Risk-based scoring and genetic identification for anisakids in frozen fish products from Atlantic FAO areas

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Abstract

Background: The presence of Anisakis larvae in fish represents a major public health concern. Effective risk management procedures should be applied to prevent heavily infected products from reaching the market. The aim of the study is to provide preliminary data on parasite exposure and risk classification in frozen fish products by applying a risk categorization scheme (site, abundance, density and epidemiology – SADE) and Fish Parasite Rating (FPR) method. Fish and cephalopods samples (N = 771) from 5 different FAO Atlantic areas were examined and categorized after an accurate visual inspection and a chloro-peptic digestion.

Results: In 25 out of 33 fish species parasite larvae were found. 10897 anisakids larvae were collected and identified to genus level. Molva dypterygia, Conger conger, Zeus faber and Aphanopus carbo were shown to be the most highly infected species. SADE and FPR scores were 1 and poor, respectively, for the referred species, because of the disseminated Anisakis infection and commercial rejection.

Conclusion: SADE/FPR method showed high specificity and accuracy. The information provided in this work could be used in early warning systems for the detection of parasites in fishery products and might help fishing industries in establishing management strategies for infected stocks in terms of cost saving decisions.

Keywords: Anisakis, food safety, risk ranking tool, zoonotic risk, fish inspection
European legislation [16] enforces an accurate visual inspection during the official control and in self-monitoring programs to prevent fish borne zoonoses: in this context Food Business Operators (FBO) must ensure that no fishery products obviously contaminated with visible parasites reach the consumers. 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” [17], a fishery product is considered obviously contaminated if visible parasites are found in edible portions; however, a maximum number of parasites was not defined. Furthermore, the application of visual inspection procedure in the fishery industry depends on the ability and training of FBO [18]. Because the presence of dead visible parasites could only represent a defect [19, 20] altering the global products quality and in order to comply with the EU prescriptions, in addition to the official control and self-monitoring procedures, the most practical procedure could be the use of a predicting scheme for evaluation of nematode larvae in the edible part of the fish batches as suggested by the European Food Safety Authority (EFSA) [21]. The use of the SADE scheme (acronym of Site of infection; Assurance of quality; Demography - density of parasites; Epidemiology of parasites) proposed by Llarena-reino et al., [22], combined with the Fish Parasite Rating (FPR) method [23], aimed at precisely evaluating the likely outcome of infected fish lots, which could be useful tools. FPR standard is a certified Community Trade Mark - Register No 012266607 at the Office for Harmonization in the Internal Market (OHIM) and provides the staging of fish lots, helping in planning manufacture, commercial, and research decisions during self-management programs. The aim of this research is to provide data on parasite risk exposure in commercial frozen fishery products collected in Atlantic FAO areas using the SADE scheme combined with FPR method in comparison with the official visual inspection procedure.

Results
Parasites frequency in fish sub lots
A total of eight fish species (24.24% of sampled fish species/sub lots) were anisakid-free. Nematode larvae were not detected in Mallotus villosus, Glyptocephalus cynoglossus, Dicologlossa cuneata, Galeoides decadactylus, Trachurus trecae, Sallolota australis, Atlantoraja castelnaui and Serranus cabrilla. A total of 10897 anisakid larvae were collected and identified to genus level in the flesh of 25 species. Among these species, M. dypterygia, C. conger, Z. faber and A. carbo, coming from FAO area 27 (Northeast Atlantic) were the most highly parasitized (Table 1) showing a total prevalence of infection (P) of 100% with a mean abundance (MA ± SD) of 204.52 ± 91.14, 115.16 ± 96.77, 44.96 ± 32.66, 74.1 ± 28.55 respectively. In these species, 90.45 % of the total larvae were detected: in particular M. dypterygia reached the highest density of parasites (102.26 larvae/kg). As much as 46.97 % of total larvae in this species were detected. The statistical analyses indicate that there was a correlation between MA and fish sample mean weight (p < 0.001).

The hypaxial region was the most infected location. In fact, in 11 fish species, anisakid larvae were found only in this region and in general the 98.53% of larvae (n. 10737) were identified in this location. In 50% of the samples, the epaxial infection took place simultaneously with hypaxial location.

Parasite frequency in fishing areas
P of infection (± CI 95%) MA and mean intensity (MI) (± SD) in the different fishing grounds of the study are reported in Table 2. No parasites were found in FAO area 34. Table 2 shows the comparison of P between different FAO areas: significative statistical differences (p < 0.0001) between FAO areas were found. In this study, regarding MA and MI, significative statistical differences between FAO area 27 and the other sampling areas were found (p < 0.001).

