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Prevalence of anisakid parasites in fish collected from Apulia region (Italy) and quantification of nematode larvae in flesh☆

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ABSTRACT

Anisakis spp. and *Hysterothylacium* spp. are nematodes that commonly parasitize several fish species. Nematode larvae can be recovered in coelomic cavity and viscera, but also in flesh and have an important economic and public health impact. A total of 1144 subjects of wild teleosts, 340 samples of cephalopods and 128 specimens of farmed fish collected from Apulia region were analysed for anisakid larvae detection by visual inspection of coelomic cavity and viscera and by digestion of the flesh. No nematode larvae were found in farmed fish and cephalopod molluscs. All examined wild-caught fish species were parasitized, except for 5 species for each of which only a few subjects belonging to the same batch were sampled, therefore the results are just indicative. A total of 6153 larvae were isolated; among these, 271 larvae were found in the muscular portion. Larvae were identified by morphological method as belonging to the genera *Anisakis* (97.2%) (type I and type II) and *Hysterothylacium* (2.8%). Both nematodes could be found in all fish species, except for round sardinella (*Sardinella aurita*), infected only by *Hysterothylacium* spp. and for Mediterranean scaldfish (*Arnoglossus laterna*), little tunny (*Euthynnus alleteratus*) and chub mackerel (*Scomber japonicus*) infected only with *Anisakis* spp.. A sample of 185 larvae was sent to the National Reference Centre for Anisakiasis (C.Re.N.A.) of Sicily for identification at the species level: 180 larvae belonged to the species *A. pegreffii* and 2 larvae to *A. physeteris*. The remaining 3 larvae were identified at genus level as *Hysterothylacium*. Statistical indices such as prevalence, mean intensity and mean abundance were calculated. Chub mackerel (*S. japonicus*) was the species with the highest prevalence and mean intensity. Moreover, the average and the median values of larvae per 100 g of edible part for each fish species were determined to estimate the consumer exposure to *Anisakis* spp.. The obtained values were then recalculated by referring to the edible part of all specimens (infected and non-infected) forming a single parasitized batch, getting more realistic and objective data useful for risk assessment. Our results indicate that the consumption of raw or undercooked wild fish caught off Apulian coasts could result in the acquisition of anisakiasis; on the contrary, farmed fish and cephalopods appear to be safer for the consumer.

1. Introduction

Anisakid nematodes from the superfamily *Ascaridoidea* (families: *Anisakidae* and *Raphidascarididae*) are parasites commonly present in marine environment. Anisakids with economic and public health importance are *Anisakis*, *Pseudoterranova* and *Contracaecum*, genera of family *Anisakidae* (EFSA, 2010) while *Hysterothylacium*, genus of family *Raphidascarididae*, is commonly considered not zoonotic (Iglesias et al., 2002), except for sporadic cases (González-Amores et al., 2015; Yagi et al., 1996).

Member of the *Anisakidae* family may infect a wide variety of aquatic organisms, marine mammals and fish-eating birds and are distributed worldwide with a complex life cycle depending on aquatic ecosystem and various intermediate, paratenic and definitive hosts (Anderson, 1992; Mattiucci and Nascetti, 2008). Larvae (L3) are consumed by krill (euphausiid) and copepods, which are intermediate

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hosts. Marine fishes and cephalopods, which constitute paratenic hosts, become infected after ingesting small crustaceans. The larvae develop into adult worms when marine mammals, primary hosts, eat infected fish. Anisakid nematodes are reported also in farmed fish, especially farmed Atlantic salmon (*Salmo salar*) (Marty, 2008; Mo et al., 2013). Humans are accidental hosts of these parasites and become infected by eating raw, salted, pickled, marinated, smoked and undercooked fish (Fumarola et al., 2009; Lee et al., 2009; Maggi et al., 2000; Mattiucci et al., 2013).

Human infection is known as anisakidosis or anisakiasis depending on if the parasite belongs to the family *Anisakidae* or to the genus *Anisakis*. Anisakiasis can cause two different clinical manifestations: gastrointestinal disorders and allergic reactions. The first one is due to traumatic damage caused by larval penetration into the gastrointestinal mucosa, while the second one is attributed to the presence of nematode allergenic determinants (AAITO-IFIACI, 2011; Fumarola et al., 2009; Mattiucci et al., 2013). In the recent years the interest for allergic forms of human anisakiasis has grown simultaneously with the increase of human cases. Several studies suggest that allergic symptoms can occur after contact with allergens released by larvae not only alive but also dead (Audicana et al., 2002; Falcao et al., 2002; Pascual et al., 1997). These hypotheses are not accepted by other authors; the most accredited theory is that allergic reaction is, in any case, subsequent to a first sensitizing contact with alive larvae only (Cuellar et al., 2012; Daschner et al., 2000; Fæste et al., 2014; Lin et al., 2013).

Anisakiasis occurs worldwide: 20,000 cases of human infection were reported in the last years, mainly from Japan (90%), where about 2000–3000 cases are registered per year, but also from Norway, Netherlands, Spain, France and USA (Audicana et al., 2002; Baird et al., 2014; Chai et al., 2005). Recently, the first case of anisakiasis has been also reported in China (Qin et al., 2013).

In Italy, human cases have been described mainly from Abruzzo, Apulia and Campania regions (Fumarola et al., 2009; Maggi et al., 2000; Mattiucci et al., 2013; Ugenti et al., 2007) where raw fish is a common ingredient in many traditional dishes. Furthermore, the prevalence of *Anisakis*-hypersensitivity in Italian people seems to be higher in seaside areas than in the inland cities and where the consumption of marinated anchovies is widely diffused (AAITO-IFIACI, 2011).

Anisakis spp. and *Hysterothylacium* spp. larvae can be recovered on and in the viscera and flesh or free in the coelomic cavity of fishery products. Eviscerating and washing fish lead to almost total parasite elimination, but larvae present in the muscular masses of fishery products represent an important and actual hazard.

Nowadays, Regulation (EC) No. 853/2004 and Regulation (EC) No. 2074/2005, with their amendments and integrations, states that food business operators (FBOs) must ensure that fishery products have to be subjected to a visual examination for the purpose of detecting visible parasites before being placed on the market. This prevention measure is not sufficient to reduce the risk to an acceptable level because it does not allow the examination of larvae in raw or processed muscle tissues (marinated, salted, etc.).

A variety of methods, including visual inspection, slicing, candling, UV-press, digestion and, recently, biomolecular analysis have been reported to recover larvae from fishery products (EFSA, 2010; Lopez and Pardo, 2010; Mossali et al., 2010). Some methods are more suitable for specific investigations: visual inspection is more effective for collecting free or encapsulated larvae in caelomic cavity (EFSA, 2010), while slicing, candling, UV-press method and digestion are better used for larval detection in muscles. The molecular techniques are adequate for screening (presence/absence) and can be applied mostly for the analysis of processed products (Cavallero et al., 2017; Herrero et al., 2011). Digestion is one of the most common techniques (Angelucci et al., 2011; Fraulo et al., 2014; Lunestad, 2003; Mo et al., 2013; Skov et al., 2009) and several procedures have been developed in the last years, starting from the historical methods by Jackson et al. (1981) and Smith and Wootten (1978) and from the Codex Stan 244-2004 method (Angelucci et al., 2011; Cammilleri et al., 2016; Fraulo et al., 2014; Llarena-Reino et al., 2013). However, nowadays none of these protocols has been accepted as the international standard and currently included in EU food hygiene and safety regulations.

