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Assessment of a Sampling Plan Based on Visual Inspection for the Detection of Anisakid Larvae in Fresh Anchovies (*Engraulis encrasicolus*). A First Step Towards Official Validation?

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Abstract The presence of anisakid 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

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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, anisakid 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, anisakid larvae may cause infection, either in a non-invasive form, generally asymptomatic, or in an invasive form with gastrointestinal symptoms (Adams et al. 1997). Anisakid 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 *Anisakis pegreffii* (Mattiucci et al. 2013), the most widespread species in the Mediterranean Sea (Mattiucci and Nascetti 2008).

Even though the impact of anisakid 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 anisakid 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 anisakid 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 anisakid larvae in fishery products is a concern for consumers and official control authorities and a large number of provisions have 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.

Materials and Methods

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 ~24 h 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 EEC 140/1993), was performed, and the number of anisakid larvae found in HV and F was registered. A decision on the marketability of the batch was issued according to the Circular n. 1 of 1997 of Liguria Region (Italy) ("Comparison of MA Values Obtained for Tested Methods").

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

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

The values of MA in italics 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

Fig. 1 Visual inspection of anchovies as performed in the present study: whole specimens (**a**), heads and viscera (**b**), and fillets (**c**)

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 ("Ultraviolet Light Press Method") and the digestion procedure ("Final Protocol and Samples Digestion") for the recovery of parasites undetected during visual inspection.

Ultraviolet Light 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 ultraviolet (UV) light at 365 nm (UltraBright UV Transilluminator, 302/365 nm, 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.

Digestion Procedure

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 mins) 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, 300, 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 anisakid larvae collected from anchovies not belonging to this study was performed.

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 600 mM 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 three to four aliquots, diluted with tap water for clarification and finally transferred in Petri dishes. Each dish was then analyzed under UV light as reported in the section "Ultraviolet Light Press Method". The visible larvae found in HV and F were collected, counted, and stored at −20 °C until molecular analysis for hazard identification ("Molecular Identification of the Larvae by Sequencing and Phylogenetic Analysis").

Mean Abundance

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.

Molecular Identification of the Larvae by Sequencing and Phylogenetic Analysis

Extraction, Amplification, and Sequencing of the mtDNA cox2 Gene

Total DNA extraction was performed from each visible anisakid 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 10× 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 100×, Bew England BIOLABS® Inc. Ipswich, MA, USA), 1.25 U PerfectTaq DNA Polymerase (5Prime, Gaithersburg, USA), and 1–2 μL 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; and final extension at 72 °C for 10 min. The amplifications were carried out using a peqSTAR 96 Universal Gradient thermocycler (Euroclone, Milan, Italy). Five microliters 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).

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 *Anisakis 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 neighbor-joining (NJ) dendrogram of 94 representative sequences was obtained using MEGA version 6 computing the distances with the Kimura two-parameter model with 2000 bootstraps re-samplings (Saitou and Nei 1987).

Results and Discussion

Definition of "Visible Parasite"

While the definition of visible parasite given by the European Union is not so detailed ("Sampling and Visual Inspection at the Wholesale Market"), 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× 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* spp. 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.

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 detection 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 EEC 140/1993). The visual inspection allowed to detect 399 total visible parasites with a variable number per batch ranging from 0 to 75 (Table 1).

Ultraviolet Light 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 1993 proposed a variation which combined the compression of frozen fillets between two acrylic plates with candling using ultraviolet (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).

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 protocol 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).

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 (Rózsa et al. 2000). 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 infection (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 infection 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 cutoff (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 four 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.

Molecular Identification of the Larvae by Sequencing and Phylogenetic Analysis

Many gene targets can be used for anisakid identification. Among these, the direct sequencing of the mitochondrial cytochrom oxidase 2 (mtDNA *cox2*) has allowed the specific identification of nine 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 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 *Hysterothylacium 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. ("Results and Discussion") 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.

Health Implications

In the light of health implications, considering that all the collected parasites are zoonotic ("Comparison of MA Values

Obtained for Tested Methods"), 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 anisakid 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 *Anisakids* 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 contrary, should be addressed by a specific legislation. In fact, despite the numerous provisions issued at European and Italian level aimed at managing the risk associated to the presence of anisakid in fish, Anisakids proteins are still not included in the list of all common allergens by the specific Community Regulation (Regulation (EU) 1169/2011).

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.

Compliance with Ethical Standards

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Conflict of Interest Lisa Guardone declares that she has no conflict of interest. Renato Malandra declares that he has no conflict of interest. Francesco Costanzo declares that he has no conflict of interest. Lorenzo Castigliego declares that he has no conflict of interest. Lara Tinacci declares that she has no conflict of interest. Daniela Gianfaldoni declares that she has no conflict of interest. Alessandra Guidi declares that she has no conflict of interest. Andrea Armani declares that he has no conflict of interest. This article does not contain any study 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.

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