Food Analytical Methods

Assessment of a sampling plan based on visual inspection for the detection of anisakids larvae in fresh anchovies (Engraulis encrasicolus). A first step towards official validation?

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Corresponding Author:	Andrea Armani, PhD ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Lisa Guardone, DVM, Ph.D
First Author Secondary Information:	
Order of Authors:	Lisa Guardone, DVM, Ph.D
	Malandra Renato
	Francesco Costanzo
	Lorenzo Castigliego
	Lara Tinacci
	Daniela Gianfaldoni
	Alessandra Guidi
	Andrea Armani
Order of Authors Secondary Information:	
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Abstract:	The presence of anisakids larvae in fish is a public health issue and effective risk management procedures are needed to avoid that heavily infected products reach the market. Currently, an official sampling plan for fresh fish defining sample size, inspection methods and criteria to accept or reject the merchandise is lacking at the European and Italian level. In this study, we compared the visual inspection proposed by the sampling plan of the Lombardy Region (Italy) to the UV press method and to an optimized digestion procedure with the aim to assess its ability in detecting visible parasites. Thirty-one batches of Engraulis encrasicolus, each composed of ~30 specimens, were collected and subsequently analyzed with the three techniques. The Mean Abundance (MA) was calculated after each procedure and compared on the basis of a threshold value. The results showed that the visual inspection performed similarly to the digestion method, with a sensitivity of 93%, a specificity of 100% and an accuracy of 97%. Overall, the comparison showed that, in the proposed sampling plan, the visual inspection is effective in rejecting unmarketable anchovies and in preventing the commercialization of unsafe products. This method is simple, less demanding than digestion in terms of time and equipment, and thus suitable as a standardized procedure to be routinely applied by Food Business Operators. The hazard

	characterization, performed by sequencing the mtDNA cox2 gene, has identified the visible larvae as Anisakis pegreffii in 98% of the cases, highlighting the zoonotic potential of the parasites found and the need for preventive measures.

Dear Editor,

We are sending you back the revised version of the manuscript entitled "*Assessment of a sampling plan based on visual inspection for the detection of anisakids larvae in fresh anchovies (Engraulis encrasicolus). A first step towards official validation?*".
The manuscript has been implemented according to the suggestions of the reviewer.
Thank you for considering the manuscript for publication after minor revision.

COMMENTS FOR THE AUTHOR:

Reviewer #1: The manuscript has been significantly improved. Nevertheless, there are some, additional minor comments for consideration before publication of this manuscript.

P.6, line 133, remove an additional article "the",
Done.
P.6, line 144, mention, which analysis do you mean?
We mean: UV press method and digestion. The analysis are explained in the next sentence "Each batch was then analyzed using the UV press method (Section 2.2) and the digestion procedure (Section 2.3.2) for the recovery of parasites undetected during visual inspection (Fig. 2)."
P.7, line 146, I am not convinced that it is necessary to incorporate figure 2 into the text. The work described is not complicated to show a schematic diagram of the analytical process.
Figure 2 has been removed from the manuscript.
P.9, line 209, change to "….the tails of M13 forward -21 and reverse-29 primers sequences…"
The sentence has been changed.
P.10, line 228-229, the Authors have only been asked to reduce the number of sequences used for generation of a phylogenetic tree, therefore change the existing sentence to: "……..were selected and, a Neighbour - Joining (NJ) dendrogram of 94 representative sequences……"
The sentence has been changed.
P.10, line 244-246, an English style requires correction
The style has been improved.
P.13, line 300-301, change to: "3.5 Comparison of MA values obtained for tested methods"
The title of paragraph 3.5 has been changed as suggested.
P.14, line 323, change to: "from viscera"
Done.
P.17, line 398-402, merge these two sentences into one.
We consider it clearer to leave the sentences as they are.
P.17, line 405-406, remove: "also considering the zoonotic potential of the visible parasites found in this study"
The sentence has been removed.
P.17, line 408, remove: "even by FBOs"
Done.
P.18, line 418-421, in a figure caption (figure 1SM), change to: "….and the reference sequences (9) retrieved from GenBank. Bootstrap values >70%, obtained from 2000 replications
using….."
Done.
line, 421: remove the sentence: "Redundant sequences have been removed"
The sentence has been removed.
Table 1SM, change to" Number of specimens used for visual inspection"

We think it is more correct to write "Number of specimens to be used" since the table is aimed at giving indications on the number of fishes to be sampled.

Table 2SM, in a table caption change to: "…on a fragment..."

Done.

in a table legend change to: "….sequence derived from…..(ITS) region. It has been …"

Done.

The prevalence of Hysterothylacium spp. should be written as 1 (0.16%)

Done.

Assessment of a sampling plan based on visual inspection for the detection of anisakids larvae in fresh anchovies (*Engraulis encrasicolus*). A first step towards official validation?

Guardone L.[a], Malandra R.[b], Costanzo F.[a], Castigliego L.[a], Tinacci L.[a], Gianfaldoni D.[a], Guidi A.[a], Armani A. [a]*

[a] *FishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy).*

[b] *Wholesale Fish Market of Milan, ASL of Milan, Viale Molise, 20, 20137 Milan, (Italy).*

***corresponding author**:

Postal address: FishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy)

Tel: +390502210207; Fax: +390502210213

Email: andrea.armani@unipi.it

Abstract

The presence of anisakids larvae in fish is a public health issue and effective risk management procedures are needed to avoid that heavily infected products reach the market. Currently, an official sampling plan for fresh fish defining sample size, inspection methods and criteria to accept or reject the merchandise is lacking at the European and Italian level. In this study, we compared the visual inspection proposed by the sampling plan of the Lombardy Region (Italy) to the UV press method and to an optimized digestion procedure with the aim to assess its ability in detecting visible parasites. Thirty-one batches of *Engraulis encrasicolus*, each composed of ~30 specimens, were collected and subsequently analyzed with the three techniques. The Mean Abundance (MA) was calculated after each procedure and compared on the basis of a threshold value. The results showed that the visual inspection performed similarly to the digestion method, with a sensitivity of 93%, a specificity of 100% and an accuracy of 97%. Overall, the comparison showed that, in the proposed sampling plan, the visual inspection is effective in rejecting unmarketable anchovies and in preventing the commercialization of unsafe products. This method is simple, less demanding than digestion in terms of time and equipment, and thus suitable as a standardized procedure to be routinely applied by Food Business Operators. The hazard characterization, performed by sequencing the mtDNA *cox2* gene, has identified the visible larvae as *Anisakis pegreffii* in 98% of the cases, highlighting the zoonotic potential of the parasites found and the need for preventive measures.

Keywords

Anisakids, UV press method, enzymatic digestion, food safety, method validation

Introduction

Ascaridoid nematodes belonging to the families Anisakidae and Raphidascarididae (Fagerholm 1991), commonly called anisakids, are of considerable public health significance worldwide (Adams et al. 1997; Chai et al. 2005). Human infection is associated with the ingestion of raw or undercooked seafood hosting viable third stage larvae (L3) of species belonging to the Anisakidae family and possibly to Raphidascarididae (Chai et al. 2005; Fagerholm 1991; Lymbery and Cheah 2007). In addition to health implications, the presence of visible parasites in the flesh affects the quality, making the fish repugnant to the consumer and reducing its commercial value (Council Regulation (EC) No 2406/1996).