The aim of this paper was to collect data (prevalence, mean intensity and mean abundance) on the presence of larvae of the genera *Anisakis* and *Hysterothylacium* in fish species collected from Apulia region, as required by the Ministry of Health in 2010 taking into account the invitation reported in Scientific Opinion by European Food Safety Authority (EFSA, 2010). Moreover, the infection level was also quantified in the edible portion by the digestion method, the first step in the consumer exposure assessment.

2. Materials and methods

2.1. Farmed fish sampling

A convenience sampling was conducted on farmed fish collected at three offshore fish farms, two located in the Gulf of Manfredonia and one in the Ionian Sea, off coasts of Gallipoli, between September 2012 and August 2013. In all three farms, fish samples were reared in floating cages placed near the coast. Fry were brought by Apulian land-based fish hatcheries. Fish was fed with dried meal. Fish of commercial size, about 18 months old, were sampled bimonthly from batches of fish soon after capture, before placing on the market. Fish were caught by hand-netting and killed by chilling with ice in holding water.

Seventy-five specimens of seabass (*Dicentrarchus labrax*) from 19 batches and 53 specimens of seabream (*Sparus aurata*) from 15 batches were analysed (Table 1).

2.2. Wild fish sampling

A convenience sampling was carried out on fish caught off Apulian coasts, in accordance with the veterinary department of Apulia region, between September 2012 and August 2013; fishing points are illustrated in Fig. 1.

Fish species were chosen including the most frequently parasitized species fished in Mediterranean Sea such as anchovy (*Engraulis encrasicolus*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus trachurus*), Mediterranean horse mackerel (*Trachurus mediterraneus*) (EFSA, 2010; Mladineo and Poljak, 2014; Pekmezci et al., 2014). Fish species with low prevalence of infection as reported in literature (e.g. red mullets and Mediterranean scaldfish) were also analysed (Ferrantelli et al., 2015; Fioravanti et al., 2006; Gutiérrez-Galindo et al., 2010; Mattiucci and Nascetti, 2008) in order to confirm or not these data. Samples of cephalopod molluscs were also included, owing to their infection with *Anisakis* larvae, although sporadic (Angelucci et al., 2011; Costa et al., 2012; Costa et al., 2016; Giuffrida et al., 2002), and to the widespread custom of eating them raw in Apulia region.

A data collecting sheet was prepared to get informations mainly on the fishing area. Samples consisted of five subjects per batch, with

Table 1
Summary of the results concerning parasitisation level in farmed teleosts (batches and fishes).

Farmed fish species	n. of infected batches/total tested 95% CI[a]	n. of infected fishes/total tested 95% CI[a]
Dicentrarchus labrax	0/19 (0–0.18)	0/75 (0–0.05)
Sparus aurata	0/15 (0–0.22)	0/53 (0–0.07)
Total	0/34 (0–0.1)	0/128 (0–0.03)

[a] CI: confidence interval.

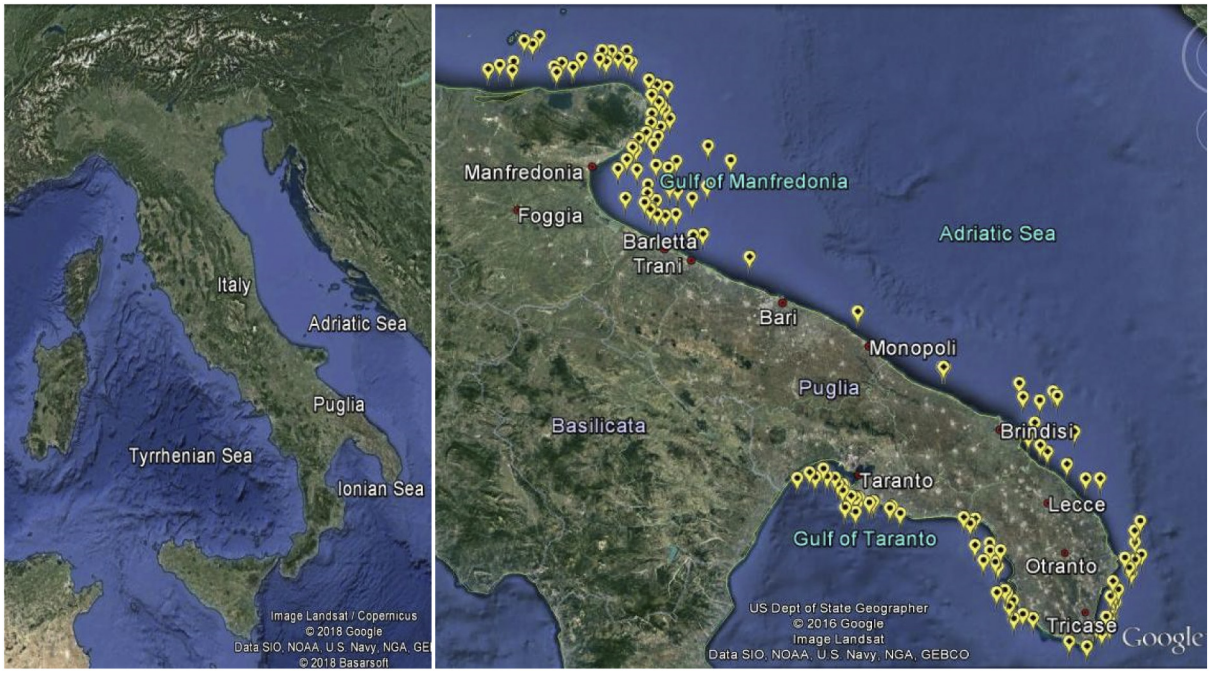

Fig. 1. Fishing points.

exceptions related to the commercial value of some fish species. They were collected during a one-year period in order to evaluate potential changes in prevalence. Samples, distributed among the different local veterinary services on the basis of the number of fishing vessels registered in the Community Fishing Fleet Register, were collected at landing ports by local veterinary services, brought at refrigerated temperature to the laboratory and examined on the day of arrival, within 24–48 h from fishing.

A total of 1144 specimens (249 batches) belonging to 19 different species of wild teleosts and 340 specimens (80 batches) of 6 species of cephalopod molluscs were analysed. More details on examined species are reported with results in Tables 2 and 3.

2.3. Larvae recovery

Each fish was subjected to species identification and measurement of length. All subjects were eviscerated and visually inspected for presence of nematode larvae in coelomic cavity. Viscera were removed and placed in a Petri dish containing 10 ml of 0.9% (w/v) NaCl solution, left at room temperature for 15 min and observed by naked eye. Subsequently, the muscle masses of all samples were submitted to digestion, except for anchovies (*Engraulis encrasicolus*) and cephalopods. Anchovy samples were subjected to direct observation because of their thin fillets. Cephalopod molluscs were examined by candling technique under stereomicroscope (Nikon SM7745T).

Table 2
Summary of the results concerning parasitisation level in cephalopod molluscs (batches and fishes).

Cephalopod molluscs	n. of infected batches/total tested 95% CI[a]	n. of infected subjects/total tested 95% CI[a]
Loligo vulgaris	0/14 (0–0.24)	0/54 (0–0.07)
Allotteuthis media	0/11 (0–0.27)	0/55 (0–0.07)
Sepia officinalis	0/24 (0–0.14)	0/88 (0–0.04)
Illex condetii	0/29 (0–0.12)	0/137 (0–0.03)
Eledone moschata	0/1 (0–0.95)	0/5 (0–0.5)
Octopus vulgaris	0/1 (0–0.95)	0/1 (0–0.95)
Total	0/80 (0–0.05)	0/340 (0–0.01)

[a] CI: confidence interval.

