan was drawn up by the Veterinary Health Institute of Sicily in collaboration with the Health Department of the Sicilian Region. The Monitoring Plan provided for a monthly sampling of fish samples.

Only samples caught in the FAO zones and sub-zones listed below were examined (**Figure 25**):

- FAO zone 37.1: Western Mediterranean
  - FAO sub-zone 37.1.3, which includes the northern coasts of Sicily and the Tyrrhenian Sea;
- FAO zone 37.2: Central Mediterranean
  - FAO sub-zone 37.2.2, which includes the southern coasts of Sicily, the Ionian Sea and the southern area of the Adriatic Sea;

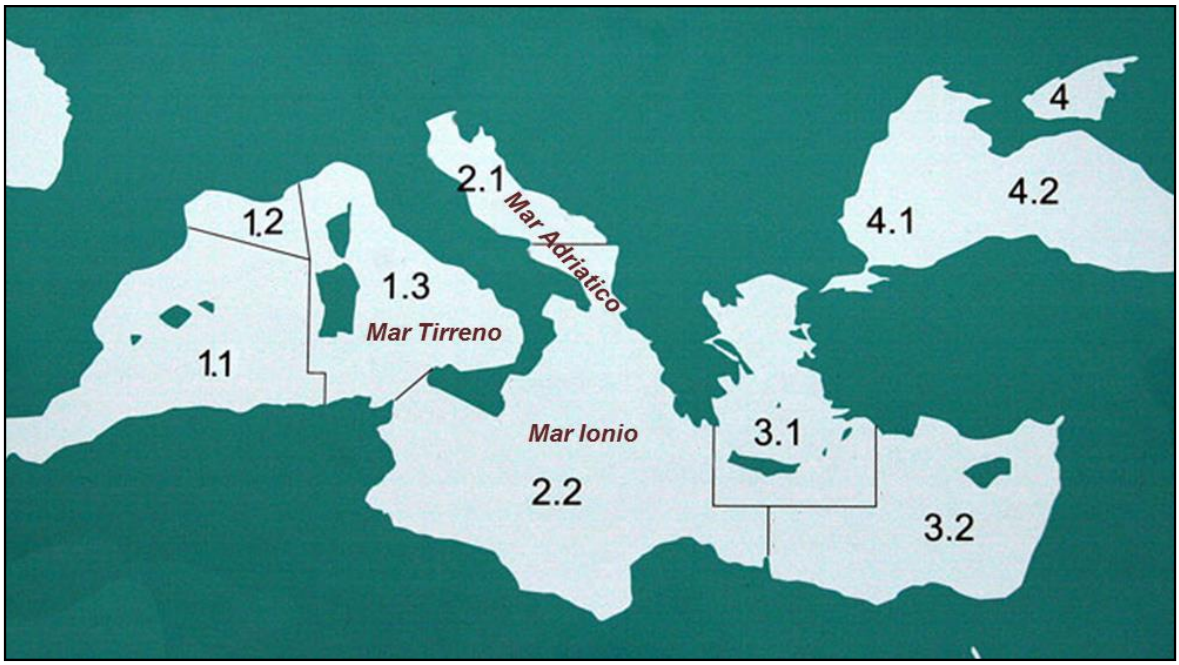


Figure 25 – Representation of FAO area 37 - Mediterranean.

A total of 7543 fish samples belonging to the species *Engraulis encrasicolus* (n = 3373), *Sardina pilchardus* (n = 3566), *Scomber scombrus* (n = 269), *Lepidopus caudatus* (n = 60)

and *Trachurus trachurus* (n = 275) were collected. All the fish samples were stored at + 4°C and transferred to the laboratories of the National Centre for the Anisakiasis for the preliminary inspection analysis.

3.1.2. Visual Inspection

The fish samples were subjected to a preliminary investigation through a visual inspection which aims to assess the presence of visible parasites.

Figure 26. Caudo-cranial opening of the sample through the use of a scalpel.

Each sample was opened with a scalpel following a caudo-cranial direction, from the anal pore to the gill to visualize the viscera, muscle tissue and the coelomic cavity, (**Figure 26**). This type of section has the ability to not compromise the integrity of the viscera and therefore avoids the transfer of any larva from the coelomic cavity to the muscle.

After dissecting the sample, the viscera and muscle tissue were visualized for the detection of any larvae present and subsequently, under a stereomicroscope (ZEISS CL 1500 ECO) for a more accurate examination (**Figure 27**).

Figure 27. Observation of the viscera and of the muscle under stereomicroscope.

Each larva found was placed in a Petri dish. A saline solution was added to the Petri dish to remove any residual of the fish and clean the larvae. (**Figure 28**).

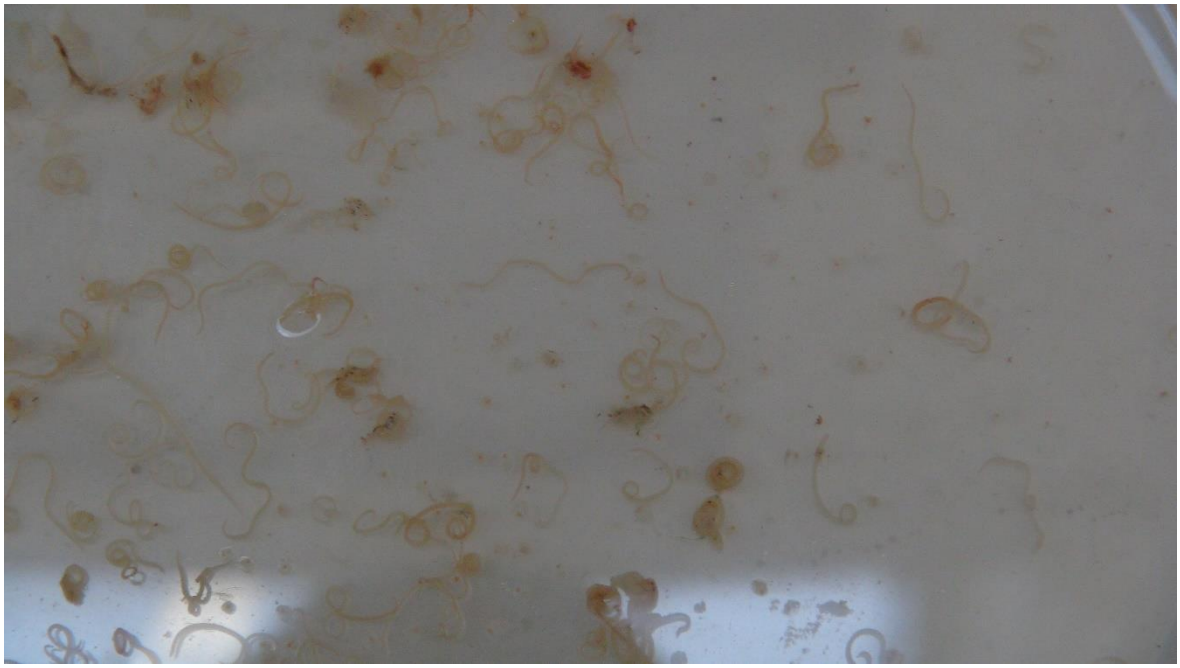


Figure 28. Washing of the larvae in physiological solution.

Subsequently, the larvae were fixed in 70% ethanol over night and stored for subsequent morphological analysis to identify the genus.

3.1.3 Chloropeptic digestion

In the event of a negative result on the visual inspection, A chloropeptic digestion was carried out. In particular, the samples were subjected to chloropeptic digestion according to Reg. (EC) n. 2075/2005; through a semi-automatic TrichinEasy digestion system (CTSV, Bruino, Italy).

The apparatus consists of a semi-automatic digestion system used to detect the presence of larvae belonging to the genus Trichinella. The digester was validated by Cammilleri et al. (2016) to allow the detection of Anisakid larvae.

The instrument consists of two main parts: the first part is composed by a homogenization system, consisting of a container with a mixer equipped with blades for shredding; the second one is composed by a filtering system consisting of a 180 µm filter and a collection cylinder joined to a tube to discharge the digestion product (**Figure 29**).

Figure 29. Semi-automatic digestion system.

One liter of water was pre-heated at 37°C (**Figure 30**); subsequently, 100 g of sample were weighed and homogenized by a stomacher (IUL Masticator).

Subsequently, the homogenized sample and the heated water were added in the homogenization sector (**Figure 30**) bypassing the mixer phase as this would risk damaging the larvae, thus compromising the veracity of the result since this instrument was designed to the detection of *Trichinella* spp. larvae, which are significantly smaller than of *Anisakis* larvae.

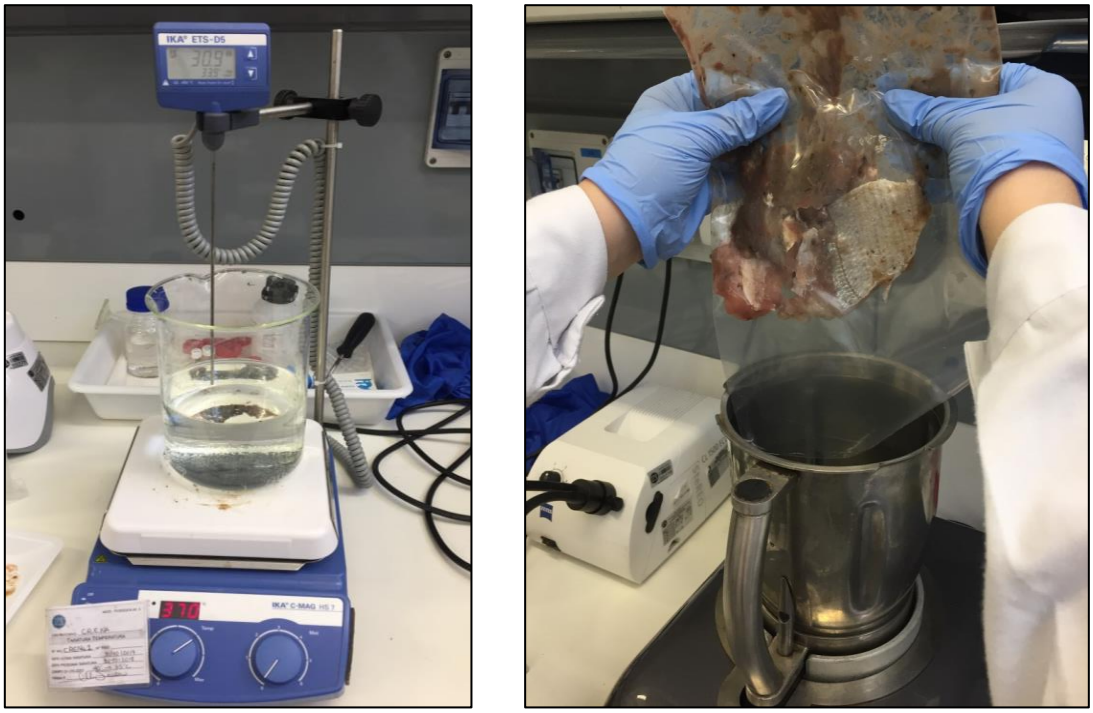


Figure 30. From left to right: heating water on a magnetic heat stirrer; Addition of the sample inside the container.

Subsequently, 50 ml of 10% hydrochloric acid and 10 g of pepsin (1: 10000 NF) were added (CTSV, Bruino, Italy)

Once the digestion phase was completed (after 20 minutes), the digested liquid was poured into the filtering system (**Figure 31**).

The filter (**Figure 31**) was then inspected under a stereomicroscope to visualize the potential presence of larvae.

Figure 31. From left to right: Filtration phase; Filter with the post-digestion larvae.

The larvae were washed in physiological solution, then fixed and stored with 70% ethanol in test tubes for subsequent morphological identification.

3.1.4 Analysis of epizootic parameters and seasonal trend evaluaion

After conducting the visual inspection and chloropeptic digestion, it was possible to obtain the number of samples infested and the number of parasites found. As a result, the epizootic parameters most used in epidemiological studies were calculated to quantify the presence of *Anisakis* parasites in the fish samples analysed: prevalence of infestation, mean infestation intensity and mean infestation abundance.

The prevalence of infestation is a value expressed as a percentage that indicates the frequency of anisakid larvae in a certain number of fish species examined. This parameter is calculated by comparing the number of infested samples to the total number of samples examined (Rózsa et al., 2000).

The mean intensity indicates the number of parasites for each individual sample infested and varies regardless of the prevalence. This index is calculated by relating the number of parasites to the number of infested samples, obtaining the average number of anisakid larvae present in an infested sample (Rózsa et al., 2000).

Finally, the mean abundance verifies the number of parasites in a certain number of fish species examined and is related to the prevalence. This parameter is obtained by dividing the number of parasites by the total number of samples analysed; it therefore expresses the average quantity of parasites present in all the samples examined (Rózsa et al., 2000). All the infestation parameters were examined using the software Quantitative Parasitology 3.0 (Rózsa et al. 2000, **Figure 32**). The prevalence and mean intensity values were broken down by month of sampling to give an estimation of the seasonal infestation trend.

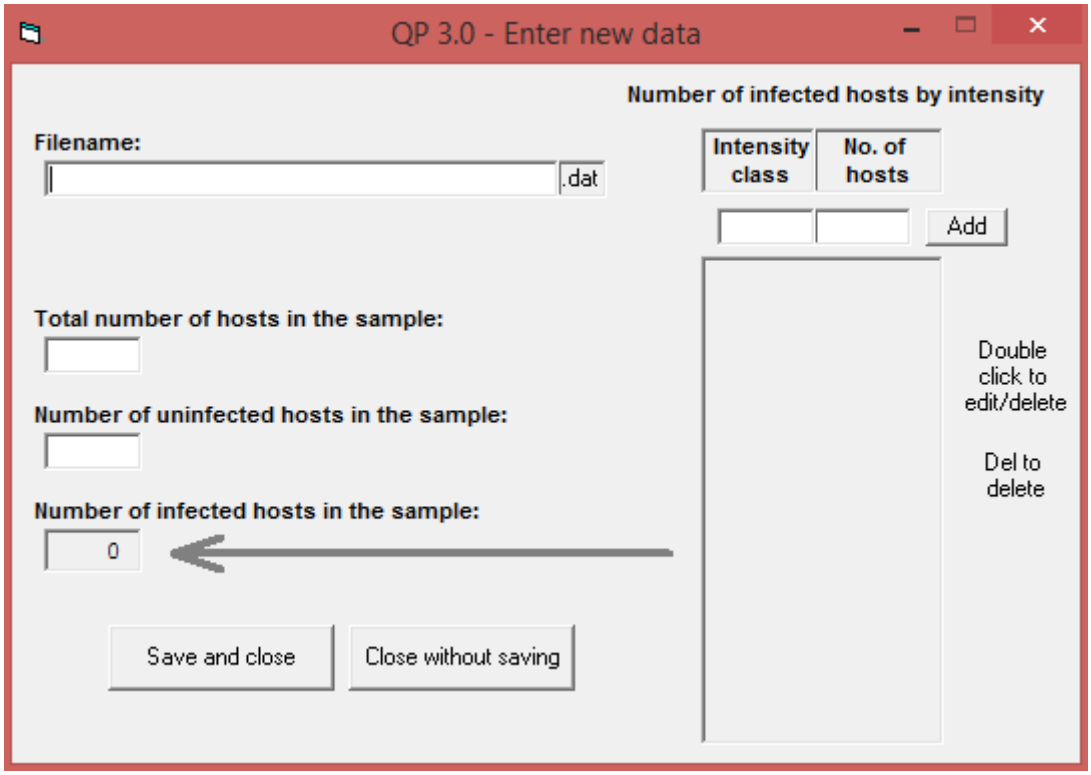


Figure 32. Quantitative Parasitology interface.

3.1.5 Morphological identification

The larvae, fixed in 70% ethanol, were clarified in 85% glycerol (Merck, USA) to be visualized by an Optika B-800 microscope (OPTIKA, Italy) (**Figure 33**).

Figure 33. Optika B-800 microscope.

The larvae were placed on a slide with 2 drops of glycerol and covered with a cover slide for clarification (**Figure 34**).

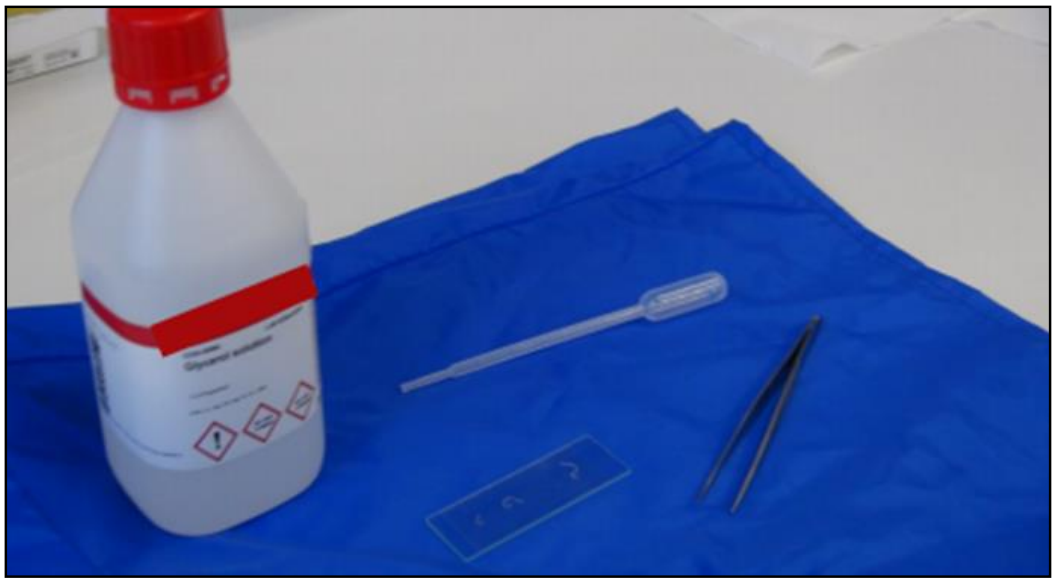


Figure 34. Clarification of parasites in glycerol.

After 15 minutes, the morphological characteristics of the anterior and posterior extremities of the larvae found were examined for morphological discrimination, according to taxonomic keys reported before (Berland, 1961). It was possible to identify whether they were anisakid larvae and, according to the morphology of the ventricle and the caudal end, the morphotype of the nematodes belonging to the genus *Anisakis*.

Once the morphological identification was completed, the larvae were again fixed in 70% ethanol for subsequent molecular analysis.

3.1.6 Molecular analysis

After the morphological identification, biomolecular methods were carried out to assess the species belonging to the genus *Anisakis*.