The life cycle of anisakids is indirect, with fish as intermediate or paratenic hosts (Anderson 1992). In fish, anisakids L3 are typically encapsulated on visceral organs, mesenteries and peritoneum, but they can also directly encyst in the edible tissues (Adams et al. 1997; Anderson 1992). In particular, L3 are able to migrate from the viscera to the muscle after the fish's death (Adams et al. 1997; Lymbery and Cheah 2007; Rello et al. 2009).

After ingestion by humans, anisakids larvae may cause infection, either in a non-invasive form, generally asymptomatic, or in an invasive form with gastrointestinal symptoms (Adams et al. 1997). Anisakids larvae, both alive and dead, may also cause allergic reactions (Nieuwenhuizen and Lopata 2013).

The vast majority of the diseases related to the ingestion of anisakids are caused by *Anisakis* spp. and *Pseudoterranova* spp. (Anisakidae family) (Chai et al. 2005; Lymbery and Cheah 2007), while *Contracaecum* spp. (Anisakidae family) and *Hysterothylacium* spp. (Raphidascarididae family) seem to be rarely involved in pathological forms (Yagi et al. 1996). In Italy, the species most frequently associated to human cases is *A. pegreffii* (Mattiucci et al. 2013), the most widespread species in the Mediterranean Sea (Mattiucci and Nascetti 2008).

Even though the impact of anisakids parasites on public health has been recognized for a long time, this infection is generally considered an emerging fish-borne zoonotic disease, due to the increased habit of eating raw fish in ethnic dishes (D'Amico et al. 2014) or in typical recipes (Mattiucci et al. 2013).

Among the Mediterranean fish species hosting anisakids larvae, the European anchovy (*Engraulis encrasicolus*) is the most fished by the Italian fleet: in 2012 it accounted alone for 22% of the national catch (ISMEA 2013). The large amount of product caught, associated with the fact that this species is a common second intermediate or paratenic host of anisakids and that it is usually sold not eviscerated, makes this fish a significant source of infection for humans. Morevoer, a widespread presence of anisakids infection is reported in this species (Mladineo and Poljak 2013; Piras et al. 2014; Rello et al. 2009), with very high values in some capture areas (Mladineo and Poljak 2013; Piras et al. 2014). In Italy, as well as in Spain, the greatest risk of acquiring anisakidosis is associated with the traditional consumption of raw marinated anchovies (Mattiucci et al. 2013).

The presence of anisakids larvae in fishery products is a concern for consumers and Official Control Authorities and a large number of provisions has been issued at the European and Italian level. Considering that no fishing area can be considered free from anisakids (EFSA 2010) and that also aquaculture products are affected (Lima dos Santos and Howgate 2011) the only prevention system is represented by the application of an effective control system by trained Food Business Operators (FBOs). In fact, while before the Hygiene Package the Veterinary Inspector was the person in charge of controls, nowadays this task is delegated to the FBOs (D'Amico et al. 2014).

Several methods, such as visual inspection (Commission Regulation (EC) No 2074/2005), candling (Butt et al. 2004), UV illumination (Adams et al. 1997), UV press method (Karl and Leinemann 1993) and pepsin digestion (Cavallero et al. 2015; Fraulo et al. 2014; Llarena-Reino et al. 2013a) have been proposed for detecting visible parasites in fish. According to Reg. No 2074/2005, the visual inspection must be performed on a representative number of samples. In

particular: "*the persons in charge of establishments on land and qualified persons on board factory vessels shall determine the scale and frequency of the inspections by reference to the type of fishery products, their geographical origin and their use*". However, currently, there are no law provisions at the European or Italian national level that define a detailed sampling plan to be used for the collection of fresh fish to be visually inspected according to the Reg. No 2074/2005. By definition, a sampling plan includes the sample size, the inspection procedure and the criteria to be used to accept or reject the lot of production based on the results of the inspection (Codex Alimentarius Commission 1969). Due to the aforementioned lacks, the sampling plan for the detection of parasites often refers to internationally accepted protocols, such as the Codex Alimentarius. However, the available protocols refer to prepackaged products, quick frozen and minced fish and salted Atlantic herring and Sprat (Codex Alimentarius Commission 1969; 1989; 2004) and not to fresh fish. In addition, a recently proposed procedure, based on a scoring system for the prediction of fish lots infection, does not take into consideration *E. encrasicolus* (Llarena-Reino et al. 2013b).

As reported by Regulation (EC) No 852/2004, "*When this Regulation, Reg. (EC) No. 853/2004 and their implementing measures do not specify sampling or analysis methods, FBOs may use appropriate methods laid down in other Community or national legislation or, in the absence of such methods, methods that offer equivalent results to those obtained using the reference method, if they are scientifically validated in accordance with internationally recognized rules or protocols*".

In this work, a visual inspection according to Reg. No 2074/2005 was performed on *E. encrasicolus* specimens collected at the Wholesale market of Milan (Italy), according to the sampling plan proposed by the Circular Letter VS8/C790/94 of the Lombardy Region (Italy). Then, the same samples of anchovies were analyzed using the UV press method (Karl and Leinemann 1993) and an enzymatic digestion procedure optimized in this study. The aim of this work was to assess the ability of the sampling plan proposed by the aforesaid Regional law in detecting visible parasite in fresh anchovies. In fact, to the best of our knowledge, this sampling plan is the most

routinely applied for the control of anchovies at fish markets in Italy (D'Amico et al. 2014). Thus, this work represents an attempt to propose a simple and rapid workflow to be used by FBOs to ensure safety and marketability of anchovies.

2. Materials and Methods

2.1. Sampling and visual inspection at the wholesale market

Thirty-one batches, made on average of 30 (±2.79 DS) *E. encrasicolus* each, for a total of 929 specimens, were randomly sampled at the Wholesale market of Milan from different lots of anchovies caught in the Western Mediterranean Sea (FAO area 37.1.3) and in the Central Mediterranean Sea (FAO area 37.2.1) (Table 1). Anchovies were sampled after ~ 24h of storage on ice (the usual average time from the catch to the market). Considering that the accuracy of a visual inspection method largely depends on the training and skills of the inspectors (Levsen et al. 2005), in this work, the sampling and the visual inspection were performed by the Official Veterinarian of the Health Local Unit of Milan, according to Reg. No 2074/2005, following the Circular Letter VS8/C790/94 of the Lombardy Region. The Head and the Viscera (HV) were separated from the Fillets (F) (including the belly flaps and the backbone carrying epaxial muscles) and both HV and F were left on a tray at Room Temperature (RT) for about 10 min (Fig. 1). Subsequently, a visual inspection for the detection of visible larvae, "*a parasite or a group of parasites which has a dimension, color or texture which is clearly distinguishable from fish tissues*" (Commission Decision No 93/140/EEC), was performed and the number of anisakids larvae found in HV and F were registered. A decision on the marketability of the batch was issued according to the Circular n. 1 of 1997 of Liguria Region (Italy) (Section 3.4).