2.4. Digestion method

Preliminarily, the digestion method to be used for larvae recovery was defined, starting from the classic methods by Jackson and Smith, also reported by EFSA's Scientific Opinions (EFSA, 2010; EFSA, 2011) and from the procedure reported in Regulation (EC) No. 1375/2015 and ISO 18743:2015 for *Trichinella* spp. detection, introducing some modifications: the sedimentation phase was avoided and the digestion temperature was decreased from 44-46 °C to 37 °C, as reported in Codex Stan 244-2004 method and also by other authors (Jackson et al., 1981; Llarena-Reino et al., 2013; Skov et al., 2009; Smith and Wootten, 1978), because it allowed a good yield of alive larvae. In fact, the viability of *Anisakis* spp. larvae decreases during digestion at high temperature (over 40 °C, related to time/temperature combination) until all the larvae die (Giarratana et al., 2012; Huang, 2005). This is even more true for *Hysterothylacium* spp. larvae that do not succeed in surviving after heat treatment at 30 °C (Huang, 1988).

Several trials were performed subjecting different fish species to digestion times ranging between 30 and 60 min in order to establish the shortest one which allowed a complete digestion (Table 4). Since there was a different optimal digestion time for each fish species, it was decided not to fix a digestion time but to continue the digestion process up to a residual debris weighing < 5% of the total examined sample.

The following procedure was finally defined: each subject was eviscerated and head, fishbone and fins were removed in order to obtain the edible portion. Flesh of each fish species was then chopped by hand, weighted and placed in a 3 l glass beaker containing 0.5% pepsin solution in HCl 0.063 M (pH 2). The weight/volume ratio was of 1:20. The solution was prepared by adding to each liter of tap water 15 ml of liquid pepsin (660 EP U/mol) and, immediately before use, 8 ml of HCl 25%. The mixture was heated on a hot plate magnetic stirrer to 37 °C

Table 3
Prevalence of batches and specimens infected with *Anisakis* spp. (*A*) and *Hysterothylacium* spp. (*H*): larvae detected in coelomic cavity and muscles.

Fish species	Prevalence of infected batches 95% CI[a] (infected/total)	Prevalence of infected specimens 95% CI[a] (infected/total)	Prevalence of specimens infected with *A* 95% CI[a] (infected/total)	Prevalence of specimens infected with *H* 95% CI[a] (infected/total)	Prevalence of specimens co-infected with *A* and *H* 95% CI[a] (infected/total)
Scomber japonicus	1.00 na (12/12)	0.67 (0.53–0.80) (35/52)	0.67 (0.53–0.80) (35/52)	0 (0–0.07) (0/52)	0 (0–0.07) (0/52)
Micromesistius poutassou	0.70 (0.48–0.85) (16/23)	0.54 (0.44–0.64) (56/104)	0.50 (0.40–0.60) (52/104)	0 (0–0.04) (0/104)	0.04 (0.01–0.09) (4/104)
Trachurus trachurus and *T. mediterraneus*	0.82 (0.67–0.91) (32/39)	0.53 (0.46–0.60) (97/183)	0.50 (0.43–0.58) (92/183)	0.005 (0.003–0.031) (1/183)	0.04 (0.01–0.1) (4/183)
Scomber scombrus	0.76 (0.57–0.88) (22/29)	0.47 (0.39–0.56) (60/127)	0.43 (0.34–0.52) (54/127)	0.016 (0.003–0.057) (2/127)	0.07 (0.02–0.16) (4/127)
Engraulis encrasicolus	0.44 (0.27–0.61) (14/32)	0.32 (0.25–0.40) (51/160)	0.09 (0.05–0.14) (14/160)	0.12 (0.08–0.18) (19/160)	0.11 (0.07–0.17) (18/160)
Trisopterus minutus capelanus	0.33 (0.11–0.55) (6/18)	0.21 (0.13–0.31) (18/85)	0.19 (0.12–0.29) (16/85)	0 (0–0.04) (0/85)	0.02 (0.004–0.08) (2/85)
Sardina pilchardus	0.33 (0.14–0.52) (8/24)	0.20 (0.13–0.28) (22/113)	0.04 (0.01–0.09) (4/113)	0.14 (0.09–0.22) (16/113)	0.02 (0.003–0.06) (2/113)
Merluccius merluccius	0.28 (0–0.56) (2/7)	0.14 (0.06–0.30) (5/35)	0.11 (0.04–0.27) (4/35)	0 (0–0.1) (0/35)	0.03 (0.001–0.15) (1/35)
Sardinella aurita	0.25 (0.01–0.75) (1/4)	0.1 (0.02–0.32) (2/20)	0 (0–0.17) (0/20)	0.1 (0.02–0.32) (2/20)	0 0–0.17 (0/20)
Arnoglossus laterna	0.15 (0.04–0.37) (3/20)	0.05 (0.02–0.11) (4/87)	0.05 (0.02–0.11) (4/87)	0 (0–0.04) (0/87)	0 (0–0.04) (0/87)
Mullus barbatus and *M. surmuletus*	0.11 (0.01–0.21) (4/35)	0.04 (0.02–0.09) (7/158)	0.013 (0.002–0.046) (2/158)	0.03 (0.01–0.07) (5/158)	0 (0–0.02) (0/158)
Euthynnus alletteratus	1.00 (0.05–1.00) (1/1)	1.00 (0.05–1.00) (1/1)	1.00 (0.05–1.00) (1/1)	0 (0–0.95) (0/1)	0 (0–0.95) (0/1)
Others[b]	0 (0/5)	0 (0/19)	0	0	0
Total	0.49 (0.43–0.55) (121/249)	0.31 (0.29–0.34) (358/1144)	0.24 (0.22–0.27) (278/1144)	0.04 (0.03–0.05) (45/1144)	0.03 (0.02–0.04) (35/1144)

[a] CI: confidence interval.
[b] Others: *Phycis blennoides*, *Ophidium barbatum*, *Alosa fallax*, *Spicara smaris*, *Diplodus annularis*.

for the appropriate time depending on the fish species, under continuous slow stirring without splashing, until the digestion was completed. The digest fluid was filtered through a sieve with a mesh size of 180 µm. The sieve was then inspected for the presence of larvae.

After, trials were carried out to evaluate the performance of the digestion protocol as finally defined.

The precision of the method was evaluated: 6 tests were performed in triplicate by two operators, under the same conditions, on mackerel (*Scomber scombrus*) samples spiked with 3 *Anisakis* spp. alive larvae (low contamination level) in order to assess repeatability and intra-laboratory reproducibility.

For the evaluation of the recovery capacity of the method and larval vitality at the end of the digest process, this method was performed 3 times on mackerel (*S. scombrus*) and mullet (*Mullus surmuletus*) samples spiked with *Anisakis* spp. alive larvae at high contamination level (10 larvae in 50 g of flesh) and then, 7 times on mackerel (*S. scombrus*) and twice on mullet (*M. surmuletus*) samples spiked at lower level of contamination (3 larvae in 50 g of flesh) (Table 5).

2.5. Morphological and molecular identification

All detected larvae were collected, noting their location, counted,

Table 4
Preliminary tests to establish the best digestion time for each fish species.

Fish species	Temperature	Time	Larvae added	Larvae recovered	% recovery	Note
Mullus surmuletus	37 °C	30 min	3	3	100%	Partial digestion
	37 °C	40 min	3	3	100%	Complete digestion
Trachurus trachurus	37 °C	40 min	3	3	100%	Partial digestion
	37 °C	60 min	3	3	100%	Complete digestion
Scomber scombrus	37 °C	40 min	3	3	100%	Partial digestion
	37 °C	60 min	10	10	100%	Complete digestion

Table 5
Performance parameters of method.