PCR-RFLP

PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) is a biomolecular method that allows the identification of species belonging to the genus *Anisakis*. The method is based on the amplification of specific DNA and the subsequent restriction by the use of restriction enzymes (HinfI and HhaI). In particular, the nuclear genomic region amplified includes the ribosomal spacers ITS1 and ITS2, plus the coding region for the 5.8S subunit. This molecular technique allows accurate identification of

species belonging to the genus *Anisakis* as it is very sensitive in detecting even minimal genetic variations (D'Amelio et al., 2012). In fact, the ITS region (ITS1 - 5.8S - ITS2) of the rDNA provides genetic markers and shows high levels of interspecific point mutations in the presence of very low intraspecific variations (Zhu et al., 2000).

Samples extraction

Before proceeding with the DNA extraction, the preserved larvae fixed in 70% ethanol, morphologically identified as *Anisakis* spp., were washed with sterile distilled water and fragmented with the use of a scalpel. The larval fragments were then placed into a 2 ml tube containing 200 µl of sterile distilled water and frozen at −20° C for 24 hours, to facilitate subsequent tissue lysis.

The extraction of nucleic acids was carried out by E.Z.N.A. Tissue DNA Kit D3396-01 (OMEGA bio-tek, Germany), based on the use of affinity columns.

The first step consits on the addition of 200 µl of Tissue Lysis Buffer and 25 µl of OB Protease Solution to degrade the tissues.

Subsequently, the samples were incubated in a thermomixer (Eppendorf, Germany) at 55°C with an agitation of 650 rpm. The incubation lasted about 2 hours, in order to ensure tissue degradation.

At the end of the incubation, 220 µl of nucleus lysis Buffer were added and the samples were incubated at 70°C for 10 minutes.

Subsequently, 220 µl of absolute Ethanol were added for DNA binding. Each sample lysed was then transferred to columns (HiBind DNA Mini Columns), assembled in collection tubes (2 mL Collection Tubes) and subjected to centrifugation at 14000 rpm for 1 minute. At the end of the centrifugation, the collection tube was emptied.

After adding 500 µl of HBC Buffer and centrifugation, two washes were performed. The washes allow the precipitation of all the lysis residues except for the DNA, which adheres to the filter of the column, thanks to the addition of ethanol.

For the first wash 700 µl of DNA Wash Buffer were added, centrifuging at 14000 rpm for 30 seconds and discarding the contents. The same operation was repeated for a second wash. The samples were centrifuged at maximum power for 2 minutes in order to eliminate other residues. Subsequently, each collection tube was discarded and the columns were assembled into 2ml tubes.

Finally, 200 µl of Elution Buffer were added, an nuclease-free water based reagent with monovalent cations able to precipitate DNA.

The tubes were placed at room temperature for 2 minutes and then subjected to centrifugation at 14000 rpm for 1 minute. Finally, the columns were discarded and the DNA extracted inside the 2 ml tubes was obtained and subsequently stored at −20°C.

The concentration of the extracted DNA was evaluated by a nanodrop spectrophotometer (Nanodrop, Thermofisher) at 260 nm, by adding 1 µl of extracted DNA.

DNA amplification

The DNA extracted was amplified by PCR reactions. Two primers were used to amplify the specific DNA region (ITS1 - 5.8S - ITS2), NC5 (forward; 5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3 ') and NC2 (reverse; 5'-TTA GTT TCT TTT CCT CCG CT-3 ') (Introvigen). Illustra ™ PuReTaq ™ Ready-To-Go ™ PCR beads (GE Healthcare) kit containing tubes, in which all the reaction reagents including nucleotides and Taq polymerase are lyophilized, were used.

The first operation was to prepare the Master Mix containing the water and the primers at the volumes shown in **Table 3**. The volumes of the various components refer to a single sample; therefore, they were multiplied by the total number of nematode samples to be analysed plus positive controls and negative control.

Reagent	Volume per sample
Water	21.5 µl
NC5	0.5 µl
NC2	0.5 µl

Table 3. Master mix composition.

After preparing the Master Mix, the numbered reaction tubes for the corresponding samples, the tubes for the positive controls and the tube for the negative control were placed in a suitable tube holder.

The positive controls are used to verify the correct functioning of the PCR reaction, while the negative control is used to verify the absence of contaminations. 22.5 µl of Master Mix was aliquoted into each reaction tube and then 2.5 µl of extracted DNA was added, obtaining

a reaction mix of 25 µl. Reference genomic DNA, extracted from larvae belonging to the genus *Anisakis* was used as positive controls, whereas nuclease-free water was used as negative control.

Finally, the test tubes were put into a 2720 Thermal Cycler (Applied Biosystems, USA) to allow the DNA amplification for about 2 hours, following the thermal profile reported in **Table 4**.

Phase	Time	Temperature	N. cycles
Initial denaturation	10:00	95°C	1
Denaturation	0:30	95°C	35
Annealing	0:30	58°C	
Polimerization	1:30	72°C	
Final polimerization	15:00	72°C	1
Maintaining	∞	10°C	1

Table 4. PCR thermal program.

Electrophoresis

The amplification products were examined using the electrophoresis technique on a 1% agarose gel (Invitrogen).

The first step was to weigh 1 g of agarose and add into a flask containing 100 ml of TBE Buffer (Invitrogen, USA), an electrophoretic running buffer, diluting at 1X.

After dissolvation of the agarose by boiling, 5 µl of SYBR® Safe DNA gel stain (Invitrogen) intercalator was added to the gel.

Then, the solution was poured into the electrophoretic cell with the relative combs, until a gel of about 4 mm thickness was obtained (**Figure 35**).

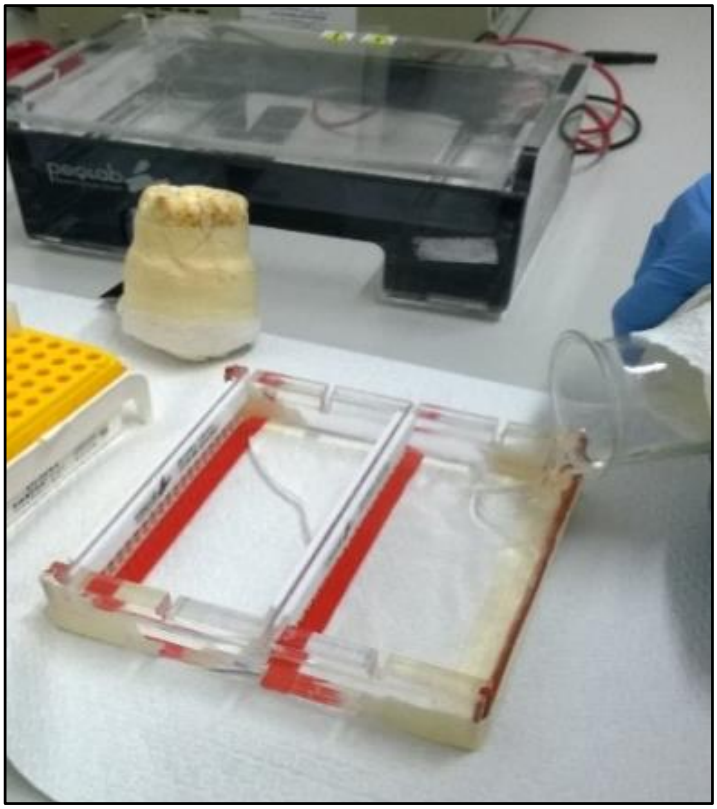


Figure 35. Pouring of the gel into the bed of the electrophoretic cell.

Once solidified at room temperature (after about 30 minutes), the bed was introduced into the electrophoretic cell (PEQLAB Biotechnologie GmbH, Holland) (**Figure 36**) containing sufficient running buffer to cover the wells fully.

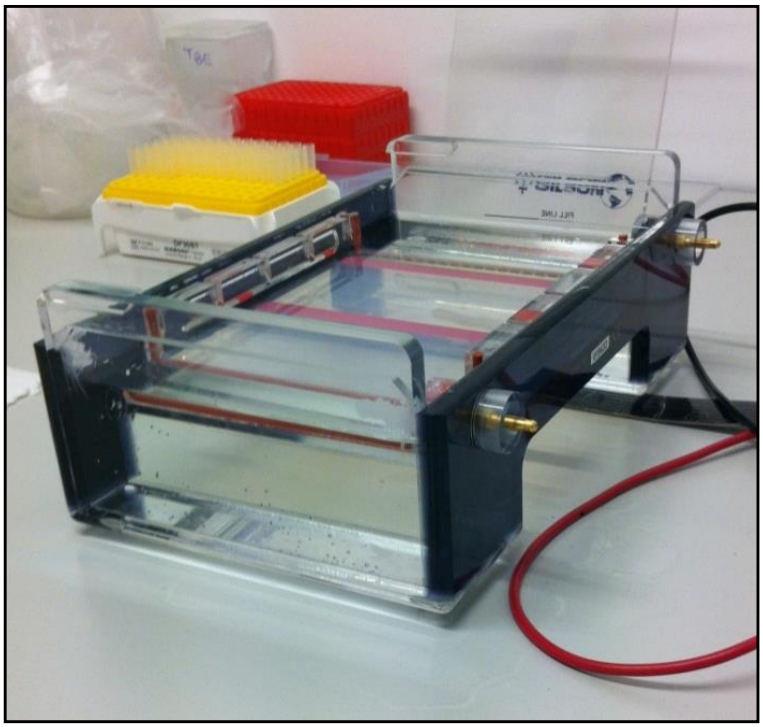


Figure 36. Electrophoresis system.

After extracting the combs, 5 µl of amplified sample mixed with 2 µl of Loading Buffer 6X (Promega, USA) were loaded into each well. The Ladder was loaded into the last well, i.e. the molecular weight marker (TrackIt ™ 100 bp DNA Ladder, Invitrogen, USA) which was prepared by adding 7 µl of TBE Buffer 1X and 2 µl of Loading Buffer 6X to 3 µl of concentrated Ladder.

The wells were loaded from left to right in the order: samples, negative control, positive controls and Ladder. Then, through a power supply (EuroClone) a current of around 60-70 V was applied and the run was interrupted after 90 min.

Finally, the gel was placed inside the UV transilluminator (GelDoc-It ™ Imaging System, UVP, USA) (**Figure 37**) to view the amplicons.

Figure 37. Transilluminator.

The size of the amplification bands were evaluated by comparison with the molecular weight marker and with the positive amplification controls (**Figure 38**).

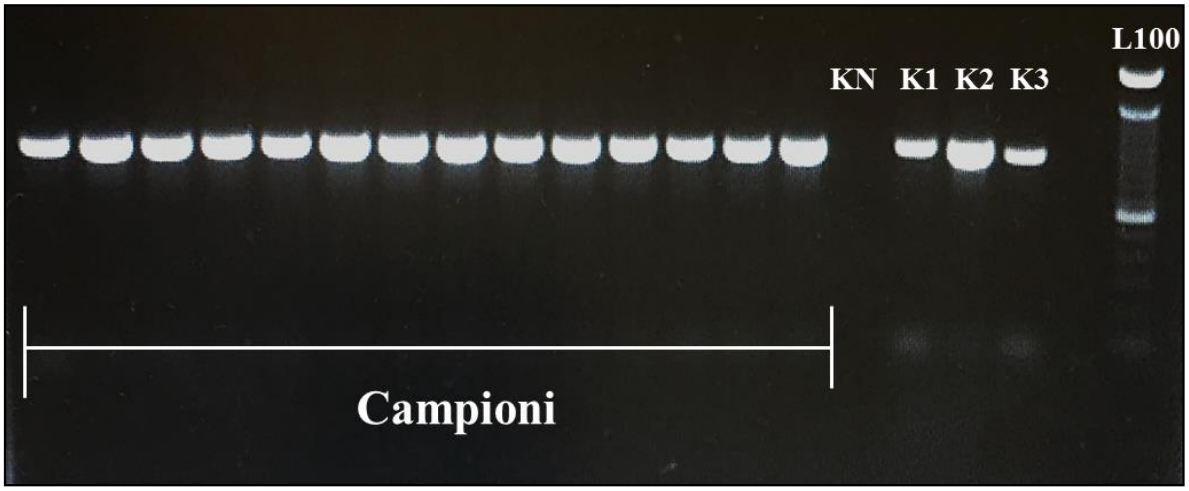


Figure 38. Amplification bands obtained after electrophoresis.

The amplification of the ITS region in *Anisakis* spp. was considered valid if the samples and positive controls showed a 960 bp band and also when the negative control showed no amplification products.

PCR-RFLP analysis

The restriction enzymes have the peculiarity of cutting in a defined region of the DNA corresponding to specific nucleotide sequences (**Figure 39**); they are called palindromic sequences, i.e. sequences that can be read in both directions on the two complementary strands of the DNA and are specific for each enzyme.

The purpose of the restriction is to obtain an identification of species thanks to the variability that the lengths of the restriction fragments have for each organism.

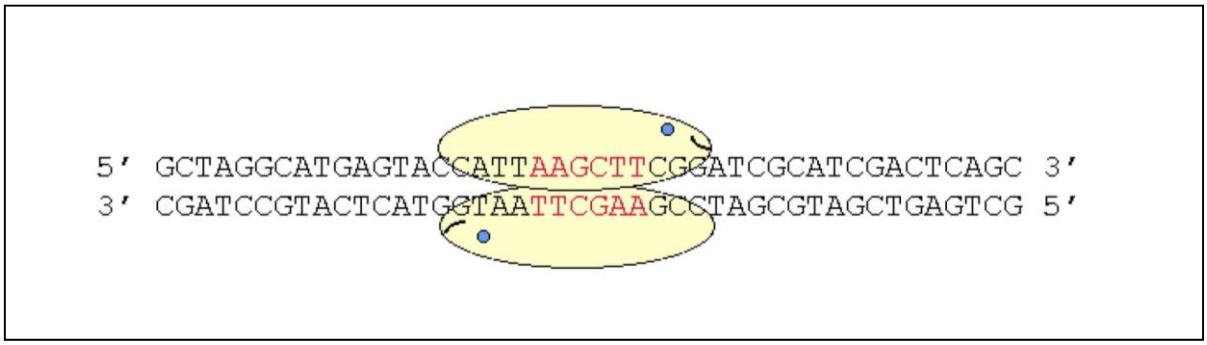


Figure 39. Mechanism of restriction enzymes.

In this study, the amplified DNA samples were subjected to enzyme restriction using two different restriction enzymes, *Hha*I and *Hinf*I (Promega, USA).

The sequences recognized for the identification of *Anisakis* species are as follows: GCG C - CGC G for HhaI and GANT C - CTNA G for HinfI.

Two different reaction mixtures were prepared, each for an enzyme, containing sterile distilled water, Buffer, BSA (Bovine Serum Albumine) and finally the enzymes (**Table 5**). The volumes of the reaction mix were then multiplied by the total number of samples to be analyzed, including positive controls and negative control.

Reagent	Volume per Sample		Reagent	Volume per sample
Water	13.8 µl		Water	13.8 µl
Buffer	2 µl		Buffer	2 µl
BSA	0.2 µl		BSA	0.2 µl
*Hha*I	1 µl		*Hinf*I	1 µl

Table 5. Components and volumes of the PCR-RFLP reaction mixtures.

Once the two mixtures were prepared, 17 µl of the reaction mixture containing the HhaI enzyme were aliquoted into each reaction tube and then 3 µl of amplified DNA were added, obtaining a final volume of 20 µl per sample. The same operation was done for the reaction mixture containing the HinfI enzyme.

The tubes were put into the thermal cycler AB 2720 (Applied Biosystems, Carlsbad, CA, USA) and incubated at 37°C overnight to allow a greater effectiveness of the enzymes.

Electrophoresis

The restriction products were analyzed by electrophoresis on 2% agarose gel (**Figure 40**). The restriction patterns of the species belonging to the genus *Anisakis* are shown in **Table 6** and represent the key to the identification (D'Amelio et al., 2000; D'Amelio et al., 2012).

Specie	*Hinf*I	*Hha*I
A. simplex s.s.	620-250-80 bp	550-430 bp
A. pegreffii	370-300-250 bp	550-430 bp
A. pegreffii/A. simplex s.s.	620-370-300-250-80 bp	550-430 bp
A. berlandi	620-250-80 bp	550-300-130 bp
A. typica	610-350 bp	320-240-180-160 bp
A. ziphidarum	370-320-290 bp	550-430 bp
A. physeteris	380-290-270 bp	540-420 bp
A. brevispiculata	1000 bp	400-320-200 bp

A. paggiae	1000 bp	520-400 bp

Table 6. Restriction p*atterns* for the *Anisakis* genre (D'Amelio *et al.*, 2012).

The electrophoresis procedure was done according to procedure described for the PCR products but on 2% agarose gel.

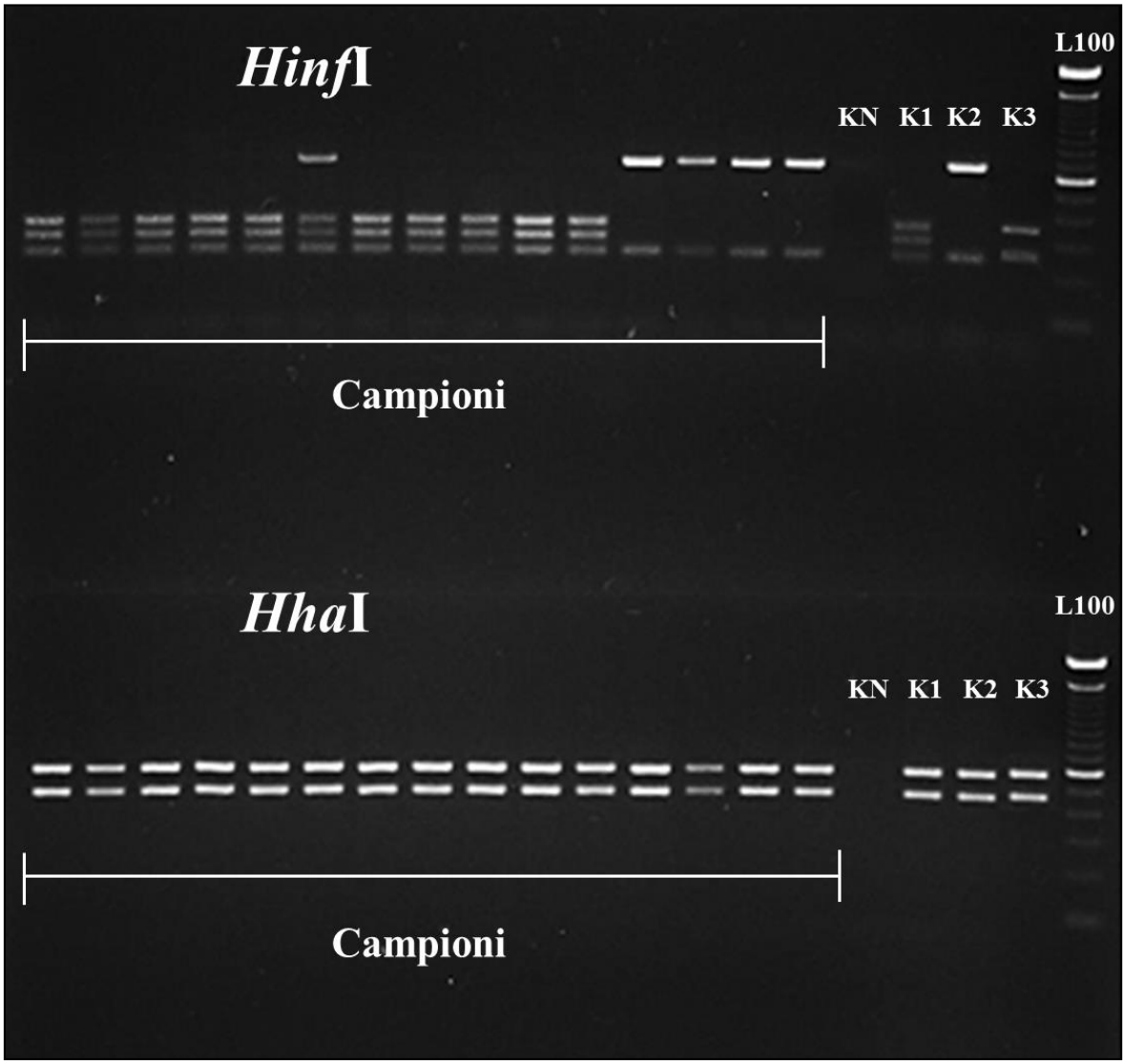


Figure 40 – Restriction profiles obtained with the enzymes H*haI* and H*infI*.