Parasite identification
The results show mixed infection in 45.83 % of the fish sub lot examined. All sequences obtained in this study shared 99-100 % nucleotide identity with other sequences of anisakid species deposited in the GenBank (accession ID and web links for each identified parasite, linked to fish species and FAO area, are indicated in the supplementary materials) belonging to Anisakis simplex sensu stricto, Anisakis pegreffii, Anisakis typica, Anisakis berlandi, Pseudoterranova cattani, Pseudoterranova decipiens s.l., Contracaecum osculatum s.l. and Hysterothylacium aduncum.

In this study A. simplex was the main parasite isolated in fishery products from FAO area 21 (100%) and from FAO area 27 (88.40%), while A. pegreffii was the main parasite isolated in fishery products from FAO area 41 (65.9%) and from FAO area 47 (63.82%). Fish collected from FAO area 41 showed the highest variability in terms of different species of parasites found.

Risk categorisation
Table 4 shows inspection data categorized by the SADE and FPR scoring systems. Using visual inspection, 36.36% of the lots didn't meet the EU standards [16] and were rejected. The rejected batches during the naked eye visual inspection present at least 1 visible larva in the edible portion. Over 66 % of fish sub lots have been accepted as they present less than one parasite in the flesh, expressed as MA [24] (Table 1). MA, due to its correlation with P and with the number of samples [25], could
Table 1 Infection values according to Bush et al. 1997

| Sub lot host species          | Parasites Found | Correctly classified | PREVALENCE (% ± CI 95%) | MEAN INTENSITY ± SD | MEAN ABUNDANCE ± SD |
|------------------------------|-----------------|----------------------|-------------------------|---------------------|---------------------|
|                              | Epaxial         | Hypaxial             | Total                   | Epaxial             | Hypaxial            | Total |
| Alepocephalus bairdii        | 14              | 5                    | 0.00                    | 16.00 ± 0.14        | 16.00 ± 0.14        | 0.00  |
| Aphanopus carbo              | 741             | 6                    | 60.00 ± 0.30            | 100.00              | 100.00              | 0.8 ± 2.29 |
| Atlanticoglossa castelnaui   | 0               | 0                    | 0.00                    | 0.00                | 0.00                | 0.00  |
| Grelbrinthus fasciolus       | 3               | 3                    | 4.00 ± 0.07             | 8.00 ± 0.10         | 12.00 ± 0.12        | 1.00  |
| Oupes harenus                | 13              | 12                   | 0.00                    | 32.00 ± 0.18        | 32.00 ± 0.18        | 0.00  |
| Conger conger                | 2879            | 12                   | 1.33 ± 2.56             | 113.56 ± 96.34      | 115.16 ± 96.77      | 1.6 ± 2.08 |
| Dicolyoglossa cuneata        | 0               | 0                    | 0.00                    | 0.00                | 0.00                | 0.00  |
| Genypterus blacoides         | 0               | 0                    | 0.00                    | 0.00                | 0.00                | 0.00  |
| Gymnocephalus bicoxoides     | 83              | 11                   | 1.57 ± 0.78             | 4.50 ± 4.93         | 4.15 ± 4.76         | 0.44 ± 0.82 |
| Hex argentinus               | 2               | 2                    | 100.00 ± 0.18           | 2.00                | 0.20 ± 0.63         | 0.00  |
| Lepidopus caudatus           | 44              | 9                    | 0.00                    | 100.00              | 100.00              | 0.00  |
| Macrurus magellanicas        | 3               | 2                    | 3.57 ± 0.06             | 7.14 ± 0.01         | 2.00                | 1.00  |
| Macrurus berglar             | 314             | 13                   | 0.00                    | 84.00 ± 0.14        | 84.00 ± 0.14        | 0.00  |
| Melanogrammus aeglefinus     | 7               | 5                    | 12.00 ± 0.12            | 0.00                | 2.33 ± 0.57         | 0.00  |
| Merluccius paradoxus         | 15              | 14                   | 2.94 ± 0.05             | 23.52 ± 0.14        | 23.52 ± 0.14        | 1.00  |
| Merluccius polli             | 3               | 3                    | 10.00 ± 0.10            | 0.00                | 1.00                | 0.00  |
| Micromesistius australis     | 61              | 12                   | 11.76 ± 0.15            | 70.58 ± 0.21        | 70.58 ± 0.21        | 1.00  |
| Molva dypterygia             | 5113            | 8                    | 36.00 ± 0.18            | 84.00 ± 0.14        | 84.00 ± 0.14        | 3.55 ± 6.57 |
| Patagonosthenus rahmsayi     | 7               | 0                    | 16.00 ± 0.14            | 0.00                | 1.75 ± 0.88         | 0.00  |
| Regalohus gilsoni            | 4               | 3                    | 36.00 ± 0.28            | 13.33 ± 0.57        | 13.33 ± 0.57        | 0.00  |
| Reinhardtius hippoglossoides | 125             | 14                   | 44.00 ± 0.19            | 8.92 ± 14.2         | 8.92 ± 14.2         | 0.00  |
| Salintra australis           | 0               | 0                    | 0.00                    | 0.00                | 0.00                | 0.00  |
| Saranus cabralia             | 0               | 0                    | 0.00                    | 0.00                | 0.00                | 0.00  |
| Todarodes angolensis         | 3               | 3                    | 100.00 ± 0.18           | 2.22 ± 1.48         | 203.72 ± 90.78      | 0.80 ± 1.38 |
| Trachurus trachurus          | 68              | 12                   | 45.45 ± 0.20            | 16.00 ± 0.14        | 16.00 ± 0.14        | 0.00  |
| Trachurus trachurus          | 0               | 0                    | 0.00                    | 0.00                | 0.00                | 0.00  |
| Trachysis sasbris            | 18              | 6                    | 32.00 ± 0.18            | 2.25 ± 1.83         | 2.25 ± 1.83         | 0.00  |
| Urophycis Chus               | 31              | 7                    | 100.00 ± 0.10           | 100.00              | 100.00              | 1.03 ± 4.52 |
| Zeus faber                  | 1124            | 12                   | 72.00 ± 0.17            | 100.00              | 100.00              | 2.05 ± 1.35 |