Species	Contamination level	N. tests	Tests condition	Sensibility (%)	Recovery (%)	Recovery of alive larvae (%)
Scomber scombrus	Low level (3 larvae)	7	37 °C/60′	100	100	100
	High level (10 larvae)	3		100	100	93*
Mullus surmuletus	Low level (3 larvae)	2	37 °C/40′	100	100	100
	High level (10 larvae)	3		100	100	90**
Overall values	Low level (3 larvae)	9		100	100	100
	High level (10 larvae)	6		100	100	92

Note *2 out of 3 tests: 9 alive larvae, 1 dead larva; **1 out of 3 tests: 6 alive larvae, 1 dead larva, 2 dead and damaged larvae, 1 alive and damaged larva.

washed in saline solution, preserved in 70% ethanol, clarified with glycerin and subjected to morphological identification at genus level by light microscopy (Nikon Eclipse 50i) according to the morphological keys of Berland (1961) and Petter and Maillard (1988).

A sample of 185 larvae, isolated from specimens coming from different fishing areas, was sent to the National Reference Centre for Anisakiasis (C.Re.N.A.) of Sicily, for molecular identification at the species level. Molecular analysis of the *Anisakis* larvae, was performed using the 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 (D'Amelio et al., 2000) using the primer pair NC5/NC2 (Zhu et al., 1998) and two restriction enzymes *Hha*I and *Hinf* I for the species identification according to genetic key (D'Amelio et al., 2000). The procedure is described in Costa et al. (2016).

2.6. Statistical elaboration

The total number of detected larvae was used for the calculation of statistical indices, regardless of localisation site (caelomic cavity, viscera and flesh).

Firstly, the Prevalence (P) of parasitized batches and respective Confidence Interval (CI) were calculated. Then, Prevalence (P), mean Intensity (MI) and mean Abundance (MA) were determined for each fish species and for total fish samples. Confidence Interval (CI) was calculated for each statistical index.

The three indices were calculated as follows:

- Prevalence (P) as the ratio between parasitized subjects/batches and the total subjects/batches analysed; the p 95% Confidence Interval (CI) was defined with the Sterne's exact method (N < 1000), modified according to Wald (N > 1000) (Reiczigel, 2003).
- Mean Abundance (MA) as the ratio between the number of larvae recovered and the number of all examined subjects (number of larvae present per examined subjects).
- Mean Intensity (MI) as the ratio between the number of larvae and the number of examined parasitized subjects (mean number of larvae present per parasitized subjects).

As regards MA and MI, the p 95% Confidence Interval (CI) was calculated with Bootstrap method (number of bootstrap replications = 2000).

The above indices and the related CIs were calculated using the program Quantitative Parasitology QP 3, available free on-line (Rózsa et al., 2000).

The trends of demographic indices, depending on fishing period, fishing area and length of the subjects have always been evaluated by construction of graphs and, when appropriate, by pairwise comparisons to state whether differences and/or similarities noted were statistically significant.

2.7. Quantitative evaluation of larvae in muscle

For each fish species, prevalence of batches infected with nematode larvae in muscle masses and, furthermore, prevalence and mean intensity of fish specimens parasitized with *Anisakis* spp. and *Hysterothylacium* spp. larvae in muscle masses were calculated. To quantify the consumer exposure to the fish parasite *Anisakis* spp., the number of larvae per 100 g of edible part was determined by the following steps. Firstly, for each infected batch, the ratio between the total number of larvae present in infected specimens and the sum of the weights of the edible part of all these specimens was calculated and then expressed by referring it to a defined quantity of edible portion (100 g). Subsequently, given these data, the average and the median values of larvae per 100 g of edible part was determined for each fish species. However, especially for some fish species with a small size, such as anchovies, the consumer meal most likely comprises infected and non-infected subjects, since a batch involves both of them. Thus, the same values were also estimated by referring to the edible part of all specimens (infected and non-infected) forming the parasitized batch.

3. Results

With regard to the performance of the digestion procedure used in the present study, it showed 100% recovery capacity, which is reduced to 92% when alive larvae only are considered (Table 5). Repeatability and intra-laboratory reproducibility were good as the 6 tests carried out by two operators (3 tests/operator) on *S. scombrus* samples spiked at low level, gave always the same results (100% recovery).

Examinations conducted on seabass (*Dicentrachus labrax*) and seabream (*Sparus aurata*) samples collected at offshore fish farms revealed no nematode larvae (Table 1).

The same result was registered in cephalopod samples involved in the project (Table 2).

As regards wild teleosts, 8 out of the 19 examined fish species (*Merluccius merluccius*, *Sardinella aurita*, *Euthynnus alleteratus*, *Phycis blennoides*, *Ophidium barbatum*, *Alosa fallax*, *Spicara smaris*, *Diplodus annularis*) were initially not included in the sampling plan and only a small number of specimens was collected. Thus, the pertinent statistical indices are just indicative.

Prevalence (P) values and their Confidence Intervals (CI) of the two parasite genera detected are reported in Table 3.

Overall, 121 batches were parasitized out of 249 examined (P 49%), for a total of 358 infected subjects out of 1144 examined (P 31%) (Table 3).

Mean Intensity (MI) and Mean Abundance (MA) values, total and

Table 6
Mean intensity and mean abundance of nematode larvae (*Anisakis* spp. and *Hysterothylacium* spp.) per fish species.

Fish species	Mean intensity of nematode larvae 95% CI[a]	Mean abundance of nematode larvae 95% CI[a]	Mean intensity of *Anisakis* larvae 95% CI[a]	Mean abundance of *Anisakis* larvae 95% CI[a]	Mean intensity of *Hysterothylacium* larvae 95% CI[a]	Mean abundance of *Hysterothylacium* larvae 95% CI[a]
Scomber japonicus	54.6 (34.8–83.1)	36.7 (23.5–57.4)	54.6 (34.8–83.1)	36.7 (23.5–57.4)	na	na
Micromesistius poutassou	35.4 (25.0–48.2)	19.1 (13.1–27.0)	35.3 (25.4–48.5)	19.0 (13.1–27.2)	2.3 (1.0–3.3)	0.09 (0.01–0.22)
Trachurus trachurus and *T. mediterraneus*	15.8 (9.6–26.9)	8.4 (5.0–14.7)	15.9 (9.8–27.3)	8.4 (5.0–14.1)	1 (uc)	0.03 (0.01–0.05)
Scomber scombrus	6.1 (4.1–10.2)	2.9 (1.8–4.9)	6.1 (4.6–10.6)	2.8 (1.8–4.8)	1.5 (1.0–2.2)	0.07 (0.02–0.15)
Engraulis encrasicolus	2.7 (2.3–3.0)	0.8 (0.6–1.0)	1.6 (1.3–1.9)	0.3 (0.2–0.4)	2.3 (1.9–2.6)	0.5 (0.4–0.7)
Trisopterus minutus capelanus	7.4 (5.0–9.9)	1.6 (0.9–2.6)	6.9 (4.9–9.6)	1.5 (0.8–2.5)	4.5 (4.0–4.5)	0.1 (0–0.3)
Sardina pilchardus	2.3 (1.9–2.7)	0.4 (0.3–0.7)	1.5 (1.0–2.2)	0.08 (0.03–0.17)	2.3 (1.8–2.8)	0.4 (0.2–0.6)
Merluccius merluccius	3.6 (1.8–6.6)	0.5 (0.1–1.4)	3.4 (1.6–6.6)	0.5 (0.1–1.4)	1 (uc)	1 (uc)
Sardinella aurita	5 (uc)	0.5 (0.0–1.3)	na	na	5 (uc)	0.5 (0.0–1.3)
Arnoglossus laterna	1.8 (1.0–2.3)	0.08 (0.01–0.21)	1.8 (1.0–2.3)	0.08 (0.01–0.21)	na	na
Mullus barbatus and *M. surmuletus*	1.7 (1.1–2.6)	0.08 (0.03–0.16)	1 (nc)	0.01 (0–0.03)	2.0 (1.2–3.0)	0.06 (0.02–0.15)
Euthynnus alletteratus	1 (uc)	1 (uc)	1 (uc)	1 (uc)	na	na
Total	17.2 (13.7–21.5)	5.4 (4.3–6.7)	19.2 (14.9–24.3)	5.2 (4.1–6.7)	2.2 (1.9–2.4)	0.2 (0.1–0.2)

na: not applicable.
uc: 95% confidence intervals are uncertain.
[a] CI: confidence interval.

related to the two genera of parasites of interest are reported in Table 6.