All the larvae were counted and, after registering the number and the site of collection, they were separately packed into plastic bags with fish HV and F according to their site of detection, and then frozen at -20°C and transferred to the FishLab of the University of Pisa for subsequent analysis.

Each batch was then analyzed using the UV press method (Section 2.2) and the digestion procedure (Section 2.3.2) for the recovery of parasites undetected during visual inspection.

2.2 Ultraviolet Light (UV) press method

Once thawed at 4°C over night inside the original plastic bags, F and HV of each batch were analyzed. F were placed in a plastic film and manually squeezed between two acrylic sheets to a thin layer of 2-3 mm. Heads (H) were cut longitudinally, in order to facilitate the visualization of the larvae, and placed, together with Viscera (V), in a petri dish. HV were not compressed and were moved with a metal rod during the analysis. F and HV were both analyzed in a darkened room under UV light at 365 nm (UltraBright UV Transilluminator, 302nm/365nm, Maestrogen, Las Vegas, USA) as proposed by Karl and Leinemann (1993). In fact, dead nematode larvae show fluorescence under UV light. The thawing liquid and the bags in which F and HV had been stored were also analyzed using the same procedure. The visible parasites found were counted and left in place, to subsequently verify their recovery with the digestion method.

2.3 Digestion procedure

2.3.1 Development and optimization of the digestion procedure. Initially, 5 batches made each of 30 anchovy's HV and F different from those used in this study were separately digested according to the procedure proposed by the Commission Regulation (EC) No 2075/2005. The pH value of the digestion solution made of 25% HCl and pepsin [2000 FIP] (Pepsina A, EuroCloneS.p.A, Milan, Italy) was measured using a pH meter (Eutech 700 – Thermo Scientific Inc. – Eutech Instruments Pte Ltd Singapore) every 5 min for 15 min after adding the fish tissues and stirring at 44°C. The temperature was monitored using a thermocouple thermometer Hanna HI92704 (Hanna Instruments, Padova, Italy). Considering the increase of the pH value (from 2.7 to more than 5 after 5 minutes) and the amount of the residual indigested tissue the HCl solution was replaced with a buffered solution of H_3PO_4 (pH 2.4) at different concentrations (150 mM, 300 mM, 600 mM) and different times of digestion (15, 30, 45, 60 min) and different temperatures (44, 46, 48°C) were

tested. These modifications were then tested in all the possible combinations. The best method was chosen considering the stability of the pH value and the amount of indigested tissue. In order to test the recovery of parasites after the digestion with the proposed method, a preliminary test using 10 frozen anisakids larvae collected from anchovies not belonging to this study was performed.

2.3.2. Final protocol and samples digestion. Aliquots of ~50 mg of F or HV were digested separately using the final digestion protocol and the procedure was repeated until the complete digestion of each batch. Fish tissues were grossly chopped with scissors and placed in a beaker containing pepsin powder (final concentration 10 FIP/ml) previously dissolved in 250 mL of 600mM H_3PO_4 buffered solution (pH 2.4). The beaker, placed on a magnetic stirrer, was maintained into a pre-heated stove at 48°C for 45 min. The temperature of the digestion solution was monitored. The digestion solution was decanted for at least 20 min (instead of sieving as proposed by Reg. No 2075/2005), then the sediment was subdivided in 3-4 aliquots, diluted with tap water for clarification and finally transferred in Petri dishes. Each dish was then analyzed under UV light as reported in paragraph 2.2. The visible larvae found in HV and F were collected, counted and stored at −20°C until molecular analysis for hazard identification (Section 2.5).

2.4 Mean Abundance (MA)

The Mean Abundance (MA) was calculated after each procedure (Bush et al. 1997) and used to assess the batch marketability according to a threshold provided by the sampling plan of the aforesaid regional laws. The criteria used to accept or reject the lot of production based on the results of the examination of the samples was the one indicated by the Liguria Region in Circular n. 1 of 1997. In particular: "*when opening the coelomic cavity numerous viable larvae appear, giving a repellent aspect to the product, the batch is withdrawn from the market; ii) if the number of visible parasites is higher than 3 per anchovy in the 10% of the examined specimens, or the number of parasitized specimens is higher than 10% of the total, the batch should be submitted to decontamination by means of freezing, according on the existing law; iii) if the number of larvae is*

≤ *3 per anchovy in maximum 10% of the examined specimens, the batch is intended to free consumption*". It derives that a MA of 0.30 corresponds to the threshold that allows to divide the batches in "non marketable" (MA > 0.3) or "marketable" (MA ≤ 0.3). MA values retrieved after the visual inspection and the UV press method were compared to the values found after the enzymatic digestion (assumed as the gold standard) in order to evaluate the sensitivity (*s*), specificity (*s'*) and accuracy (*ac*) of the tests. Finally, Pearson's correlation coefficient was calculated to check the existence of a relationship of linearity between MA values obtained with the three procedures.

2.5 Molecular identification of the larvae by sequencing and phylogenetic analysis

2.5.1 Extraction, amplification and sequencing of the mtDNA cox2 gene. Total DNA extraction was performed from each visible anisakids larvae recovered following the procedure described by Armani et al. (2014), with the addition of proteinase K. DNA concentration and purity were determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

A 629 bp fragment of mtDNA *cox2* gene was amplified using the primers 211F and 210R from Nadler and Hudspeth (2000), linked to the tails M13 forward -21 and reverse M13-29 (Messing 1983). PCR amplifications were set up in a 20 µl reaction volume containing 2 µl of a 10x buffer (5Prime, Gaithersburg, USA), 200 µM of each dNTP (dNTPmix, EurocloneS.p.A-Life Sciences Division, Pavia, Italy), 200 nM primers, 25 ng/µL of BSA (Purified BSA 100X, Bew England BIOLABS® Inc. Ipswich, MA, USA), 1.25 U PerfectTaq DNA Polymerase (5Prime, Gaithersburg, USA), 1-2µL of DNA and DNase free water (Water Mol. Bio. Grade, DNase-RNase and Protease free, 5Prime GmbH, Hamburg, Germany) with the following cycling program: denaturation at 94 °C for 3 min; 40 cycles at 94 °C for 20 s, 45 °C for 20 s, 72 °C for 25 s; final extension at 72 °C for 10 min. The amplifications were carried out using a peqSTAR 96 Universal Gradient thermocycler (Euroclone, Milan, Italy). Five µL of PCR products were checked by gel electrophoresis and the presence of fragments of the expected length was assessed by comparison with the marker

SharpMass™50-DNA ladder (Euroclone, Wetherby, UK). Purification and sequencing were performed by the High-Throughput Genomics Center (Washington, USA).

2.5.2 Sequences assembling, BLAST and phylogenetic analysis. All the obtained sequences were analyzed using Clustal W in MEGA version 6 (Tamura et al. 2013). Adjustments were made after visual checking and the sequences were analyzed on GenBank by using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Since the sequences were produced from unidentified parasites they were not deposited. Finally, 622 mtDNA *cox2* gene fragments of 576 bp (613 sequences of *A. pegreffii* and *A. simplex* from this work and 9 reference sequences of the *Anisakis* species genetically characterized so far as proposed by Cipriani et al. (2015) were selected and a Neighbour-Joining (NJ) dendrogram of 94 representative sequences was obtained using MEGA version 6 computing the distances with the Kimura 2-parameter model with 2000 bootstraps re-samplings (Saitou and Nei 1987).

