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# 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)

*Laura Serracca* [a,*], *Roberta Battistini* [a], *Irene Rossini* [a], *Annalaura Carducci* [b], *Marco Verani* [b], *Marino Prearo* [c], *Laura Tomei* [a], *Gabriella De Montis* [a], *Carlo Ercolini* [a]

[a] *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Sezione La Spezia, Marine Microbiology Laboratory;*

[b] *Hygiene and Environmental Virology Laboratory, Department of Biology, University of Pisa, Pisa, Italy;*

[c] *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Laboratorio di Ittiopatologia, Torino;*

*Corresponding author at: Marine Microbiology Laboratory, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Sezione La Spezia. Via degli Stagnoni 96, 19126 La Spezia, Italy. Tel.: +39 0187 507370; fax: +39 0187 500308.

E-mail address: laura.serracca@izsto.it (L. Serracca).

**Abstract**

*Engraulis encrasicolus* and *Sardina pilchardus* are pelagic fishes of notable economic and gastronomic importance in the northwest Mediterranean (Ligurian Sea, Italy). The consumption of thermally unprocessed or lightly processed, marinated or salted anchovies and sardines presents a potential risk to acquire anisakiasis, a fish-borne parasitic disease in humans. Prevalence and abundance of *Anisakis* larvae in *Engraulis encrasicolus* and *Sardina pilchardus* from the Monterosso fishing grounds (Cinque Terre National Park, Ligurian Sea, Italy) were assessed, and

the larvae were identified by morphological and PCR-RFLP methods. *Anisakis* larvae, all belonging to *Anisakis pegreffii* spp. were found in the visceral mass of 1050 anchovies (0.8% overall prevalence), whereas no *Anisakis* larvae were found in the 750 sardines examined. According to these data, the risk of acquiring anisakiasis from the consumption of raw or undercooked anchovies and sardines caught in the fishing area we investigated is very low.

**Keywords:** Anisakiasis; PCR-RFLP; anchovy; sardines; Ligurian Sea.

## 1. Introduction

The consumption of raw or undercooked seafood presents the risk for anisakiasis, a fish-borne parasitic infection in humans. The disease is caused by the accidental ingestion of anisakid nematode third-stage larvae of the genus *Anisakis* present in parasitized fishes or cephalopods (Audícana and Kennedy, 2008). Initial manifestations of infection include general gastrointestinal symptoms such as diarrhoea, vomiting, abdominal pain, and nausea (Hochberg and Hamer, 2010); subsequent sensitization to *Anisakis*-derived allergens can raise the risk of allergic exacerbation on secondary exposure to contaminated seafood (Baird et al., 2014). The life cycle of *Anisakis* parasites involves marine mammals as definitive hosts, fish, squid and other invertebrates as intermediate or paratenic hosts, and crustaceans as first intermediate hosts (Baird et al., 2014). Nematode worms of the genus *Anisakis* have been found in several economically important fish species in the Mediterranean, including anchovies and sardines (Manfredi et al., 2000; Chaligiannis et al., 2011; Mladineo et al., 2012; De Liberato et al., 2013; Serracca et al., 2013). European pilchard (= Sardine) *Sardina pilchardus* (Walbaum 1792) and European anchovy *Engraulis encrasicolus* (L.) are the most abundant pelagic fishes in the northwest Mediterranean, accounting for 22% (46,273 tons in 2011) and 7% (14,377 tons in 2011) of total seafood production, respectively (INEA, 2012). Although *S. pilchardus* is more abundant, fishing pressure on the European anchovy is greater due to its higher commercial value (García and Palomera, 1996).

