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

Corresponding Author: Dr. Andrea Armani,
Corresponding Author's Institution: University of Pisa

First Author: Lisa Guardone
Order of Authors: Lisa Guardone; Daniele Nucera; Laura B Lodola; Lara Tinacci; PierLuigi Acutis; Alessandra Guidi; Andrea Armani

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


*aFishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy).

bDepartment of Agriculture, Forest and Food Science, University of Turin, Largo Braccini 2, 10095, Grugliasco - Torino (Italy).

cExperimental Institute of Zooprophylaxis Piedmont, Liguria and Aosta Valley, 10154 Turin, Italy;

*corresponding author:
Postal address: FishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy)
Tel: +390502210207; Fax: +390502210213
Email: andrea.armani@unipi.it
Abstract

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%, density 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 (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.
Keywords
Anisakid larvae, anchovies, *Engraulis encrasicolus*, artificial digestion, contamination, semi-preserved seafood products, *Anisakis pegreffii, Anisakis simplex*, 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 legislation requires preventive freezing treatment also in case of domestic preparation of raw, marinated or not fully cooked fish (D’Amico et al., 2014), 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; Serracca et al., 2014; 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 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 that 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). Although 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, therefore, 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 anisakid parasites in these kind of preparations (Fraulo et al., 2014; Sospedra et al., 2015), the aim of this study was to assess the 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, 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 anchovies, the number of specimens was counted. 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 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. 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). All the larvae were recovered with this procedure, which was then applied to all the samples. A maximum of 200 g of tissue was digested per time.

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. PCR products were sequenced 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: 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 $\chi^2$ 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 $\chi^2$ (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 the number of larvae per product, frequency of products contaminated by at least one larva and larval density per gram were also analysed in respect to the product being composed by whole anchovies or by fillets. The analyses were carried out using $\chi^2$ (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 and the value obtained was used to issue a marketability judgement. The MA threshold was calculated by applying an approach widely used throughout Italy (D’Amico et al., 2014) which defines the maximum number of tolerated larvae in fresh batches of anchovies (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. As mentioned, trials were performed using dead Anisakis spp. larvae. Since all the larvae were recovered after the digestion, the procedure was considered suitable for semi-preserved products.

At least one visible larva was found in 58 (54.2%) of the total 107 products analysed. A total of 1283 visible larvae were collected, which were all morphologically identified as Anisakis sp. 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, although emission of fluorescence is known to not always discriminate between live and dead larvae (Tejada et al., 2006; Vidaček et al., 2009), spontaneous and stimulated movements of the larvae were absent in this study. Among the subset of 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, 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”
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 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 the 33 products (100%). Totally, 1139 larvae were collected in this category, corresponding to 88.8% of the total collected larvae. The mean number of larvae per product was 34.5 (±29.3 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.

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).

The MA varied from 0.0 to 3.9. Twenty-nine products (87.9%) exceeded the MA threshold previously set for fresh anchovies. No differences in MA values were observed 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 24 (49.0%) products in oil and a total of 143
larvae 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 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. In 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 the 5 products of brand 1. The high
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 were observed in whole and filleted products (at least
one larva was found in 61.1% of the whole products and 41.9% of the fillets). Within whole
products, differences were also observed between red and white fish: 83.9% 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 the 25 marinated products (4.0%). The larva 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 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’ $\chi^2$=69.95; p<0.001), frequency of contaminated products ($\chi^2$=50.34; p<0.001) and density of larvae per gram ($\chi^2$=58.89; p<0.001).

The average number of larvae per product was around 35, 3 and 0 for salted, in oil and marinated products, respectively (Table 1). Similarly, the frequency of contaminated products 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.

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 contrary, 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 each analysed salted product. Similarly, all the whole salted anchovies in oil were contaminated with a high number of larvae. Overall 1259 larvae were found in whole salted anchovies in brine and 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 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 anisakid 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.,
Salted-ripened anchovies undergo a ripening process after salting that takes at least 2-3 months (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

*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 coelomic cavity of fish species. This is particularly true in case of fresh fish containing live larvae 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). In fact, the presence of dead larvae can represent a reason to disqualify the fish product (Council Reg. EC No 2406/1996) and to consider it not fit for human consumption (Council Reg. EC No 2406/1996; 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, 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-Gómez 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 on the basis of the current knowledge it is not possible, on the basis of the current knowledge, to consider dead larvae cannot be considered as a proven hazard, appropriate measures should be implemented to avoid commercialization of obviously contaminated products must be avoided. This would require FBOs processing involved in processing of salted, in oil or marinated anchovies; at industrial or artisanal level, to should 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. In particular, batches with a higher level of contamination should be destined to the production of marinated or filleted 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.

Conclusion
The present work showed that semi preserved anchovy products heavily contaminated with Anisakis spp. larvae 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.