3. Results and discussion

3.1. Definition of "visible parasite"

While the definition of visible parasite given by the European Union is not so detailed (Section 2.1), the Codex Alimentarius Commission considers as visible parasite all the parasites with a capsular diameter of at least 3 mm or, if not encapsulated, longer than 10 mm (Codex Alimentarius Commission 1971). However, this definition is only focused on the dimension of the parasite and does not take into consideration its zoonotic potential (D'Amico et al. 2014). In relation to this aspect, it must be underlined that the larvae of *Hysterothylacium* spp. (3–19 x 0.1-05 mm) (Borges et al. 2012; Shamsi et al. 2011; Shamsi et al. 2013) can often co-infect fish together with the L3 of *Anisakis* spp. (14–44 mm in length and 0.4–0.9 mm in diameter) (Murata et al. 2011; Shamsi et al. 2011; Pardo-Gandarillas et al. 2009). Although on average *Anisakis* spp. larvae are larger than *Hysterothylacium* spp., overlapping sizes may occur, so they are not easily distinguishable macroscopically. In this regard it is important to point out that, even though zoonotic infections by

Hysterothylacium are rare (Yagi et al. 1996), *Hysterothylacium* spp. falls within the definition of visible parasite. Interestingly, also the recent Commission Regulation (EU) No 1276/2011 does not consider all the "*nematode larvae*", as the previous Reg. No 853/2004, but only the visible parasites. For the aforesaid reason, in this study, only the larvae approximately longer than 10 mm (visible parasite) were collected, counted and molecularly identified.

3.2 Sampling and visual inspection at the wholesale market

According to the Lombardy Region circular (Circular Letter VS8/C790/94), knowing the total weight of the fish lot, it is possible to calculate the total number of specimens and then, by means of conversion rates and using an appropriate table, the number of subjects to be examined in each case. In the case of fish species caught in large batches (> 600 specimens), the number of subjects to collect is, at least, 29 (Table 1SM). Although this protocol had been intended for the Veterinary Inspector, it represents a simple and valid method also for the self-monitoring procedure. In fact, the Regulation No 853/2004 established that FBOs must ensure that fishery products have been subjected to a visual examination for the detecting of visible parasites before being placed on the market. This method, based on the fact that the room temperature provokes the mobilization of the larvae allowing their visualization, is in agreement with the definition of "*visual inspection*" as "*a non-destructive examination of fish or fishery products without optical means of magnifying and under good light conditions for human vision, including, if necessary, candling*" (Commission Decision No 93/140/EEC). The visual inspection allowed to detect 399 total visible parasites with a variable number per batch ranging from 0 to 75 (Table 1).

3.3 Ultraviolet Light (UV) press method

Candling procedures are a valuable aid in the search for parasitic larvae in fishery products. Although the white light candling is the method of choice for the detection of nematodes in blocks of frozen fish fillets (Codex Alimentarius 1989), it presents some limits in the case of not skinned fillets (Karl and Leinemann 1993; Lymbery and Cheah 2007). Karl and Leinemann, in 1993,

proposed a variation, which combined the compression of frozen fillets between two acrylic plates with candling using UV light ("UV press method"). In fact, the compression facilitates the penetration of the UV light that causes the emission of fluorescence. In order to use this method the F must be frozen for some hours to kill the larvae and promote fluorescence. The UV method has been chosen and applied both on F and HV, since the combination of UV and compression is more sensitive than the classical candling technique (Karl and Leinemann 1993) and the pigmented muscle of the anchovy prevents candling with white light. The UV press method allowed to detect 355 total visible parasites with a variable number per batch ranging from 0 to 86 (Table 1).

3.4 Digestion procedure

The digestion is a destructive technique that dissolves fish tissues in order to detect the larvae, by exploiting the high degree of resistance of the cuticle of nematodes to the digestive processes. Despite the excellent results that can be obtained, the use of the digestion is limited by the relatively small number of samples that can be digested at each time and by long reaction times (Karl and Leinemann 1993). While this technique is not routinely used as an inspection tool, it is largely applied in epidemiological studies (Bernardi et al. 2011; Piras et al. 2014). In this study, the method described by Reg. No 2075/2005 for the detection of *Trichinella* spp. larvae in meat was initially selected. However, it was considered necessary to introduce some modifications to the original official method, also considering that protocols digestion optimization may differ according to the material to be digested (Llarena-Reino et al. 2013a). In fact, the digestion process can be considered satisfactory only if no more than 5% of the original weight of the sample remains undigested. In particular, considering that the enzymatic action of pepsin is expressed at best in an acid environment, to overcome the observed rise in the pH, the solution of HCl was replaced with a buffered solution of H_3PO_4 (pH 2.4) at a concentration of 600 nM. Finally, the digestion test performed on 10 *Anisakis* spp. dead larvae allowed us to verify that the digestive procedure did not determine the destruction of the parasites, in contrast with previous suggestions (Fraulo et al. 2014;

Karl and Leinemann 1993) and despite the use of a temperature of 44°C. In fact, other parameters such as the pH value and the pepsin concentration could influence the recovery of the larvae (Bernardi et al. 2011; Llarena-Reino et al. 2013a). Overall, the digestion method retrieved 640 visible parasites with a variable number per batch ranging from 0 to 115 (Table 1).

3.5 Comparison of MA values obtained for tested methods

The MA is among the most important descriptors to quantify parasite numbers in a host sample or population. MA carries the same information of mean intensity, but it correlates with prevalence (Rozsa et al. 2002). Especially in the case of small fish, which are not sold individually but in batch, the MA could be used to estimate the degree of infestation. Considering that the provisions established by the Region Lombardia, which states the rejection of the batch if in a sample of less than 1 kg even a single specimen is found parasitized (MA=0), are not applicable in the light of the diffusion of anisakids in anchovies (Angelucci et al. 2011; Mladineo and Poljak, 2013; Rello et al. 2009), we took into consideration the protocol indicated by the Liguria Region, which tolerates a certain levels of infestation (Circular n. 1 of 1997). In fact, it is essential to identify the percentage of parasitized subjects that can be tolerated, or rather that is not perceptible to the observation of the consumer. Calculating the MA on the basis of the reported criteria it is clear that a MA of 0.30 corresponds to the threshold that allows the distinction between a product that can be intended for free consumption and a product that requires a sanification treatment. In fact, food shall not be placed on the market if unsafe (injurious to health or unfit for human consumption) (Regulation (EC) No 178/2002). In this regard, noteworthy is the fact that even at the Wholesale fish market of Milan a certain level of tolerance on the non marketability of the product has been introduced, especially considering the high degree of infestation often present in anchovies.

According to some authors (Angelucci et al. 2011) the visual method would not ensure an appropriate level of safety of the product, as it is strictly dependent on the experience of the operator and on the environment light. For others, the visual inspection has a low efficiency for gut

parasites (Llarena-Reino et al. 2012). Moreover, according to Bernardi et al. (2011), digestion would allow a more efficient recovery of L3 larvae from viscera compared to the visual inspection. On the contrary, in this study, the visual inspection showed a sensitivity (s) of 93%, a specificity (s') of 100% and an accuracy of 97%. The results of the visual inspection on the marketability of each batch were in agreement with the digestion in 30 cases out of 31. In fact, in only one case, which had a MA found with the digestion method very close to the threshold (0.33), the inspection detected a MA lower than the cut-off (Table 1). Moreover, as expected, the MA values found after the three tests were found to be highly correlated (coefficients of correlation always higher than 0.94).