The identification of the *Anisakis* species was done comparing the size of the bands obtained from the samples with those of the reading key for *Anisakis* spp. shown in the previous Table.

3.2 WORK PACKAGE 2

3.2.1 Sampling plan and sample collection

83 European sea bass samples (*Dicentrarchus labrax*) from 2 farms located in Sicily (Southern Italy) were sampled for the detection of *Anisakis* larvae. The farm sited in Licata (37°05'N 13°56'E) adopt offshore floating cages and compound feed supply as a rearing technique.

The farm sited in Pachino (36°42'N 15°07'E) adopt an indoor rearing technique for hatchery and open sea floating cages for fattening. The European sea bass of this farm are fed exclusively with fishmeal. Further, 68 farmed European sea bass samples were collected from 13 fish markets of North-Western Sicily. Based on what was stated on the label, all the 68 farmed fish samples came from farms located in Greece (FAO 37.3). All the fish samples described above were intact. The samples were stored at +4 °C and transferred to the laboratories of the Centro di Referenza Nazionale per le Anisakiasi for the morphometric and inspection analysis.

3.2.2 Inspection of the fish samples

The fish samples were initially submitted to morphometric analysis by the calculation of maximum standard length and Fulton's condition index - K. Standard length (SL) is the length of a fish measured from the tip of the snout to the posterior end of the last vertebra or to the posterior end of the midlateral portion of the hypural plate. Simply put, this measurement excludes the length of the caudal fin (http://www.fishbase.org/Glossary/Glossary.php?q=standard+length&sc=is).

The value of *K* is calculated from the weight and length, and can be used to estimate changes in nutritional condition (Nash et al. 2006). Proposed by Fulton in 1904, it assumes that the standard weight of a fish is proportional to the cube of its length by the formula:

$$K = 100\ (\frac{W}{L^3})$$

Where W correspond to the weight of the fish (g) and L correspond to the standard length of the fish (cm).

A parasitological exam was performed on fish samples examining accurately the coelomic cavity and muscle by visual and stereoscopic inspection according to section 3.1.2. For visual. Subsequently, a chloro-peptic digestion of viscera and muscles was carried out

according to the protocol reported by Cammilleri et al. (2016) and described in section 3.1.3 (**Figure 41**). All the nematode larvae collected were washed in saline solution, fixed in 70 % ethanol and cleared with glycerol for morphological identification by light microscopy Leica DM 2000 (Wetzlar, Germany), following the taxonomic keys (Berland, 1961).

Figure 41 – Sample of *Dicentrarchus labrax* subjected to chloro-peptic digestion.

3.2.3 Molecular analysis and data analysis

All the larvae were submitted to molecular identification at species level by PCR- based restriction fragment length polymorphism (PCR-RFLP) analysis of the rDNA comprising the internal transcribed spacers ITS (ITS-1, 5.8S gene, and ITS-2) region according to the protocol reported in section 3.1.6. The Infestation parameters such as prevalence and mean intensity were examined using the software Quantitative Parasitology 3.0, according to the section 3.1.4.

3.3 WORK PACKAGE 3

The third work package consisted in the optimization and validation of a commercial LAMP assay for *Anisakis* spp. DNA detection in order to obtain a simple, fast and cheap tool, which can identify possible risks to consumer health due to the presence of these organisms in processed fish products.

3.3.1 Loop-mediated Isothermal Amplification (LAMP)

Loop mediated Isothermal amplification was published and invented by ICANN Chemical Company in 2000 (Notomi et al. 2000). The method, as the name suggests, runs on a single amplification and single temperature. The method uses a polymerase with high strand displacement activity, which allows it to be isothermal not requiring a denaturation of DNA at 95°C as in the conventional PCR; it is rapid (positive reactions even under 30 min) and factorial as opposed to exponential in PCR. The LAMP requires six primers, and its yield is much higher than conventional PCR, with 10 to 20 milligrams which makes it amenable to visual detection based on two grid and tends to be more tolerant of sample matrix inhibitors. LAMP is particular useful in diagnostic and particularly in molecular diagnostics and food testing.

Lamp workflow is very easy with very low complexity with instrumentation, because of the isothermal nature; it either as the sample can be directly added or a simple nucleic acid extraction heat lysis can be used, and the results can be visualized with different modes.

LAMP requires a minimum of four primers (six with the inclusion of loop primers which allow for faster reactions). The primer set (**Figure 42**) is traditionally composed by 2 internal primers (FIB and BIP), 2 external primers (F3 and B3) and 2 loop primers (FL and BL).

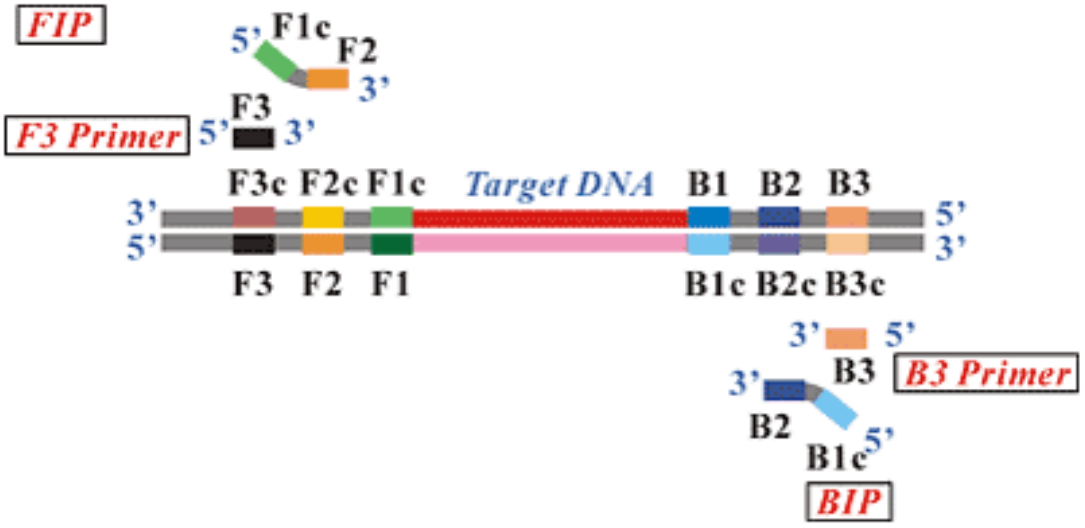
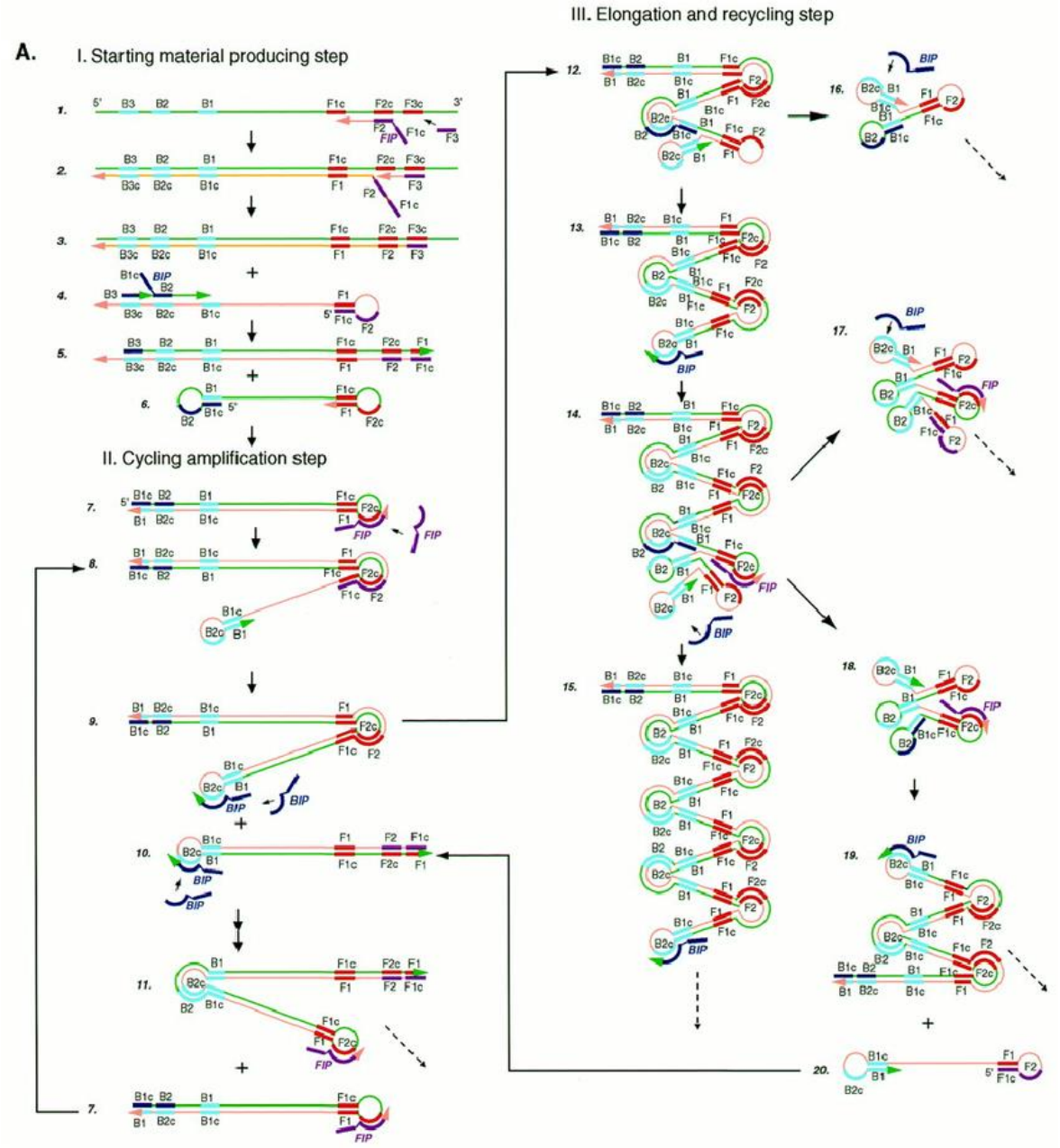


Figure 42 – LAMP primers representation (Eiken Chemical Co., Ltd).

Amplification initiates from strand invasion by one of the inner primers and the strand displacing DNA polymerase extends the primer and separates the target DNA duplex.

The first product is then displaced by synthesis initiating from an outer primer which anneals to an upstream target region as it is displaced the end of the product forms a self hybridizing loop structure due to the inclusion of a reverse complementary sequence in the inner primer sequence. This annealing and displacement cycle repeats on the opposite end of the target sequence and the resulting product is a short dumbbell structure that forms a seed for factorial lamp amplification.

The dumbbell structure contains multiple sites for initiation of synthesis from the three prime ends of the open loops and annealing sites for both the inner and loop primers. His amplification proceeds from these multiple sites. The products grow and form long concatamers each with more sites for initiation. The result is a rapid accumulation of double-stranded DNA and amplification byproducts that can be detected by a variety of methods. **Figure 43** shows a schematic representation of the LAMP process (Eiken Chemical Co., Ltd).



3.3.2 Fish samples and Anisakis larvae collection

All the processed fish samples used for the LAMP method optimisation and validation came from a large-scale distribution, in order to reduce any bias from local food specialities and extend the range of validation. Homogenised farmed trout (*Oncorhynchus mykiss;* n=40), homogenised farmed sea bream (*Saprus aurata;* n=40) and homogenised farmed salmon (*Salmo salar;* n=40) were chosen as naturally negative (non-contaminated by *Anisakis*) samples (**Figure 44**) (Lunestad 2003; EFSA 2010; Peñalver et al. 2010) whereas anchovy paste (n=40), anchovy in oil (n=40) and salted sardines (*Sardina pilchardus;* n=40)

samples were chosen as positive samples for the validation of the method and for matrix effects evaluation.

Figure 44. Homogenised farmed salmon samples subjected to LAMP validation.

All the processed fish samples came from Italian supermarkets. The Anisakidae larvae used for the artificial contamination of the samples were collected from *Lepidopus caudatus*, *Clupea harengus* and *Merluccius merluccius* samples after visual inspection and modified chloro-peptic digestion method described in the previous sections.

The larvae isolated were washed in physiological saline serum (pH 7) and morphologically identified by B-800 light microscopy (Optika, Ponteranica, Italy). Furthermore, *Anisakis* morphotype II, *Hysterothylacium* sp., *Contracaecum* sp. and *Pseudoterranova* sp. larvae were taken from the reference materials of the Centro di Referenza Nazionale per le Anisakiasi (Palermo, Italy). The number of *Anisakis* spp. larvae used for artificial contamination has been defined according to the prevalence of infestation of the fish species examined reported in the literature (Ciccarelli et al. 2011; Cavallero et al. 2015; Cipriani et al. 2016; Cammilleri et al. 2020). The larvae were cut into pieces and then were carefully mixed with the processed fish samples.

3.3.3 Optimization of the LAMP assay

The method has been optimised for DNA extraction phase by testing in triplicate initial weight of the samples at 50, 100, 250 and 350 mg with 0.5, 2, 4 and 8 ml of extraction buffer (Enbiotech S.r.l., Palermo, Italy). The extract has been tested with undiluted and diluted 1:5 and 1:10 for matrix effect assessment. An initial weight of 250±50 mg with 4 ml of extraction buffer and a 1:5 dilution after the extraction were found to be the best conditions for effective real-time detection of DNA amplification by the LAMP method proposed, giving the fluorescence intensity required for detection.

DNA extraction

Genomic DNA was extracted from positive and negative fish samples, contaminated or not with *Anisakis* spp., respectively. The extraction was also carried out for the samples artificially infested by *Contracaecum* sp., *Pseudoterranova* sp. and *Hysterothylacium* sp. The DNA extraction was performed using a ready to use buffer contained in the Anisakis Screen Glow kit (Enbiotech S.r.l., Palermo, Italy) as stated above. A portion of 250±50 mg of sample was directly placed into 15 mL tubes containing 4 mL of the ready for use extraction buffer (Enbiotech S.r.l., Palermo, Italy) and then incubating for 40±5 minutes at room temperature.

Primers design and optimization

To design the primer set targeting *Anisakis* spp. gene, the genomic sequences of internal transcribed spacer 2 gene from various species were collected from GenBank™ (EU624342.1, AY826720.1, AB277823.1, AB196671.1, AB277821.1, AB551660.1, HF911524.1, AY826723.1, EU718479.1, JQ912690.1, KF512840.1, JX535521.1, KF032062.1, EU327691.1), taking into account conserved regions. Closely related organisms were evaluated to avoid false positive. Different set of six primers, two outer (F3 and B3), two inner (FIP and BIP) and two loop (LF and LB) were designed using the Primer Explorer software.

The primers were carried out taking into account the different assumption to obtain an optimized design. The design process considered a distance between F2 and B2 of 140 nucleotides (nt), a distance between F1C and F2 of 50 nt; a distance between F2 and F3 of 15 nt and 50 nt for the distance between F1c and B1c. The distance between F2 and F1c is important because this is the site where the loop primers sit. The primers optimized showed

a GC content between 40 and 60%. Furthermore, the primer designing was carried out in order to have an equal melting temperature (Tm) and the absence of complementarity avoiding regions of secondary structure (intra/inter-primer homology) and mutation within the primers The different set of primers produced were also compared taking into account the assay temperature that will provide solid resolution and fast results.

The time to result (TTR) parameter, expressed in minutes, was considered for this purpose by comparing positive samples and no template control (NTC) samples trying to get faster TTR for positive samples and decreased non-specific amplification (slower TTR from NTC). . The optimized primer set was synthetized by the Enbiotech ® Company (Palermo, Italy) and added as a component of the Anisakis Screen Glow commercial kit (Enbiotech Group S.r.l., Palermo, Italy). The Anisakis Screen Glow commercial kit includes ready-to-use reaction tubes (containing primers, fluorescent dye and other reagents.) to achieve a rapid amplification of DNA template.

Lamp assay

As described above the method was optimised by real time monitoring of the time and temperature of reaction. The optimal reaction temperature and time for the LAMP assay has proved to be 65°C and 35 minutes, respectively giving the fluorescence intensity required for detection.

The analytical and diagnostic assays to recognise *Anisakis* spp. DNA has been performed using the Anisakis Screen Glow commercial kit (Enbiotech Group S.r.l., Palermo, Italy) with ICGENE mini portable instrument (**Figure 45**) (Enbiotech Group s.r.l., Palermo, Italy), consisting of a real-time fluorimeter regulated by the the ICGENE application (Enbiotech Group s.r.l., Palermo, Italy), downloadable on various smart devices.

Figure 45. ICGENE mini portable instrument.

The protocol to obtain the specific amplification of the target *Anisakis* spp. DNA was carried out in a mixture of a final volume of 55 µl, including 22 µl of Anisakis Screen Glow LAMP mix (Enbiotech Group s.r.l., Palermo, Italy), 30 µl of mineral oil and 3 µl of the extracted DNA samples. The mineral oil was added to the top of the reaction mixture to prevent evaporation. The amplification was performed at 65°C for 35 minutes.

3.3.4 Specificity of the LAMP assay

Based on the evolutionary relationships and their feasible genetic similarity, parasitic material belonging to the Anisakidae and Raphidascaridadae family were screened to have evidence on the diagnostic specificity of the LAMP assay. The parasitic material was previously characterised as belonging to *Hysterothylacium*, *Contracaecum* and *Pseudoterranova* genera using specific molecular diagnostic keys and methods reported in the literature (Cavallero et al. 2011).

