Anisakis spp. larvae in different kinds of ready to eat products made of anchovies (Engraulis encrasicolus) sold in Italian supermarkets.

DOI:10.1016/j.ijfoodmicro.2017.12.030

Elsevier Editorial System(tm) for International Journal of Food Microbiology
Manuscript Draft

Manuscript Number: FOOD-D-17-00670R2

Title: Anisakis spp. larvae in different kinds of ready to eat products made of anchovies (Engraulis encrasicolus) sold in Italian supermarkets.

Article Type: Full Length Article

Keywords: Anisakid larvae, anchovies, Engraulis encrasicolus, artificial digestion, contamination, semi-preserved seafood products, Italy

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Abstract: In this study the occurrence of visible anisakid larvae in semi-preserved anchovy products sold on the Italian market was investigated. Totally, 107 ready to eat products (33 salted-ripened, 49 in oil and 25 marinated) were sampled. Each sample was digested, then the digested material was observed under natural and UV light. Parasites were counted, collected and microscopically identified to genus level. A representative subset was molecularly identified using the cox2 gene. At least one visible Anisakis sp. larva was found in 54.2% of the total 107 products analysed. Totally 1283 dead larvae were collected. Anisakis sp. larvae were found in all the 33 salted products and 1139 (88.8%) larvae were collected, with a range of 1-105 parasites per product. Larval density per gram was 0.13. Anisakis sp. larvae were found in 49.0% of the products in oil and 143 (11.1%) larvae were isolated, with a range of 0-28 and a density of 0.03. Only 1 larva was found in the 25 marinated products (4.0%), the density was 0.00. A highly significant difference between all the product categories in respect of number of larvae per product, frequency of products contaminated by at least one larva and larval density per gram was found. Within the subset of larvae molecularly analysed (n=122), 92 larvae (75.4%) were identified as A. pegreffii and 30 (24.6%) as A. simplex. This study showed that semi-preserved anchovy products heavily contaminated with Anisakis spp. larvae reach the market. Beyond the negligible risk for anisakidosis, the presence of dead visible parasites may cause immediate rejection in consumers. In addition, the potential risk related to allergic reactions in sensitized individuals needs to be further assessed. In order to avoid commercialization of obviously contaminated products , fresh anchovies' batches intended for the production of such products should be accurately selected by the processing industry applying inspection methods.

Pisa, 14th July 2017

Dear Editor,

Please find enclosed the manuscript entitled "***Anisakis* spp. larvae in different kinds of ready to eat products made of anchovies: a defect or a hazard?"** to be considered for publication in the International Journal of Food Microbiology.

The European anchovy (*Engraulis encrasicolus*) is one of the most important fish resources of Mediterranean countries, where it is commonly used to produce traditional semi-preserved products, such as salted-ripened, in oil, marinated/pickled anchovies. Among the most important biohazards related to the consumption of raw anchovies is the presence of viable zoonotic nematode larvae belonging to the *Anisakis* genus. The parasitological risk associated to the presence of viable larvae in semi-processed seafood products can be prevented applying a freezing treatment or an appropriate brining or pickling process for a sufficient time. Nevertheless, the presence of dead visible parasites in processed products represents a defect that alters the overall quality, causes immediate consumers' rejection and may damage the reputation of the brand. In addition, although it is generally believed that sensitization with live *Anisakis* spp. larvae is required prior to the development of a clinical allergic responses, the allergenic potential of dead larvae is still debated.

Taking into account the increasing market request of ready to eat semi-preserved anchovies, their high prices and the scarcity of data on the presence of anisakid parasites in these preparations, the aim of this study was to assess the presence of visible anisakid larvae in different commercial categories of the most appreciated types of these semi-preserves on the Italian market.

Totally, 107 ready to eat products (33 salted-ripened, 49 in oil and 25 marinated) were sampled and separately submitted to artificial digestion. Parasites were counted, collected and microscopically identified to genus level. A subset was molecularly identified using the *cox2* gene.

Of the total 107 products analysed, 54.2% were positive for the presence of at least one visible *Anisakis* sp. larva and a total of 1283 larvae were collected. All the parasites found were dead. All the 33 salted products were positive and 1139 (88.8%) *Anisakis* spp. larvae were collected, with a range of 1-105 parasites per product. Among products in oil, 48.9% were positive with 143 (11.1%) *Anisakis* spp. larvae isolated and a range of 0-28. Only 1 out of the 25 marinated products (4%) was positive, with the presence of one larva. Within the larvae subset (n=122), 92 larvae (75.4%) were molecularly identified as *A. pegreffii* and 30 (24.6%) as *A. simplex*.

The present results showed that semi preserved anchovy products heavily contaminated with *Anisakis* spp. larvae can reach the market. In particular, the level of contamination was different depending on the products typology, being linked to the processing procedure and to the preliminary preparation of the fish, especially depending on the removal of the viscera. Beyond the negligible risk for anisakidosis, the presence of dead parasites may cause immediate rejection in consumers. In addition, the risk related to allergic reactions in sensitized individuals is still an open issue.

The manuscript has not been published elsewhere nor is it being considered for publication elsewhere. All authors have approved this manuscript, agree to the order in which their names are listed, declare that no conflict of interests exists and disclose any commercial affiliation.

Yours sincerely,

Andrea Armani

Dear editor,

thank you very much for considering our manuscript for publication in International Journal of Food Microbiology.

Please find below our answers to the reviewers' comments. We would like to thank both reviewers for their constructive suggestions. We are sorry they were not completely satisfied by the first revision. Some of the issues were probably due to the fact that the reviewers' comments were partly contrasting.

We hope that we have now succeeded in making all the requested modifications and clarifications.

Reviewer #1:

Line 231. Sorry but this is still unclear to me. Does it mean that the MA threshold is calculated as 3/N where N is 10% of the batch, 10% of the samples collected for analysis or a maximum of 3 larvae in all the samples within 10% of the sample size? It is unclear what is the value of the MA threshold and how the MA is calculated at individual level to define the samples exceeding this threshold.

The mean abundance threshold was calculated on the basis of the Liguria Region Circular n. 1 of 1997. This regulation states that: "if when opening the coelomic cavity numerous viable larvae appear, giving a repellent aspect to the product, the batch is withdrawn from the market; ii) <u>if the number of visible parasites is higher than 3 per anchovy in the 10 % of the examined specimens</u>, 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.

As regards the number of examined specimens, we referred to the Lombardy Region circular (Circular Letter VS8/C790/94) which states that: "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, such as anchovies), <u>the number of subjects to collect is, at least, 29</u>".

Therefore:

Number of examined specimens = 29

10% of 29 specimens = 2,9

3 (maximum number of tolerated parasites) * 2,9 = 8,7 maximum (theoretical) number of parasites in 29 specimens → 9 maximum (real) number of parasites in 29 specimens

9/29 = 0.3 mean abundance threshold

It derives that a MA of 0.3 corresponds to the threshold that allows to divide the batches in "non-marketable" (MA>0.3) or "marketable" (MA≤0.3).

The combination of the described sampling plan and mean abundance threshold is the most applied approach in Italy (D'Amico et al., 2014 Food research international, 64, 348-362).

The proposed MA threshold was first used in a work comparing the performance of three different techniques (visual inspection, UV press method and digestion) (Guardone et al. 2016 Food Analytical Methods 9.5, 1418-1427) and subsequently in a work aimed at assessing the reliability of the digestion of a subsample of 150 g (± 30 g) of viscera and adjacent muscles, randomly collected from 29 specimens, in estimating the marketability of fresh anchovies' batch.

We added more details also in the text (lines 222-227). We really hope it is clear now. If you think it is necessary, we can add further explanations.

Finally, we would like to point out that, although we have used the MA threshold also in this publication, it was only applied to a part of the samples (those composed by whole specimens for which the MA could be calculated). The paper focuses on the number of larvae per product and on the contamination (presence of at least one larva). Following the suggestion of reviewer 2 we have also introduced another epidemiological index, the larval density per gram.

Discussion

In the discussion section, aspects related to batch selection and practical meaning of this study for food industry has been removed from the previous version. I really think it would be important to discuss this and demonstrate how this work could be used in practice to inform decision making in fishery industry in long term.

Except for the above aspect of the MA, which I am sure will be clarified, the manuscript is well written but as presented, it remains a descriptive study. Considering the level of the journal, including some strong final remarks on the practical utility of the findings, would noticeably increase the quality of the work.

Therefore, I propose again what I wrote in the first revision:

"what 'a precautionary approach' would mean in practice for decision makers and the food industry? is it related to the batch selection reported in the conclusion? what options the food industry would have in practice to select the batches (e.g. fishing area?)? furthermore, from the results it appears that many samples are above the threshold in terms of MA. In the light of your results, it would be good to discuss the role of this threshold on the economic impact on the food industry if this value is used, as suggested, for batch selection."

We thank the reviewer for the suggestion. These points have been added to the discussion (lines 432-451).

Reviewer #2:

1. I think the title should be considered for revision; the title 'Anisakis spp~ a defect or a hazard ?' sounds like the authors investigated whether the Anisakis species in anchovy products are hazardous to humans. But the authors assessed the prevalence of anisakid larvae in different kinds of anchovy products, as mentioned in the end of the INTRODUCTION. Of course, the authors can mention the dead larvae have the potential risks of allergic reactions to humans in DISCUSSION. But this is not the main focus (aim) of this study (if the authors want to keep the aim of this study as described in the INTRODUCTION). So if the authors also want to emphasize the potential risk of the larvae (or want to keep the title), I suggest that they should at least raise enough evidence that dead Anisakis larvae are allergic to humans, particularly dead Anisakis larvae in ANCHOVIES can cause allergic reactions to humans, not in pink salmon.

The title has been changed, we preferred to maintain the aim as it is.