The UV candling method was less sensitive (s = 71%) and less accurate (ac = 87%) than visual inspection, although the specificity was 100%. In fact, the results of this method on the batches marketability disagreed with the digestion in 4 cases (Table 1). However, it has to be remarked that the UV candling was also applied to the HV, even though it is usually intended for muscle tissue since HV only allows the passage of a small part of ultraviolet rays.

From the analysis of the data obtained in this study, it appears that, in spite of a difference in absolute terms of parasite detection between the two techniques (which can be observed also comparing the average MA after visual inspection and after digestion, 0.85 and 1.33 respectively), the comparison of the tests showed that visual examination, if performed by a skilled and scrupulous operator, is sufficiently sensitive to discriminate marketable from unmarketable batches. Similarly, Huang (1990) reported that the visual examination of the fish can allow the detection of 90% of the larvae in little fish, such as *Clupea harengus*, *Scomber scombrus* and *Trachurus trachurus*.

Considering that FBOs have to issue a judgment on the marketability for a high number of batches in a limited period of time, the described sampling plan will be helpful in the simplification and standardization of FBOs controls.

3.6 Molecular identification of the larvae by sequencing and phylogenetic analysis

Many gene targets can be used for anisakids identification. Among these, the direct sequencing of the mitochondrial cytochrom oxidase 2 (mtDNA *cox2*) has allowed the specific identification of 9 different species of the genus *Anisakis* (Mattiucci and Nascetti 2008) and thus it can be successfully applied for identification purposes (Cipriani et al. 2015).

In this study a BLAST analysis supported by a phylogenetic analysis performed using a fragment of the the mtDNA *cox2* were used to identify the visible parasite collected and characterize the hazard. In fact, the epidemiological relevance of the species represents a pivotal criteria in defining the risk associated to the fish consumption (Llarena-Reino et al. 2013b).

Overall, 640 visible (longer than ~10 mm) anisakid larvae were collected. Totally, 613 readable mtDNA *cox2* sequences of on average 578 bp (range 524-582 bp) were obtained and submitted to a BLAST analysis: 597 (97.39%) retrieved a maximum identity of 99-100% with sequences of *A. pegreffii*, 14 (2.28%) with sequences of *A. simplex* sensu stricto (99-100% max identity), 1 (0.16%) with the sequence of a hybrid *A. simplex* x *A. pegreffii* (100% max identity) and 1 with a sequence of *Hysterothylacium* spp. (96% max identity with *H. aduncum*) (Table 2SM). The phylogenetic analysis performed on 622 sequences of *Anisakis* spp. (Fig. 1SM) confirmed the results obtained by BLAST (Table 2SM).

Our results confirm the widespread diffusion of *A. pegreffii*, the dominant *Anisakis* species in the Mediterranean Sea. To the best of the authors' knowledge, this is the first report of *A. simplex* s.str. in anchovies. All the positive batches originated from FAO area 37.1.3. In fact, even though *A. simplex* s.str. is the dominant species in Atlantic and Pacific Oceans, it is also occasionally present in southwestern Mediterranean waters (Mattiucci and Nascetti 2008).

Finally, it has to be noted that in many studies co-infections of *Anisakis* spp. and *Hysterothylacium* spp. have been found (Angelucci et al. 2011; Cavallero et al. 2015). Also in the present study some batches presented probable co-infection with *Hysterothylacium* (in fact, in many

anchovies larvae shorter than ~10 mm were found). However, due to the collection and analysis of the visible larvae only, the most part of the specimens of *Hysterothylacium* spp. which are smaller than *Anisakis* spp. (Section 3) may have been disregarded. In fact, only one of the specimens was identified as *Hysterothylacium* spp. In the light of consumers' protection, this result confirms the importance of focusing the inspection of fishery products on visible larvae.

3.7 Health implications.

In the light of health implications, considering that all the collected parasites are zoonotic (Section 3.5), an aspect to be stressed is the presence of parasites in the edible portions (fillets). In addition, parasitic larvae have the ability to migrate in the fish muscle (edible portion) even during the *post mortem*. In this study, we observed anisakids larvae coming out through the natural orifices and through the skin from the muscle. Other recent studies reported the presence of L3 larvae in the muscles of *E. encrasicolus* (Angelucci et al. 2011; Rello et al. 2009). Considering that small fish species are generally sold ungutted, the presence of anisakids must be assessed not only in the fish flesh but also in the viscera.

According to the working document SANCO/10137/2013-rev1 (2013) a fishery product is considered obviously infested if visible parasites are detected in edible portions. On the contrary, if the parasites are found in non edible parts, the raw material may be considered suitable for consumption. Obviously, this definition considers only sanitary implications (zoonotic potential) but not the commercial ones. In fact, even though the parasites are confined to the viscera, heavily infested products induce consumers' repulsion and must be considered as not suitable (Reg. No 178/2002).

Finally, the health implications related to allergic reactions after ingestion of Anisakids should be taken into account (Daschner et al, 2000; Dominguez-Ortega et al., 2001). Even though the ingestion of alive larvae is usually required for sensitization and allergic reactions (Alonso-Gomez et al., 2004; Audicana et al., 2002; Daschner et al., 2000), also the exposure to Anisakis proteins

alone may suffice to elicit allergic reactions in sensitized individuals (Nieuwenhuizen et al., 2006). Obviously, the present approach cannot prevent the risk of allergy in sensitive subjects which, on the countrary, should be addressed by a specific legislation. In fact, despite the numerous provisions issued at European and Italian level aimed at manage the risk associated to the presence of Anisakid in fish, Anisakid proteins are still not included in the list of all common allergens by the specific Community regulation (Regulation (EU) 1169/2011).

4. Conclusions

The need of a reliable sampling plan to search for visible larvae in fishery products represents a priority for both official authorities and FBOs. The obtained results showed that in the proposed sampling plan the visual inspection performed similarly to the digestion procedure, while being simpler and less time-consuming and thus suitable to be routinely applied. This is especially important in the case of anchovies that are sold ungutted and often consumed raw. In fact, the possible migration of the larvae from the viscera to the muscle highly increases the health risk for consumers and decreases the final quality of the products. Finally, this study represents a first step to validate the proposed sampling plan and to standardize the inspection process on fresh fish.

Figure captions

Fig. 1. Visual inspection of anchovies as performed in the present study: whole specimens (A), heads and viscera (B) and fillets (C).

Fig. 1SM. Neighbour-joining (NJ) tree obtained using 622 mtDNA *cox2* gene sequences (576 bp) of the *Anisakis* spp. larvae found in this study (613) and the reference sequences (9) retrieved from Genbank. Bootstrap values >70%, obtained from 2000 replications using the Kimura 2-parameter genetic distance are shown in the tree.

Compliance with Ethical Standards:

Funding:

The research was performed with funds granted from the University of Pisa.