Two different experiments were carried out for the specificity evaluation: i) in the first experiment performed using genomic DNA, *P. decipiens sensu stricto, P. krabbei, P. cattani, P. azarasi, C. rudolphii A* and *Hysterothylacium aduncum* were tested in duplicate, also including internal positive and negative controls of the Anisakis Screen Glow commercial kit (Enbiotech S.r.l., Palermo, Italy); ii) in the second experiment processed fish samples artificially contaminated with *Pseudoterranova* sp., *Contracaecum* sp. and *Hysterothylacium* sp. larvae were subjected to DNA extraction described above and analysed in duplicate, together with positive and NTC. Twenty samples of each processed fish product

type, of which ten artificially infested (5 with genomic DNA and 5 with larvae), were tested for the specificity assessment.

3.3.5 Sensitivity of the LAMP assay

The sensitivity or inclusivity was established as the ability of the LAMP method to detect DNA of *Anisakis* spp. (expressed as a percentage; Feldsine et al. 2002). Two different experiments were carried out for the inclusivity evaluation: i) in the first experiment performed using genomic DNA, *A. pegreffii, A. simplex sensu stricto, A. typica, A.ziphidarum and A. physeteris* were tested in duplicate, also including internal positive and negative controls; ii) in the second experiment processed fish samples artificially contaminated with *Anisakis* spp. type I and type II larvae, were subjected to DNA extraction and analysed in duplicate, together with positive and NTC. Twenty samples of each processed fish products type were tested for the inclusivity assessment.

3.3.6 Limit of detection

The limit of detection (LOD) was established as the lowest concentration of DNA of *Anisakis* species which provides a signal significantly different from the negative template control. The determination of the LOD of the LAMP method was assessed by serial 10-fold dilution of the DNA extracted from *Anisakis* spp. larvae with nuclease-free water. All measurements were performed in ten replicates from each sample type independently.

The range of the DNA extracted varied between 2.22 ng μl^{-1} and 8.40 ng μl^{-1} with good 260/280 and 260/230 ratios (1.8 to 2.1) tested by a Nanodrop Spectrophotometer (Thermo, Waltham, USA). A test was considered acceptable when it ensures the detection of positive samples successfully with DNA content equal to or greater than LOD.

3.3.7 PCR Real-Time

Real-Time PCR (RT-PCR) assays of the same samples analysed by LAMP were also carried out for a comparative purpose and as a confirmation method for the results of the LAMP assays.

The amplification reaction is highly sensitive and specific, therefore it allows to detect even very small quantities of DNA extracted from the food matrix (Cavallero et al., 2017). The amplification trend is detectable in real time, by the use of a thermal cycler (LineGene 9660, BIOER) (**Figure 46**) connected to a computer.

Figure 46. PCR Real-Time Thermocycler.

DNA extraction

The ION Force DNA Extractor Fast kit (Generon, Modena, Italy) was used for the extraction of nucleic acids, which is based on the use of affinity columns.

The first step was to weigh 2.5 g of each sample in 50 ml tubes and then 20 ml of Solution A was added.

After vortexing, the tubes were incubated for 1 hour at 85°C in a water bath (PolyScience, USA) (**Figure 47**), taking care to vortex 2-3 times during incubation.

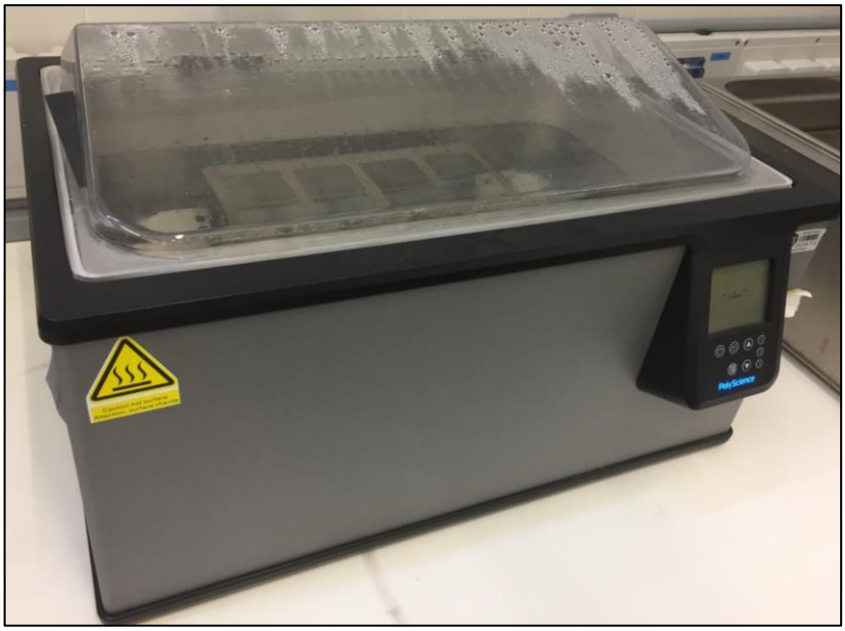


Figure 47. Water bath used for the incubation.

After incubation, the tubes were placed into a centrifuge (Heraeus Biofuge Stratos, Germay) and were centrifuged at 8,000 rpm for 10 minutes. After centrifugation, 1 ml of supernatant (aqueous phase) was transferred into a 2 ml tube and 0.8 ml of Buffer E was added.

Once vortexed for about 1 minute, the tubes were centrifuged at 10,000 rpm for 5 minutes and 0.5 ml of the subnatant (aqueous phase below) was transferred into a propylene tube containing 5 ml of Buffer T.

The propylene tubes were stirred gently by inversion to avoid DNA stresses. Subsequently, the solution was filtered through a 0.45 microns cellulose acetate membrane filter, inserted into a 10 ml syringe (**Figure 48**).

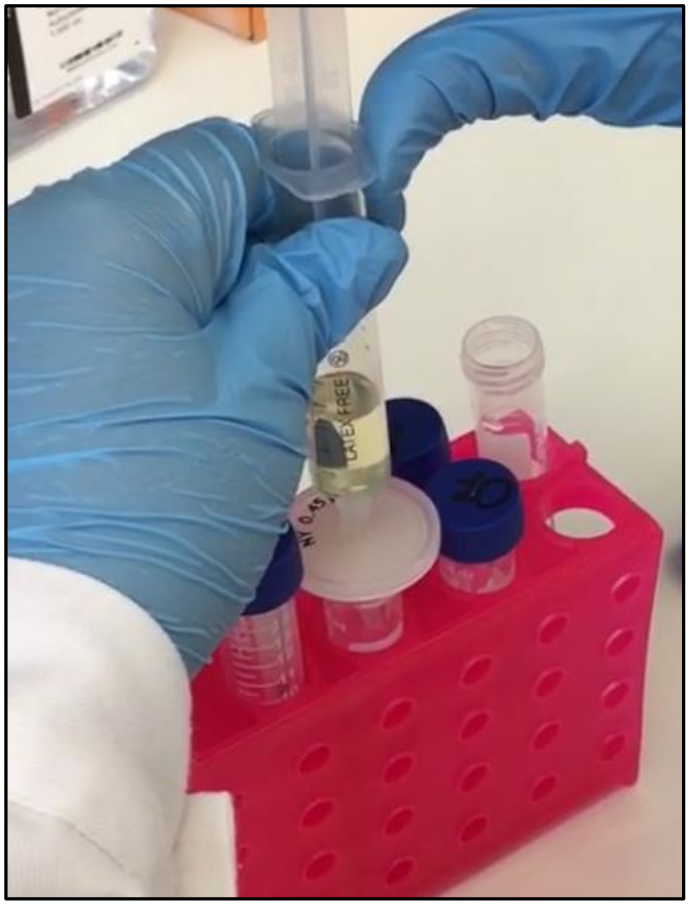


Figure 48. Solution filtration.

After filtering, the stopcocks, the purification columns, the adapters for the vacuum chamber and finally the 10 ml syringes were mounted on the vacuum chamber (**Figure 49**).

Figure 49. Disposition of the samples in the vacuum chamber.

Once the vacuum pump was turned on, the filtered content of each tube was gradually poured into the respective syringe, then the stopcock was opened to let one drop per second go down and finally 0.75 ml of Buffer P was added three times for washing.

After washing, the columns were removed from the vacuum chamber and placed in a collection tube to be subjected to centrifugation at 7,000 rpm for 5 minutes, in order to eliminate the Buffer P.

Subsequently, the collection tube was removed and the columns were transferred to 2 ml tubes, followed by adding 150 µl of Solution D.

The 2 ml tubes, containing the columns, were placed at room temperature for 2 minutes and two centrifugations were carried out to elute the DNA from the column: one at 500 rpm for one minute and the next at 14,000 rpm for 5 minutes. Finally, the columns were discarded and the extracted DNA was stored at −20°C.

DNA amplification

The PATHfinder Anisakis/Pseudoterranova Assay (Generon, Modena, Italy) kit was used for amplification. The kit includes the Positive and Negative Controls. The positive control contains DNA of nematodes belonging to the *Anisakis* genus and allows to verify the correct functioning of all reagents. Finally, the amplification control or internal control, present inside the GENERase ULTRA PLUS Mastermix together with all the other reagents, contains generic eukaryotic DNA allowing to check if the amplification was done correctly.

The reaction mix always contains all the reaction reagents including the primers, the Taq polymerase and in this case the probes. The probes used emit a certain fluorescence which is detected in real time by dedicated softwares. Those used were FAM and HEX. The fluorescent hybridization probe labeled with the FAM fluorophore specifically recognizes DNA belonging to the *Anisakis* genus. The internal control is detected by a fluorescent hybridization probe labeled with HEX fluorophore.

After preparing the reaction tubes for the samples, the positive control and the negative control, 15 µl of PATHfinder WORKING M-MIX (Generon, Modena, Italy) were aliquoted into each tube. 5 µl of extracted DNA was added to the sample tubes, resulting in a total reaction volume of 20 µl. Instead, 5 µl of Positive Control were added to the positive control tube and 5 µl but of Negative Control to the negative control tube.

Subsequently, the tubes were introduced into the thermal cycler and the amplification program was set using the LineGene 9600 software connected to the thermal cycler.

The related fluorophores (FAM and HEX) corresponding to the probes contained in the kit have been inserted into the software. Then, the samples and controls were set and then the wells for each sample were selected respecting their real position in the thermal cycler. Finally, the thermal cycle shown in **Table** 7 was set.

Step	Time	Temperature	N cycles
Taq Activation	3:00	95°C	1
DNA Denaturation	0:10	95°C	
Annealing/Extension + Plate Reading	0:16	57°C	45

Table 7. PCR Real-Time thermal programme.

The results elaborated by the software were interpreted by comparing the fluorescence signals of the samples with those of the positive and negative control as well as verifying the presence of the HEX and FAM curves, which indicate that the reaction was successfully done and the samples were positive for *Anisakis*.

4. RESULTS

4.1 WORK PACKAGE 1

4.1.1 Inspection analysis: visual inspection and chloro-peptic digestion

The visual inspection and chloropeptic digestion carried out on the fish samples verified the presence of 8172 larvae, infesting 13.78% of all the samples analysed.

The presence of anisakid nematodes was found in all the species examined. **Table 8** describes the data relating to the total number of samples examined, the number of samples that presented larvae and the total number of larvae detected by inspection analysis.

Of the total 7543 samples, the presence of larvae was detected in 720 samples by visual inspection (**Figure 50 and 51**).

Fish samples that did not show larvae during the visual inspection were subjected to chloropeptic digestion. This second investigation revealed positivity in 28 samples through the TrichinEasy® digestion system, detecting a success rate of 2.7% for chloropeptic digestion.

Specie	N. samples	N. samples infested	N. larvae	Visual inspection	Chloropeptic digestion
Engraulis encrasicolus	3373	586	586	586	-
Lepidopus caudatus	60	60	4888	60	-
Sardina pilchardus	3566	96	96	92	4
Scomber scombrus	269	87	671	72	15
Trachurus trachurus	275	133	1931	124	9

Table 8. Results of the inspection analysis sorted by fish species. Visual inspection and chloropeptic digestion correspond to the number of positive samples detected by the different inspection methods.

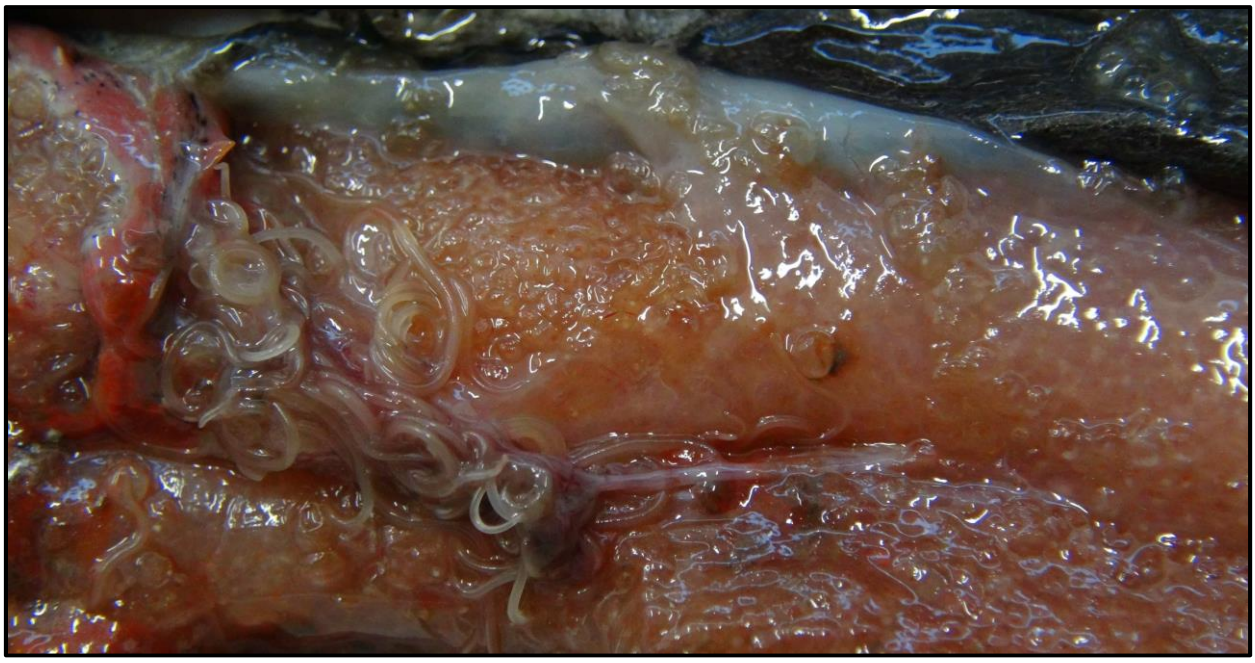


Figure 50. Anisakidae larvae found in a silver scabbardfish sample after visual inspection.

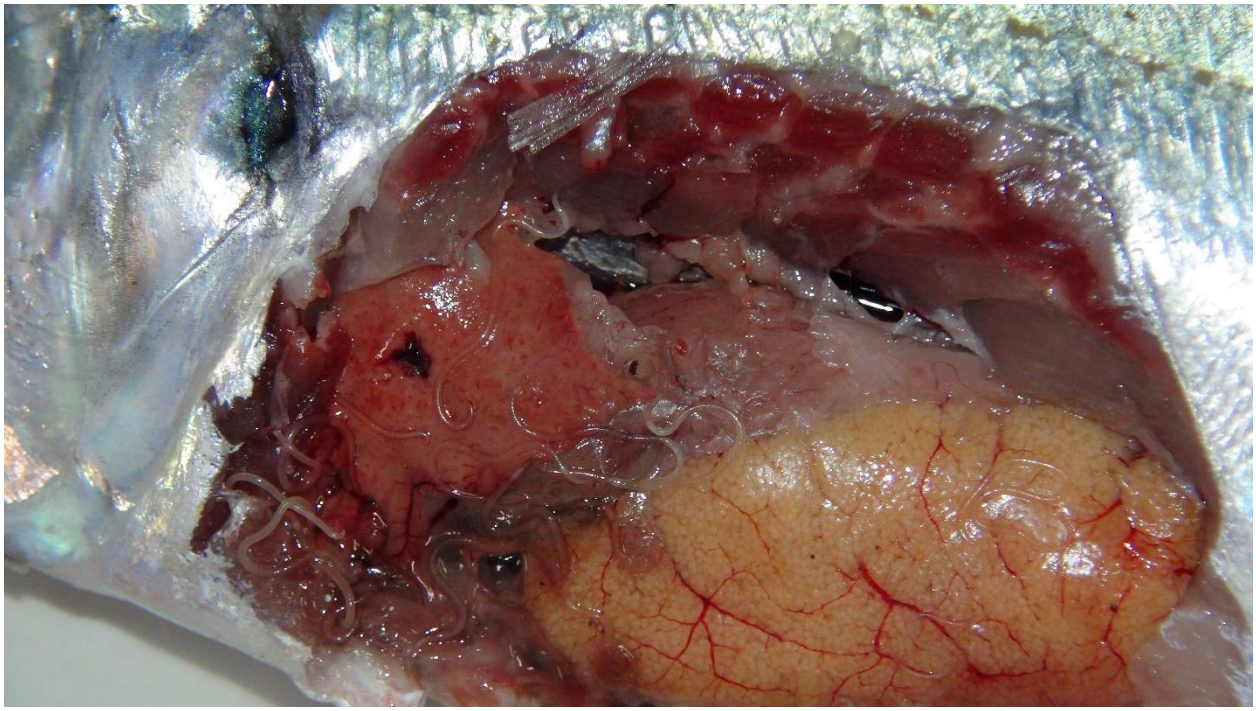


Figure 51. Anisakidae larvae found in a horse mackerel sample after visual inspection.

In particular, the chloropeptic digestion allowed to find 4 larvae in sardine samples, 15 in mackerel samples and 9 in horse mackerel samples.

Furthermore, over 90% of the larvae were found viable, verifying motile activity up to 30 minutes after collection.

Regarding the location of the larvae, these have been found in the viscera, in the muscle and also in the superficial portions of the fish. Only in one mackerel sample the larvae were found in the swim bladder. All fish species examined showed larvae in the viscera and specifically, in the pyloric caecum, gonads, liver, stomach and intestines. Furthermore, all the fish samples analysed showed the presence of Anisakidae larvae in edible parts (muscle). In particular, 28, 77, 17, 54, 61 larvae were found in the muscle of anchovies, silver scabbardfish, sardines, mackerel and horse mackerel, respectively.

Once the number of infested samples and the number of parasites were obtained through the inspection analysis, the epizootic parameters were calculated (prevalence of infestation, mean intensity of infestation and mean infestation abundance) to quantify the presence of the parasites in the samples examined.