*Table created by a large language model.*
be used to estimate the degree of infestation [26], partic- 
larly in the case of fishery products sold in batches. 
With regard to the naked eye rejected products, 33.33% 
of the total rejections belong to FAO 27. Not infected 
fish batches in FAO area 34 were found.

Using the SADE/FPR schemes, 27.27 % of fish sub lots 
(M. hubbsi, M. australis, U. Chus, G. blacoides, M. para-
doxus, A. carbo, Z. faber, C. conger, M. dypterygia) were 
assigned a low SADE score (from 1 to 3) corresponding to 
a “poor” FPR standard. Hence, these fish lots must be dis-
carded. The lowest score (SADE 1) was assigned to A. 
carbo, Z. faber, C. conger and M. dypterygia, belonging to 
FAO 27, corresponding to the 44.44 % of the total rejec-
tion. No statistical differences ($p = 0.3711$) between 
SADE/FPR outcomes and visual inspection were observed.

Finally, the non-zoonotic H. aduncum (Raphidiascar-
dae) was also detected in I. argentinus; this parasite is 
generally considered not zoonotic, even if a case of inva-
sive gastro-allergic infection was recently reported [27]. 
This result did not show differences in the application of 
SADE scoring system because a co-infection with the 
zoonotic A. simplex was noticed.

**Discussion**

**Parasites distribution**

The high frequency of parasites and MA observed in this 
study supports that *Anisakis* has the status of compo-
ent parasite of many fish species and FAO fishing areas. 
Among the different species of *Anisakis* isolated, *A. sim-
plex* commonly occurred in various ecologically and 
economically important fish species from Atlantic FAO
areas 21, 27 and 41 (Atlantic Northwest, Northeast and 
Southwest) as reported by Mattiucci et al. [28]. *A. pegref-
fii* was found in southern Atlantic Ocean (FAO areas 41 
and 47) and in FAO area 27 in agreement to previous 
studies [29–33]. According to Mattiucci et al. [34], *A. typica* can occur from 30° S to 35° N in warmer temper-
ate and tropical waters and this data were confirmed by 
our findings (FAO area 47). Unlike Mattiucci [30] who 
highlighted a discontinuous range of distribution of this 
species including Pacific Canada, Chile, New Zealand 
waters and the Atlantic South African coast, *A. berlandii*
was found only in South-Atlantic (FAO area 41 and 
FAO area 47). *P. decipiens* s. l., as reported by Szosta-
kowska et al., [35], occurs sporadically and in our work 
only in 2 fish species were found (C. conger and Z. faber 
from FAO area 27) confirming that only parasites be-
longing to the *P. decipiens* complex are present in the 
NE Atlantic Ocean. *P. cattani* was found in *G. blacoides* 
from FAO area 41, in agreement with Timi et al. [36]. *H. 
aduncum* and *C. osculatum* s.l were found only in FAO 
area 41 with low prevalence, in contrast to data reported 
by Niklitschek et al. [37] in the same sampling area in 
N= 41 samples of *M. australis*. Furthermore, in the same 
fish species caught in this area were found only parasites 
belonging to *Anisakis* genera.