Conflict of Interest:

Guardone Lisa, Malandra Renato, Costanzo Francesco, Castigliego Lorenzo, Tinacci Lara, Gianfaldoni Daniela, Guidi Alessandra, Armani Andrea declare that they have no conflict of interest.

Ethical Approval:

This article does not contain any studies with human or animal subjects. In particular, the fish included in the present study were intended for human consumption and they were bought at the Wholesale market of Milan.

Informed Consent:

Not applicable.

References

1. Adams AM, Murrell KD, Cross JH (1997) Parasites of fish and risks to public health. Revue scientifique et technique (International Office of Epizootics) 16:652-660.
2. Anderson RC (1992) Nematode Parasites of Vertebrates: Their Development and Transmission. Cambridge: CAB International.
3. Angelucci G, Meloni M, Merella P, Sardu F, Madeddu S, Marrosu R, Petza F, Salati F (2011) Prevalence of *Anisakis* spp. and *Hysterothylacium* spp. Larvae in Teleosts and Cephalopods Sampled from Waters off Sardinia. J Food Protect 74:1769-1775.
1. Alonso-Gomez A., Moreno-Ancillo A., Lopez-Serrano M. C., Suarez-de-Parga J. M., Daschner A., Caballero M. T., et al. (2004) Anisakis simplex only provokes allergic symptoms when the worm parasitises the gastrointestinal tract. Par Res, 93, 378–384.
2. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410.
3. Armani A, Tinacci L, Xiong X, Titarenko E, Guidi A, Castigliego L (2014) Development of a simple and cost-effective bead-milling method for DNA extraction from fish muscles. Food Analytical Methods 7:946–955.
4. Audicana M. T., Ansotegui I. J., Corres L. F., Kennedy M. W. (2002). Anisakis simplex: Dangerous — Dead and alive? Trend Parasitol, 18, 20–25.
5. Bernardi C, Gustinelli A, Fioravanti ML, Caffara M, Mattiucci S, Cattaneo P (2011) Prevalence and mean intensity of *Anisakis simplex* (*sensu stricto*) in European sea bass (*Dicentrarchus labrax*) from Northeast Atlantic Ocean. Int J Food Microbiol 148:55-59.
6. Borges JN, Cuhna LFG, Santos HLC, Monteiro-Neto C, Santos CP (2012) Morphological and molecular diagnosis of anisakid nematode larvae from cutlassifish (*Trichiurus lepturus*) off the Coast of Rio de Janeiro, Brazil. PloS one 7:e40447.
7. Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997) Parasitology meets ecology on its own terms: Margolis et al. revisited. J Parasitol 83:575-583.
8. Butt AA, Aldridge KE, Sander CV (2004) Infections related to the ingestion of seafood. Part II: parasitic infections and food safety. Lancet infect dis 4:294-300.

9. Cavallero S, Magnabosco C, Civettini M, Boffo L, Mingarelli G, Buratti P, Giovanardi O, Fortuna CM, Arcangeli G (2015) Survey of *Anisakis* sp. and *Hysterothylacium* sp. in sardines and anchovies from the North Adriatic Sea. Int J Food Microbiol 200:18-21.
10. Chai JY, Murrell KD, Lymbery AJ (2005) Fish-borne parasitic zoonoses: status and issues. Int J Parasitol 35:1233-1254.
11. Cipriani P, Smaldone G, Acerra V, D'Angelo L, Anastasio A, Bellisario B, Palma G, Nascetti G, Mattiucci S (2015) Genetic identification and distribution of the parasitic larvae of *Anisakis pegreffii* and *Anisakis simplex* (ss) in European hake *Merluccius merluccius* from the Tyrrhenian Sea and Spanish Atlantic coast: Implications for food safety. Int J Food Microbiol 198:1-8.
12. Circular Letter VS8/C 790/94 of the Lombardy Region.
13. Circular n. 1 of 1997 of Liguria Region.
14. Codex Alimentarius Commission (1969) Codex sampling plans for prepackaged foods. CODEX STAN 233-1969. Available at: http://down.40777.cn/stardard/10/CODEX%20STAN%20233-1969%20CODEX%20SAMPLING%20PLANS%20FOR%20PREPACKAGED%20FOODS%20%28AQL%206.5%29.pdf Accessed 14/05/2015.
15. Codex Alimentarius Commission (1971) Report of the eighth session of the joint FAO/WHO Codex Alimentarius Commission: Recommended international standard for quick frozen filet of cod and haddock. CAC/RS-50-1971. Available at: http://www.fao.org/docrep/meeting/005/c0531e/C0531E09.htm Accessed 16/02/2015
16. Codex Alimentarius Commission (1989) Codex standard for quick frozen blocks of fish fillet, minced fish flesh and mixtures of fillets and minced fish flesh. CODEX STAN 165-1989. Available at: http://www.codexalimentarius.org/standards/list-of-standards/ Accessed 09/06/2015.
17. Codex Alimentarius Commission (2004) Standard for salted atlantic herring and salted sprat CODEX STAN 244-2004 Available at: http://www.codexalimentarius.org/standards/list-of-standards/ Accessed 09/06/2015.
18. Commission Decision 93/140/EEC of 19 January 1993 laying down the detailed rules relating to the visual inspection (or the purpose of detecting parasites in fishery products. OJEC L56:42.
19. Commission Regulation (EC) No 2074/2005 of 05 December 2004. Laying down implementing measures for certain products under Regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official control under Regulation (EC) No. 854/2004 of the European Parliament and of the Council and Regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No. 853/2004 and (EC) No. 854/2004. OJEU L338:27–59.
20. Commission Regulation (EC) No 2075/2005 of 5 December 2005 laying down specific rules on official controls for *Trichinella* in meat. OJEU L338:60.
21. Commission Regulation (EU) No 1276/2011 of 8 December 2011 amending Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council as regards the treatment to kill viable parasites in fishery products for human consumption. OJEU L327:39-41.
22. Council Regulation (EC) No 2406/96 of 26 November 1996. Laying down common marketing standards for certain fishery products. OJEC L334:1-15.