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


Cipriani, P., Acerra, V., Bellisario, B., Sbaraglia, G. L., Cheleschi, R., Nascetti, G., Mattiucci, S., 2016. Larval migration of the zoonotic parasite *Anisakis pegreffii*


Changes in bacterial counts and biogenic amines during the ripening of salted anchovy
Ascaridida) based on three genes and morphology: hypotheses of structural and sequence
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
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 specific hygiene rules for on the hygiene of foodstuffs. OJEU L139, 55.
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.,
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.
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., Mendizábal, A., 2006. Scanning electron microscopy of
Anisakis larvae following different treatments. J. Food Prot. 69(6), 1379-1387.
Table 1 Summary of the results concerning contamination, number of collected larvae, range of larvae per product, mean number of larvae per product and density for each analysed category and overall

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

SD: Standard Deviation
**Table 2** Summary of the results obtained analysing the 107 products divided per product category and per brand.

<table>
<thead>
<tr>
<th>Commercial name of the product</th>
<th>Product codes</th>
<th>Geographical origin</th>
<th>Product presentation</th>
<th>Mean net weight/product (g)</th>
<th>N analysed products</th>
<th>Total n L3 Anisakis spp.</th>
<th>Total n L3/total net weight</th>
<th>Range</th>
<th>N positive products</th>
<th>N products exceeding MA threshold</th>
<th>Molecular analysis (n analyzed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salted anchovies Brand 1</td>
<td>RTE25, RTE33, RTE72, RTE73, RTE74</td>
<td>Atlantic Ocean NE FAO 27.VIII.C, Cantabrian Sea</td>
<td>whole</td>
<td>153.8</td>
<td>5</td>
<td>105</td>
<td>0.137</td>
<td>5-51</td>
<td>5</td>
<td>4</td>
<td>A. pegreffii (1), A. simplex (11)</td>
</tr>
<tr>
<td>Salted anchovies Brand 2</td>
<td>RTE12, RTE36, RTE42, RTE63, RTE69, RTE70</td>
<td>South Gulf of Biscay, Cantabrian Sea</td>
<td>whole</td>
<td>195.8</td>
<td>6</td>
<td>165</td>
<td>0.140</td>
<td>1-42</td>
<td>6</td>
<td>5</td>
<td>A. pegreffii (11), A. simplex (2)</td>
</tr>
<tr>
<td>Salted anchovies Brand 3</td>
<td>RTE46, RTE48, RTE49, RTE87, RTE88, RTE89</td>
<td>Mediterranean Sea FAO 37.2.1</td>
<td>whole</td>
<td>100</td>
<td>6</td>
<td>55</td>
<td>0.092</td>
<td>3-15</td>
<td>6</td>
<td>5</td>
<td>A. pegreffii (12), A. simplex (2)</td>
</tr>
<tr>
<td>Salted anchovies Brand 4</td>
<td>RTE53, RTE75, RTE76, RTE93, RTE94</td>
<td>FAO 37</td>
<td>whole</td>
<td>221.4</td>
<td>5</td>
<td>146</td>
<td>0.132</td>
<td>20-48</td>
<td>5</td>
<td>5</td>
<td>A. pegreffii (13), A. simplex (2)</td>
</tr>
<tr>
<td>Salted anchovies Brand 5</td>
<td>RTE5, RTE6, RTE7, RTE24, RTE69, RTE70</td>
<td>FAO 37.2.1</td>
<td>whole</td>
<td>427.9</td>
<td>6</td>
<td>439</td>
<td>0.171</td>
<td>39-105</td>
<td>6</td>
<td>6</td>
<td>A. pegreffii (16), A. simplex (2)</td>
</tr>
<tr>
<td>Salted anchovies Brand 6</td>
<td>RTE32, RTE110, RTE121, RTE126, RTE149</td>
<td>FAO 37.2</td>
<td>whole</td>
<td>571.8</td>
<td>5</td>
<td>229</td>
<td>0.080</td>
<td>2-87</td>
<td>5</td>
<td>4</td>
<td>A. pegreffii (12), A. simplex (1)</td>
</tr>
<tr>
<td>Total salted anchovies (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>33</td>
<td>1139</td>
<td></td>
<td>1-105</td>
<td>33</td>
<td>29</td>
<td>A. pegreffii (65), A. simplex (19)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 7</td>
<td>RTE23, RTE40, RTE65, RTE67, RTE71</td>
<td>FAO 37</td>
<td>whole (red)</td>
<td>73.2</td>
<td>5</td>
<td>40</td>
<td>0.109</td>
<td>3-13</td>
<td>5</td>
<td>5</td>
<td>A. pegreffii (5), A. simplex (4)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 2</td>
<td>RTE37, RTE58, RTE64, RTE65, RTE95</td>
<td>Not reported</td>
<td>whole (red)</td>
<td>109.5</td>
<td>5</td>
<td>80</td>
<td>0.146</td>
<td>8-28</td>
<td>5</td>
<td>5</td>
<td>A. pegreffii (13)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 1</td>
<td>RTE54, RTE59, RTE61, RTE68, RTE92</td>