*Anisakis* and *Pseudoterranova* are generally most 
abundant in European NE Atlantic waters [8]. These are 
traditionally some of the most productive fishing areas 
in Europe and the abundance of different hosts at all 
 trophic levels presumably accounts for the overall abun-
dance of the parasites. Differences in infection levels 
could also be related to the presence of definitive hosts 
or to host’s feeding habits [1] and to the abundance of 
obligate intermediate crustacean and/or cephalopods 
hosts. *M. dypterygia*, *C. Conger* and *Z. faber* were the 
most highly infected species (rejected after visual inspec-
tion and with the lowest SADE/FPR scores), probably 
because of their relatively high trophic level in FAO area 
27 ecosystems, their size ($p < 0.05$) and high quantity of 
food intake confirm that this fishing area had the stron-
gest effect on larva infection [9, 38].

Worst results corresponded to this fishing grounds with 
significant differences in P, MA and MI match this area 
and the others ($p < 0.0001$). No statistical differences ($p > 
0.05$) between FAO areas with low MA and MI (FAO 21, 
FAO 34, FAO 41 and FAO 47, Table 3) were observed.

Moreover, the different spatial distribution in fish body of 
*Anisakis* infecting the same fish species could be in-
fluenced by *Anisakis* species. Cipriani et al. [7] noted 
that in *M. merluccius* from FAO area 27, *A. simplex* lar-
vae outnumbers *A. pegreffii* larvae in the flesh of the 
same fish host; on the other hand, in the viscera the 
mean abundance of two larvae species was superimpos-
able. This phenomenon could be the result of different 
resource utilization or linked to the different migrating

| FAO areas | Individuals | Infected | P (%) | IC (±) 95 % | $\chi^2$ | p | MA ± SD | MI ± SD |
|-----------|-------------|----------|-------|-------------|--------|---|--------|--------|
| Total sampling areas | 771 | 251 | 32.55 | 3.30 | 106.25 | $p < 0.0001$ | 14.13 | 77.18 | 43.41 | 48.46 |
| 21 | 130 | 41 | 31.53 | 7.98 | 3.61 | 10.07 | 11.46 | 15.33 |
| 27 | 196 | 111 | 56.63 | 6.93 | 50.57 | 85.89 | 89.30 | 97.91 |
| 34 | 100 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 41 | 190 | 63 | 33.15 | 6.69 | 1.97 | 4.75 | 5.95 | 6.68 |
| 47 | 155 | 36 | 23.22 | 6.64 | 0.89 | 4.10 | 3.86 | 7.89 |

Table 2 FAO areas infection values according to Bush et al. 1997. Comparison of prevalence ($\chi^2$) of infection between different FAO areas
ability of the *Anisakis* species because of different abilities of the two species to respond to the fish host’s immune system [39].

**Safety and quality considerations**

Our study confirms the presence of anisakid species with public health implications in lots of fishery products from different FAO areas. Although freezing condition and other treatments as salting and spicing assure no viable larvae in the fish products [40–43], the risk of allergens in the edible part of fish for hypersensitive individuals should be highlighted. EU legislation [44] recognizes that any parasitized fish under a visual inspection scheme should be unfit for human consumption. Comparing predictive schemes and visual inspection, in general the highest scores were associated with the acceptance of the fish batches as stated by the EU legislation. A different situation was found in the case of some batches: 7 fish sublots reached SADE score 4, corresponding to a “fair” FPR standard. “Fair” fish batches have neither pathological nor commercial problems (A2 SADE code – Table 4) and FBO have the possibility to give different final destinations to these fish lots, as processing, assuring safety and cost saving. Under visual inspection 4 “fair” fish batches were rejected because of the number of parasites detected (MA over 3, high parasite density – D0 SADE code) despite the absence of flesh alterations. This approach matches the precautionary principle set by Reg. EU 178/02 [20] but was restrictive in terms of economics gain. As stated by EU Reg. 853/04, FBO have to ensure that the product to