2. Please include the scientific name of anchovy in the title. **done**

3. Please make all percentage data round off to one decimal place (e.g., 88.8%, not 88.98%; 4.0%, not 4%) throughout the whole MS including Tables. **done**

4. 'positivity' and 'number of larvae' do not seem to be the terms generally used in parasitology. The authors should refer Bush et al. (1997)'s reference and select appropriate terms which fit into the definitions.

We are aware of the terminology defined in Bush et al., 1997. However, most of these terms refer to animal hosts, while in this study we have analysed seafood products which were composed of many individual hosts. This is the reason why we used the Mean Abundance, in the case of products composed of whole anchovies for which the number of individuals was countable. Now, we also added the larval density per gram as a further epidemiological index.

We have removed the term "positivity" and, where possible, we have replaced it with the term "contaminated", in accordance with the EU regulations, EFSA (https://www.efsa.europa.eu/en/topics/topic/parasites-food) and with previous works (Audicana & Kennedy 2008 *Clinical microbiology reviews*, *21*(2), 360-379; Fæste et al., 2015 *Food Analytical Methods*, *8*(6), 1390-1402; Llarena-Reino et al., 2012 *Food Control*, 23(1), 54-58).

The term "number of larvae" was kept since it is a measure and not a parasitological index and therefor it cannot be replaced. Besides, it has been used in similar recent studies:

- **Cipriani, P., Acerra, V., Bellisario, B., Sbaraglia, G. L., Cheleschi, R., Nascetti, G., & Mattiucci, S. (2016). Larval migration of the zoonotic parasite Anisakis pegreffii (Nematoda: Anisakidae) in European anchovy, Engraulis encrasicolus: Implications to seafood safety. Food Control, 59, 148-157.**
- **Pierce, G. J., Bao, M., MacKenzie, K., Dunser, A., Giulietti, L., Cipriani, P., ... & Hastie, L. C. (2017). Ascaridoid nematode infection in haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*) in Northeast Atlantic waters. *Fisheries Research*, in press**

5. Keywords in the MS should be selected for being detected by DB (e.g., PUBMED) as much as possible. I think the keywords in this MS are not useful for being detected in PUBMED or other DBs. I suggest that the keywords should be carefully selected again. **done**

6. Line 114~115: it has been supposed that also ingestion ~ -> it has been also suspected that ingestion ~ **done**

7. Line 121~ : Taking into ~EU. the high prices of ~ -> Taking into ~ EU and the high prices of ~ .

It is not possible to change the sentence, as we cannot substitute the comma with "and" because there is another element listed and there would be two "and" in the sentence

8. Line 144 : ~ University of Pisa, Department ~ -> Department of ~ University of Pisa **done**

9. Line 149: ~ their number -> their number of what? **The sentence has been modified**

10. Line 151: ~ rinsed in a glass beaker. -> rinsed with what ? **with tap water, the indication has been added**

11. Line 152: The oil was carefully removed also ~ -> The oil was also carefully removed **done**

12. Line 154: With the aim to test the recovery ~ -> To test the recovery ~ **done**

13. Line 159~162 : Considering that ~ per time -> I do not understand what the authors want to describe. Please revise this sentence. **Done**

14. Line 196~197: by the Experimental ~ (Turin, Italy) -> Delete **done**

15. Line 252: trials ->What trials ? **Those mentioned at line 156. However, the sentence has benne modified to make it more clear.**

16. Line 261~264: All the parasites ~dead. In fact, even though ~and might be ~ treatment -> All the parasites ~dead. Although In fact, even though the emission of fluorescence are known to not always discriminate between live and dead larvae, might be related to ~ by the treatment spontaneous and stimulated movements of the larvae were absent in this study**. We found very hard to understand this point. However, we have modified the sentence hoping to have correctly interpreted the meaning.**

17. Among those ~ analyzed (n=122) 92 (75.4%) -> Among these ~ analyzed larvae (n=122), 92(75.4%)~ **we have modified the sentence trying to clarify it. However, we cannot use "these", instead of "those", because not all the larvae were moleculary analysed.**
18. Line 296~297: see section 3.2 -> delete **done**

19. Line 299: ~A. simplex (22.6%) -> A. simplex (22.6%) (Table 2). **Done**

20. Line 305: The MA~ 3.92 -> **Delete. The sentence was wrong and did not make sense. However, this sentence cannot be deleted because this information is not present elsewhere in the text. Therefore, the sentence was corrected.**

21. Line 313: corresponding to 11.1% of the ~ -> corresponding to 11.1% (00/00) of the ~ **Done**

22. Line 323: ~ for 15 product, in fact although other ~ -> ~ for 15 products. in fact although Other 3 products ~ **done**

23. Line 351: Analogously -> Similarly **done**

24. Line 353: As known, most anisakid larvae are located ~ -> As known, most anisakid larvae are known to be located ~? **The sentence has been modified assuming you wanted to move "known" from the beginning to the middle of the sentence. Otherwise it would be repeated.**

25. Line 357: ~ after the capture especially ~ -> ~ after the capture, especially ~ **done**

26. Line 364: ~ were positive. -> positive for what? **For the presence of at least one larva. The sentence has been modified.**

27. Line 389: ~ inactivates Anisakidae larvae -> inactivates anisakid larvae. **done**

28. Line 392~395: In addition, salted-ripened anchovies ~ for E. encrasicolus -> This sentence dose not make sense; The scientific name of European anchovy is E. encrasicolus. Delete 'for E. encrasicolus'. **Done**

29. Line 417~418: ~ live larvae that can actively ~ the external surface. -> ~ live larvae which can actively move and become evident also on the external surface. **done**

30. Line 419: However, also the presence ~ -> However, also the presence ~ **we don't understand this point.**

31. Line 467~476: I think this paragraph is not helpful for discussion and better to be deleted. As mentioned in the INTRODUCTION, the aim of this study is that the assessment of the presence of visible anisakid larvae in different commercial categories of products. But this paragraph contains how the consumers and authorities should react with the presence of the larvae in fish products. And what is FBO? I suggest the authors should keep the MS clear, concise, readable, not too wordy.

Conclusions have been modified and shortened. FBO stands for Food Business Operators, as stated at line 96.

32. Line 478~479: I think the authors should make it clear what the aim of this study is. The authors described that 'The present work highlighted how semi-preserved anchovy products heavily ~ can reach the market'. I think the authors should discuss the distribution structure, marketing system regarding the fisheries products, to highlight the issue written in this sentence. But there is no mention regarding 'how ~ can reach the market' in this MS, and as in the INTRODUCTION, the authors assessed the prevalence of anisakid larvae in different kinds of anchovy products. Furthermore, the title also includes the debate about allergic issue of dead larvae. All of these make the readers confused and the MS difficult to understand.

We think the word "how" was probably misleading. We did not intend to describe the products' distribution on the market but to point out that highly contaminated products are already sold in Italian supermarkets. We have change the sentence hoping to clarify our idea and to better agree with the aim of our work.

33. I strongly suggest that the authors should replace the references which cannot be read nor accessed by international readers. If the references cannot be accessed or cannot be obtained by the readers who want to have a look, those references should not be referred. Unfortunately, there are so many references which I cannot read nor access in this MS.

The references have been revised. All the documents are now accessible to international readers.

Highlights (for review)

- Different kinds of anchovy ready to eat products were analysed by digestion
- 54.2% of the products were positive for at least one visible *Anisakis* spp. larva
- A total of 1283 dead larvae were collected
- The product category influenced the number of larvae and positivity rate
- Salted products were found to be the most contaminated (positivity 100%)

Anisakis spp. larvae in different kinds of ready to eat products made of anchovies (*Engraulis encrasicolus*) sold in Italian supermarkets~~: a defect or a hazard?~~

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Abstract

~~The aim of~~In this study ~~was to assess~~ the ~~presence~~ occurrence of visible anisakid larvae in semi-preserved anchovy products sold on the Italian market was investigated. Totally, 107 ready to eat products (33 salted-ripened, 49 in oil and 25 marinated) were sampled. Each sample was digested, then the digested material was observed under natural and UV light. Parasites were counted, collected and microscopically identified to genus level. A representative subset was molecularly identified using the *cox2* gene. At least one visible *Anisakis* sp. larva was found in 54.2% of the total 107 products analysed. ~~Of the total 107 products analysed, 54.2% were positive for the presence of at least one visible *Anisakis* sp. larva and a total of and t~~Totally 1283 dead larvae were collected. ~~All~~ *Anisakis* sp. larvae were found in all the 33 salted products ~~were positive~~ and 1139 (88.8%) ~~*Anisakis* sp.~~ larvae were collected, with a range of 1-105 parasites per product. Larval density per gram was 0.13. *Anisakis* sp. larvae were found in 49.0% of the products in oil ~~Among products in oil, 49.08.98% were positive~~ and 143 (11.1%) ~~*Anisakis* spp.~~ larvae were isolated, with a range of 0-28 and a density of 0.03. Only 1 larva was found in ~~out of~~ the 25 marinated products (4.0%), the density was 0.00 ~~was positive, with the presence of one larva~~. A highly significant difference between all the product categories in respect of ~~both~~ number of larvae per product, frequency of products contaminated by at least one larva and larval density per gram was found ~~and positivity was found~~. Within the subset of larvae molecularly ~~identified~~ analysed (n=122), 92 larvae (75.4%) were identified as *A. pegreffii* and 30 (24.6%) as *A. simplex*. This study ~~highlighted how~~ showed that semi-preserved anchovy products heavily contaminated with *Anisakis* spp. larvae ~~can~~ reach the market. Beyond the negligible risk for anisakidosis, the presence of dead visible parasites may cause immediate rejection in consumers. In addition, the potential risk related to allergic reactions in sensitized individuals needs to be further assessed. In order to avoid commercialization of obviously contaminated

products ~~corrective measures on the final products~~, fresh anchovies' batches intended for the production of such products should be accurately selected by the processing industry applying inspection methods.