The number of collected larvae organized depending on both the fish species and their localization is reported in Table 7.

Hysterothylacium spp. and *Anisakis* spp. were present in all parasitized species, except for round sardinella (*S. aurita*), parasitized only by *Hysterothylacium* spp. and for Mediterranean scaldfish (*Arnoglossus laterna*), little tunny (*Euthynnus alleteratus*) and chub mackerel (*Scomber japonicus*) infected only with *Anisakis* spp.. However, it must be highlighted that results related to sardinella and little tunny derive from analysis of a few subjects.

A total of 5980 *Anisakis* spp. larvae and 173 *Hysterothylacium* spp. larvae were detected: larvae were found mainly in coelomic cavity and/or on viscera (5754 *Anisakis* spp. and 128 *Hysterothylacium* spp.), while 271 larvae (226 *Anisakis* spp. and 45 *Hysterothylacium* spp.) were detected from muscle tissues after chloro-peptic digestion. Therefore, larvae localized in the flesh represented 4.4% of total larvae recovered (*Anisakis* spp. 3.8% and *Hysterothylacium* spp. 26%). Out of the 19 fish species studied, 11 resulted positive to the presence of larvae in muscles.

The prevalence values of batches and specimens with muscular *Anisakis* infection (Table 8) were lower than the overall ones (related to *Anisakis* spp. infection in both coelomic cavity, viscera and muscles). Blue withing (*Micromesistius poutassou*) was the fish species with highest level of infection, with prevalence of 0.30 and 0.22 for infected batches and specimens respectively and mean intensity of 5.

Regarding the 185 larvae sent to the C.Re.N.A., 180 larvae, morphologically recognised as *Anisakis* type I larvae, were identified by biomolecular PCR-RFLP analysis as *A. pegreffii*; 2 larvae, identified as

Table 7
Number of larvae recovered from each fish species segregated by nematode genus and site of localization.

Fish species	Total number of larvae	Number of *Anisakis* spp. larvae		Number of *Hysterothylacium* spp. larvae	
		Overall value	Muscular masses	Overall value	Muscular masses
Scomber japonicus	1910	1910*	20	0	0
Micromesistius poutassou	1983	1974	114	9	1
Trachurus trachurus and *T. mediterraneus*	1529	1524**	32**	5	0
Scomber scombrus	364	355	16	9	0
Engraulis encrasicolus	137	53	3	84	27
Trisopterus minutus capelanus	134	125	37	9	0
Sardina pilchardus	50	9	0	41	12
Merluccius merluccius	18	17	1	1	0
Sardinella aurita	5	0	0	5	5
Arnoglossus laterna	7	7	1	0	0
Mullus barbatus and *M. surmuletus*	12	2	2	10	0
Euthynnus alletteratus	4	4	0	0	0
Total	6153	5980	226	173	45

Note *1 *A. physeteris* larva in coelomic cavity. **1 *A. physeteris* larva in muscle.

Table 8
Prevalence of infected batches and prevalence and mean intensity of specimens infected with *Anisakis* spp. (*A*) and *Hysterothylacium* spp. (*H*) in muscular masses.

Fish species	Prevalence of infected batches 95% CI[a] (infected/total)	Prevalence of infected specimens 95% CI[a] (infected/total)	Prevalence of specimens infected with *A* 95% CI[a] (infected/total)	Prevalence of specimens infected with *H* 95% CI[a] (infected/total)	Mean intensity of specimens infected with *A*	Mean intensity of specimens infected with *H*
Scomber japonicus	0.25 (0.07–0.54) (3/12)	0.15 (0.07–0.28) (8/52)	0.15 (0.07–0.28) (8/52)	0 na	2.5	0
Micromesistius poutassou	0.30 (0.15–0.52) (7/23)	0.22 (0.15–0.31) (23/104)	0.22 (0.15–0.31) (23/104)	0.01 (0.001–0.051) (1/104)	5	1.0
Trachurus trachurus, *T. mediterraneus*	0.15 (0.07–0.31) (6/39)	0.05 (0.03–0.09) (9/183)	0.05 (0.03–0.09) (9/183)	0 na	3.6	0
Scomber scombrus	0.20 (0.09–0.4) (6/29)	0.08 (0.04–0.14) (10/127)	0.08 (0.04–0.14) (10/127)	0 na	1.6	0
Engraulis encrasicolus	0.21 (0.11–0.39) (7/32)	0.09 (0.06–0.15) (15/160)	0.02 (0.005–0.055) (3/160)	0.08 (0.05–0.13) (13/160)	1.0	2.3
Trisopterus minutus capelanus	0.17 (0.05–0.41) (3/18)	0.12 (0.06–0.21) (10/85)	0.12 (0.06–0.21) (10/85)	0 na	3.7	0
Sardina pilchardus	0.04 (0.002–0.204) (1/24)	0.04 (0.02–0.1) (5/113)	0 na	0.04 (0.02–0.1) (5/113)	0	2.4
Merluccius merluccius	0.14 (0.007–0.55) (1/7)	0.03 (0.002–0.15) (1/35)	0.03 (0.002–0.15) (1/35)	0 na	0	1
Sardinella aurita	0.25 (0.01–0.75) (1/4)	0.1 (0.02–0.32) (2/20)	0 na	0.1 (0.02–0.32) (2/20)	0	1
Arnoglossus laterna	0.05 (0.003–0.244) (1/20)	0.01 (0.001–0.061) (1/87)	0.01 (0.001–0.061) (1/87)	0 na	0	0
Mullus barbatus, *M. surmuletus*	0.03 (0.002–0.152) (1/35)	0.01 (0.002–0.046) (2/158)	0.01 (0.002–0.046) (2/158)	0 na	1	0

na: not applicable.
[a] CI: confidence interval.

Anisakis type II, were molecularly confirmed as *A. physeteris*. They were found in the coelomic cavity of one chub mackerel (*S. japonicus*) and in the flesh of one horse mackerel (*T. trachurus*) caught in the Strait of Otranto, an area where different water masses, originating from the Adriatic Sea, the Ionian Sea and the Eastern Mediterranean, are exchanged and where cetacean sightings are fairly common. In both cases, larvae type II were detected in co-infection with larvae type I identified as *A. pegreffii*. Of the remaining 14 larvae, not conclusively identified by morphological examination, 11 were identified as *A. pegreffii* while 3 as *Hysterothylacium* L4 larvae by PCR RFLP.