23. Daschner A., Alonso-Gomez A., Cabanas R., Suarez-de-Parga J. M., Lopez-Serrano M. C. (2000) Gastroallergic anisakiasis: borderline between food allergy and parasitic disease: Clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by Anisakis simplex. J Allergy Clin Immunol, 105, 176–181.
24. D'Amico P, Malandra R, Costanzo F, Castigliego L, Guidi A, Gianfaldoni D, Armani A (2014) Evolution of the *Anisakis* risk management in the European and Italian context. Food Res Int 64:348-362.
25. Dominguez-Ortega J., Alonso-Llamazares A., Rodriguez L., Chamorro M., Robledo T., Bartolome J.M., et al. (2001) Anaphylaxis due to hypersensitivity to Anisakis simplex. Int Arch Allergy Immunol, 125, 86–88.
26. EFSA (2010) Scientific Opinion on risk assessment of parasites in fishery products. EFSA Journal 8:1543 Available at: http://www.efsa.europa.eu/it/search/doc/1543.pdf Accessed 10/05/2015.
27. Fagerholm HP (1991) Systematic implications of male caudal morphology in ascaridoid nematode parasites. Syst Parasitol 19:215-229.
28. Fraulo P, Morena C, Costa A (2014) Recovery of Anisakid larvae by means of chloro-peptic digestion and proposal of the method for the official control. Acta Parasitol 59:629-634.
29. Huang W (1990) Methods for detecting anisakid larvae in marine fish. Possibilities of application to the inspection of fish sold in the Paris region. Recueil de Médecine Vétérinaire 166:895-900.
30. ISMEA (2013) Il settore ittico in Italia. Check-up – 2013. Available at: http://www.ismea.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/8845 Accessed 20/04/2015.
31. Karl H, Leinemann M (1993) A fast and quantitative detection method for nematodes in fish fillets and fishery products. Arch Lebensmittelhyg 44:124–125.
32. Levsen A, Lunestad BT, Berland B (2005) Low detection efficiency of candling as a commonly recommended inspection method for nematode larvae in the flesh of pelagic fish. J Food Protect 68:828-832.
33. Lima dos Santos CAM, Howgate P (2011) Fishborne zoonotic parasites and aquaculture: A review. Aquaculture 318:253–261.
34. Llarena-Reino M, González ÁF, Vello C, Outeiriño L, Pascual S (2012) The accuracy of visual inspection for preventing risk of *Anisakis* spp. infection in unprocessed fish. Food Control 23:54-58.
35. Llarena-Reino M, Piñeiro C, Antonio J, Outeriño L, Vello C, González AF, Pascual S (2013a) Optimization of the pepsin digestion method for anisakids inspection in the fishing industry. Vet Parasitol 191:276-293.
36. Llarena-Reino M, Abollo E, Pascual S (2013b) A scoring system approach for the parasite predictive assessment of fish lots: a proof of concept with Anisakids. Foodborne pathog dis 10:1067-1074.
37. Lymbery AJ, Cheah FY (2007) Anisakid Nematodes and Anisakiasis. In: K. D. Murrell and B. Fried (Eds.), Food-borne parasitic zoonoses (pp. 185-206). New York: Springer.
38. Mattiucci S, Nascetti G (2008) Advances and trends in the molecular systematics of anisakid nematodes, with Implications for their evolutionary ecology and host-parasite co-evolutionary processes. Adv Parasitol 66:47-148.
39. Mattiucci S, Fazii P, De Rosa A, Paoletti M, Megna AS, Glielmo A, De Angelis M, Costa A, Meucci C, Calvaruso V, Sorrentini I, Palma G, Bruschi F, Nascetti G (2013) Anisakiasis and gastroallergic reactions associated with *Anisakis pegreffii* infection, Italy. Emerg Infect Dis 19:496–499.
40. Messing J (1983) New M13 vectors for cloning. Methods Enzymol 101:20-78.

41. Mladineo I, Poljak V (2013) Ecology and genetic structure of zoonotic *Anisakis* spp. from adriatic commercial fish species. Appl Environ Microbiol 80:1281–1290.
42. Murata R, Suzuki J, Sadamasu K, Kai A (2011) Morphological and molecular characterization of Anisakis larvae (Nematoda: Anisakidae) in *Beryx splendens* from Japanese waters. Parasitol Int 60:193-198.
43. Nadler AS, Hudspeth DSS (2000) Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. J Parasitol 86:380–393.
44. Nieuwenhuizen N., Lopata A. L., Jeebhay M. F., De'Broski R. H., Robins T. G., Brombacher F. (2006). Exposure to the fish parasite Anisakis causes allergic airway hyperreactivity and dermatitis. J Allergy Clin Immunol, 117, 1098–1105.
45. Nieuwenhuizen NE, Lopata AL (2013) *Anisakis* – A food-borne parasite that triggers allergic host defences. Int J Parasitol 43:1047-1057.
46. Pardo-Gandarillas MC, Lohrmann KB, Valdivia AL, Ibáñez CM (2009) First record of parasites of *Dosidicus gigas* (d'Orbigny, 1835) (Cephalopoda: Ommastrephidae) from the Humboldt Current system off Chile. Rev Biol Mar Oceanogr 44:397-408.
47. Piras MC, Tedde T, Garippa G, Virgilio S, Sanna D, Farjallah S, Merella P (2014) Molecular and epidemiological data on *Anisakis* spp. (Nematoda: Anisakidae) in commercial fish caught off northern Sardinia (western Mediterranean Sea). Vet Parasitol 203:237-240.
48. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJEC L31:1-24.
49. Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. OJEU L139:1.
50. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. OJEU L139:55.
51. Rello FJ, Adroher FJ, Benitez R, Valero A (2009) The fishing area as a possible indicator of the infection by Anisakids in anchovies (*Engraulis encrasicolus*) from southwestern Europe. Int J Food Microbiol 129:277–281.
52. Rózsa L, Reiczigel J, Majoros G (2000) Quantifying parasites in samples of hosts. J Parasitol 86:228-232.
53. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.
54. SANCO/10137/2013-rev1 (2013) Guidance on the term 'obviously contaminated' in relation to parasites in fishery products. Working document SANCO/10137/2013-rev1. Available at: http://multimedia.food.gov.uk/multimedia/pdfs/obviouslycontaminated. Accessed 16/04/2015
55. Shamsi S, Eisenbarth A, Saptarshi S, Beveridge I, Gasser RB, Lopata AL (2011) Occurrence and abundance of anisakid nematode larvae in five species of fish from southern Australian waters. Parasitol Res 108:927-934.
56. Shamsi S, Gasser R, Beveridge I (2013) Description and genetic characterisation of *Hysterothylacium* (Nematoda: Raphidascarididae) larvae parasitic in Australian marine fishes. Parasitol Int 62:320-328.
57. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725-2729.

58. Yagi K, Nagasawa K, Ishikura H, Nagasawa A, Sato N, Kikuchi K, Ishikura H (1996) Female worm *Hysterothylacium aduncum* excreted from human: a case report. Japanese J Parasitology 45:12-23.

59. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. OJEU L304:18.