Keywords

~~Salted-ripened Processed seafood products, anchovies, anchovies in oil, marinated anchovies, visible parasite, anisakid dead larvae, product quality, defect, risk assessment~~

Anisakid larvae, anchovies, *Engraulis encrasicolus*, artificial digestion, contamination, semi-preserved seafood products, Italy

1. Introduction

The European anchovy (*Engraulis encrasicolus*) is an economically important fish species particularly appreciated in Mediterranean countries, where it is commonly used to produce traditional salted-ripened, in oil and marinated/pickled products (Anastasio et al., 2016; Felix et al., 2016; Triqui and Reineccius, 1995).

In the presence of salt, anchovies undergo physicochemical modifications giving origin to a product called "ripened" or "matured" (Codex Alimentarius, 2012). Usually, salting-ripening involves a preliminary operation of brining, where the whole fish is immersed in saturated brine. Following this, anchovies are beheaded and gutted, placed in barrels, alternating layers of fish and salt, and pressed (Czerner et al., 2011; Felix et al., 2016). In some cases, fish are beheaded and gutted immediately at the beginning of the process (Granata et al., 2012). The curing process takes several months and the final product is characterized by firm consistency, reddish colour, juicy texture and characteristic odour and flavour (Felix et al., 2016; Granata et al., 2012; Sospedra et al., 2015). Salted-ripened anchovies may be packed in brine or preserved in oil. For preservation in oil, fish are generally skinned, washed, dried and filleted (Mohamed et al., 2016).

The term ‘‘marinades’’ or ‘‘marinated fish’’ is used to define products consisting of fish processed with an edible organic acid, usually acetic acid, and salt, which gives them a characteristic white colour of the flesh, and put into brines, sauces, or oil (McLay, 1972). Pickled anchovies are very popular in Spain as *boquerones en vinaigre* and in Italy as *alici marinate.* Traditionally, homemade marinated anchovies are prepared with fresh fish eviscerated and de-boned by hand, then pickled in lemon juice or vinegar and salt for less than 24h before consumption. Although the Italian ~~and Spanish~~ legislation requires preventive freezing treatment also in case of domestic preparation of raw, marinated or not fully cooked fish (D’Amico et al., 2014~~Decreto Legislativo 17 Luglio 2013; Real Decreto 1420/2006~~), this is frequently not applied (Serracca et al., 2014), because it alters the texture and the taste of fish meat (Sánchez-Monsalvez et al., 2005; Vidaček et al., 2009).

Among the most important biohazards related to the consumption of raw anchovies is the presence of viable zoonotic nematode larvae belonging to the genus *Anisakis*, as their ingestion is responsible for a zoonotic disease known as anisakiasis (Mattiucci et al., 2013). Of the nine genetically characterized species of the genus *Anisakis*, only *A. pegreffii* and *A. simplex* (*s. s.*) have been reported as causative agents of human gastric, intestinal and gastro-allergic anisakiasis (Cipriani et al., 2017). *A. simplex s.l.* and *A. pegreffii* are frequently found in European anchovies (Bao et al., 2017; Costa et al., 2016).

The ~~presence~~ occurrence of anisakid larvae in fish is a natural condition throughout the supply chain and their complete elimination from fishery products is not feasible (EFSA, 2010). Food Business Operators (FBOs) must ensure that fishery products obviously contaminated with visible parasites are not placed on the market for human consumption, by conducting a visual inspection of fresh fish products (Commission Reg. EC No 2074/2005). In addition, the parasitological risk associated to the presence of viable larvae in semi-processed seafood products can be prevented by applying a freezing treatment or an appropriate brining or pickling process for a sufficient time (AESAN, 2007; Anastasio et al., 2016; Sánchez-Monsalvez et al, 2005). Nevertheless, the presence of dead visible parasites in processed products represents a defect that alters the overall quality (Codex Alimentarius, 2012; Council Reg. EC No 2406/1996) making them unfit for human consumption (Reg. EC No 178/2002). In fact, the finding of parasites in fish products causes immediate consumers' rejection and may damage the reputation of the brand. Moreover, although it is generally believed that sensitization with live *Anisakis* spp. larvae is required prior to the development of a clinical allergic responses, it has been also suspected ~~supposed~~ that ~~also~~ ingestion (and inhalation) of dead larvae or their allergens might induce allergic reactions (Bao et al., 2017; EFSA, 2010; Mattiucci et al., 2017).

In a preliminary phase of this study 44 ready to eat products made of anchovies, herrings, mackerel and sardines were analysed (Guardone et al., 2016a). Considering that all the samples made of mackerel and sardines were negative, while larvae were found in 80.0% of the products made of anchovies, the present study specifically addressed this type of product. Taking into account the increasing request of ready to eat seafood products from the EU (EUMOFA, 2017), the high prices of semi-preserved anchovies and the scarcity of data on ~~on the presence of~~ anisakid parasites ~~associated to~~in these kind of preparations (Fraulo et al., 2014; Sospedra et al., 2015), the aim of this study was to assess the ~~presence~~ occurrence of visible anisakid larvae in different commercial categories of products sold in Italian supermarkets. The most appreciated types of semi-preserves on the national market, such as salted-ripened, in oil and marinated anchovies, were collected and analyzed.

2. Materials and methods

2.1 Sampling

A total of 107 ready to eat products made of anchovies, belonging to 17 different brands and to different lots were sampled between April 2015 and May 2017 in Tuscany (Northern

Italy), at different points of sale of a large national purchasing consortium. A convenience, non-probabilistic sampling was conducted, structured to include a proportional number of products per type and brand. Three different types of commercial products were collected: salted-ripened, in oil and marinated (Fig. 1). In 51 products, the fishes were only beheaded and (partially) gutted, but the bones were not removed and the structure of the body was maintained unaltered ("whole" anchovies) (Fig. 1a), while in the remaining 56 products the anchovies were deboned and opened to become flat ("fillets") (Fig. 1b-c). Thirty-three products were salted-ripened anchovies (all whole fishes), 49 products were in oil (18 whole fishes and 31 fillets) and 25 products were marinated anchovies (all fillets). The samples were then transferred to the FishLab, ~~University of Pisa,~~ Department of Veterinary Sciences, University of Pisa, and analysed.

2.2 Parasitological analysis

2.2.1 Digestion procedure. Each sample was registered with an internal unique code. Photos of the external packaging with the labelling information and of the internal content were taken. In the case of whole ~~specimens~~anchovies, the~~ir~~ number of specimens w~~ere~~as counted~~, and the Mean Abundance (MA) was calculated (see Section 2.4)~~. Salt, brine and oil were carefully removed from the products. Salted products were also lightly rinsed with tap water in a glass beaker. The oil was also carefully removed ~~also~~ with the aid of absorbent paper. Then, the edible part was weighted. Considering that the whole content of the collected products is edible, the full weight of each sample was digested. ~~With the aim t~~To test the recovery rate of parasites from semi-preserved anchovy products, preliminary trials were performed. Larvae collected from products analysed in the preliminary phase of this study (Guardone et al., 2016a) were submitted to artificial digestion using the Trichineasy®, according to the manufacturer's instructions (CTSV, 2007). ~~according to the procedure described in Guardone et al., (2017).~~ ~~Considering that a~~All the larvae were recovered with thisset procedure, which was then applied to all the samples. , digesting. A aA maximum of 200 g of tissue was digested per time. was digested according to the manufacturer's instructions (CTSV, 2007http://www.ctsv.biz/image-ctsv/PDF/TrichinEasy-anisakis.pdf).

At the end of the digestion the material retained in the filter was rinsed with water and divided in Petri dishes to create a thin layer of a few mm. The Petri dishes were observed under natural and UV light (UltraBright UV Transilluminator, 302/365 nm, Maestrogen, Las Vegas, USA) for the detection of anisakid larvae. During this step, spontaneous and stimulated movements of the larvae were assessed to evaluate viability. In consideration of the provisions of the Regulation EC No 853/2004 and subsequent amendments, only the visible larvae (non-encapsulated nematodes longer than 1 cm or parasites with a capsular diameter of at least 3 mm according to the definition given by the Codex Alimentarius Commission, 1971) were counted and collected. The residual salt and oil and the water used to rinse the anchovies were inspected as described above. The larvae found during this step were collected and summed to those found after the complete digestion. All the larvae were identified to genus level following Sakanari and McKerrow (1989) and Berland (1989) by observation under a microscope (Nikon Eclipse E200) and then stored in 70% alcohol for molecular analysis.

2.2.2 Molecular identification. A subset of *Anisakis* larvae (from 1 to 4 larvae per product) was submitted to molecular identification. Total DNA extraction was performed according to the protocol used in Guardone et al., (2016b). DNA concentration and purity were determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

A 629-bp fragment of the mitochondrial cytochrome *c* oxidase subunit II (*cox2*) gene was amplified using the primers 211F (5'-TTT TCT AGT TAT ATA GAT TGR TTY AT-3') and 210R (5'-CAC CAA CTC TTA AAA TTA TC-3') (Nadler & Hudspeth, 2000). PCR amplifications were set up in a 20 µl reaction volume containing 2 µl of a 10× buffer (biotechrabbit GmbH, Hennigsdorf, Germany), 200 µM of each dNTP (dNTPmix, EurocloneS.p.A-Life Sciences Division, Pavia, Italy), 200 nM primers, 1.25 U PerfectTaq DNA Polymerase (biotechrabbit GmbH, Hennigsdorf, Germany), and 50-100 ng of DNA and DNase free water (Water Mol. Bio. Grade, DNase-RNase and Protease free, 5Prime GmbH, Hamburg, Germany) with the following cycling program: initial 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, as in Guardone et al., (2016b).