As regards larvae found in 100 g of edible part, values are reported in Table 9. The results show that the median number of larvae per 100 g of edible part of infected subjects is always far greater than that one calculated by referring to 100 g of edible part which includes both parasitized and not parasitized subjects forming a single batch. Blue withing (*M. poutassou*) was the fish species with the highest median value per 100 g of edible part related to the whole parasitized batch (9.3 larvae). Lower values were obtained from chub mackerel (*Scomber japonicus*) and poor cod (*Trisopterus minutus capelanus*): 3.6 and 3.8, respectively. Anchovies (*E. encrasicolus*) showed median values of 3.5 for *Anisakis* spp. and 5.8 for *Hysterothylacium* spp.. Median values of 2.1, 0.9 and 0.5 were found in red mullet (*M. surmuletus and M. barbatus*), horse mackerel (*T. mediterraneus* and *T. trachurus*) and hake (*Merluccius merluccius*), respectively. *Hysterothylacium* spp. was the only nematode present in round sardinella (*Sardinella aurita*) samples with a median value of 8.3. A particular result was registered from sardine (*S. pilchardus*): median value of *Hysterothylacium* spp. larvae was very high (30 larvae/100 g of edible part of parasitized batch). It must be highlighted that all larvae were detected in one batch comprising 5 specimens.

4. Discussion

The risk connected with the presence of *Anisakis* spp. larvae in fishery products and the related contamination level is of great relevance and economic impact, especially for farmed fish species.

The scientific studies present in literature till date of publication of the first EFSA Scientific Opinion (EFSA, 2010) were conducted exclusively on Atlantic salmons farmed in Norway (Lunestad, 2003) and in Canada (Marty, 2008) and on rainbow trout (*Oncorhynchus mykiss*) farmed in Denmark (Skov et al., 2009); among these, only Marty (2008) reported the finding of one larva in one out of the 894 examined subjects. Subsequent studies have shown the presence of larvae in farmed salmon (Mo et al., 2013), tuna (Mladineo and Poljak, 2014) and cobia (Shih et al., 2010). It should be highlighted that analysed samples were wild young tuna fished in Adriatic Sea and then farmed in sea cages (Mladineo and Poljak, 2014), while cobia (Shih et al., 2010) were frequently fed with wild fish species with high prevalence of infection. A different situation was reported by Mo et al. (2013) that found, in the same fish plant, *Anisakidae* larvae in 20% of discarded runts but no larva in salmons ready for marketing. Our results on farmed seabass and seabream coincide with those reported in literature (Gustinelli et al., 2011; Pekmezci et al., 2014; Peñalver et al., 2010). However, the presence of *Anisakis* spp. larvae in wild seabream and especially in wild

Table 9
Average and median number of larvae per 100 g of edible portion calculated for both the nematode genera (*Anisakis* spp. and *Hysterothylacium* spp.) and referred only to the infected subjects composing a batch or to the whole parasitized batch.

Fish species	Edible part of subjects infected with *Anisakis* spp.		Edible part of batch infected with *Anisakis* spp.		Edible part of subjects infected with *Hysterothylacium* spp.		Edible part of batch infected with *Hysterothylacium* spp.			
	Average number of larvae/100 g edible part	Median number of larvae/100 g edible part (min–max)	Average number of larvae/100 g edible part		Median number of larvae/100 g edible part (min–max)	Average number of larvae/100 g edible part	Median number of larvae/100 g edible part (min–max)	Average number of larvae/100 g edible part	Median number of larvae/100 g edible part (min–max)	
Micromesistius poutassou	16.5	15.5 (2.0–34)	11.2		9.3 (0.8–22.9)	0.8	0.8 nc	0.5	0.5 nc	
Scomber japonicas	10.5	9.1 (4.6–18.0)	6.3		3.6 (2.7–12.5)	0	0	0	0	
Trisopterus minutus capelanus	13.8	11.1 (4.4–25.8)	10.5		3.8 (1.7–25.8)	0	0	0	0	
Scomber scombrus	9.5	6.0 (2.1–30)	2.1		1.5 (0.4–5.7)	0	0	0	0	
Engraulis encrasicolus	17.5	17.5 (10–25)	3.5		3.5 nc	23.0	15.3 (13.6–50)	6.5	5.8 (5.5–10)	
Merluccius merluccius	2.6	2.6 nc	0.5		0.5 nc	0	0	0	0	
Trachurus trachurus and *T. mediterraneus*	15.0	4.7 (1.7–65.7)	3.9		0.9 (0.3–18.4)	0	0	0	0	
Sardina pilchardus	0	0	0		0	30	30 nc	30	30 nc	
Sardinella aurita	0	0	0		0	20.8	20.8 nc	8.3	8.3 nc	
Mullus barbatus and *M. surmuletus*	10.5	10.5 nc	2.1		2.1 nc	0	0	0	0	
Arnoglossus laterna	1.3	1.3 nc	0.3		0.3 nc	0	0	0	0	

nc: not calculable.

seabass seems to be almost frequent (Culurgioni et al., 2011).

Our findings confirm that *Anisakis* infection is quite low in farmed fish. This is true if fry come from land based hatcheries, where they are fed with farmed live food not contaminated with viable parasites, and adults are fed avoiding the use of infected fish or fish scraps (Crotta et al., 2016). In fact, with these precautions, the chances that infected secondary or paratenic hosts enter the cages and are eaten by farmed fish are modest, given that they prefer habitat with low population density (Crotta et al., 2016; Kapota, 2012; Skov et al., 2009). This does not exempt from the need to respect the national and EU legislation (Italian Ministerial Decree D.M. 17.07.2013; Regulation (EC) No. 853/2004; Regulation (EC) No. 2074/2005), since the risk of infection can not be excluded.

Concerning cephalopods, the detection of no nematode larvae in all the examined subjects contrasts with prevalence of 30.5% reported in shortfin squid (*I. coindetii*) captured in the central eastern part of the Adriatic Sea by Petrič et al. (2011) and with prevalence of 20% reported for *Anisakis* spp. in flying squid (*T. sagittatus*) fished in the Tyrrhenian Sea by Angelucci et al. (2011). Other studies on cephalopods (Ferrantelli et al., 2015; Giuffrida et al., 2002; Serracca et al., 2013) fished in Adriatic, Mediterranean and Tyrrhenian Seas, reported very low values of prevalence (less than or close to 1%).

With regard to wild teleosts, nematode larvae were found in almost all examined fish species. They were mainly *Anisakis* L3 larvae (97.2%) and to a lesser extent *Hysterothylacium* L3 and L4 larvae (2.8%). Chub mackerel (*S. japonicus*) is the species with the highest infection level and mean intensity in the Mediterranean Sea (Chaligiannis et al., 2012; Mladineo and Poljak, 2014; Pekmezci et al., 2014; Piras et al., 2014), in the Atlantic (Paladini et al., 2009) and the Pacific Oceans (Quiazon et al., 2011; Suzuki et al., 2010). According with these findings, also in the present study this species showed the highest prevalence with 100% of parasitized batches and 67.3% of parasitized subjects. Mean intensity and mean abundance were also very high (Table 6). All larvae isolated from this species (1910) were *Anisakis* spp. L3, while no *Hysterothylacium* spp. larvae were collected. All larvae were type I, identified at the C.Re.N.A as *A. pegreffii* by biomolecular method, except for one larva found to be type II and identified as *A. physeteris*.

As regards anchovies (*E. encrasicolus*), frequently implicated in cases of human anisakiasis, mainly in the countries of southern Europe (AAITO-IFIACI, 2011; Angelucci et al., 2011; Mattiucci et al., 2013; Rello et al., 2009; Serracca et al., 2014) where they are eaten raw and/or mild treated (marinated, salted) (Baird et al., 2014; Brutti et al., 2010; Pozio, 2004), the prevalence of nematode infection was 44% and 32% in batches and specimens respectively. The isolated larvae were mainly *Hysterothylacium* spp. (84 out of 137) (Table 7), therefore the prevalence of infection with *Anisakis* spp. larvae alone or associated with *Hysterothylacium* spp. was 20% (Table 3). Although this value is certainly high (20%), it is lower than expected, given the frequent alarms triggered in Apulia region for parasitized batches and for confirmed or suspected cases of human anisakiasis (AAITO-IFIACI, 2011; Fumarola et al., 2009; Maggi et al., 2000; Polimeno et al., 2010; Ugenti et al., 2007).