Uncooked anchovies and sardines are common ingredients in many traditional Mediterranean fish dishes, particularly in Spain and Italy where the majority of anisakiasis cases have been associated with the consumption of marinated anchovies (Maggi et al., 2000; Pampiglione et al., 2002; Fumarola et al., 2009; Mattiucci et al., 2013; Bucci et al., 2013). Anchovies and sardines are both economically and gastronomically important in Liguria, a region in northwestern Italy, where the anchovy fishery accounts for 56% of the total catches. A characteristic ingredient in the Ligurian cuisine, marinated or salted anchovies and sardines are prepared without thermal processing because freezing and thawing alters the texture and taste of fish fillets. The freshly fished anchovies and sardines are eviscerated and boned by hand, then pickled in lemon juice or vinegar and salt, usually for less than 24 h before consumption. However this marinating process appears not to kill the *Anisakis* larvae (Sanchez-Monsalvez et al., 2005). Anchovies and sardines are usually consumed fresh during spring and summer, but they can also be preserved in salt for fall and winter dishes. Furthermore, the high salinity of the seawater along the Ligurian coast (37.9-38.5 PSU) lends the anchovies their unique tangy taste appreciated by consumers. Commercially processed anchovies from the Ligurian Sea were awarded Protected Geographical Indication (PGI) status by the European Union (EU) in 2008. While the bulk of the fresh, salted or marinated products is consumed locally, exportation to EU markets is closely regulated. The aim of this study was to identify *Anisakis* spp. and determine their prevalence and abundance in European anchovies and sardines from the fishing grounds off the coast of Monterosso (Cinque Terre National Park) in the Ligurian Sea (NW Mediterranean).

## 2. Materials and methods

### 2.1 Sampling

A total of 1050 anchovies (*Engraulis encrasicolus*) and 750 sardines (*Sardina pilchardus*) were sampled between April 2012 and November 2012, all fished from the waters off the coast of Monterosso (NW Mediterranean). The area extends from Punta Mesco (44°01’ N, 9°06’ E) to Punta

Cavo (44°05’ N, 09°06’ E) along 20 km of the coastline of the Cinque Terre National Park (Fig. 1). Samples of 150 fishes were randomly obtained from commercial catches and private fishermen, depending on weather and maritime conditions and fish availability, in order to cover as best as possible the fishing period of both species (1 April to 15 October for anchovies and May to November for sardines). The samples were then transported in refrigerated boxes to the laboratory for analysis.

2.2 Parasite isolation

The fishes were measured for total length and weight, then examined for the presence of *Anisakis* larvae using the candling method as described in Regulation EC 2074/05. The fishes were eviscerated and filleted into butterfly fillets. Viscera and fillets were pressed to 1-2 mm thickness with a commercially available pressing devise to observe the larvae more easily. The flattened fillets or viscera were bagged and then observed under a stereomicroscope (Nikon SMZ 800, Nikon, Tokyo, Japan) with an underneath light source (Mladineo et al., 2012). Larvae were isolated and identified at the genus level according to morphological characters, then fixed in 70% ethanol (Koyama et al., 1969; Shih, 2004; Zhang et al., 2007; Quiazon et al., 2008). The larvae morphologically identified as belonging to the genus *Anisakis* underwent molecular characterization by PCR-based restriction fragment length polymorphism (PCR-RFLP) to identify the species.

2.3 DNA extraction and PCR-RFLP analysis

DNA was extracted from 30 mg of each larva using a commercial kit (PureLink Genomic DNA Kits, Invitrogen, Carlsbad, CA) and eluted in a final volume of 100 µl according to the manufacturer’s protocol. Nucleic acids were stored at -20°C until use. The nuclear rDNA region containing ITS-1, 5.8 rRNA gene, and ITS-2 were amplified by PCR using the primers NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3')

(Zhu et al., 1998). PCR was performed using a mixture containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs each, 0.5 µM of each primer, 1.25 U of FastStart Taq DNA polymerase (Roche, Mannheim, Germany), 5 µl of DNA sample and water to a final volume of 50 µl. The reaction was carried out on an automated thermocycler (GeneAmp2720, Applied Biosystems, Foster City, CA) as follows: for 4 min at 95°C, then 35 cycles of 30 sec at 95°C, 30 sec at 58°C, and 90 sec at 72°C, followed by a final extension for 15 min at 72°C. Negative and positive controls were included in all amplifications. PCR products were separated by electrophoresis on 2% agarose gel stained with 1000 X Gel Green (Biotium, Hayward, CA) and visualized using ultraviolet (UV) illumination. The amplified products were analyzed by the RFLP technique using the restriction enzymes *Hha*I, *Hinf*I and *Taq*I (Invitrogen), which identifies the members of *Anisakis* genus according to the genetic markers described by D'Amelio et al. (2000) and implemented by Pontes et al. (2005). Digestion of the PCR products was carried out according to the manufacturer's recommendations. Finally, the digested products were analyzed by electrophoresis on 2% agarose gel stained with 10000 X Gel Green (Biotium) and the bands visualized with a UV transilluminator (Gel Doc; Bio-Rad S.A., Ivry-sur-Seine, France). The size of the restriction fragments was compared with a molecular size marker (AmpliSize Molecular Ruler 50-2000 bp Ladder, Bio-Rad).