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). PCR products were purified with EuroSAP PCR Enzymatic Clean-up kit (EuroClone Spa, Milano) and stored at -80°C prior to the sequencing. ~~The sequencing of the~~ PCR products were sequenced ~~was carried out by the Experimental Institute of Zooprophylaxis of Piedmont, Liguria and Aosta Valley (Turin, Italy)~~ to obtain forward and reverse direction sequences for each PCR product. The sequencing reaction was performed by the use of a 4-capillary 3130 Genetic Analyzer (Applied Biosystems) and the BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technology, Thermo Fisher Scientific Inc.).

All the obtained sequences were analyzed using Bioedit version 7.0.9 (Hall, 1999). Adjustments were made after visual checking and the sequences were analysed on GenBank by using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

2.4 Statistical analysis

2.4.1 Comparison of the three product categories. Salted, in oil and marinated products were compared in respect to: ~~positivity (presence of at least one larva, (nominal variable) and number of total larvae in each analysed sample (counting variable).~~ presence of at least one larva (nominal variable), number of larvae per product and density (larvae/gram) (quantitative variables). To assess differences among groups two tests were applied: the χ^2 test for the nominal variable and the Kruskal-Wallis test for the counting variable. The non-parametric tests were chosen given the unequal sample size, the presence of categories with less than 30 products and, not least, the violation of the ANOVA assumptions, mostly the homogeneity of variance. For all the analyses, significant results were those associated with $p<0.05$. If overall significance was observed, pair-wise comparisons were analysed using χ^2 (for nominal variables) and Mann-Whitney (for quantitative data) tests. In these comparisons, in order to protect for type I error increase, a threshold of $\alpha=0.01$ was chosen for the interpretation of the results. Analyses were performed using SPSS v 15 (R).

2.4.2 Comparison between products made of fillets and whole anchovies. Differences in positive samples the number of larvae per product, frequency of products contaminated by at least one larva and larval density per gram were also analysed the occurrence , in the number of larvaedetected wereas also analyzed in respect to the product being composed by whole anchovies or by fillets. The analyses were carried out using χ^2 (for nominal variables) and Mann-Whitney (for quantitative data) tests. These comparisons were performed only for products preserved in oil, the only category containing both fillets and whole fishes.

2.4.3 Mean abundance (MA). The mean abundance (MA) (total number of individuals of a particular parasite species in a sample of a particular host species divided by the total number of hosts of that species examined, Bush et al., 1997) was calculated after the complete digestion of products made of whole specimens whole products and the value obtained was used to issue a marketability judgement. The MA threshold was calculated by applying an approach regional law widely used throughout Italy (D'Amico et al., 2014) which defines the maximum number of tolerated larvae in fresh batches of anchovies (three 3 larvae in 10% of the sampled fish). Considering that in the case of fish species caught in large batches, such as anchovies, the number of subjects to collect for a significant sampling is, at least, 29, the maximum number of parasites tolerated is 9 and therefore the MA threshold is 0.3 (Guardone et al., 2016b, Guardone et al., 2017).

3. Results and discussion

3.1 Parasitological analysis

The official method for the detection of parasites in fish is the visual inspection (Commission Reg. EC 2074/2005). The pressing method of frozen fillets followed by the examination under ultraviolet light is also frequently used (Gómez-Morales et al., 2017). Moreover, the artificial digestion may also be applied to isolate larvae from fish and it is considered the gold standard for its higher sensitivity (Guardone et al., 2016b; Llarena-Reino et al., 2013). The cuticle of parasitic nematodes has been reported as highly resistant to strong acids and digestive enzymes, regardless of whether the nematodes are live or have been killed by freezing or conventional heating (Tejada et al., 2006). However, damages to the cuticle occurring during processing (Anastasio et al., 2016; Tejada et al., 2006; Vidacek et al., 2009) can affect the resistance of the larvae to the artificial digestion. ~~For this reason~~As mentioned, trials were performed using dead *Anisakis* spp. larvae~~ collected from products (salted and in oil) analysed in a preliminary phase of this study (Guardone et al., 2016a)~~. Since ~~A~~all the larvae were recovered after the digestion, ~~therefore, the procedure applied to fresh anchovies (Guardone et al., 2017), already proven to be able to recover live larvae, ~~the procedure was considered suitable ~~also ~~for semi-preserved products.

At least one visible larva was found in 58 (54.2%) of ~~Of ~~the total 107 products analysed~~, 58 (54.2%) were positive for the presence of at least one visible larva~~. A total of 1283 visible larvae were collected, which were all morphologically identified as *Anisakis* sp.~~Overall a total of 1283 anisakid larvae were collected.~~ Strong differences were observed between the various categories of products and also between whole and filleted products (Table 1). All the parasites found during the analysis were dead. In fact, ~~even~~ ~~A~~although emission of fluorescence is known ~~did~~to not always ~~allow to~~ discriminate between live and dead larvae ~~and might be related to the stress produced in the larvae by the treatment~~ (Tejada et al., 2006; Vidaček et al., 2009), spontaneous and stimulated movements of the larvae were absent in this study. ~~All the visible parasites were morphologically identified as *Anisakis* sp.~~ Among the subset of ~~ose~~ molecularly analysed larvae (n=122), 92 (75.4%) were identified as *A. pegreffii* and 30 (24.6%) as *A. simplex* (Table 2). Larvae of the genus *Hysterothylacium* were found very rarely (4 samples) and were always shorter than 1 cm,~~ and~~ thus they were not counted as visible larvae. The low prevalence of *Hysterothylacium* spp. may be due to the fact that these parasites are generally smaller and thinner than *Anisakis* spp. and might be less resistant to processing techniques.

The complete elimination of parasites from fishery products is not feasible (EFSA, 2010), therefore it is necessary to establish a threshold to discriminate between fit and unfit products (Reg. EC 178/2002). In particular, it is essential to identify the number of larvae that can be tolerated in a product and to adopt a criterion for taking decisions on the marketability of fishery products. According to the "*Guidance document on the implementation of certain provisions of Regulation (EC) No 853/2004 on the hygiene of food of animal origin*" (European Commission, 2014) a fishery product is considered obviously contaminated if visible parasites are detected in edible portions. However, such document does not define a maximum number of parasites. Therefore, in a previous work (Guardone et al., 2016b), a MA threshold ~~calculated as described above,~~ was used to assess the marketability of fresh batches of anchovies. Especially in the case of small fish, which are not sold individually, the MA could be used to estimate the degree of infestation.

3.1.1 Salted anchovies. At least one visible *Anisakis* sp. larva was found in all ~~All~~the 33 products (100%)~~ were found positive for the presence of at least one larvae~~. Totally, 1139 larvae ~~of *Anisakis* sp.~~ were collected in this category, corresponding to 88.8% of the total collected larvae. The mean number of larvae per product was 34.5~~2~~ (±29.3~~3~~ standard deviation), with great variability (range: 1- 105). The mean density (larvae per gram) was 0.13 (Table 1). The highest number of larvae (439) was found in the products belonging to brand 5 (Table 2). The results show that salted anchovies are the most contaminated type of products, which is likely due to the type of processing ~~(see Section 3.2)~~.

Parasites recovered from these products were molecularly identified as *A. pegreffii* (77.4%) and *A. simplex* (22.6%) (Table 2). The majority of the larvae of *A. simplex* found in these samples was collected from anchovies declared to be fished in the Cantabrian sea (FAO area 27), while *A. pegreffii* was the dominant species in samples declared as fished in the Mediterranean Sea, confirming previous epidemiological data (Costa et al., 2016 and references therein).

T~~t~~he MA ~~, which~~ varied from 0.0~~4~~ to 3.9~~2~~. Twenty-nine products (87.9~~8~~%) exceeded the MA threshold ~~of positivity~~ previously set for fresh anchovies. No differences in MA values were observed ~~in the distribution of the positivity to the MA threshold~~ in relation to the different brands (Table 2).

3.1.2 Products in oil. Among the 49 products, 18 were made of whole anchovies and the remaining 31 of fillets. The 18 whole products belonged to 4 different brands. Two of them consisted of previously salted anchovies (red flesh, brand 2 and 7), while the other two presented a white meat (brand 1 and 8) (Table 2).

At least one *Anisakis* sp. larva was found in~~Overall~~ 24 (49.0~~8.98~~%) products in oil ~~were found positive for the presence of at least one larva~~ and a total of 143 larvae ~~of *Anisakis* sp.~~ were collected, corresponding to 11.1% (143/1283) of the total larvae collected. A mean number of 2.9 larvae per product was detected (± 5.8~~0~~ standard deviation) with a great variability (range: 0-28 larvae). The larval density per gram was 0.03 (Table 1 and 2).

Parasites recovered from products in oil were molecularly identified as *A. pegreffii* (70.3%) and *A. simplex* (29.7%). The geographical origin is not compulsory for fishery products in oil (D'Amico et al., 2016) and it was not reported for 5 of the 14 brands. All the larvae molecularly identified from these products were *A. pegreffii*. Most of the remaining indicated FAO 37 or FAO 37.2.1 and the dominant species was *A. pegreffii*. Only the products of one brand were claimed to originate from FAO area 27. In these samples the majority of the identified larvae were *A. simplex*.

It was possible to calculate the MA for 15 products.~~;~~ I~~i~~n fact, although other 3 products (brand 8) were originally prepared with whole anchovies it was not possible to count them due to the loss of integrity of the specimens induced by the processing (Table 2). Of these 15 samples, all the 10 products made of salted anchovies (brand 2 and 7) exceeded the set MA threshold. On the contrary, no larvae were found in ~~all~~ the 5 products of brand 1~~ were negative~~. The high ~~positivity~~ contamination level in whole salted in oil anchovies confirms the results obtained for salted-ripened anchovies. The MA varied from 0.0 to 2.8.