| Table 3 Number and percentage of parasites well sequenced collected in fishery products from different Atlantic areas |
|-----------------------------------------------|
| A. simplex (n./%) | A. pegreffii (n./%) | P. decipiens s. l. (n./%) | H. aduncum (n./%) | A. berlandii (n./%) | A. typica (n./%) | C. osculatum (n./%) | P. cattani (n./%) |
|------------------|------------------|-----------------|----------------|----------------|----------------|----------------|----------------|
| FAO 21           | 34/100%          | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Urophycis Chus    | 7/100%           | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Reinhardtius hippoglossoides | 14/100% | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Macrurus berglax  | 13/100%          | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| FAO 27           | 61/68.40%        | 2/2.89          | 6/8.69         | 0%             | 0%             | 0%             | 0%             |
| Alepocephalus bairdii | 5/100%    | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Conger conger     | 6/50%            | 1/8.33%         | 5/41.66%       | 0%             | 0%             | 0%             | 0%             |
| Molva dypterygia  | 8/100%           | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Zeus faber       | 11/91.66%        | 0%              | 1/8.33%        | 0%             | 0%             | 0%             | 0%             |
| Aphanopus carbo   | 6/100%           | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Trachyrhinchus scabrus | 6/100% | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Clupea harengus  | 12/100%          | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Melanogrammus aeglefinus | 5/100% | 0%              | 0%             | 0%             | 0%             | 0%             | 0%             |
| Regalecus glesne  | 2/66.66%         | 1/33.33%        | 0%             | 0%             | 0%             | 0%             | 0%             |
| FAO 41           | 1/2.27%          | 29/65.9%        | 0%             | 1/2.27%        | 8/18.18%       | 0%             | 4/9.09%        | 1/2.27%        |
| Micromesistius australis | 0%        | 8/66.66%        | 0%             | 0%             | 3/25%          | 0              | 1/8.33%        | 0%             |
| Genypterus blacoideus | 0%          | 7/63.63%        | 0%             | 0%             | 3/27.27%       | 0%             | 0%             | 1/9.09%        |
| Merluccius hubbsi | 0%              | 10/71.42%       | 0%             | 0%             | 1/7.14%        | 0%             | 3/21.42%       | 0%             |
| Caelorinchus fasciatus | 0%          | 2/66.66%        | 0%             | 0%             | 1/33.33%       | 0%             | 0%             | 0%             |
| Macroronous magellanicus | 0%          | 2/100%          | 0%             | 0%             | 0%             | 0%             | 0%             | 0%             |
| Illex argentinus  | 1/50%            | 0%              | 0%             | 1/50%          | 0%             | 0%             | 0%             | 0%             |
| FAO 47           | 0%              | 30/63.82%       | 0%             | 0%             | 5/10.63%       | 12/25.53%      | 0%             | 0%             |
| Trachurus trachurus | 0%            | 12/100%         | 0%             | 0%             | 0%             | 0%             | 0%             | 0%             |
| Lepidopus caudatus | 0%             | 0%              | 0%             | 0%             | 0%             | 9/100%         | 0%             | 0%             |
| Merluccius paradoxus | 0%             | 13/92.86%       | 0%             | 0%             | 0%             | 1/7.14%        | 0%             | 0%             |
| Merluccius capensis | 0%             | 5/83.33%        | 0%             | 0%             | 1/16.66%       | 0%             | 0%             | 0%             |
| Todarodes angolensis | 0%             | 0%              | 0%             | 0%             | 3/100%         | 0%             | 0%             | 0%             |
| Merluccius polli  | 0%              | 0%              | 0%             | 0%             | 1/33%          | 2/66.66%       | 0%             | 0%             |
be presented to the consumer is not obviously contaminated with parasites by visual inspection. Since there were different interpretations of concepts like “viable parasite” and “obviously contaminated” [16, 44–46] with a lack of standard regarding the maximum parasite limit allowed in a fish, the implementation of this risk-based surveillance according to the system developed by Llarena-Reino et al. [22] matching SADE scheme with FPR standards, should make it easier to categorize the public health and economic risk of anisakids in the flesh of commercial fish.

In this study the highest rejection rate of fish lots was under visual inspection, penalizing in some cases the FBO. SADE/FPR rejection was higher than visual inspection only in a few cases, especially for products heavily infected with deep embed larvae in which parasites were difficult to detect because of the fat percentage and colour of the viscera. However, this should be important for fish industry: in our findings, in fact, among lots with low scores, there are several fish species used for processed products of high value.
In fact, *M. dypterygia* is used for deep or light salted products and *M. hubbsi*, *M. capensis* and *M. paradoxus* are the most used species for fish sticks. According to EU legislation [16], *M. paradoxus* would not have been rejected because of the low number of deep embed larvae not detectable by naked eye inspection. These sub lots, according to the scheme adopted, were rejected to prevent food business operator to suffer serious commercial losses.