The prevalence values of infestation of all the samples examined range from 2.69% to 100% (**Figure 52**). The results obtained show a higher prevalence rate in the scabbardfish samples, followed by the horse mackerel (48.4%). The presence of anisakid nematodes was found to a lesser extent in sardines, showing a prevalence of infestation of 2.69%.

The mean intensity values ranged between 1 and 157.67 larvae per sample (**Figure 53**). The silver scabbard fish was found to be the species with the highest average intensity value followed by the horse mackerel which presented an average intensity value of 14.5. Anchovies and sardines showed an average intensity value of 1, noting that only one larva was detected in all the fish sample infested.

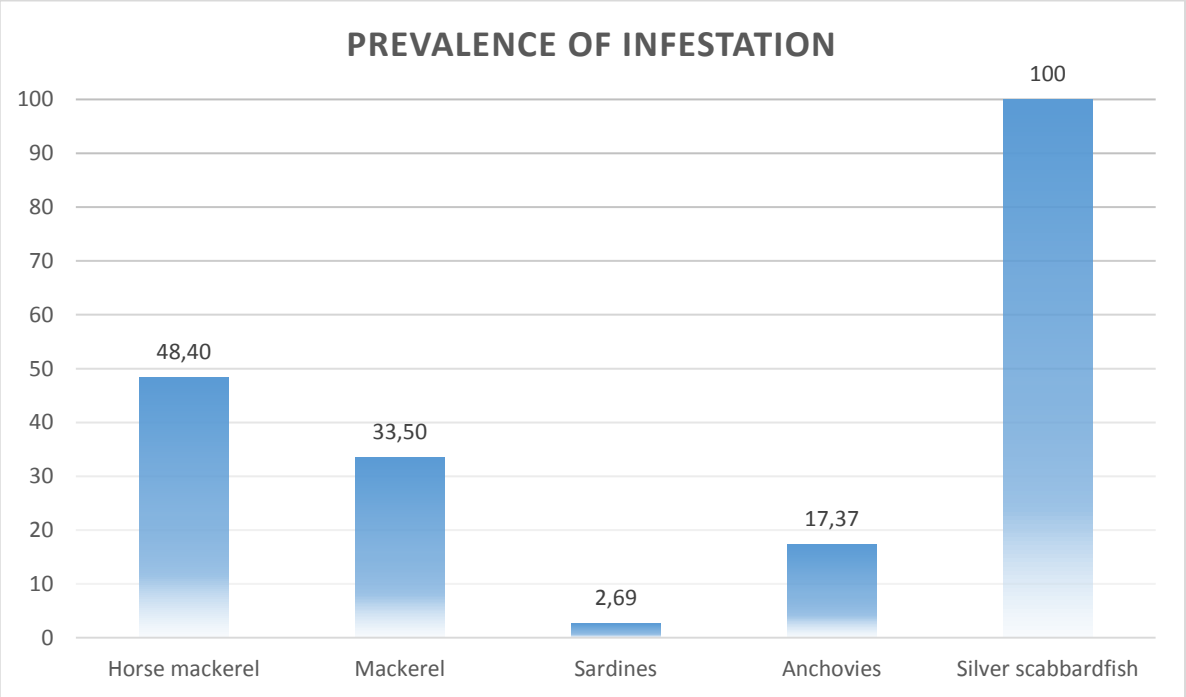


Figure 52. Prevalence of infestation comparison sorted by fish species examined.

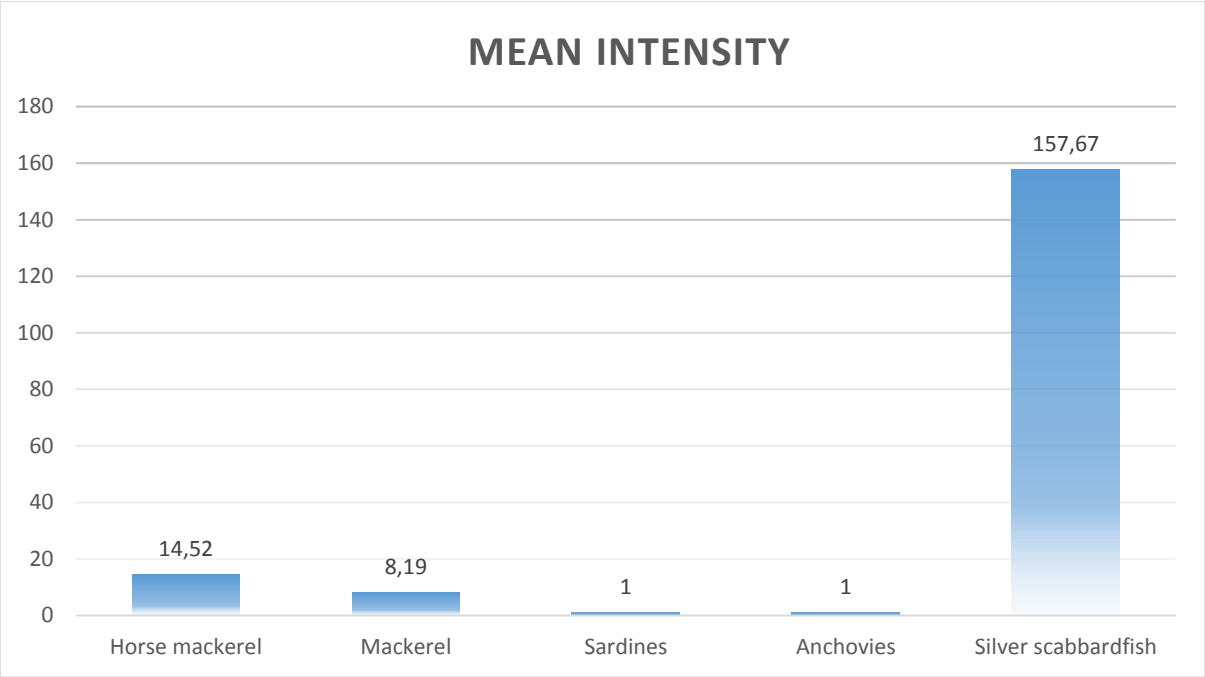


Figure 53. Mean intensity comparison sorted by fish species examined.

Finally, the fish samples examined showed mean abundance values ranging between 0.03 and 157.68 (**Figure 54**). The highest value belongs to the silver scabbardfish, this means that more than 157 anisakid larvae were found in all the samples examined. The lowest value of mean abundance was found in the sardine samples (0.03).

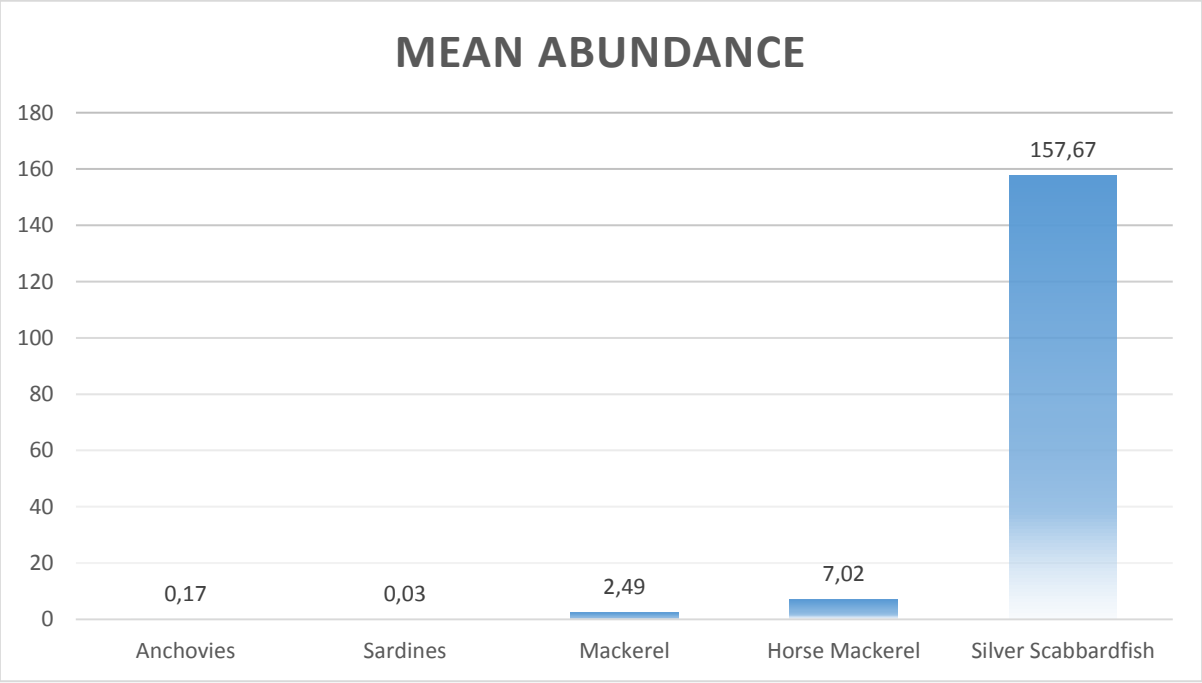


Figure 54. Mean abundance comparison sorted by fish species examined.

4.1.2 Morphological identification

All the larvae found during the inspection analysis were identified morphicologically. All the larvae examined by optical microscopy belonged to the morphotype I of *Anisakis* spp., presenting a terebrant tooth in the cephalic end, a cylindrical ventricle and a mucron in the caudal end.

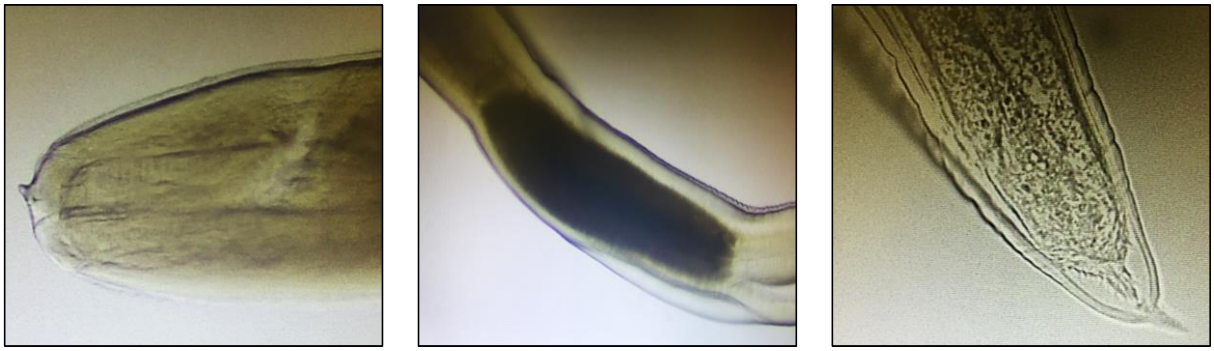


Figure 55. From left to the right: Cephalic extremity with terebrant tooth; cylindrical ventricle; caudal end with mucron.

4.1.3 Molecular analysis

To identify the larvae found at species level, a total of 410 samples were subjected to PCR-RFLP analysis. The digestion of the ITS amplified region with the HhaI enzyme by PCR-RFLP showed the restriction patterns referable to the various species belonging to the genus *Anisakis*.

In particular, the following restriction profiles were shown:

- 550-430 bp common to several species such as *A. pegreffii*, the hybrid *A. pegreffii/simplex s.s.* and *A. simplex s.s.*;

- 320-240-180-160 bp associated with the *A. typica* species.

The HinfI enzyme is instead discriminant and the digestion of the larval samples amplified with this enzyme produced the different restriction patterns associated with the following species belonging to the genus *Anisakis*: *A. pegreffii*, the *A. pegreffii*/*simplex ss* hybrid form, *A. simplex s.s.*, *A. typica* and A. *physeteris* (**Figures 56 and 57**).

Specifically, the restriction profiles detected are:

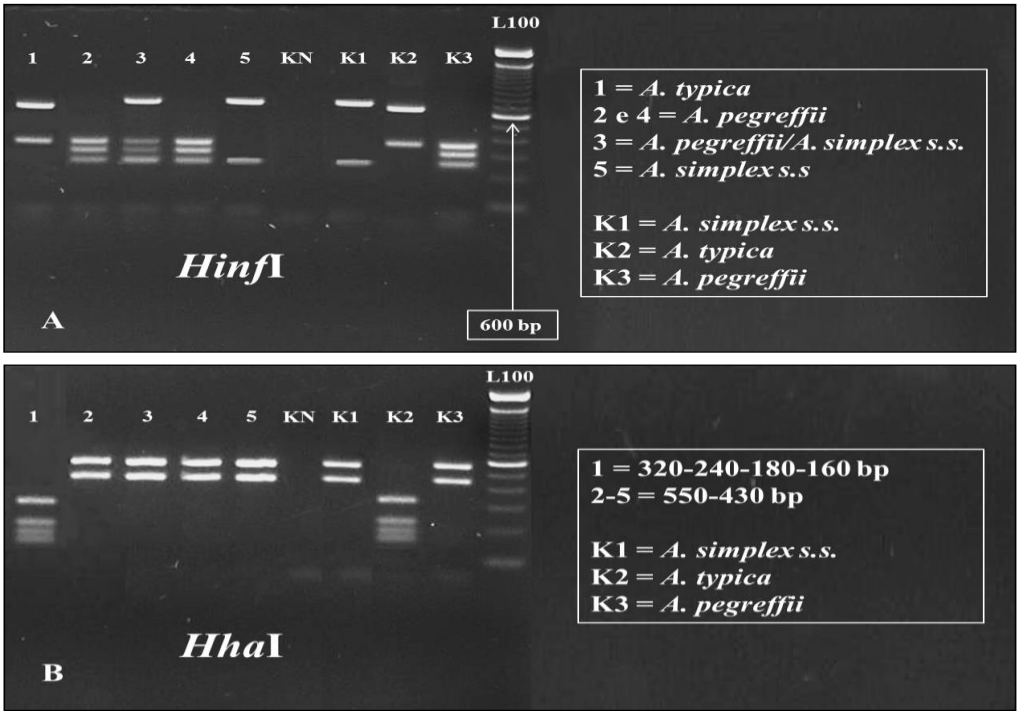
- 370-300-250 bp associated with the *A. pegreffii* species;

- 620-250 bp associated with the *A. simplex s.s.* species;

- 620-370-300-250 bp associated with the hybrid *A. pegreffii/A. simplex s.s.*;

- 610-350 bp associated with the *A. typica* species;

Regarding the *A. pegreffii/A. simplex s.s.* hybrid form, this has in fact presented the fragments that possess both *A. simplex s.s.* and *A. pegreffii* species.



Figures 56 and 57. Restriction patterns *obtained with Hinf*I (**A**) e *Hha*I (**B**) enzymes.

The results obtained with the morphological identification were confirmed by the molecular analysis. In particular, of the 410 larvae subjected to molecular investigation: 334 larvae belonging to morphotype I were identified as *A. pegreffii* (81.5%), 21 as the hybrid *A. pegreffii / simplex s.s.* (5.1%), 54 as *A. simplex s.s.* (13.2%) and 1 as *A. typica* (0.2%); (**Figure 58**).

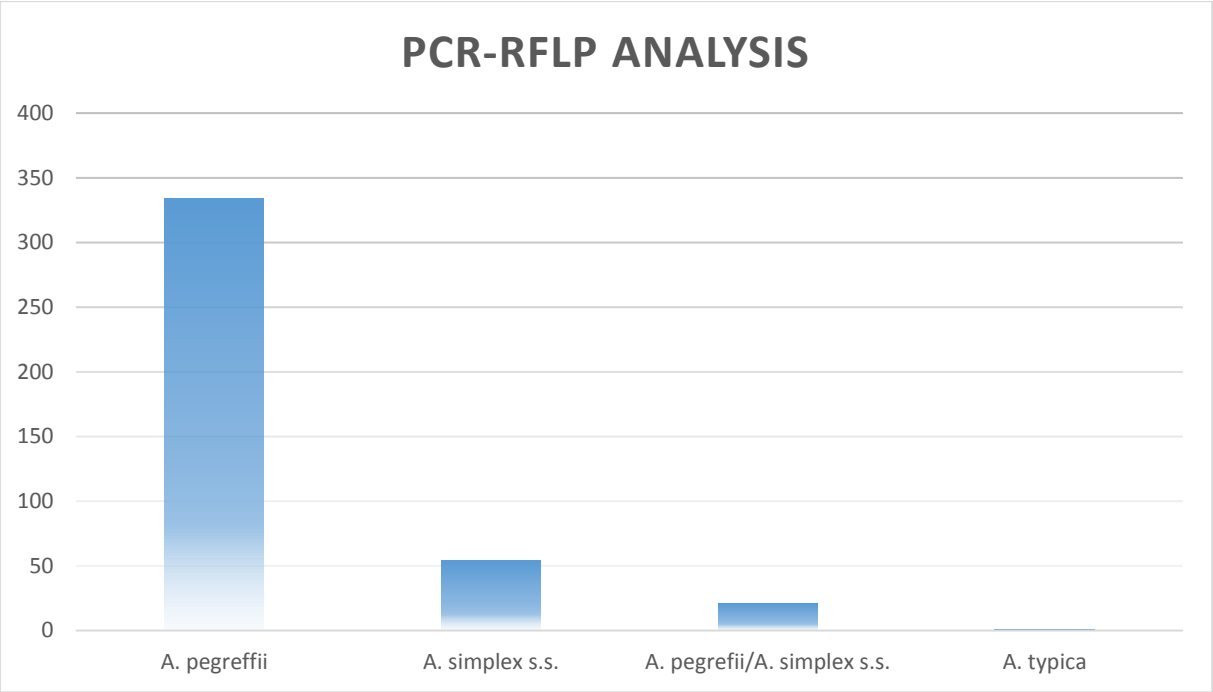


Figure 58 – Results of molecular identification by PCR-RFLP analysis.

The high percentage of *A. pegreffii* obtained by the molecular analysis conducted, gave further confirmation of the widespread distribution of this species in the Mediterranean (Fig. 60). In fact, this nematode was found in all the fish species infested. Larval specimens identified as the *A. pegreffii/simplex s.s.* hybrid genotype, described by Abollo et al. (2003), were found in silver scabbardfish, mackerel and horse mackerel specimens. The *A. simplex s.s.* was found in samples of mackerel and horse mackerel, whereas the presence of *A. typica* was found only mackerel.

4.1.4 Seasonal trend of infestation

The seasonal infestation trend showed an increase in mean prevalence values during the summer season until October for the anchovies, with a peak of 27,35% in July (**Figure 59**).