The numerous surveys carried out on this species show extremely variable results among different geographical areas but also within the same investigated area (Ciccarelli et al., 2011; Gutiérrez-Galindo et al., 2010; Mladineo et al., 2012; Piras et al., 2014; Serracca et al., 2014). Rello et al. (2009), for example, conducted a survey on anchovies coming from eastern Atlantic Ocean (Gulf of Cadiz and Strait of Gibilterrra) and from 4 Mediterranean areas (Gulf of Alboran, Catalonia coast, Gulf of Lion and Ligurian Sea), finding prevalences for *Anisakis* spp. and *Hysterothylacium* spp. larvae of 13% and of 4.3%, respectively, in the eastern Atlantic Sea and lower values for *Anisakis* spp. (P 5.6%) but higher ones for *Hysterothylacium* spp. (P 44.7%) in the western Mediterranean Sea. Moreover, significant differences were found among the 4 Mediterranean areas, with lower prevalences both for *Anisakis* spp. and *Hysterothylacium* spp. larvae in the Gulf of Alboran, a

gradual increase moving north in Gulf of Lion, to reach the highest values in the Ligurian Sea. A certain variability in prevalence of infection with *Anisakis* spp. among very close fishing areas is also confirmed in the present study, where anchovies from northern coasts of Gargano showed high prevalence values (P 33%), no nematode larvae were found in the Gulf of Manfredonia while the prevalence in the fishing area of Barletta-Trani exceeded 30%.

The published data, although few and old, on the presence of *Anisakis* spp. larvae in anchovies sampled in Apulia region show discordant results: Fioravanti et al. (2006) found no *Anisakis* spp. but only *Hysterothylacium* spp. larvae in anchovies fished in the Gulfs of Manfredonia and Taranto, with prevalence of 9.8% and 8%, respectively. Conversely, Dambrosio et al. (2005) in a study carried out on anchovy samples collected at retailers in Apulia region, found a high percentage of infection with *Anisakis* spp. (P 83.3%); but this could have been influenced by the reduced number of samples (5 infected out of 6 examined subjects).

As regards sardine (*S. pilchardus*) the prevalence of infected subjects with nematode larvae was 19.5% (22 out of 113 subjects): *Anisakis* spp. larvae were present in 4 out of 113 subjects (P 3.5%) while 2 subjects were co-infected with *Anisakis* spp. and *Hysterothylacium* spp. larvae (P 1.8%), for a total prevalence of *Anisakis* spp. of 5.3%; *Hysterothylacium* spp. larvae alone were found in further 16 subjects (P 14.2%). These data coincide with those reported by Mladineo and Poljak (2014) that show prevalence of *Anisakis* infection of 3.3% in sardines caught off eastern coasts of the central Adriatic Sea. Prevalence values even higher for *Hysterothylacium* spp. larvae (P 58.5% in Cesenatico, P 29.3% in Manfredonia) but lower for *Anisakis* spp. larvae (P 0.3% in Ancona, P 0.2% in Manfredonia, absence in the Gulf of Taranto) are reported by Fioravanti et al. (2006).

It is important to highlight the lower levels of infection found in red mullets (*Mullus barbatus* and *Mullus surmuletus*), fish species widespread in the waters of Apulia region and widely consumed by the local population. Two *Anisakis* spp. larvae were found in 2 out of the 158 examined subjects (P 1.3%), while other 5 subjects were parasitized with 10 *Hysterothylacium* spp. larvae (P 3.2%). This is in agreement with the finding reported by Dambrosio et al. (2005) on 4 fish samples collected from Apulian fish markets. Variable data on prevalence of *Anisakis* spp. larvae are also reported in literature with low or null values in some surveys (Costa et al., 2008; Serracca et al., 2013) but higher in others (P 13.2% in *M. barbatus*, eastern Mediterranean) (Pekmezci et al., 2014). However, higher prevalence values of *Hysterothylacium* spp. were reported in other works (P 25.6% in *M. barbatus*, P 61.5% in *M. surmuletus*) (Costa et al., 2008), (P 25.4% in *M. barbatus*) (Serracca et al., 2013), (P 80–90% in *M. barbatus*) (Carreras-Aubets et al., 2012).

The evaluation of statistical indices for each fish species put in relation to the length of specimens and the fishing period did not allow to highlight a clear correlation as opposed to data reported in literature (Serracca et al., 2014); this is probably due to the low number of subjects for each fish species available in our study. By analyzing the trends of the statistical indices calculated from the data referred to all fish species, the largest number of available samples allows more reliable considerations in relation to the fishing period. In particular, the prevalence of *Anisakis* spp. shows not clear statistically significant oscillations, while mean intensity values show a significant increase which starts in January, becomes maximum in March–April, and then decreases. This is accompanied by a parallel, but more limited, increase of mean abundance values. This situation indicates an increase, not only of the number of parasitized subjects but mostly of the number of larvae present in each infected specimen, maybe related to recent infections due to an huge presence of larvae and/or intermediate hosts of L2 larvae (copepod and euphausiid).

Several scientific studies report that statistical indices vary significantly among different fish species and among different geographical areas within the same species (Chai et al., 2005; EFSA, 2010; Mattiucci and Nascetti, 2008; Rello et al., 2009). The present survey shows that nematode larvae are widespread and that the variability in prevalence is related not so much to the geographical area but rather to the different fish species. For example, in anchovies caught off the Gulf of Manfredonia, contrary to findings reported for other fishing areas, it could be noted the absence of infection with both *Anisakis* spp. and *Hysterothylacium* spp. larvae; in the same area, these nematodes are present with significant infection level in the other fish species studied. Furthermore, anisakid infection in red mullet (*M. barbatus* and *M. surmuletus*) was found only in the Ionian Sea.

As regards the identification at species level, all *Anisakis* spp. larvae subjected to biomolecular analysis in the laboratories of C.Re.N.A. of Sicily, were identified as *A. pegreffii*, except for 2 larvae, morphologically identified as type II and belonging to the species *A. physeteris*. Our data are in agreement with literature data that show how *A. pegreffii* is the prevalent species in the Adriatic Sea and more generally in Mediterranean Sea (Cavallero et al., 2012; Costa et al., 2016; De Liberato et al., 2013; Mattiucci and Nascetti, 2008; Mladineo and Poljak, 2014; Pekmezci et al., 2014; Piras et al., 2014; Pozio, 2004; Serracca et al., 2014) with sporadic reports of *A. physeteris* (Cavallero et al., 2012; Costa et al., 2016; Pekmezci et al., 2014) and even less of *A. simplex* sensu stricto (Chaligiannis et al., 2012; Costa et al., 2016; Pekmezci et al., 2014) and *A. tipica* (Pekmezci et al., 2014). This situation is different from that reported in northern European Sea, Atlantic and Pacific Oceans where the predominant species seems to be *A. simplex s.s.* (Abollo et al., 2003; Mattiucci and Nascetti, 2008).