This work aims to present the application of the above mentioned method on fishery products coming from several Atlantic FAO areas. Recently Rodriguez et al. [23], according to the SADE/FPR scheme, examined fish caught from 3 different ICES areas (ICES VII – Grand Sole, ICES VIII – Galician coast and ICES IX – Portuguese coast) located in the same FAO area (NE Atlantic areas – FAO 27). These authors gave “poor” FPR score to only 2 fish species, *M. merluccius* (ICES VII and ICES VIII) and *Lophius budegassa* (ICES VII), of the 9 examined, differently from our results where several fish species (*A. carbo*, *Z. faber*, *C. conger*, *M. dypterygia*), caught in NE Atlantic areas, reached SADE 1 and consequently “poor” FPR score.

The combined scoring systems are less restrictive than visual inspection: results compared between the different methods could be helpful to analyse an appropriate balance in terms of consumer’s safety and FBO interests. The SADE/FPR method has an acceptable sensitivity (66.7%; CI95% 34.8 – 90.1%) but a high specificity (95.2%; CI95% 76.2 – 99.9%). The accuracy of 84.85% (CI95% 68.1 – 94.9%) indicates that the SADE/FPR method has a high capacity to correctly classify fishery products. This predictive scheme, proposing corrective measures within HACCP procedures, proved to be very useful for fish lots with the lowest FPR rating particularly and offers a crucial food safety device for assessing risks associated with parasites.

**Conclusion**

SADE score combined with FPR standard may represent a specific low-cost tool in fish inspection, ensuring both safety and quality, that could be useful for competent authorities and fish industry operators to establish standard management strategies. The categorization of lots in 5 quality batches, allowing the possibility of calculating accurately both parasitic load and flesh integrity, could give a unique language and *modus operandi* during self-control inspections in HACCP procedures and programs addressing fish lots in different ways depending on the score. The high specificity and accuracy of the applied predictive tests guarantees its correct applicability during the fish inspection procedures.

**Methods**

** Sampling**

Between May and October 2013, a total of 771 fish and cephalopods frozen samples belonging to several commercial frozen lots (33 different species) from 5 different Atlantic FAO fishing areas (Table 5) were examined in the laboratories of the Instituto de Investigaciones Marinas de Vigo – Ecobiomar Department. FAO Fishing areas (Fig. 1) where fishery products were sampled as FAO 21 (Atlantic, Northwest, N= 130 individuals – 5 different species), FAO 27 (Atlantic, Northeast, N= 196 individuals – 9 different species), FAO 34 (Atlantic, Eastern Central, N= 100 individuals – 3 different species), FAO 41 (Atlantic, Southwest, N= 190 individuals – 9 different species) and FAO 47 (Atlantic, Southwest, N= 139 individuals – 7 different species). According to Reg. EC 2074/05, a representative number of samples underwent visual inspection; for each lot (number of fish/box and number of boxes harvested by vessels that compose the lot is shown in Table 5) a representative sample (sub lot) was taken, ranging from 12% for *Merluccius polli* to 100% for *Aphanopus carbo*.

**Visual inspection**

After thawing, each sub lot was weighed, each sample was weighed individually, and the abdominal cavity of each sample was opened and kept under a down-light source. The samples were gutted and the celomacial cavity was briefly inspected by the naked eye, for the presence of nematodes. According to EFSA recommendations [8], the presence of potentially zoonotic anisakid species was evaluated only in the edible parts of fishery products that is considered the main exposure risk factor for the consumer. Guts are usually discarded during fish-processing procedures. The presence of parasites on visceral peritoneum was checked but not considered a serious factor because the freezing condition prevents the migration of parasites in the flesh. For this reason, viscera were not analyzed, only the edible part of the products were considered.

**Artificial peptic digestion**

After visual inspection heads and tails were removed. The remaining musculature was dissected in left and right fillets and then separated into the hypaxial (ventral) and epaxial (dorsal) regions following the horizontal septum. In case of cephalopods, the body cavity was opened and a macroscopic visual inspection was carried out.