Different levels of contamination ~~ces in positivity~~ were observed in ~~relation to the~~ whole and filleted products (at least one larva was found in 61.1~~1~~% of the whole products and 41.9~~4~~% of the fillets~~ was positive for at least one larvae~~). Within whole products, differences were also observed between red and white fish: 83.9~~2~~% of the parasites (n=120) were found in the 2 products made of red whole anchovies.

3.1.3 Marinated anchovies. Only 1 visible *Anisakis* spp. larva was found in ~~out of~~ the 25 marinated products (4.0%). The larva ~~was found positive with the presence of one *Anisakis* spp. larva that~~ was subsequently molecularly identified as *A. pegreffii*. The larval density per gram was 0.00 (Table 1 and Table 2). Considering that all these products consisted in filleted anchovies it was not possible to calculate the MA.

3.2 Comparison between product categories: influence of the processing technology on the ~~presence~~ occurrence and viability of anisakid larvae

The processing technology can influence the presence of parasites in the final products. The present study showed a significant difference between all the product categories in respect of both number of larvae per product (Kruskal-Wallis' χ^2=69.~~,~~95; p<0.001), ~~and positivity number of contaminated products (χ^2=50.34; p<0.001).~~ frequency of contaminated products (χ^2=50.34; p<0.001) and density of larvae per gram (χ^2=58.89; p<0.001).

The average number of larvae per product was ~~about~~ around 35, 3 and 0 for salted, in oil and marinated products, respectively (Table 1). ~~Analogously~~ Similarly, the frequency of contaminated products ~~being positive~~ in each category was 100.0%, 49.0% and 4.0% (Table 1). In addition the density was different across products: mean density of 0.13 (s.d.= 0.09) in salted products, 0.03 (s.d=0.06) in products conserved in oil and 0.0 (s.d.=0.001) in marinated products.

~~As known, for other fish species, also in the case of anchovies most a~~Anisakid larvae are known to be located in the fish visceral cavity and/or embedded in the visceral organs and in the adjacent muscles (belly flap) (EFSA, 2010). Larval migration to the muscles may occur after the capture~~,~~ especially in the case of an inappropriate refrigeration (Cipriani et al., 2016). When visible parasites are only found in non-edible parts of the fishery product, processing procedures, such as gutting, ensure that the raw materials are not obviously contaminated (European Commission, 2014). On the countrary, when the viscera removal is not complete, the final product may harbour a high number of parasites. This is the case of salted-ripened anchovies, where the gut is not completely removed as intestinal enzymes seem to play an essential role in ripening (Czerner et al., 2011). In fact, at least one larva was found in ~~all the~~each analysed salted product~~s were positive.~~. Similarly, all the whole salted anchovies in oil were ~~positive~~ contaminated with~~for~~ a high number of larvae ~~and exceeded all~~

~~thresholds~~. Overall 1259 larvae were found in whole salted anchovies in brine and in oil ~~these products (1139 in 33 salted products and 120 in 2 salted whole anchovies in oil). Another larva was found in one of the "white" whole anchovies in oil~~ (Table 1 and 2). As concerns the fillets in oil, these are generally previously treated as whole salted anchovies for the maturation process, and only after this phase they are filleted and put under oil. The lower presence of parasites in this kind of products can be explained by the fact that parasites are removed together with the gut residual during filleting. Statistical analyses revealed the significance of the differences (Z=-2.98; p<0.01) observed between whole and filleted anchovies in oil. The same differences were found when the larval density was evaluated (Z=-2.98; p<0.02), with a value of 0.07 (s.d.=0.08) in whole fish compared to 0.01 (s.d=0.02) in fish fillets. The analyses were performed only in products preserved in oil considering that the salted products were all whole fish and the marinated ones were all filleted.~~-~~ The presence of ~~very low positivity found for marinated products (~~only one larva in the 25 marinated products~~)~~ analysed may be explained by the fact that this kind of products are usually filleted as fresh, hampering the parasitic migration from the viscera to the muscle. The very low contamination of industrially marinated anchovies sampled in this study agrees with the results of Sospedra et al., (2015) who analysed the same products from Spanish restaurants, while it is well known that domestically prepared marinated anchovies are one of the products most at risk for human anisakiasis (Bao et al., 2017; Mattiucci et al., 2013).

As concerns the viability of the larvae in semi processed anchovy products, it is known that salting may reduce the parasite hazard by killing anisakid larvae if salt content and time are adequate (Codex Alimentarius, 2012; Karl et al., 1994). Recently, the opinion No. 2007-SA-0379 of the French Food Safety Agency (AFSSA, 2007), reported that salting inactivates ~~A~~anisakid~~ae~~ larvae within 21 or 28 days depending on the final salt concentration in fish. In a recent work, all the larvae collected from anchovies salted according to a traditional Italian procedure (final salt concentration of 24.5%) were found dead after 15 days (Anastasio et al., 2016). ~~In addition, s~~Salted-ripened anchovies undergo a ripening process after salting that takes at least 2-3 months ~~for *E. encrasicolus*~~ (Anastasio et al., 2016). Therefore, the processing time in this kind of products is much longer than the one required to effectively kill the larvae.

Nematodes have been reported as highly resistant to the conditions created by traditional marinating methods, being able to survive for periods of a few days up to several weeks, depending on the concentration of salt, acetic acid and marinating times (AESAN 2007; Anastasio et al., 2016; Karl et al., 1994). In the traditional marinating process, the fish is left in a solution of vinegar and salt for less than 24 h. However, in a study the death of all larvae in fillets exposed to vinegar did not occur until day 13 (Sánchez -Monsalvez et al., 2005).

Considering that all the larvae found were dead, the processing technologies (including the preventive freezing treatment applied by FBOs according to the European legislation) for the production of semi preserved anchovy products analysed in this study seem to be effective to nullify the risk of contracting human gastrointestinal anisakiasis.

3.3 Dead anisakid larvae in semi-preserved anchovies: a potentially hazardous defect ~~and hazard?~~

~~Dead visible larvae can be considered a defect according to the definition of the Codex Alimentarius: "*A condition found in a product that fails to meet essential quality, composition and/or labelling provisions of the appropriate Codex product standards*" (Codex Alimentarius, 2012).~~ *Anisakis* sp. larvae are whitish to transparent and are not easily detected by the naked eye when they reside deeply embedded in fish muscles. On the contrary, they are evident when they infect in high number the celomatic cavity of fish species. This is particularly true in case of fresh fish containing live larvae ~~that~~ which can actively move and become evident also on the external surface (Guardone et al., 2016b). However, dead visible larvae can also be considered a defect according to the definition of the Codex Alimentarius: "*A condition found in a product that fails to meet essential quality, composition and/or labelling provisions of the appropriate Codex product standards*" (Codex Alimentarius, 2012). ~~also~~In fact, the presence of dead larvae ~~can~~ represents a reason to disqualify the fish product (Council Reg. EC No 2406/1996) and to consider it not fit for human consumption ~~according to~~(-Reg. (EC) No 178/2002).

The finding of parasitized products on the European market has elicited numerous RASFF (Rapid Alert System for Food and Feed) notifications over the years. Between 2010 and 2016, 409 notifications for the presence of anisakid larvae in fishery products were issued. Among these, the state of the product was indicated in 327 cases: besides fresh or chilled products (n=254), 81 referred to non-fresh products (frozen, smoked, salted, marinated and in oil) and thus probably involving dead larvae. In some of the heavily contaminated products found in this study, visible parasites were evident at visual inspection even before opening the packet or simply observing the fish edible tissue (Fig. 2). The observation of a similar contamination by consumers might result in disgust and rejection of the product and may also damage the brand reputation.

The ingestion of live *Anisakis* spp. worms may cause hazardous allergic reactions, including anaphylaxis, generally in association with gastrointestinal forms (EFSA, 2010; Daschner et al., 2012; Mattiucci et al., 2013). On the contrary, ~~T~~the potential of dead larvae to induce allergies in sensitized subjects is still debated (Daschner et al., 2012). Oral challenges performed in clearly allergic subjects with non-infective frozen or lyophilized larvae (Alonso-Gòmez et al., 2004; Sastre et al., 2000) and parasitic antigens (Baeza et al., 2004; Daschner et al., 2000) did not elicit any adverse effect. However, according to different authors, allergic reactions may also occur after ingestion of processed fish or parasite proteins alone (Audicana and Kennedy, 2008; Nieuwenhuizen et al. 2006) and it has been supposed that no-viable larvae or related antigens could be involved in chronic urticarial reactions (Mattiucci et al., 2017). Accordingly, the high prevalence (72.5%) of *Anisakis* larvae in frozen fillets of pink salmon was considered a public health issue due to the potential risk for allergic reactions in sensitized persons (Bilska-Zajac et al., 2016). The issue of allergic reactions is also related to different fish-eating habits, which probably account for different sensitization rates or the frequency of allergic symptoms in the different regions of the world (Mattiucci et al., 2017).

Therefore, even though it is not possible, on the basis of the current knowledge, to consider dead larvae as a proven hazard, appropriate measures should be implemented to avoid commercialization of obviously contaminated products. This would require FBOs involved in processing of salted, in oil or marinated anchovies, at industrial or artisanal level, to include appropriate risk management measures in theirs self-checking programs. In practice, FBOs should implement a system, based on the sampling method associated with a visual inspection as usually applied in Italy (D'Amico et al. 2014), or others of similar efficiency, to inspect batches of fresh anchovies. This would allow to select the most appropriate kind of processing (salting, preparation in oil or marinating) on the basis of the level of contamination detected. In fact, in this study, the level of contamination depended on the products' typology, being high in salted-ripened, medium in fillets in oil and very low for industrially marinated anchovies. The observed differences are linked to the preliminary preparation of the fish, in particular to the complete or incomplete removal of the viscera. Batches with a higher level of contamination should be destined to the production of marinated products. This would be economically advantageous for industries to reduce the costs arising from the discard of heavily contaminated batches of fresh anchovies and from the withdrawal of unfit product from the market. The continuously growing awareness of consumers and food authorities as to the occurrence of parasites in seafood, emphasises the importance of providing the fish processing industries with procedures able to reduce hazards and defects.