Figure
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Figure

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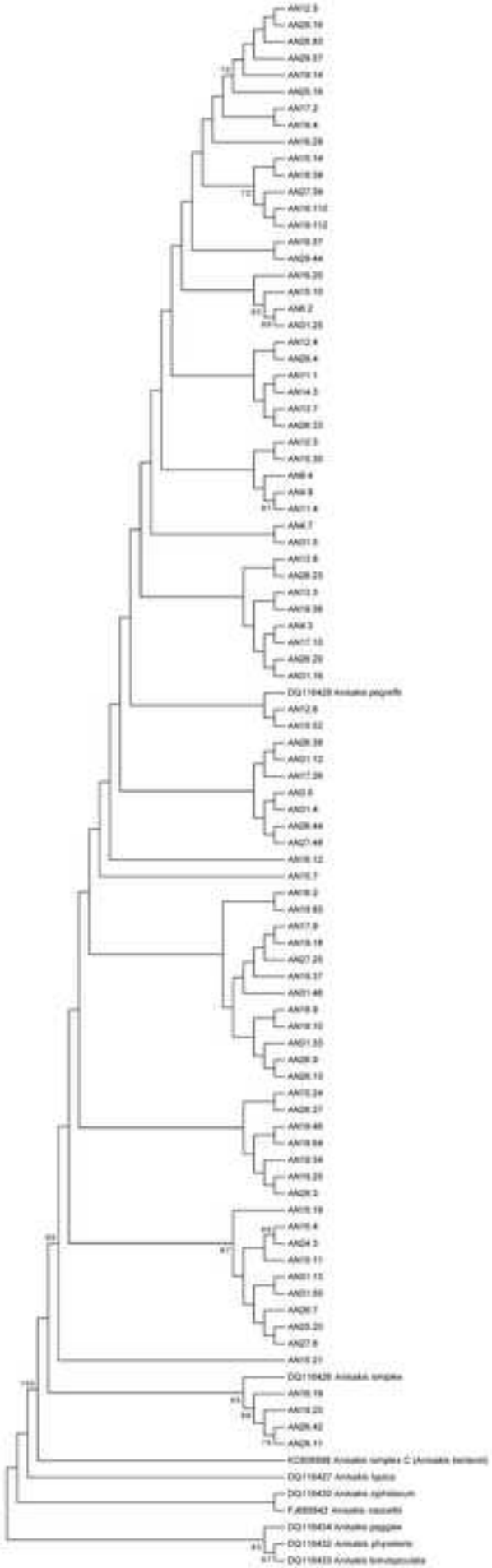

Table

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Batch	Specimens per batch	Origin	Visual inspection		UV-press method		Digestion	
			n L3	MA	n L3	MA	n L3	MA
1	29	FAO area 37.2.1	1	0.03	1	0.03	1	0.03
2	29	FAO area 37.2.1	0	0.00	0	0.00	0	0.00
3	29	FAO area 37.2.1	2	0.07	5	0.17	6	0.21
4	29	FAO area 37.2.1	9	**0.31**	11	**0.38**	12	**0.41**
5	29	FAO area 37.2.1	0	0.00	0	0.00	0	0.00
6	31	FAO Area 37.1.3	3	0.10	3	0.10	3	0.10
7	33	FAO Area 37.1.3	2	0.06	2	0.06	2	0.06
8	34	FAO Area 37.1.3	3	0.09	4	0.12	4	0.12
9	29	FAO Area 37.1.3	0	0.00	0	0.00	0	0.00
10	27	FAO Area 37.1.3	0	0.00	1	0.04	2	0.07
11	29	FAO area 37.2.1	4	0.14	2	0.07	4	0.14
12	24	FAO area 37.2.1	4	0.17	4	0.17	8	**0.33**
13	29	FAO area 37.2.1	13	**0.45**	8	0.28	14	**0.48**
14	29	FAO area 37.2.1	7	0.24	2	0.07	7	0.24
15	29	FAO Area 37.1.3	24	**0.83**	25	**0.86**	61	**2.10**
16	29	FAO Area 37.1.3	24	**0.83**	16	**0.55**	38	**1.31**
17	29	FAO Area 37.1.3	27	**0.93**	16	**0.55**	35	**1.21**
18	34	FAO area 37.2.1	11	**0.32**	5	0.15	12	**0.35**
19	34	FAO Area 37.1.3	75	**2.21**	86	**2.53**	115	**3.38**
20	27	FAO Area 37.1.3	0	0.00	1	0.04	1	0.04
21	29	FAO Area 37.1.3	0	0.00	1	0.03	1	0.03
22	29	FAO Area 37.1.3	0	0.00	0	0.00	1	0.03
23	29	FAO area 37.2.1	0	0.00	0	0.00	0	0.00
24	29	FAO Area 37.1.3	10	**0.34**	5	0.17	10	**0.34**
25	31	FAO area 37.2.1	13	**0.42**	18	**0.58**	26	**0.84**
26	27	FAO Area 37.1.3	57	**2.11**	62	**2.30**	89	**3.30**
27	27	FAO Area 37.1.3	50	**1.85**	29	**1.07**	76	**2.81**
28	31	FAO Area 37.1.3	0	0.00	0	0.00	1	0.03
29	31	FAO Area 37.1.3	26	**0.84**	33	**1.06**	58	**1.87**
30	34	FAO Area 37.1.3	0	0.00	0	0.00	0	0.00

31	40	FAO area 37.2.1	34	**0.85**	15	**0.38**	53	**1.33**
Total	**929**		**399**	**0.43**	**355**	**0.38**	**640**	**0.69**

Table 1 Samples collected (31 batches for a total of 929 specimens of anchovies) and results of the three methods (visual inspection, UV-press method and digestion) applied. The values of MA in bold in shaded boxes are those above the threshold of 0.3, which discriminated the marketability of the batches. The batches are in order of arrival at the FishLab, University of Pisa. n L3: number of anisakid larvae; MA: Mean Abundance.

Table
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Number of items in the lot	Number of specimens to be used for visual inspection	
	Sensitivity10 %	Sensitivity 5 %
10	All	All
20	16	19
30	19	26
40 – 45	21	31
46 – 50	22	35
51 – 60	23	38
61 – 70	24	40
71 – 80	24	42
81 – 90	25	43
91 – 100	25	45
101 – 120	26	47
121 – 140	26	48
141 – 161	27	49
161 – 180	27	50
181 – 200	27	51
201 – 250	27	53
251 – 350	28	54
351 – 450	28	55
451 – 600	28	56
601 – 1200	29	57
1201 - 4000	29	58
> 4000	29	59

Table 1SM. Sampling for the visual inspection to detect Anisakis larvae in fish batches, according to the Circular Letter VS8/C790/94 of the Lombardy region (adapted and published in D'Amico et al. 2014).

D'Amico, P., Malandra, R., Costanzo, F., Castigliego, L., Guidi, A., Gianfaldoni, D., & Armani, A. (2014). Evolution of the *Anisakis* risk management in the European and Italian context. Food Research International, 64, 348-362

Table
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Table 2SM. Molecular results of the BLAST analysis performed on a fragment of ~582 bp of the *cox2* gene.

Batch	number of visible larvae collected	number of visible larvae sequenced	*Anisakis pegreffii*	*Anisakis simplex*	*A. simplex* x *A. pegreffii*[a]	*Hysterothylacium* spp.
1	1	1	1			
2	0	0				
3	6	6	6			
4	12	12	12			
5	0	0				
6	3	3	3			
7	2	2	2			
8	4	4	4			
9	0	0				
10	2	2	2			
11	4	4	4			
12	8	8	8			
13	14	13	13			
14	7	7	7			
15	61	60	58	2		
16	38	36	33	3		
17	35	31	30		1	
18	12	12	12			
19	115	111	107	3		1
20	1	1	1			
21	1	1	1			
22	1	1	1			
23	0	0				
24	10	10	10			
25	26	25	25			
26	89	86	84	2		
27	76	72	72			
28	1	0				
29	58	55	53	2		
30	0	0				
31	53	50	50			
Tot	640	613	597 (97.39%)	14 (2.28%)	1 (0.16%)	1 (0.16%)

[a]The hybrid sequence derived from a parasite previously identified by PCR-RFLP and sequencing of the ribosomal internal transcribed spacer (ITS) region. It has been deposited by Pekmezci et al. (2014). In fact, the mtDNA cox2 fragment, due to its maternal inheritance does not allow to identify hybrids species.