The whole muscle (hypaxial and epaxial regions separately) of each fish sample was digested in an ACM-11806 Magnetic Stirrer Multiplate in pepsin solution [47]. For cephalopods, the mantle muscle was used to perform the process. Digestions were performed for 30 minutes at
incubation temperature of 37° C in an acid solution (pH = 1.5) with HCl 0.063 M. Assays using liquid pepsin at concentration of 0.5 % and a ratio 1:20 sample weight/solution volume were used. The digestion solution was decanted through a sieve and the rests of digestion and nematodes were inspected under stereomicroscope. All anisakids were placed in individual eppendorf with ethanol 70% for further molecular diagnosis.

**Molecular analysis**

All anisakid larvae were identified at genus level by microscopic examination of diagnostic characters. The biomolecular identification was performed by randomly choosing 15 larvae per species; in sub lots/species with a number of parasites lower than 15, all larvae were analyzed. A total of 275 anisakid larvae, previously identified at genus level, were used for molecular identification but only 194 were correctly classified by biomolecular analysis (Table 1). DNA extractions were performed using the commercial kit NucleoSpin® Tissue kit (Macherey-Nagel) following the manufacturer’s recommended protocols. DNA quality and quantity were checked in a spectrophotometer Nanodrop® ND-1000 (Nanodrop technologies, Inc). The entire ITS (ITS1, 5.8S rDNA Table 5

| FAO fishing areas | Coordinates | Host | N. boxes / total fish count | Individuals sampled (N) |
|------------------|-------------|------|-----------------------------|-------------------------|
| FAO 21 Atlantic, Northwest | 48°38 ´N 45°43´W | Rhipicephalus sanguineus | 1/60 25 |
| FAO 21 Atlantic, Northwest | 46°51 ´N 47°20´W | Mallotus villosus | 1/50 25 |
| FAO 21 Atlantic, Northwest | 48°33 ´N 45°45´W | Reinharditus hippoglossoides | 8/109 25 |
| FAO 21 Atlantic, Northwest | 48°38 ´N 45°42´W | Urophycis chus | 4114 30 |
| FAO 27 Atlantic, Northeast | 56°13 ´N 17°34´W | Alepecephalus bairdi | 7/52 25 |
| FAO 27 Atlantic, Northeast | 56°13 ´N 17°35´W | Aphanopus carbo | 1/10 10 |
| FAO 27 Atlantic, Northeast | FAO 27 Ilia | Clupea harengus | 2/100 25 |
| FAO 27 Atlantic, Northeast | FAO 27/ VII | Conger conger | 6/100 25 |
| FAO 27 Atlantic, Northeast | 54°34 ´N 17°59´W | Melanogrammus aeglefinus | 1/50 25 |
| FAO 27 Atlantic, Northeast | 58°38 ´N 15°94´W | Galeodes decodactylus | 5/50 25 |
| FAO 27 Atlantic, Northeast | 12°00 ´N 17°15´W | Trachurus trecae | 2/60 50 |
| FAO 41 Atlantic, Southwest | 13°37,09S 12°17,38´E | Lepidopus caudatus | 1/25 9 |
| FAO 41 Atlantic, Southwest | 13°53,1´S 13°41,7W | Serranus cabrilla | 1/50 25 |
| FAO 41 Atlantic, Southwest | 27°03,85 ´N 14°14,7E | Todarodes angolensis | 3/50 10 |
| FAO 41 Atlantic, Southwest | 24°10,95 ´S 13°31,0E | Trachurus trachurus | 2/50 22 |
Fig. 1 Global map of FAO Major Fishing Areas. (https://commons.wikimedia.org/w/index.php?search=fao+areas&title=Special%3ASearch&go=Go
&ns0=1&ns6=1&ns12=1&ns14=1&ns100=1&ns106=1#/media/File:FAO_Major_Fishing_Areas.svg)

Fig. 2 Flow diagram for the Site of infection, Assurance of quality, Demography, Epidemiology (SADE) modified according to Llarena-reino et al., 2013
gene and ITS2) was amplified using the forward primer NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') and the reverse primer NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3'). PCR assays were carried out in a total volume of 25 μl containing 100 ng of genomic DNA, 0.3 μM of each primer, 2.5 μl of 10x buffer, 1.5 mM of MgCl2, 0.2 mM of dNTPs and 0.625 U of Taq DNA polymerase (Roche Mannheim, Germany). PCR cycling parameters included denaturation at 94°C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 75 s, and a final extension at 72 °C for 7 min. PCR products were purified for sequencing using ExoSAP-IT © following recommended protocol by the manufacturer. Sequencing was performed by Secugen (Madrid, Spain) and the electropherograms were analysed using the program ChromasPro version 1.41 Technelysium Pty LtdA. All sequences were searched for similarity using BLAST (Basic Local Alignment Search Tool) through web servers of the National Center for Biotechnology Information (USA).