In fact, an incorrect risk communication can influence consumers' trust and even lead to a significant reduction of fish consumption.

A study conducted in Italy showed that the highest prevalence was detected along the Adriatic and Tyrrhenian coasts where marinated anchovies are a frequently consumed traditional food, often prepared at home. In seaside areas of Southern Italy, where anchovies are generally eaten fried rather than marinated, *Anisakis* hypersensitivity was much less commonly found (AAITO-IFIACI, 2011). The same association between *Anisakis* hypersensitivity and marinated seafood was observed in studies in Spain (Garcia et al., 1997; Valinas et al., 2001).

Therefore, even though it is not possible, on the basis of the current knowledge to consider the dead larvae as a proven hazard, a precautionary approach should be adopted. In practice, this would mean to adopt appropriate measures to reduce the risk of ingestion of dead larvae. … In fact, when the available supporting information and data are not sufficiently complete to enable a comprehensive risk assessment, official authorities may take measures based on the precautionary principle, while seeking more complete scientific and other data (Reg. EC 178/2002).

Incorrect risk communication can influence consumers' trust and even lead to a significant reduction of fish consumption. Therefore, the finding of contaminated products by FBOs within their self-control programs requires corrective actions to avoid that products heavily contaminated with dead larvae reach the market. The HACCP approach, usually aimed at ensuring food safety and preventing risks, can also be applied to cover food quality aspects, if instead of identifying the hazards of the process, potential defects are considered. The continuously growing awareness of consumers and food authorities as to the possible

~~presence of parasite or parasite-related quality defects in seafood emphasises the importance of providing the fish processing industries with feasible procedures able to monitor hazard and defect.~~

Conclusion

The present work ~~highlighted how~~ showed that semi preserved anchovy products heavily contaminated with *Anisakis* spp. larvae ~~can~~ reach the market and that the processing technology can influence the occurrence of parasites in semi-preserved products. Therefore, the batches intended for the production of these products (whole or filleted) should be accurately selected by industries, at the initial phases of the fish supply chain, according to the industrial fate of the raw material. Beyond the negligible risk for anisakidosis, due to the inactivation of larvae by freezing and processing technologies, the occurrence of dead parasites may cause immediate rejection in consumers. In addition, the risk related to allergic reactions in sensitized individuals is still an open issue. Providing the fish processing industries with procedures able to reduce hazards and defects is particularly important in the light of the continuously growing awareness of consumers and food authorities as to the occurrence of parasites in seafood. ~~and that the processing technology can influence the occurrence of parasites in semi-preserved products. Therefore, the batches intended for the production of these products (whole or filleted) should be accurately selected by industries, at the initial phases of the fish supply chain, according to the industrial fate of the raw material. Providing the fish processing industries with procedures able to reduce hazards and defects is particularly important in the light of the continuously growing awareness of consumers and food authorities as to the occurrence of parasites in seafood. In particular, the level of contamination depended on the products' typology, being high in salted-ripened, medium in fillets in oil and very low for industrially marinated anchovies. The observed differences are strictly linked to the processing procedure and to the preliminary preparation of the fish, in~~

particular to the complete or incomplete removal of the viscera. Beyond the negligible risk for anisakidosis, due to the inactivation of larvae by freezing and processing technologies, the presenceoccurrence of dead parasites may cause immediate rejection in consumers. In addition, the risk related to allergic reactions in sensitized individuals is still an open issue.

This study demonstratedshowed that the processing technology can influence the presence of parasites in the final semi-preserved products. Therefore, the batches intended for the production of these products (whole or filleted) should be accurately selected by industries, at the initial phases of the fish supply chain, according to the industrial fate of the raw material.

Acknowledgments

The authors wish to thank the Quality Office of UNICOOP Firenze for its contribution to the research activities, which were carried out in the framework of a survey aimed at assessing the overall quality of seafood products. The authors wish to thank Maria Vittoria Riina for the technical support in molecular analysis.

Captions

Figure 1 Presentation of the most part of the products analysed in the present study: whole salted anchovy (left), salted fillet preserved in oil (centre), marinated fillet (right).

Figure 2 From left to right: (a) salted anchovies heavily contaminated, one of the larvae was already visible from outside the glass jar before opening; (b) detail of another heavily contaminated salted product, the larva was visible from the external of the package; (c) larva in the muscle (edible part) of a salted anchovy; (d-e) parasites collected from the one of the most contaminated products: (d) natural light, (e) UV light.

References

AESAN, Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición, AESAN, 2007. Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición sobre medidas para reducir el riesgo asociado a la presencia de

Anisakis. Revista del Comité Científico de la AESAN, 6, 59–65. http://aesan.msssi.gob.es/AESAN/docs/docs/publicaciones_estudios/revistas/comite_cientifico_6.pdf. Accessed 10/07/2017. http://www.aecosan.msssi.gob.es/AECOSAN/docs/documentos/seguridad_alimentaria/evaluacion_riesgos/informes_comite/TRATAMIENTOS_ANISAKIS.pdf Accessed 24/10/2017

AFSSA, 2007. Opinion of the French Food Safety Agency (AFSSA) on a risk assessment request concerning the presence of Anisakidae in fishery products and the extension of the exemption from the freezing sanitary obligation of fishery products whose feeding is under control and for certain species of wild fish. Request no. 2007-SA-0379. https://www.anses.fr/fr/system/files/MIC2007sa0379EN.pdf Accessed 24/09/2017.

AAITO-IFIACI, 2011. *Anisakis* hypersensitivity in Italy: prevalence and clinical features: a multicenter study. Allergy 66, 1563-1569.

Alonso-Gómez, A., Moreno-Ancillo, A., López-Serrano, M. C., Suarez-de-Parga, J. M., Daschner, A., Caballero, M. T., Barranco, P., Cabanas, R., 2004. *Anisakis simplex* only provokes allergic symptoms when the worm parasitises the gastrointestinal tract. Parasitol. Res. 93(5), 378-384.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J., 1990. Basic Local Alignment Search Tool. J. Mol. Biol. 215, 403–410.

Anastasio, A., Smaldone, G., Cacace, D., Marrone, R., Voi, A. L., Santoro, M., Cringoli, G., Pozio, E., 2016. Inactivation of *Anisakis pegreffii* larvae in anchovies (*Engraulis encrasicolus*) by salting and quality assessment of finished product. Food Control, 64, 115-119.

Arcangeli, G., Galuppi, A., Bicchieri, M., Gamberini, R., Presicce, M., 1996. Prove sperimentali sulla vitalità di larve del genere *Anisakis* in semiconserve ittiche. Industria Conserve, 71(4), 502-507.

Armani, A., D'Amico, P., Cianti, L., Pistolesi, M., Susini, F., Gasperetti, L., Guarducci, M., Guidi, A., 2017. Assessment of Food Business Operators' training on parasitological risk management in sushi restaurants: a local survey in Florence, Italy. J. Environ. Health. In press.

Audicana, M. T., Kennedy, M. W., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. Rev. 21(2), 360-379.

Baeza, M. L., Rodríguez, A., Matheu, V., Rubio, M., Tornero, P., De Barrio, M., Herrero, T., Santaollala, M., Zubeldia, J. M. 2004. Characterization of allergens secreted by *Anisakis simplex* parasite: clinical relevance in comparison with somatic allergens. Clin. Exp. Allergy 34(2), 296-302.

Bao, M., Pierce, G. J., Pascual, S., González-Muñoz, M., Mattiucci, S., Mladineo, I., Cipriani, P., Bušelić, I., Strachan, N. J., 2017. Assessing the risk of an emerging zoonosis of worldwide concern: anisakiasis. Sci. Rep. 7, 43699.

Berland, B., 1989. Identification of larval nematodes from fish. In: Möller, H. (Ed.), Nematode Problems in North Atlantic Fish. Report from a Workshop in Kiel 3–4 April 1989.Int. Counc. Explor. Sea CM/F, 6.

Bernardi, C., Gustinelli, A., Fioravanti, M. L., Caffara, M., Mattiucci, S., Cattaneo, P., 2011. Prevalence and mean intensity of *Anisakis simplex* (*sensu stricto*) in European seabass (*Dicentrarchus labrax*) from Northeast Atlantic Ocean. Int. J. Food Microbiol. 148(1), 55–59.

Besteiro, I., Rodriguez, C. J., Tilve- Jar, C., Pascual, C., 2000. Selection of attributes for the sensory evaluation of anchovies during the ripening process. J Sens. Stud. 15(1), 65-77.

Bilska-Zając, E., Lalle, M., Różycki, M., Chmurzyńska, E., Kochanowski, M., Karamon, J., Sroka, J., Pozio, E., Cencek, T., 2016. High prevalence of Anisakidae larvae in marketed frozen fillets of pink salmons (*Oncorhynchus gorbuscha*). Food Control, 68, 216-219.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. J. Parasitol. 575–583.

Cipriani, P., Acerra, V., Bellisario, B., Sbaraglia, G. L., Cheleschi, R., Nascetti, G., Mattiucci, S., 2016. Larval migration of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in European anchovy, *Engraulis encrasicolus*: implications to seafood safety. Food Control, 59, 148-157.