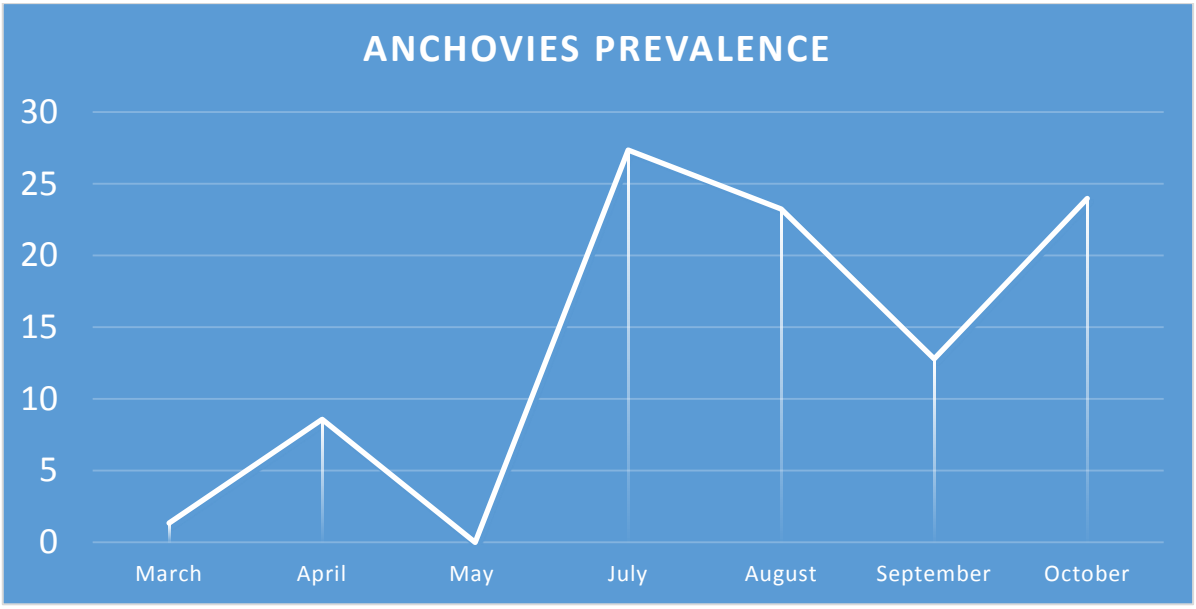


Figure 59. Prevalence of infestation trend of *Anisakis* larvae for anchovies' samples sorted by month of sampling.

Statistical differences between sampling season were verified (Kruskal-Wallis chi-squared = 10.233, df = 2, p-value = 0.005996). Regarding the sardines, there were two infestation peaks in August (6.86%) and March (8.19%) and a minimum of infestation in September (0) (**Figure 60**). No significant differences were found between sampling season, confirming a heterogeneous trend (Kruskal-Wallis chi-squared = 2.0882, df = 2, p-value = 0.352).

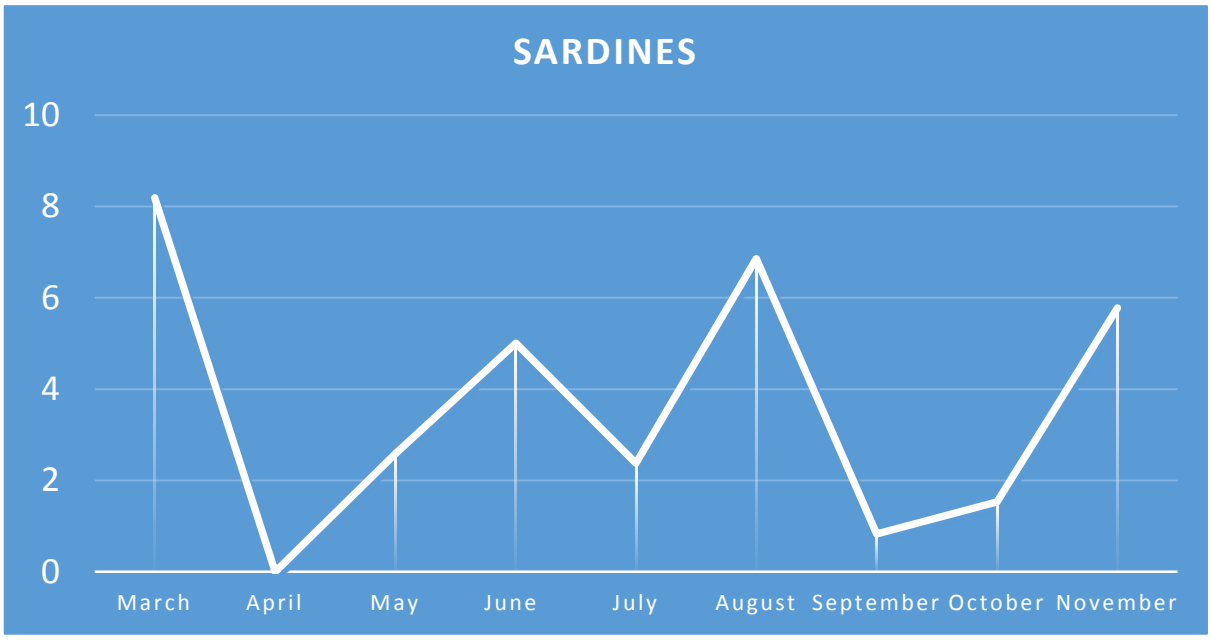


Figure 60. Prevalence of infestation trend of *Anisakis* larvae for sardines' samples sorted by month of sampling.

In the case of Atlantic mackerel, the prevalence of infestation increased in spring with a peak of 100% in May and decreased gradually during summer season up to October, showing a mean prevalence of 14.8% (**Figure 61**). However, the statistical analysis showed no significant differences between the seasons of sampling (Kruskal-Wallis chi-squared = 5.3611, df = 2, p-value = 0.06853)

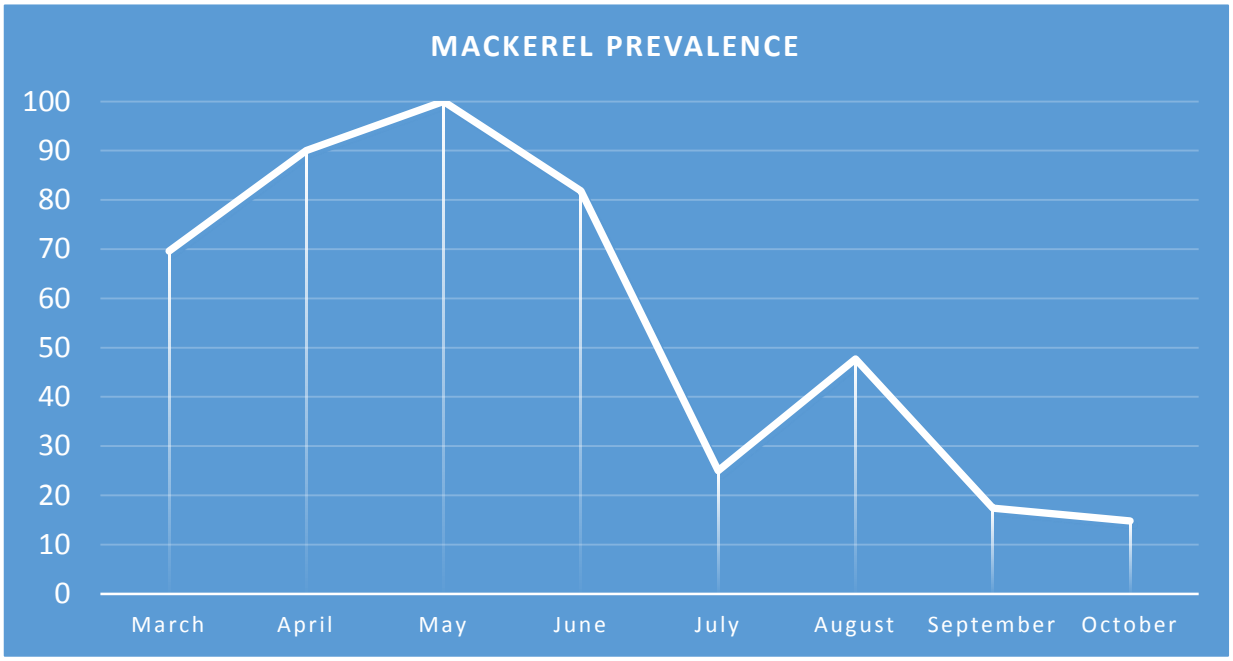


Figure 61. Prevalence of infestation trend of *Anisakis* larvae for mackerels' samples sorted by month of sampling.

Regarding the mean intensity, the trend seems to emulate that reported for the prevalence of infestation, with the same peak during May (**Figure 62**). Contrary to the prevalence of infestation, the mean intensity showed significant differences (Kruskal-Wallis chi-squared = 53.452, df = 2, p-value = 2.471e-12).

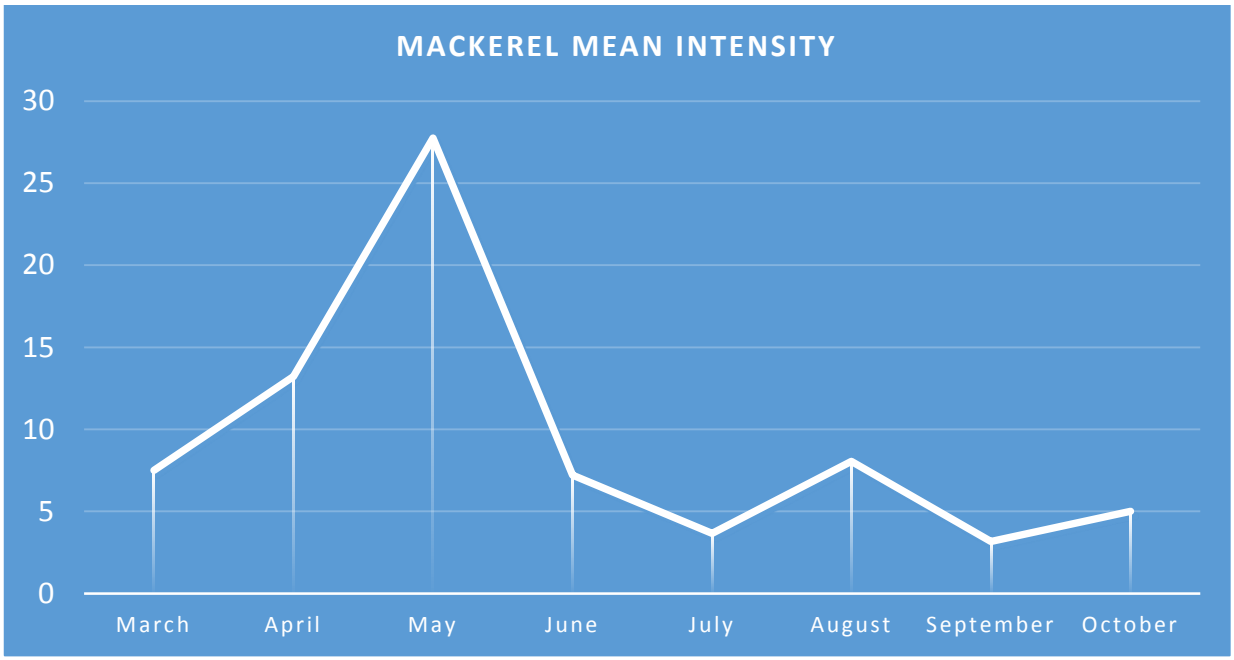


Figure 62. Mean intensity of infestation trend of *Anisakis* larvae for mackerels' samples sorted by month of sampling.

The horse mackerel samples showed a peak of mean prevalence in the spring season (100%) and a progressive decrease during summer (**Figure 63**). No significant differences were found between season of sampling (Kruskal-Wallis chi-squared = 2, df = 3, p-value = 0.3679).

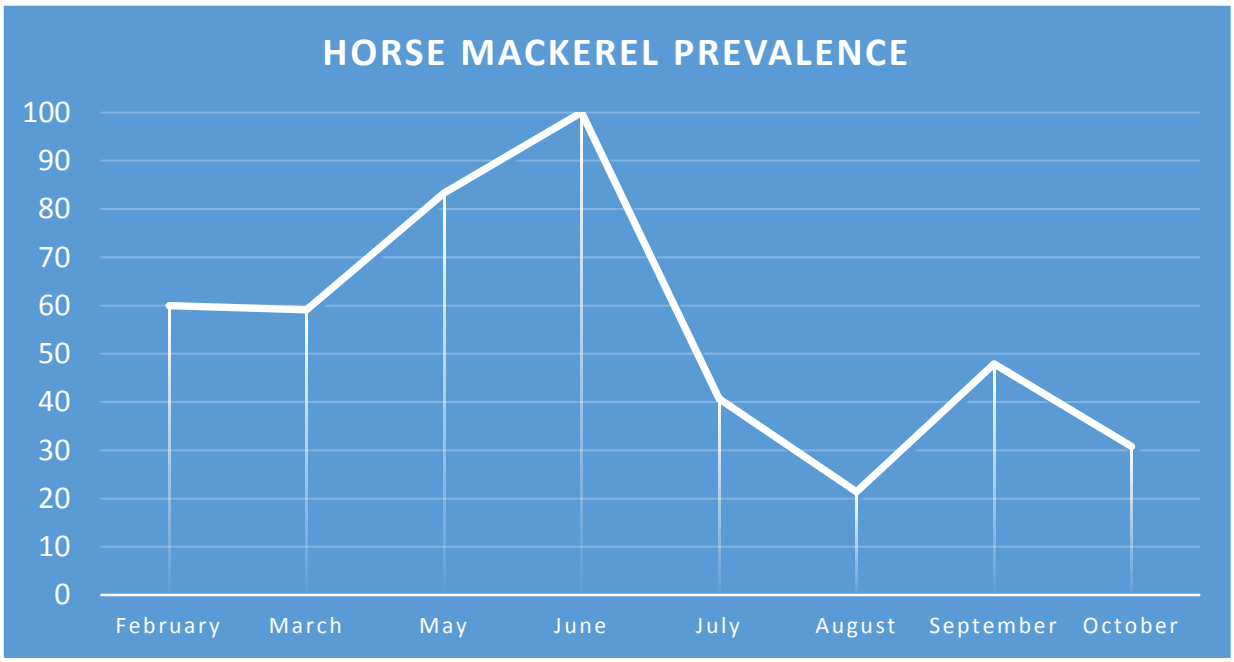


Figure 63. Prevalence of infestation trend of *Anisakis* larvae for horse mackerels' samples sorted by month of sampling.

The analysis of mean intensity verified even a peak during June but showing a different trend compared to the mean prevalence (**Figure 64**).

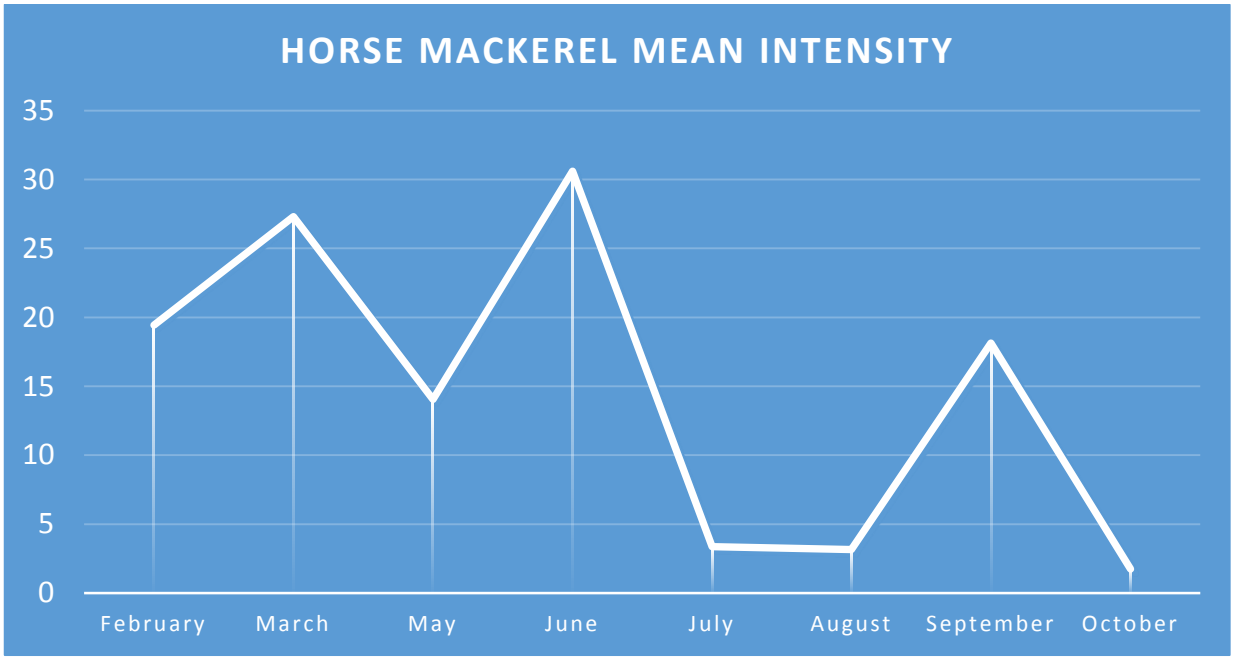


Figure 64. Mean intensity trend of *Anisakis* larvae for horse mackerels' samples sorted by month of sampling.

Statistical differences between sampling seasons were verified (Kruskal-Wallis chi-squared = 38.504, df = 3, p-value = 0.00000002211).

Given the constant value of prevalence for the silver scabbardfish (*Lepidopus caudatus*) samples (100%), I decided to assess the seasonal trend according to the mean intensity values. For the silver scabbardfish a bimodal trend with a peak during March (m.i. = 302.5) and November (m.i. = 250) was found. No statistical differences between sampling seasons were verified (Kruskal-Wallis chi-squared = 2.332, df = 3, p-value = 0.5064).

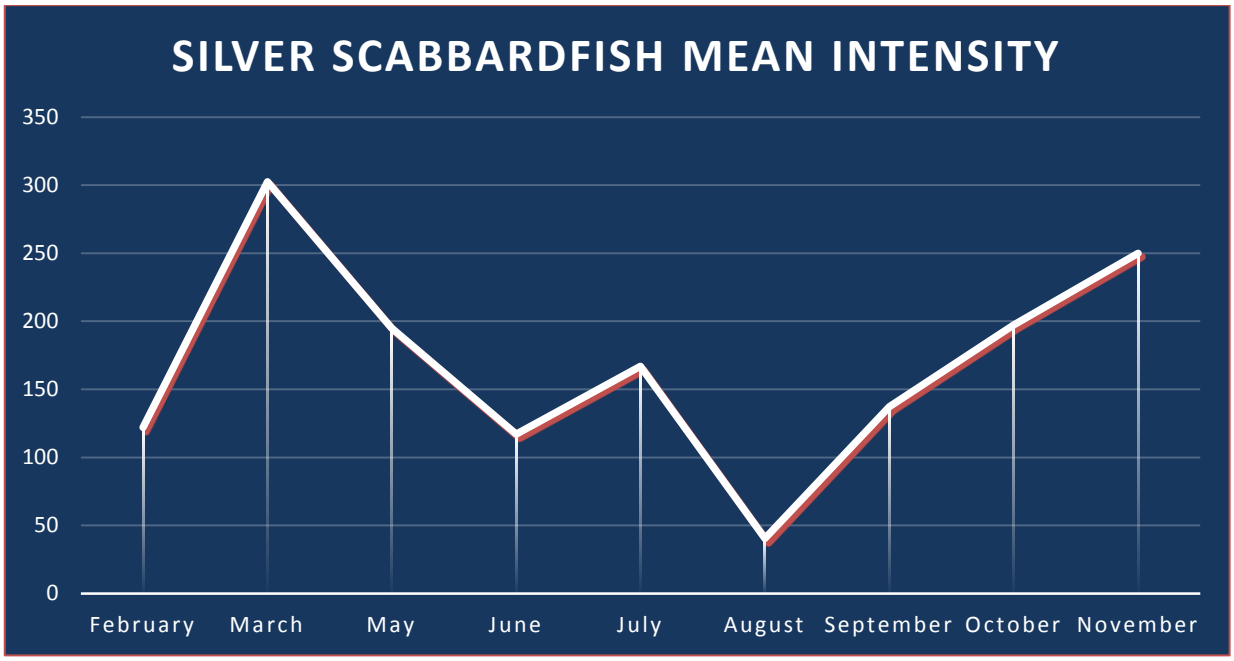


Figure 65. Mean intensity trend of *Anisakis* larvae for silver scabbardfish's samples sorted by month of sampling.