Risk categorisation
Briefly sub lots are grouped according to four homogeneous categories named S, A, D, and E, that are afterwards split into numeric subcategories by the means of a flow diagram (Fig. 2 modified according to Llarena-reino et al., [22]): each inspected fish lot was categorized according to the localization of parasites (S – hypaxial or epaxial musculature flesh), the presence/absence of pathological or unaesthetic signs in the edible part (A), the density of infection (D – number of larvae/kg of fish) and finally the epidemiological relevance of the parasites (E – zoonotic or not). By adding the numeric values of each categories, the SADE system adopts a 10-point scale: as a result, a SADE code and a final score are obtained for each lot checked, in order to decide the final destination.

The highest values indicate no risk connected to the examined lots, whereas the lowest score suggests serious issues connected to fish parasites.

These preliminary results were translated to the standard Fish Parasite Rating (FPR) score, which allows the classification of fish lots into five categories (Fig. 3):

- Poor: final score 0-3;
- Fair: final score 4-5;
- Good: final score 6-7;
- Very good: final score 8-9;
- Excellent: final score 10.

Since the SADE scheme is used for the evaluation of homogeneous commercial fish lots, the scientists who took part in the study did not proceed to the individual measurement of the sample. The products were only weighed in order to assess the parasitic density (n. Parasites / kg).
Finally, according to the European Hygiene Rules (Reg. EC 853/2004, Section VIII, Chapter V, Pt. D), the percentage of fishery products that should be unfit for human consumption was calculated and compared with SADE – FPD scores.

Statistical analysis
To describe parasite population the following parameters were used, according to Bush et al. [24]: P (the ratio between parasitized subjects/sub lots and the total subjects/ sub lots analysed), MA (the ratio between the number of larvae recovered and the number of all examined subjects of sub lots) and MI (the ratio between the number of larvae recovered and the number of exam- ined parasitized subjects of sub lots). Infection indexes were calculated regardless of parasites’ localisation site (Epaxial/Hypaxial flesh), species/sub lot and for each FAO areas. The differences in the P between FAO areas were assessed by the two-sided chi-square test.

Statistical significance between MA and MI of different FAO Areas was performed using One-way ANOVA with Bonferroni’s post-test.

In order to assess statistical association between MA and fish sample mean weight a simple regression analysis was used. Finally, statistical comparisons between SADE/FPR and Visual Inspection were performed by McNemar’s chi-square test [48]. Moreover, sensitivity, specificity and accuracy values of the SADE/FPR relative to Visual Inspection were calculated. Statistical analyses were performed using GraphPad InStat Version 3.0 (GraphPad Software, San Diego California USA) and MedCalc for Windows, version 18.11.3 (MedCalc Software, Ostend, Belgium); p < 0.05 was considered significant for all statistical tests.

Supplementary information
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Additional file 1. Identified parasites in fish species,Accession ID related to the aligned sequences and web links (https://www.ncbi.nlm.nih.gov/ pubmed/).

Abbreviations
EFSA: European Food Safety Authority; EU: European Union; EU: European Union; FBO: Food and Agriculture Organization of the United Nations; FPR: Part of European Food and Agriculture Organization; FPR: Fish Parasite Rating; FPR: Hazard Analysis and Critical Control Points; HA: Hazard Analysis and Critical Control Points; ICES: International Council for the Exploration of the Sea; MA: Mean abundance; MI: Mean intensity; OHIM: Office for Harmonization in the Internal Market; P: Prevalence of infection; RASFF: Rapid Alert System for Food and Feed; SADE: Site, abundance, density and epidemiology

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Authors’ contributions
GS and SP designed the overall study; EA and CC developed the molecular protocol. GS performed parasites inspection in fishery products and morphological identification of anisakid samples. AA, RM and SP analysed the results and drafted the paper. SP, CB and GS contributed to the ideas behind the study and the writing of the paper. All authors critically reviewed the paper and agreed the final content of the version to be published. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Sequencing was performed by Secugen (Madrid, Spain) and the electropherograms were analysed using the program ChromasPro version 1.41 Technelysis Pty LtdA. All sequences were searched for similarity using BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Accession ID were in the supplementary materials.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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