Cipriani, P., Sbaraglia, G.L., Palomba, M., Giulietti, L., Bellisario, B., Bušelić, I., Mladineo, I., Cheleschi, R., Nascetti, G., Mattiucci, S., 2017. *Anisakis pegreffii* (Nematoda: Anisakidae) in European anchovy *Engraulis encrasicolus* from the Mediterranean Sea: Fishing ground as a predictor of parasite distribution. Fish. Res., in press.

Circular n. 1/97 of Liguria Region, 1997. Norme di comportamento ispettivo relativo alla presenza di larve della famiglia Anisakidae nell'acciuga (*Engraulis encrasicolus*). Published 24 March 1997.

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. http://www.fao.org/docrep/meeting/005/c0531e/C0531E09.htm Accessed 10/07/2017.

Codex Alimentarius, 2012. Code of practice for fish and fishery products. World Health Organization and Food and Agriculture Organization of the United Nations, Rome. ftp://ftp.fao.org/codex/Publications/Booklets/Practice_code_fish/CCFFP_2012_EN.pdf Accessed 18/09/2017.

Commission Regulation (EC) (2005) No 2074/2005 Laying down implementing measures for certain products under regulation (EC) No.853/2004oftheEuropeanparliamentandofthe 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.

Costa, A., Cammilleri, G., Graci, S., Buscemi, M.D., Vazzana, M., Principato, D., Giangrosso, G., Ferrantelli, V., 2016. Survey on the presence of *A. simplex* ss and *A. pegreffii* hybrid forms in Central-Western Mediterranean Sea. Parasitol. Int., 65(6), 696-701.

Council Regulation EC No 2406/1996 Laying down common marketing standards for certain fishery products. OJEC L334, 1–15.

Council Directive 91/493/EEC of 22 July 1991 laying down the health conditions for the production and the placing on the market of fishery products. OJ L 268, 15-34.

CTSV, 2007. TrichinEasy & PLYTricons *Anisakis* procedure. http://www.ctsv.biz/image-ctsv/PDF/TrichinEasy-anisakis.pdf Accessed 14/09/2017.

Czerner, M., Tomás, M. C., Yeannes, M. I., 2011. Ripening of salted anchovy (*Engraulis anchoita*): development of lipid oxidation, colour and other sensorial characteristics. J Sci. Food Agr. 91(4), 609-615.

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.

D'Amico, P., Armani, A., Gianfaldoni, D., Guidi, A., 2016. New provisions for the labelling of fishery and aquaculture products: Difficulties in the implementation of Regulation (EU) n. 1379/2013. Mar. Policy, 71, 147-156.

Daschner, A., Alonso-Gómez, A., Cabañas, R., Suarez-de-Parga, J. M., López-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(1), 176-181.

Daschner, A., Cuéllar, C., Rodero, M., 2012. The *Anisakis* allergy debate: does an evolutionary approach help? Trends Parasitol. 28(1), 9-15.

~~Decreto Legislativo 17 Luglio 2013 Informazioni obbligatorie a tutela del consumatore di pesce e cefalopodi freschi e di prodotti di acqua dolce, in attuazione dell'articolo 8, comma 4, del decreto-legge 13 settembre 2012 No. 158, convertito, con modificazioni, dalla legge 8 novembre 2012, No. 189. GU, 187.~~

EFSA, 2010. Scientific opinion on risk assessment of parasites in fishery products. EFSA Journal 8, 1543. http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2010.1543/epdf Accessed 24/10/2017.

EUMOFA, 2017. EU consumer habits regarding fishery and aquaculture products. Final report. https://www.eumofa.eu/documents/20178/84590/EU+consumer+habits_final+report+.pdf/5c61348d-a69c-449e-a606-f5615a3a7e4c Accessed 14/09/2017.

European Commission, 2014. Guidance document on the implementation of certain provisions of Regulation (EC) No 853/2004 on the hygiene of food of animal origin https://ec.europa.eu/food/sites/food/files/safety/docs/biosafety_fh_legis_guidance_reg-2004-853_en.pdf Accessed 18/09/2017.

Felix, M. M., Czerner, M., Ameztoy, I., Ramírez, E., Yeannes, M. I., 2016. Investigation of *Halococcus morrhuae* in salted-ripened anchovy products. Int. Food Res. J. 23(6), 2668-2674.

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(4), 629-634.

~~García, M., Moneo, I., Audicana, M.T., del Pozo, M.D., Muñoz, D., Fernández, E., Díez, J., Etxenagusia, M.A., Ansotegui, I.J. and de Corres, L.F., 1997. The use of IgE immunoblotting as a diagnostic tool in *Anisakis simplex* allergy. J Allergy Clin. Immunol. 99, 497–501.~~

Granata, L. A., Flick Jr, G. J., Martin, R. E., 2012. The seafood industry: species, products, processing, and safety. John Wiley & Sons, New Delhi.

Gómez-Morales, M. A., Castro, C. M., Lalle, M., Fernández, R., Pezzotti, P., Abollo, E., Pozio, E., Trial, T.R., 2017. UV-press method versus artificial digestion method to detect Anisakidae L3 in fish fillets: Comparative study and suitability for the industry. Fish. Res., in press.

Guardone, L., Lodola, L. B., Guidi, A., Armani, A. 2016a. *Anisakis* spp. in ready-to-eat fish products. XXIX SOIPA National Congress, Bari 21-24 June 2016. Available at: http://soipa.web.mtncompany.it/wp-content/uploads/2017/09/soipa-abstract-2016.pdf Accessed 24/10/2017

Guardone, L., Malandra, R., Costanzo, F., Castigliego, L., Tinacci, L., Gianfaldoni, D., Guidi, A., Armani, A., 2016b. 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? Food Anal. Method., 1-10.

Guardone, L., Nucera, D., Pergola, V., Costanzo, F., Costa, E., Guidi, A., Gianfaldoni, D., Armani, A., 2017. A rapid digestion method for the detection of anisakid larvae in European anchovy (*Engraulis encrasicolus*): visceral larvae as a predictive index of the overall level of fish batch infestation and marketability. Int. J. Food Microbiol. 250, 12-18

Hall, T. A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series, 41 pp. 95e98.

~~Hernández-Herrero, M. M., Roig-Sagués, A. X., López-Sabater, E. I., Rodríguez-Jerez, J. J., Mora-Ventura, M. T., 2000. SDS-PAGE of salted anchovies (*Engraulis encrasicholus* L) during the ripening process. Eur. Food Res. Technol. 212(1), 26-30.~~

Karl, H., Roepstorff, A., Huss, H. H., Bloemsma, B., 1994. Survival of *Anisakis* larvae in marinated herring fillets. Int. J. Food Sci. Technol. 29(6), 661-670.

Llarena-Reino, M., Piñeiro, C., Antonio, J., Outeriño, L., Vello, C., González, Á.F., Pascual, S., 2013. Optimization of the pepsin digestion method for anisakids inspection in the fishing industry. Vet. Parasitol. 191 (3), 276–283.

Mattiucci, S., Fazii, P., De Rosa, A., Paoletti, M., Megna, A.S., Glielmo, A., De Angelis, M., Costa, A., Meucci, C., Calvaruso, V., Sorrentini, I., 2013. Anisakiasis and gastroallergic reactions associated with *Anisakis pegreffii* infection, Italy. Emerg. Infect. Diseases, 19(3), 496.

Mattiucci, S., Colantoni, A., Crisafi, B., Mori- Ubaldini, F., Caponi, L., Fazii, P., Nascetti, G., Bruschi, F., 2017. IgE sensitization to *Anisakis pegreffii* in Italy: comparison of two methods for the diagnosis of allergic anisakiasis. Parasite Immunol. 39, 12340.

McLay, R., 1972. Marinades. Ministry of Agriculture, Fisheries and Food. http://www.fao.org/wairdocs/tan/x5932e/x5932e01.htm Accessed 16~~4~~/10~~9~~/2017.

~~Mladineo, I., Šimat, V., Miletić, J., Beck, R., Poljak, V., 2012. Molecular identification and population dynamic of *Anisakis pegreffii* (Nematoda: Anisakidae Dujardin, 1845) isolated from the *European anchovy* (*Engraulis encrasicolus* L.) in the Adriatic Sea. Int. J. Food Microbiol. 157 (2), 224–229.~~

Mohamed, S. B., Mendes, R., Slama, R. B., Oliveira, P., Silva, H. A., Bakhrouf, A., 2016. Changes in bacterial counts and biogenic amines during the ripening of salted anchovy (*Engraulis encrasicholus*). J. Food Nutr. Res. 4(5), 318-326.

Nadler, A. S., Hudspeth, D. S. S., 2000. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. J. Parasitol. 86, 380–393.

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(5), 1098-1105.

~~Real Decreto 1420/2006, de 1 de Diciembre, sobre prevencion de la parasitosis por anisakis en productos del la pesca suministrados por establicimientos que sirven comida a los consumidores finales o a colectividades. Boletin Oficial del Estado. https://www.boe.es/boe/dias/2006/12/19/pdfs/A44547-44549.pdf Accessed 18/09/2017.~~

Regulation EC No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and 1426 Food Anal. Methods, 2016. 9: 1418–1427 requirements of food law, establishing the European food safety authority and laying down procedures in matters of food safety. OJEC L31, 1–24.

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.

Sakanari, J.A., McKerrow, J.H., 1989. Anisakiasis. Clin. Microbiol. Rev. 2(3), 278–284.