<td>Not reported</td>
<td>whole (whitish)</td>
<td>88.4</td>
<td>5</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Anchovies in oil Brand 8</td>
<td>RTE127, RTE128, RTE129</td>
<td>Not reported</td>
<td>whole (whitish)</td>
<td>285</td>
<td>3</td>
<td>1</td>
<td>0.001</td>
<td>0-1</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Anchovies in oil Brand 9</td>
<td>RTE103, RTE104, RTE105, RTE106</td>
<td>Mediterranean Sea FAO 37</td>
<td>fillets</td>
<td>88</td>
<td>4</td>
<td>1</td>
<td>0.003</td>
<td>0-1</td>
<td>1</td>
<td>ND</td>
<td>A. simplex (1)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 5</td>
<td>RTE8, RTE142, RTE143</td>
<td>FAO 37</td>
<td>fillets</td>
<td>140</td>
<td>3</td>
<td>2</td>
<td>0.005</td>
<td>0-1</td>
<td>2</td>
<td>ND</td>
<td>A. pegreffii (1)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 10</td>
<td>RTE34, RTE108, RTE109</td>
<td>FAO 37</td>
<td>fillets</td>
<td>60</td>
<td>3</td>
<td>2</td>
<td>0.011</td>
<td>0-1</td>
<td>2</td>
<td>ND</td>
<td>A. simplex (1)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 11</td>
<td>RTE116, RTE117, RTE118</td>
<td>FAO 37</td>
<td>fillets</td>
<td>50.7</td>
<td>3</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Anchovies in oil Brand 11</td>
<td>RTE136, RTE137, RTE138</td>
<td>Not reported</td>
<td>fillets</td>
<td>82.3</td>
<td>3</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Anchovies in oil Brand 12</td>
<td>RTE133, RTE134, RTE135</td>
<td>FAO 27</td>
<td>fillets</td>
<td>64.7</td>
<td>3</td>
<td>10</td>
<td>0.052</td>
<td>2-5</td>
<td>3</td>
<td>ND</td>
<td>A. pegreffii (2), A. simplex (4)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------</td>
<td>--------</td>
<td>---------</td>
<td>------</td>
<td>---</td>
<td>---</td>
<td>-------</td>
<td>-----</td>
<td>---</td>
<td>----</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Anchovies in oil Brand 13</td>
<td>RTE130, RTE131, RTE132</td>
<td>FAO 37.2.1</td>
<td>fillets</td>
<td>51.2</td>
<td>3</td>
<td>4</td>
<td>0.026</td>
<td>0-1</td>
<td>2</td>
<td>ND</td>
<td>A. pegreffii (3)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 14</td>
<td>RTE119, RTE124, RTE125</td>
<td>FAO 37.2</td>
<td>fillets</td>
<td>112.4</td>
<td>3</td>
<td>2</td>
<td>0.006</td>
<td>0-1</td>
<td>2</td>
<td>ND</td>
<td>A. pegreffii (1), A. simplex (1)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 15</td>
<td>RTE139, RTE140, RTE141</td>
<td>Not reported</td>
<td>fillets</td>
<td>59.3</td>
<td>3</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>A. pegreffii (1)</td>
</tr>
<tr>
<td>Anchovies in oil Brand 6</td>
<td>RTE120, RTE122, RTE123</td>
<td>FAO 37.2.1</td>
<td>fillets</td>
<td>22.3</td>
<td>3</td>
<td>1</td>
<td>0.015</td>
<td>0-1</td>
<td>1</td>
<td>ND</td>
<td>A. pegreffii (1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total anchovies in oil (%)</th>
<th>49</th>
<th>143</th>
<th>0-28</th>
<th>24 (48.98)</th>
<th>A. pegreffii (26), A. simplex (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marinated anchovies Brand 9</td>
<td>RTE39, RTE102, RTE107, RTE150, RTE151</td>
<td>Mediterranean Sea FAO 37</td>
<td>fillets</td>
<td>70.1</td>
<td>5</td>
</tr>
<tr>
<td>Marinated anchovies Brand 12</td>
<td>RTE52, RTE60, RTE62, RTE98, RTE99</td>
<td>Adriatic Sea</td>
<td>fillets</td>
<td>124.4</td>
<td>5</td>
</tr>
<tr>
<td>Marinated anchovies Brand 2</td>
<td>RTE55, RTE56, RTE57, RTE96, RTE97</td>
<td>Not reported</td>
<td>fillets</td>
<td>108</td>
<td>5</td>
</tr>
<tr>
<td>Marinated anchovies Brand 16</td>
<td>RTE113, RTE114, RET115, RTE152, RTE153</td>
<td>Adriatic Sea</td>
<td>fillets</td>
<td>135.6</td>
<td>5</td>
</tr>
<tr>
<td>Marinated anchovies Brand 17</td>
<td>RTE9, RTE10, RTE146, RTE147, RTE148</td>
<td>Not reported</td>
<td>fillets</td>
<td>121.9</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total marinated anchovies (%)</th>
<th>25</th>
<th>1</th>
<th>0-1</th>
<th>1 (4)</th>
<th>A. pegreffii (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (%)</td>
<td>107</td>
<td>1283</td>
<td>0-105</td>
<td>58 (54.20)</td>
<td>A. pegreffii (92), A. simplex (30)</td>
</tr>
</tbody>
</table>

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