4.2 WORK PACKAGE 2

All the European sea bass samples were of commercial size with a mean weight and standard deviation (SD) of 402.78 ± 49.72. **Table 9** shows the weight, maximum standard length and Fulton's condition index of the fish samples examined.

	N	**MSL (mean ± SD)**	**Weight (mean ± SD)**	**Fulton's K index (mean ± SD)**	**Fulton's K index (min-max)**
Pachino	43	29.3 ± 2.09	406.3 ± 53.10	1.6 ± 0.38	1.21-2.27
Licata	40	29.5 ± 1.77	405.9 ± 39.27	1.5 ± 0.25	1.29-2.09
Greece (FAO 37.3)	68	29.4 ± 2.32	398.6 ± 60.97	1.6 ± 0.45	0.76-2.45

Total	151	29.4 ± 2.01	402.7 ± 49.72	1.6 ± 0.36	0.76-2.45

Table 9. Morphometric data of the European sea bass samples analysed.

A Fulton's condition - K index above 2 was found in 12 samples from Sicilian and Greek farms. Five samples came from Greek farms showed a K index below 1.

Two nematode larvae were found in a European sea bass sample from farms of Greece after visual inspection, giving a prevalence of infestation of 0.7%, a mean abundance of 0.01 and a mean intensity of 2. The larvae were located under the visceral serosa of intestine. No additional larvae were found after chloro-peptic digestion. No co-infestation with other parasites was found. The weight of the affected fish was lower than the mean weight, detecting a K index of 0.83. The morphological analysis allowed to identify these parasites as L3 *Anisakis* larvae belonging to the morphotype I (*sensu* Berland 1961; **Figure 66**).

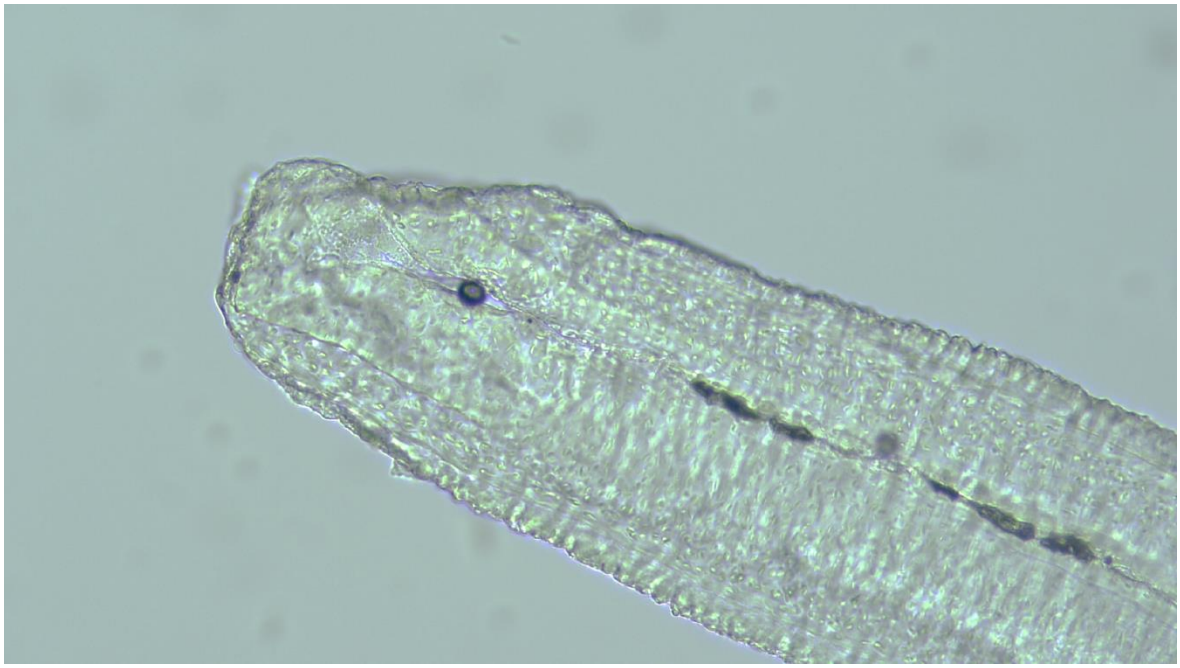


Figure 66. Microscope picture of one larva found in the *D. labrax* sample.

The amplification of ITS region produced a single band of ≈1000 bp for all the specimens. The PCR- RFLP analysis of ITS region, obtained with H*inf*I restriction enzyme produced one pattern with three strong bands at 370, 300 and 250 bp corresponding to *Anisakis*

pegreffii. The digestion with H*ha*I produced one pattern with two bands (550-430 bp) (**Figure 67**).

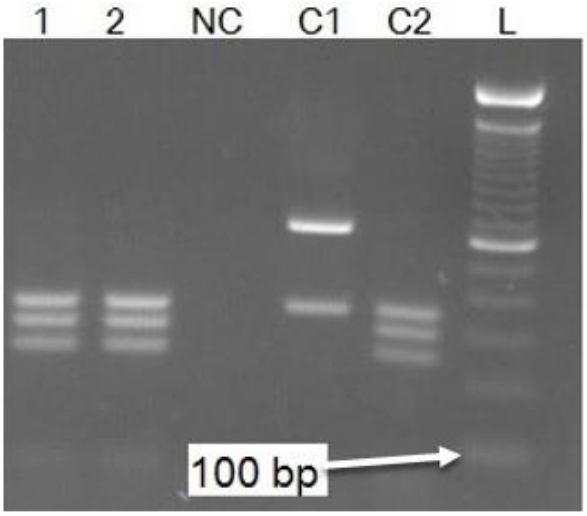


Figure 67. RFLP pattern of PCR products of the larvae infected sea bass after digestion with H*inf*I (lanes 1-2: *Anisakis pegreffii*; lane NC: Negative control; lane C1: positive control *Anisakis typica*; lane C2: positive control *Anisakis pegreffii*; lane L: 100 bp DNA size marker.

4.3 WORK PACKAGE 3

4.3.1 Sensitivity and Specificity

The LAMP assay proposed was able to amplify *Anisakis* spp. DNA from artificially infested fish samples, giving a sensitivity of 100% for each sample type analysed (**Figure 68**). Moreover, the method was also able to detect each sample contaminated with *A. simplex s.s., A. pegreffii, A. physeteris, A. ziphidarum* and *A. typica* DNA.

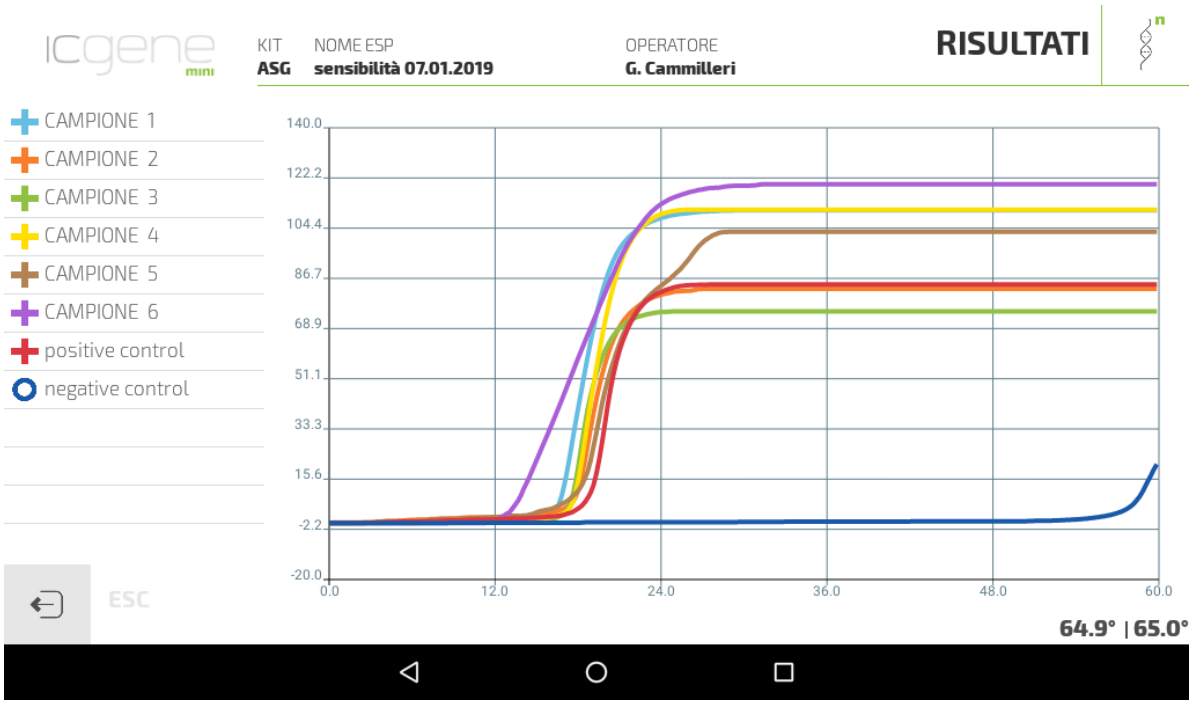


Figure 68. Monitoring of LAMP amplification for sensitivity test from homogenized farmed salmon samples. The analysis shows the DNA amplification detection in real-time by measuring the increasing fluorescence of DNA binding to the dye.

The assay detected *Anisakis* spp. DNA with a LOD of 10^{-4} (0.00022 ng µl-1), giving an amplification for all the replicates, with fluorescence values necessary for detection **(Figure 69)**.

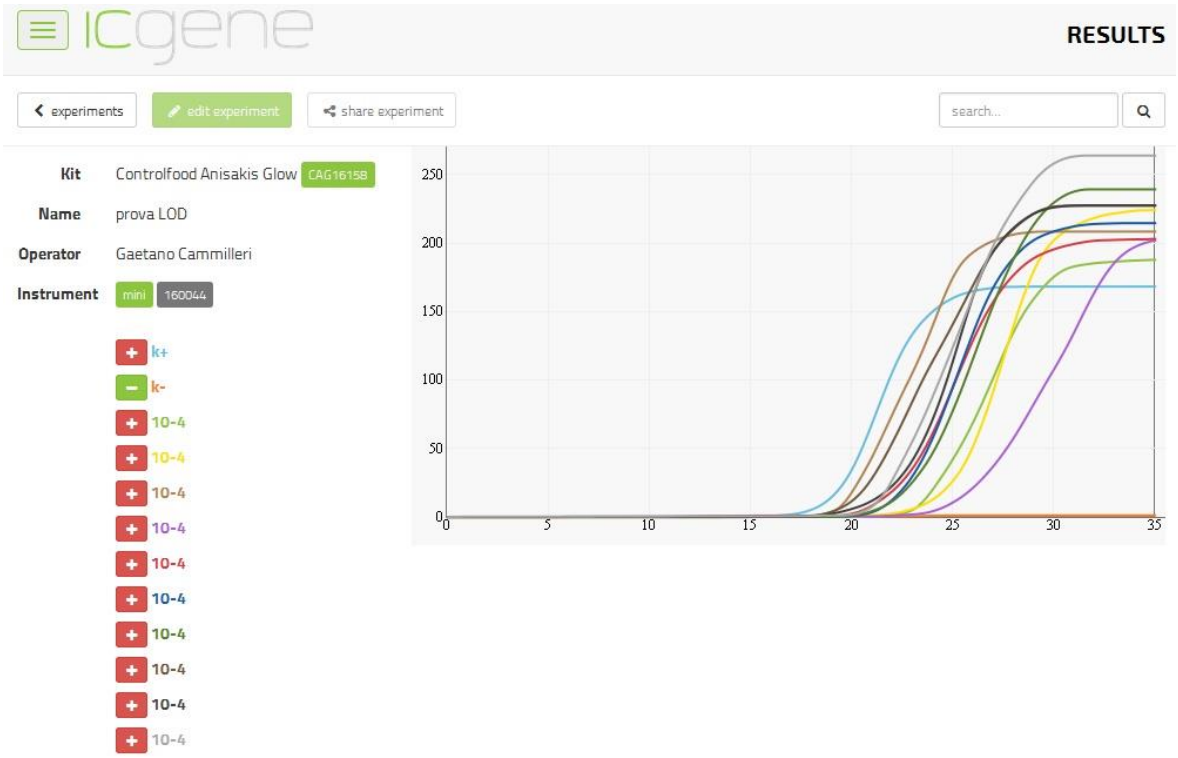


Figure 69. Monitoring of LAMP amplification for Limit of detection test from homogenized sea bream samples. The analysis shows the DNA amplification detection in real-time by measuring the increasing fluorescence of DNA binding to the dye.

All the LAMP analysis were carried out using positive and negative controls contained in the kit. No amplification products were detected in uninfected samples, giving a specificity rate of 100%. Furthermore, no amplification was obtained on processed fish samples contaminated with *Contracaecum* sp. *Pseudoterranova* sp. and *Hysterothylacium* sp. larvae and DNA (**Figure 70**). The RT-PCR assay showed a sensitivity rate of 100% for the fish samples artificially infested by *Anisakis* sp. larvae and DNA. Whereas the analysis of uninfected samples and samples contaminated with *Pseudoterranova* sp. and *Hysterothylacium* sp. and *Contracaecum* sp. larvae and DNA for specificity test did not obtain an amplification.

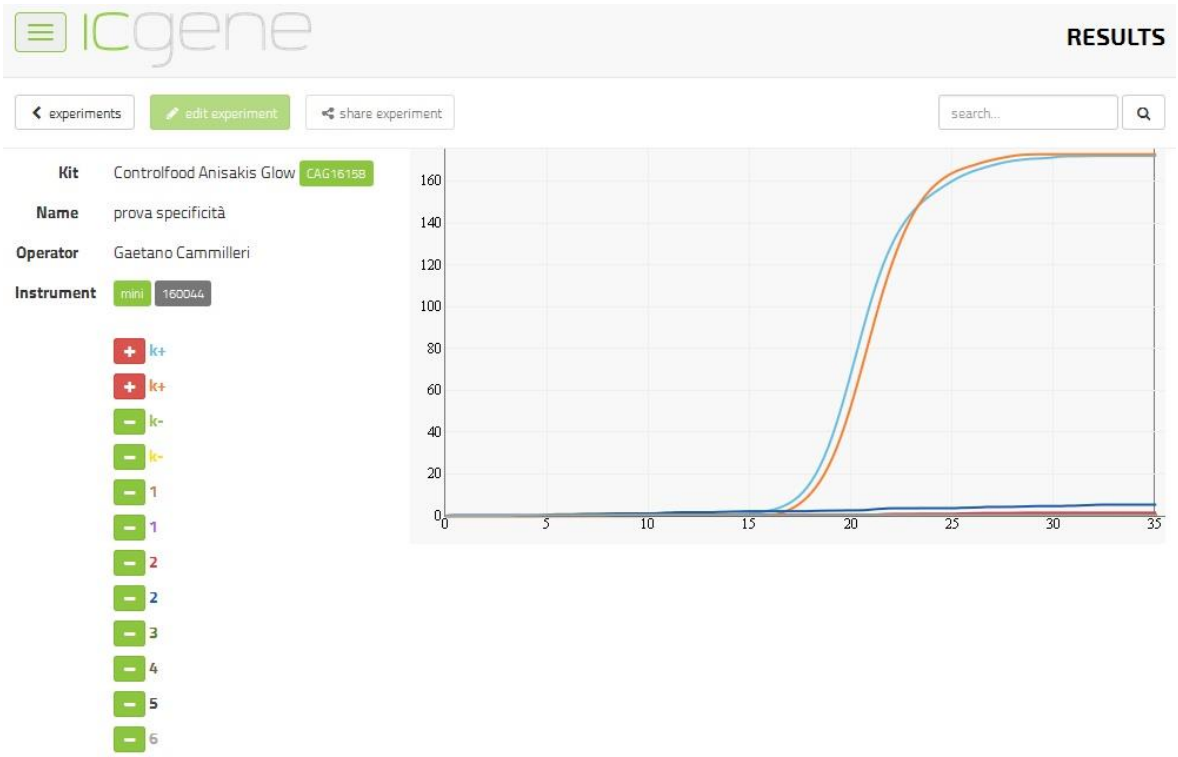


Figure 70. Monitoring of LAMP amplification for specificity tests from homogenized farmed salmon samples. The amplification plots displayed in the specificity correspond to the positive control (duplicate).

On agarose gel analysis, the LAMP amplicons revealed the ladder-like pattern, according to what was reported in the literature, with many bands of different molecular weights indicating the production of stem-loop DNA with inverted repeats of the target sequence (Parida et al., 2008; Li et al., 2012; **Figure 71**).