Before evaluating the results concerning the enumeration of larvae in the edible portion, it should be noted that the digestion method used in this study showed an excellent performance, as demonstrated by favorable results on repeatability, reproducibility and recovery capacity, which are in accordance with those reported in literature (Cammilleri et al., 2016; Fraulo et al., 2014). This technique seems to be the best one for the routinely official checks as it can be easily performed by both Competent Authority and FBOs. Digestion procedure shows higher sensitivity for larval recovery from muscle masses (EFSA, 2010; Marty, 2008) than the other available methods, giving more reliable values to calculate demographic indices and also provides additional information on larval viability. However, other techniques such as visual inspection, candling and UV-press method are more recommended for exact localization of larvae (Levsen and Karl, 2014). Compared to UV-press method, the digestion procedure allows to examine simultaneously many fish specimens and seems to be less laborious and readily adaptable routinely in diagnostic laboratories. On the other hand molecular techniques can be easily and rapidly performed (Cavallero et al., 2017; Espiñeira et al., 2010; Herrero et al., 2011) and allow a more accurate species identification, however they cannot provide information on larval viability.

With regard to the statistical indices related to the muscle masses, the prevalence values of infected batches and specimens are lower than expected, if they are compared to the overall values (related to larval detection in both coelomic cavity and muscles) (Tables 3 and 8). Our results coincide with those reported in literature for some fish species as *S. japonicus colias*, *S. scombrus* and *M. poutassou* in the Mediterranean Sea (Gutiérrez-Galindo et al., 2010; Piras et al., 2014). Otherwise, Piras et al. (2014) found prevalence and mean intensity (P 7.9% and MI 2.0) in anchovies (*E. encrasicolus*) caught off the Mediterranean Sea significantly higher than those observed in the present study. The presence of *Anisakis* spp. larvae in muscle mass was described in anchovies (*E. encrasicolus*) fished off Tyrrhenian coast of central Italy also by De Liberato et al. (2013), but the results of this survey, expressed in percentage, cannot be compared to our data because prevalence and mean intensity were not calculated. Actually, further comparison data are not available because only few studies have been carried out on the prevalence of *Anisakis* spp. larvae in muscle portion of the same fish species examined in the present survey.

Compared to our results and generally to those registered in the Mediterranean area, the prevalence of infection in muscles of samples

fished in Atlantic Ocean and in northern European Seas appears considerably higher, close to 100%, with significant differences about the localization of larvae in the abdominal muscles (belly flaps) and in the epiassial and hypoassial muscles (fillets) (Bernardi et al., 2011; Levsen and Karl, 2014; Levsen and Lunestad, 2010; Skov et al., 2009).

These different findings can be explained taking into account both the higher penetration ability, in vivo and in vitro, of *A. simplex* rather than *A. pegreffii* (Quiazon et al., 2011; Suzuki et al., 2010), and the greater values of overall prevalence and mean intensity observed in those fishing areas and fish species (Levsen and Karl, 2014; Levsen and Lunestad, 2010). These assessments should not give the impression that there is a close correlation between the total parasitization rate and the infestation level in muscle mass. Therefore, the evaluation of the first parameter alone is not sufficient to establish the sanitary and product quality of a fish batch (Llarena-Reino et al., 2012).

It should be also highlighted the presence of *Hysterothylacium* spp. larvae in muscle masses that was the only nematode detected in some fish species (*S. pilchardus and S. aurita*). This circumstance was already indicated in previous surveys (Felizardo et al., 2009; Karl and Levsen, 2011) and suggests the necessity to perform the identification of larvae recovered in muscle masses, at least to genus level, since *Hysterothylacium* spp. has mainly an impact on commercial and organoleptic quality rather than a sanitary relevance.

Aim of this survey was also to determine the *Anisakis* hazard on a defined quantity of edible part (100 g) since the studies reported in literature until nowadays expressed the infection level only by calculating the prevalence and/or the mean intensity (Cipriani et al., 2018; Costa et al., 2009; Costa et al., 2016; Karl and Levsen, 2011; Levsen and Karl, 2014; Llarena-Reino et al., 2012; Mladineo and Poljak, 2014). The average and median values of larvae per 100 g of edible portion registered in this study are not negligible, but they decrease if we realistically consider that the consumer meal is composed by the edible part of many subjects belonging to the same batch. Despite a batch is reasonably supposed to be taken from the same fish stock, with regard to *Anisakis* infection there is a certain variability among subjects concerning the presence and the number of larvae in flesh. In fact, the intramuscular migration of larvae is not homogeneous for all subjects, probably because it is influenced by physiological (nutrient availability) and/or immune states of the host.

According to these results, the exposure level to *Anisakis* spp. larvae and allergens, after the ingestion of a parasitized meal, could sometimes be alarming, in particular for some fish species; fortunately, this possibility is not very frequent, since the prevalence of parasitized batches with larvae in muscle masses is significantly lower than the overall one. For example, if we consider anchovies (*E. encrasicolus*), the percentage of subjects infected with *Anisakis* spp. larvae in the edible part was 2% (3 subjects out of 160) and fishes belonged to 2 batches out of 32: which means that the ingestion of a contaminated meal is possible in 2 cases out of 32. In particular, muscular nematode infection was found in 7 out of 32 batches of which, one batch was infected only with *Anisakis* spp. larvae (2 positive subjects, each of them infected with one larva); 5 batches were infected only with *Hysterothylacium* spp. larvae (12 positive specimens); one batch contained one specimen co-infected with both nematodes. However, the risk for the consumer after eating 100 g of anchovies (*E. encrasicolus*) is to ingest 3.5 *Anisakis* larvae.

Moreover, it should be noted that most fish species which were found with a high prevalence of batches with muscular infection (blue whiting, poor cod, chub mackerel, horse mackerel) are usually consumed cooked, at least in our country. The situation concerning anchovies is different because they are often eaten raw and/or undercooked, therefore they represent a high risk for the consumer and the safety measures established by current legislation, such as freezing treatment at −20 °C for at least 24 h, should necessarily be applied.

Limitation of this study is the low representativeness of samples, resulting from the compromise between costs and the purpose to collect data on several fish species taken along the entire Apulia coastline. Despite the large quantity of overall samples, the number of subjects for each fish species was consequently too low for a significant statistical evaluation. However, not truly representative sample schemes is an issue affecting most of the studies conducted on wild fish, since the wild population constantly changes owing to climate, environmental and many other factors and costs for sampling of so many fish species would be excessive and not sustainable.

In conclusion, our results confirm that *Anisakis* infection is widespread in wild teleosts also in the investigated sea area, while the consumption of raw and/or undercooked cephalopods and farmed fish originating from the Apulia region seems to imply a low risk of acquiring infection, as no larvae were found in these products. However, since it is not possible to exclude the presence of *Anisakis* spp. in farmed fish it would be advisable to introduce an official systematic monitoring of the health status of offshore fish farms in relation to "*Anisakis*" hazard, extending it to all fish products and not only to those destined to be consumed raw or almost raw, as already established by Regulation (EC) No. 853/2004 and its subsequent amendments. With regard to wild teleosts, since no fishing area can be considered free from *Anisakis* spp., in addition to ensuring compliance with the current legislation, it is suggested to encourage the practice of a rapid and correct evisceration as soon as possible and also to inform consumers and FBOs about the benefits of this control measure. A further safety measure for the consumer protection could be the introduction of a national surveillance programme, at least for certain fish species, and the establishment of criteria, within the European or national legislation, for acceptance or rejection of a fish batch, based on cost/benefit analysis. Moreover, FBOs should use a unique official sampling protocol provided by European or national laws and a more accurate analytical method, such as the digestion procedure or UV-press method, should be associated with the visual inspection already provided by law.

In addition to further epidemiological surveys, useful to evaluate the situation about other wild and farmed fish species in Italy and in Apulia region, it is necessary to collect more quantitative data on the infection level in muscle masses for the consumer exposure assessment to *Anisakis* spp..

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