Sánchez-Monsalvez, I., de Armas-Serra, C., Martinez, J., Dorado, M., Sanchez, A., Rodriguez-Caabeiro, F., 2005. A new procedure for marinating fresh anchovies and ensuring the rapid destruction of *Anisakis* larvae. J. Food Prot. 68(5), 1066-1072.
Sastre, J., Lluch- Bernal, M., Quirce, S., Arrieta, I., Lahoz, C., Del Amo, A., Fernández- Caldas, E., Marañón, F., 2000. A double- blind, placebo- controlled oral challenge study with lyophilized larvae and antigen of the fish parasite, *Anisakis simplex*. Allergy 55(6), 560-564.
Serracca, L., Battistini, R., Rossini, I., Carducci, A., Verani, M., Prearo, M., Tomei, L., De Montis, G., Ercolini, C., 2014. Food safety considerations in relation to Anisakis pegreffii in anchovies (*Engraulis encrasicolus*) and sardines (*Sardina pilchardus*) fished off the Ligurian Coast (Cinque Terre National Park, NW Mediterranean). Int. J. Food Microbiol., 190, 79-83.
Sospedra, I., Rubert, J., Soriano, J. M., Mañes, J., Fuentes, M. V., 2015. Prevalence of bacteria and absence of anisakid parasites in raw and prepared fish and seafood dishes in Spanish restaurants. J. Food Prot. 78(3), 615-618.
Tejada, M., Solas, M. T., Navas, A., Mendizabal, A., 2006. Scanning electron microscopy of *Anisakis* larvae following different treatments. J. Food Prot. 69(6), 1379-1387.
Triqui, R., Reineccius, G. A, 1995. Flavor development in the ripening of anchovy (*Engraulis encrasicholus* L.). J. Agric. Food. Chem., 43. 453-458.
~~Valinas, B., Lorenzo, S., Eiras, A., Figueiras, A., Sanmartin, M.L., Ubeira, F.M., 2001. Prevalence of and risk factors for IgE sensitization to *Anisakis simplex* in a Spanish population. Allergy 56, 667–671.~~
~~Velasco, A., Aldrey, A., Pérez-Martín, R. I., Sotelo, C. G., 2016. Assessment of the labelling accuracy of spanish semipreserved anchovies products by FINS (forensically informative nucleotide sequencing). Heliyon, 2(6), e00124~~.
Vidaček, S., de las Heras, C., Solas, M. T., Mendizábal, A., Rodriguez- Mahillo, A. I., González- Muñoz, M., Tejada, M., 2009. *Anisakis simplex* allergens remain active after conventional or microwave heating and pepsin treatments of chilled and frozen L3 larvae. J. Sci. Food Agr. 89(12), 1997-2002.

Table

Table 1 Summary of the results concerning ~~positivity~~contamination, number of collected larvae, range of larvae per product~~, and~~ mean number of larvae per product and density for each analysed category and overall

Product category (n)	~~Positivity (to~~ Products with at least one larvae~~):~~ n (% of ~~the total of~~contaminated products for each category)	~~Total n~~Number of ~~collected~~ larvae (% of the total ~~of~~ collected larvae~~products~~)	Range	Mean number of larvae per product	Density (larvae/gram)
Salted (33)	33 (100.0%)	1139 (88.8%)	1-105	34.5 (± 29.3 SD)	0.13
In oil (49)	24 (49.0%)	143 (11.1%)	0-28	2.9 (± 5.8 SD)	0.03
Marinated (25)	1 (4.0%)	1 (0.1%)	0-1	0.0 (± 0.2 SD)	0.00
Total (107)	**58 (54.2%)**	**1283**	**0-105**	**12.0 (± 22.5 SD)**	**0.05**

SD: Standard Deviation

Table

Table 2 Summary of the results obtained analysing the 107 products, sub-divided per product category and per brand. NA: Not Available

Commercial name of the product	Product codes	Geographical origin	Product presentation	Mean net weight/ product (g)	N analysed products	Total n L3 *Anisakis* spp.	Density (larvae/gram)	Range	N ~~positive~~ contaminated products[a]	N products exceeding MA threshold[b]	Molecular identification (n analyzed)
Salted anchovies Brand 1	RTE25, RTE33, RTE72, RTE73, RTE74	Atlantic Ocean NE FAO 27.VIII.C, Cantabrian Sea	whole	153.8	5	105	0.1	5-51	5	4	*A. pegreffii* (1) *A. simplex* (11)
Salted anchovies Brand 2	RTE12, RTE36, RTE42, RTE63, RTE69, RTE70	South Gulf of Biscay, Cantabrian Sea	whole	195.8	6	165	0.1	1-42	6	5	*A. pegreffii* (11) *A. simplex* (2)
Salted anchovies Brand 3	RTE 46, RTE48, RTE49, RTE87, RTE88, RTE89	Mediterranean Sea FAO 37.2.1	whole	100.0	6	55	0.1	3-15	6	5	*A. pegreffii* (12) *A. simplex* (2)
Salted anchovies Brand 4	RTE53, RTE75, RTE76, RTE93, RTE94	FAO 37	whole	221.4	5	146	0.1	20-48	5	5	*A. pegreffii* (13) *A. simplex* (1)
Salted anchovies Brand 5	RTE5, RTE6, RTE7, RTE24, RTE144, RTE145	FAO 37.2.1	whole	427.9	6	439	0.2	39-105	6	6	*A. pegreffii* (16) *A. simplex* (2)
Salted anchovies Brand 6	RTE32, RTE110, RTE121, RTE126, RTE149	FAO 37.2	whole	571.8	5	229	0.1	2-87	5	4	*A. pegreffii* (12) *A. simplex* (1)
Total salted anchovies (%)					**33**	**1139**		**1-105**	**33 (100.0)**	**29 (87.9)**	***A. pegreffii* (65) *A. simplex* (19)**
Anchovies in oil Brand 7	RTE23, RTE40, RTE65, RTE67, RTE71	FAO 37	whole (red)	73.2	5	40	0.1	3-13	5	5	*A. pegreffii* (5) *A. simplex* (4)
Anchovies in oil Brand 2	RTE37, RTE58, RTE64, RTE66, RTE95	Not reported	whole (red)	109.5	5	80	0.1	8-28	5	5	*A. pegreffii* (13)
Anchovies in oil Brand 1	RTE54, RTE59, RTE61, RTE68, RTE92	Not reported	whole (whitish)	88.4	5	0	0.0	0	0	0	
Anchovies in oil Brand 8	RTE127, RTE128, RTE129	Not reported	whole[c] (whitish)	285.0	3	1	0.0	0-1	1	NA[c]	
Anchovies in oil Brand 9	RTE103, RTE104, RTE105, RTE106	Mediterranean Sea FAO 37	fillets	88.0	4	1	0.0	0-1	1	NA	*A. simplex* (1)
Anchovies in oil Brand 5	RTE8, RTE142, RTE143	FAO 37	fillets	140.0	3	2	0.0	0-1	2	NA	*A. pegreffii* (1)
Anchovies in oil Brand 10	RTE34, RTE108, RTE109	FAO 37	fillets	60.0	3	2	0.0	0-1	2	NA	*A. simplex* (1)
Anchovies in oil Brand 7	RTE116, RTE117, RTE118	FAO 37	fillets	50.7	3	0	0.0	0	0	NA	
Anchovies in oil Brand 11	RTE136, RTE137, RTE138	Not reported	fillets	82.3	3	0	0.0	0	0	NA	

Anchovies in oil Brand 12	RTE133, RTE134, RTE135	FAO 27	fillets	64.7	3	10	0.0	2-5	3	NA	*A. pegreffii* (2) *A. simplex* (4)
Anchovies in oil Brand 13	RTE130, RTE131, RTE132	FAO 37.2.1	fillets	51.2	3	4	0.0	0-1	2	NA	*A. pegreffii* (3)
Anchovies in oil Brand 14	RTE119, RTE124, RTE125	FAO 37.2	fillets	112.4	3	2	0.0	0-1	2	NA	*A. pegreffii* (1) *A. simplex* (1)
Anchovies in oil Brand 15	RTE139, RTE140, RTE141	Not reported	fillets	59.3	3	0	0.0	0	0	NA	
Anchovies in oil Brand 6	RTE120, RTE122, RTE123	FAO 37.2.1	fillets	22.3	3	1	0.0	0-1	1	NA	*A. pegreffii* (1)
Total anchovies in oil (%)					**49**	**143**		**0-28**	**24 (49.0)**		***A. pegreffii* (26)** ***A. simplex* (11)**
Marinated anchovies Brand 9	RTE39, RTE102, RTE107, RTE150, RTE151	Mediterranean Sea FAO 37	fillets	70.1	5	0	0.0	0	0	NA	
Marinated anchovies Brand 12	RTE52, RTE60, RTE62, RTE98, RTE99	Adriatic Sea	fillets	124.4	5	0	0.0	0	0	NA	
Marinated anchovies Brand 2	RTE55, RTE56, RTE57, RTE96, RTE97	Not reported	fillets	108.0	5	0	0.0	0	0	NA	
Marinated anchovies Brand 16	RTE113, RTE114, RET115, RTE152, RTE153	Adriatic Sea	fillets	135.6	5	1	0.0	0-1	1	NA	*A. pegreffii* (1)
Marinated anchovies Brand 17	RTE9, RTE10, RTE146, RTE147, RTE148	Not reported	fillets	121.9	5	0	0.0	0	0	NA	
Total marinated anchovies (%)					**25**	**1**		**0-1**	**1 (4.0)**		***A. pegreffii* (1)**
Total (%)					**107**	**1283**		**0-105**	**58 (54.2)**		***A. pegreffii* (92)** ***A. simplex* (30)**

[a]presence of at least 1 larva; MA: mean abundance; L3: third stage larvae; [b]MA threshold~~LpG 1~~ proposed in Guardone et al., 2016b~~2017~~; [c]despite the fact that the product was originally prepared with whole anchovies it was not possible to count the~~ir~~ number of specimens due to the loss of anatomical integrity~~ of the specimens~~ induced by processing.

Figure
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