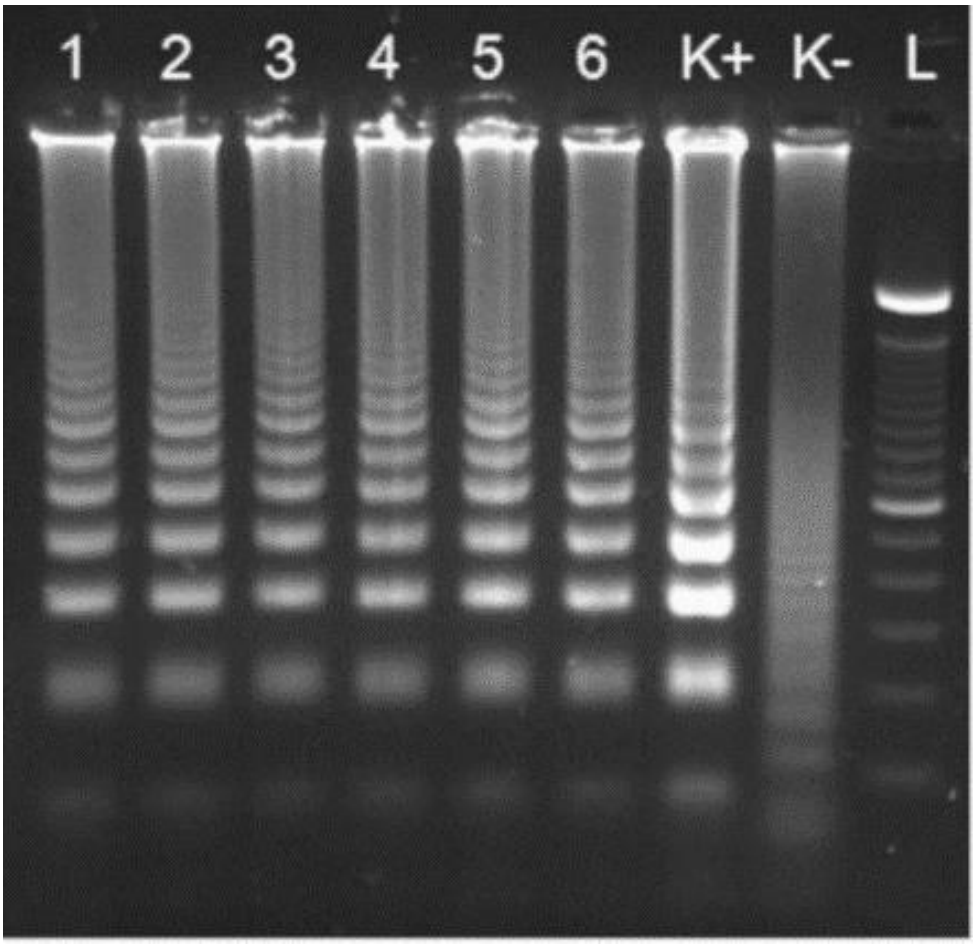


Figure 71. Amplification of LAMP for DNA extracted from anchovy paste samples experimentally infected with Anisakis spp. larvae (Lane 1-6). Lane K+: positive control, Lane K-: negative control, Lane L: 100-bp DNA ladder.

5. DISCUSSIONS

In recent years, the demand for fish products is constantly growing, therefore their marketing occupies an important position in the international market. For this reason, it is essential to guarantee food safety in order to protect the consumer by monitoring the presence of parasites in these foods.

Parasites belonging to the Anisakidae family are one of the most studied nematodes since they involve a high number of hosts in their life cycle and some species have immediate repercussions on human health. In fact, an increase in the consumption of raw and/or undercooked fish has led to a significant worldwide increase in the cases of anisakiasis. However, in the Mediterranean area, this disease is associated with the traditional consumption of raw and processed fish products such as marinated anchovies or salted sardines are not able to devitalize the larvae.

Considering this, some of the fish samples most consumed in the Mediterranean were examined in this study for the presence of these parasites using different methodological approaches. The presence of anisakid nematodes was found in all the fish species examined.

The prevalence of infestation found in the anchovy samples (17.4%) demonstrates the need to pay greater attention to this fish species, as the consumption of anchovies, subjected to treatments such as marinating, is very frequent in the Southern European countries. Consequently, the possible infestation could represent a risk of transmission of *Anisakis* to humans through the consumption of raw preparations based on this fish species.

The prevalence of infestation values in anchovies are higher than those found in the southern Tyrrhenian Sea (1.6%) and southern Sicily (0% Cipriani et al. 2018) but significantly lower than those reported by Piras et al. 2014 in North Tyrrenian. The values of average intensity and average abundance were instead similar to those found in the aforementioned area (Cipriani et al., 2018).

The prevalence values of infestation found in the sardines (2.7%) and horse mackerel (48.8%) samples were much lower than those found in the same species from the north of Sardinia (13.1% and 100% respectively) (Piras et al. , 2014).

Furthermore, the prevalence of infestation in anchovies (2.7%) and horse mackerel (48.4%) has verified contrasting results with those reported by Chaligiannis et al. (2012) on the Greek coast. In particular, the study by Chaligiannis et al. (2012) showed prevalence values of 3.9% and 40% for anchovies and horse mackerel, respectively.

The study by Serracca et al. (2014) reported prevalence values in samples of anchovies and sardines clearly different from those found in the present study. The analysis conducted by Serracca et al. (2014) reported a prevalence of infestation in anchovies equal to 0.8% whereas sardines did not show any larvae (0%) compared to the 96 larvae found in the sardines examined in this work (prevalence = 2.7%).

Finally, the prevalence value obtained for the silver scabbardfish samples (100%) is complementary to that reported by Mattiucci and Nascetti (2007) and Costa et al. (2016), confirming a high prevalence of infestation in this fish species (80% and 100%, respectively).

The larvae analysed by molecular investigation were subjected to PCR-RFLP as it is a very sensitive molecular technique that allows an accurate identification of the species belonging to the genus *Anisakis* as well as to detect even minimal genetic variations (D'Amelio et al., 2012).

The results obtained from the analysis demonstrated the presence of *A. pegreffii* in all the fish species infested, confirming that this parasite belonging to the Anisakidae family is the most widespread in the Mediterranean Sea.

The second parasite mainly found in the fish samples examined was *A. simplex s.s.* followed by the hybrid form with *A. pegreffii*. According to the study by Abollo et al. (2003), this hybridization could derive from migratory phenomena of some hosts that have determined the spread of Anisakis simplex s.s. from the Atlantic to the Mediterranean. In particular, the presence of the hybrid form *A. pegreffii/A. simplex s.s.* was reported in the Strait of Gibraltar which represents a transition zone between the Atlantic and the Mediterranean, constituting an area of sympatry for the two anisakid species (Abollo et al., 2003). Therefore, these geographical areas represent sites in which natural interspecific recombination occurs between two distinct species (Abollo et al., 2003; Costa et al., 2016; Martín-Sánchez et al., 2005).

Results from the seasonal infestation trend analysis revealed an overlap with the ecological aspects of the fish species examined (Cammilleri et al. 2020a). The anchovies have a reproductive period ranging from the beginning of summer until October. In many small pelagic species of Osteichthyes group, the deposited energy is mainly derived from feeding rather than energy reserves (Wang & Houde 1994), and consequently there is an increase in infestation by *Anisakis* parasites due to intense feeding activity during summertime. Considering their annual reproductive strategy and the results of this work, sardines seem to have a bimonthly feeding behavior, consisting of a month of intense

predation activity and a month of energy storage. Despite their different evolution, the results seem to confirm that Scombridae and Carangidae reproduce mostly during the summer period by storing energy before the breeding period. For this reason, in the period before the summer season there was an increased *Anisakis* infestation. My results on the seasonal trend of infestation of *Trachurus trachurus* seem to be in accordance with what was found by on specimens of Moroccan Atlantic coasts for prevalence, showing an increase of prevalence during spring and summer for prevalence (Shawket et al. 2017; Abattouy et al. 2013). Conversely, the mean intensity values were conflicting, revealing a peak during the winter season compared to this work. The results obtained for silver scabbardfish showed a trend of infestation similar to the other two big pelagic predators examined in this work (horse mackerel and Atlantic mackerel). The results suggest an intense predatory activity at March, before the spawning period. The results showed a spawning period from April to September, in contrast of Northwestern Mediterranean specimens (Demestre et al. 1993).

As far I know, this was the first ecological studies on silver scabbardfish population of South Mediterranean Sea. This gives interesting epidemiological data on the prevalence of *Anisakis* infestation in south Mediterranean fish.

The findings seem to confirm the role of *Anisakis* as ecological tag to deepen the ecology of their hosts as confirmed previously for other species (Pascual et al. 1999; Madrid et al. 2012). Furthermore, the data obtained and the significant differences found in the seasonal prevalence of infestation values for anchovies, and of seasonal mean intensity for horse mackerel, mackerel and silver scabbardfish could be useful to plan a seasonal fishing strategy in order to reduce the risks related to the presence of *Anisakis* in fish products.

The parasites belonging to the *Anisakis* genus are no longer related only to wild fish but also to aquaculture ones. The aquaculture sector is growing rapidly and assumes an important importance as it helps to satisfy, together with fishing, the growing world demand for fish products. For these reasons, it was decided to examine the presence of these parasites also in aquaculture fish samples. Several studies, including the work of Peñalver et al. (2010), verified the absence of anisakid parasites in sea bass and sea bream samples from Iberian farms. No *Anisakis* larvae were found even in 1040 European sea bass samples from North Italy (Menconi et al., 2017) farms but a *Hysterothylacium fabri* larva was detected in only one sample, suggesting the possibility for European sea bass in open-net cages to feed on live food in addition to the feed offered.

However, the present study revealed the presence of *Anisakis* larvae albeit in low values, evaluating for the first time the presence of *Anisakis* spp. in sea bass farmed and marketed

in the Mediterranean (Cammilleri et al. 2018), in order to obtain a detailed risk assessment on the presence of anisakid larvae in farmed fish. Of the 151 sea bass samples examined, two *A. pegreffii* larvae were found in only one sample, giving a prevalence of infestation of 0.7%.

It is assumed that this positivity is due to the method of cage farming that can expose farmed fish to crustaceans or small-infested fish capable of entering the cages. Unfortunately, I was unable to identify the farming practice used for the imported European sea bass sample that showed the presence of *Anisakis* parasites.

Based on what is reported on the National Aquaculture Sector Overview of the Food and Agriculture Organization of the United Nations (FAO), Greek aquaculture is dominated by the farming of marine finfish in offshore cages, specifically of gilthead sea bream and European sea bass (FAO, 2005). Therefore, it can be assumed that this sample infected by *Anisakis* was reared in offshore open-net cages. These farming practices could allow the exposure of fish to the crustacean intermediate host (Peñalver et al., 2010).

Furthermore, the European sea bass sample infected by *Anisakis* showed a low Fulton's K index (0.83). Given that the Fulton's K condition index is considered a good indicator of the general welfare of the fish (Lambert and Dutil, 1997), it is possible to give particular stressed condition that lead the fish to feed on infected crustaceans and small fish entering the cage.

Most of the studies on the presence of anisakid parasites in farmed fish have focused on salmonids. Several authors have demonstrated the absence of anisakids in farmed Atlantic salmon (*Salmo salar*) (Angot and Brasseur, 1993; Lunestad, 2003). Marty (2008) reported the presence of an anisakid larva in a farmed Atlantic salmon, although molecular identification was not carried out. The presence of these parasites was verified even in runts of farmed Atlantic salmon (Mo et al., 2013). Based on the prevalence values reported in literature (Bernardi et al., 2011), the relative risk of anisakids in European sea bass is estimated to be 136 times greater in wild forms than in farmed forms.

At present, there are few data on the prevalence of anisakid parasites in farmed European sea bass (Menconi et al., 2017; Mladineo et al., 2010; Peñalver et al., 2010). This could be because these fish are not usually consumed raw and consequently are not considered a potential risk in terms of zoonosis. Nevertheless, several studies that point out the allergic potential of *Anisakis* spp. in clinical reports and the presence of thermally stable allergens in processed fish products (Fæste et al., 2015; Audicana and Kennedy, 2008) encourage to increase the monitoring data on farmed fish of Mediterranean, in order to clarify the effects

of different farming practices on the prevalence of Anisakidae nematodes and fulfil the EFSA Panel on Biological Hazards' requests (EFSA, 2010).

The results obtained in this study, compared with the scientific data previously mentioned suggest that the risk of exposure to anisakid nematodes in Mediterranean farmed fish remains very low. Consequently, the risk of transmission of the anisakid parasites to humans is also insignificant, although this statement should not exclude the conduct of continuous and careful monitoring in the aquaculture sector.

Given the few studies that focus on examining the possible infestation of anisakid larvae in farmed fish, it is necessary to conduct further investigations in order to have exhaustive epidemiological data and a detailed analysis of the risks in these productive realities.

Regarding the LAMP assay, the set of primers employed was able to amplify *Anisakis* spp. DNA in 35 min at 65°C from a considerable initial weight of samples, giving satisfactory sensibility and specificity values. The time needed for the LAMP assay optimized is much lower than the RT-PCR method (about 94 min) used for comparison purposes.

The use of 2 loop primers and the ability of the sets of primers (F3 – B3, FIP – BIP) to recognise two distinct regions on the target DNA allowed to accelerate the reaction time and provide extremely high specificity. The specificity test has demonstrated that the LAMP assay optimized can discriminate the *Anisakis* genera with respect to other genera of the anisakidae family which are not present in the Mediterranean Sea, such as *Pseudoterranova* spp. Furthermore, the method was able to discriminate the *Anisakis* genera with respect to non-allergic parasites such as *Hysterothylacium* spp, which proved that the LAMP primers are highly specific for the detection of *Anisakis* spp (Cammilleri et al. 2020b).

The LAMP primers recognise specifically eight independent regions in comparison with PCR primers that can only recognise two independent regions, enhancing the sensitivity and specificity and decreasing the probability of false-positive results (Li et al. 2012).

Generally, the fluorescence-based real-time monitoring of LAMP reaction is considerably faster than that performed by a real-time turbidimeter (Zhang et al. 2014).

Besides, compared to the real time turbidity method, the real time fluorescence method possesses two further advantages: the first is the higher sensitivity; the second is that the sensitivity is less affected by the presence of substances in the mixture, such as proteins (Francois et al. 2011). Furthermore, the LAMP assay optimised was able to amplify *Anisakis* spp. DNA from different matrix type, suggesting that the type of fish processing does not

affect the quality of the assay by matrix interferences (Cammilleri et al. 2020), as confirmed in other works based on LAMP method (Kong et al. 2012).

However, given the large amount of the LAMP products, a high risk of aerosol contamination could be verified; the use of a mineral oil inside the reaction is proved useful in overcoming this problem.

The LAMP assay proposed can detect the concentration of *Anisakis* spp. DNA up to 100 times lower than the RT-PCR method (Cammilleri et al. 2020). The high sensitivity of a method is undoubtedly an advantage but needs special care to avoid false positive; the utilisation of ready-to-use reagents, expecially for the control analysis in the fishery sector, allowed to minimise any operator error and the occurrence of possible false positives.

Besides, the presence of non-target DNA and inhibitors in the LAMP reaction has been shown not to affect the amplification results. The present method in combination with the ICGENE mini portable instrument has proved to be accurate as of the Real-Time PCR method but more rapid, cheap, easy to use and with a lower limit of detection.

Furthermore, given the satisfactory results obtained during the validation and the ease of use, the samples do not need to be analyzed in duplicate.

Anisakis spp. nematodes are considered one of the most critical biological hazards present in fish products (EFSA 2010). These food-borne parasites are also a hidden food allergen (Nieuwenhuizen et al. 2014).

Concerning the *A. simplex* complex, there is no information about the minimum allergen concentration that causes allergic diseases. In this respect, Lopez et al. (2010) concluded that a single larva contains sufficient allergen to induce an antibody response in sensitive individuals. The exposure to this parasite seems to increase due to the increasing consumption of fish products worldwide and the advent of new gastronomic trends based on the consumption of raw and undercooked fish.

Since all the *Anisakis* species reported in literature can provoke the same disease and allergic reactions, the species identification, in this context, is less relevant (Herrero et al. 2011). Given this, it is necessary the use of more efficient, simply, cost- and time-effective methods.

6. CONCLUSIONS

The growing worldwide demand for fish products and the increase in the consumption of raw and/or undercooked foods has led to the consolidation of continuous monitoring of the presence of parasites in these products and led the field of research towards new methodological approaches for the detection of such parasites. Therefore, the purpose of this work was to investigate the presence in the Mediterranean Sea of nematode parasites belonging to the Anisakidae family in fishery and aquaculture products, evaluating new procedures for the prevention of related diseases.

Furthermore, given the possibility of allergic diseases in humans due to the presence of these parasites in processed fishery products, the study was integrated with the application and validation of a rapid method possibly also useful to fish sector operators.

The results obtained confirmed the presence of parasites belonging to the genus Anisakis in the fish samples most consumed in the Mediterranean Sea. In addition, this work has expanded the epidemiological data on the presence of these parasites in the fish fauna, defining the prevalence of infestation and the mean infestation intensity values for each species of fish examined. Based on the results obtained, it was also possible to evaluate a seasonal change in the parameters mentioned above. This type of survey is useful for planning a species-specific temporal fishing strategy useful for reducing the possible risks associated with these parasites. The molecular analysis carried out allowed an accurate identification at the species level of the parasite samples collected. The PCR-RFLP analysis confirmed that the species most commonly found in Mediterranean fish is *A. pegreffii*, followed by *A. simplex s.s.*

In relation to the farmed fish samples, the results indicated the presence of Anisakis in a single sea bass, confirming what has already been highlighted in the past regarding the very low risk of infestation in Mediterranean farmed fish (Peñalver et al., 2010). However, epidemiological data relating to the risk of Anisakis spp. in aquaculture fish species are very low; therefore, it is desirable to conduct further studies in order to have a more comprehensive picture of the risk of infestation in the aquaculture sector.

The infestation parameters found in some fish species suggest the need to pay particular attention to prevention, since by implementing the appropriate preventive measures it is possible to reduce or eliminate the risk of anisakiasis, a zoonotic disease that can have serious repercussions on human health.

In particular, in addition to the evisceration that operators must carry out immediately after capture, the most effective methods to devitalize the larvae are thermal treatments (cooking and freezing). For this reason, Regulation (EC) no. 853/2004 establishes that all fish products consumed raw or intended for treatments such as marinating, salting and smoking must be compulsorily frozen immediately after capture at −20 ° C for at least 24 hours in order to reduce the risk of consuming fish products infested with larvae of anisakids.

However, the procedures mentioned above are not able to prevent allergological pathologies related to *Anisakis* spp. allergens present even in post-mortem conditions of the parasite. Several studies showed that immunological and molecular methodologies yield comparable results concerning the detection of allergens in processed foods as sensitive and specific tools.

The LAMP assay optimised has the advantages to be more straightforward and more sensitive than real-time PCR (Parida et al. 2008). The LAMP method also has the characteristics of not requiring special reagents and sophisticated temperature control devices so the detection of LAMP products is also suitable for on-site conditions.

Furthemore, the preservation of the reagents used for the LAMP assay require only a storage at +4°C. Therefore, the LAMP assay proposed should be considered a new and reliable tool for the food quality and security control for the prevention of *Anisakis* allergy (Cammilleri et al. 2020).

The present system can also be suited to be used by operators of fish product processing industry for self-monitoring purpose, given its extremely ease and speed in order to verify the correct evisceration practices of fish sector operators, following the suggestion of the Italian Ministry of Health. Furthermore, due to its capability of discriminate *Anisakis* spp. DNA with respect to species belonged to *Pseudoterranova* genre, the LAMP system proposed in this work can be a valuable tool to trace Mediterranean fish products.

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