## 3. Results

Table 1 presents the average length and weight of anchovies and sardines. A total of eight *Anisakis* spp. larvae were isolated from the 1050 anchovies analysed (*E. encrasicolus*) (overall prevalence 0.8%), whereas no *Anisakis* spp. larvae were found in the 750 sardines (*Sardina pilchardus*). All larvae were viable and found in the visceral mass but not in the musculature. All infected fish samples harboured only a single larva. All eight larvae morphologically referred to the genus *Anisakis* showed the restriction profiles corresponding to *A. pegreffii*: three fragments of about 370, 300 and 250 bp using the *Hinf*I enzyme and three fragments of about 400, 320 and 150 bp using the *Taq*I enzyme. *Anisakis* infestation was very low across the entire sampling period: the prevalence of

infection was highest in anchovy samples collected in September (1.3%), whereas no larvae were detected in the samples collected in April and August (Table 1).

## 4. Discussion

Anchovies and sardines represent a large portion of Italian fisheries production and human seafood consumption; however, information on *Anisakis* spp. infection of these two fish species from Italian waters is limited. This is the first report on a sizeable sample of anchovies and sardines from the Monterosso fishing grounds in the Ligurian Sea. The overall prevalence of *Anisakis* larvae in anchovies was 0.8%, i.e far lower than that reported by Rello et al., (2009) for other areas in the Ligurian Sea (21.7%) and the Mediterranean (de la Torre Molina et al., 2000; Rello et al., 2009; Chaligiannis et al., 2011), confirming that the prevalence of *Anisakis* varies considerably between different geographical areas/fishing grounds and seasons in the same fish host species (Mattiucci et al., 2002; Tejada et al., 2006). Our findings are similar to previous studies of sampling locations along the Tyrrhenian coast of Latium (Central Italy) (De Liberato et al., 2013) (prevalence 1% in anchovies) but, again, far lower than that reported by Angelucci et al., (2011) for Sardinia (prevalence 34.6% in anchovies) and Cavallero et al., (2012) for Tuscany (prevalence 37.7%). In the Adriatic, a wide prevalence range (9.8-56.5%) was reported along the coast of Marche Region (Central Italy), depending on the fishing area (Fioravanti et al. 2004; Fioravanti et al., 2006). Prevalences ranging 71.4 to 100% have been reported for Croatian Adriatic fishing grounds (Mladineo et al. 2003; Mladineo et al. 2012; Mladineo and Poljak, 2013). We found only *A. pegreffi*, further confirming the widespread occurrence of this parasitic nematode in fish inhabiting Italian coastal waters and that it more frequently infects pelagic fish (Mattiucci et al., 1997; Paggi et al., 2001; Abollo et al., 2001). In contrast, no *Anisakis* larvae were found in the sardines which is in agreement with previous studies on the infection dynamics of anisakid larvae in fish from the Atlantic and Mediterranean coasts of Spain (Rello et al., 2008; Gutiérrez-Galindo et al., 2010). To our knowledge, *Anisakis* infections in sardines from the Ligurian Sea have not been reported so far,

whereas infections were found in sardines from other areas along the Adriatic coast of Italy (prevalence 3.3 %) (Mladineo and Poljak, 2013) and the Tyrrhenian around northern Sardinia (prevalence 13.1-20%) (Angelucci et al., 2011; Tedde et al., 2011). The prevalence of *Anisakis* infection in sardines from the Aegean Sea (Chaligiannis et al., 2011) is reportedly far lower than in sardines from the West coast of Portugal (5.5% vs. 28.1%) (Silva and Eiras, 2003). The low *A. pegreffii* infection rate of anchovies and sardines in our study seems unusual because the Ligurian Sea is included in the Pelagos Sanctuary for Mediterranean Marine Mammals, a vast marine protected area with a notable cetacean population (Notarbartolo di Sciara at al., 2008) which, according to many authors, would normally be associated with a high presence of nematodes (Arthur et al., 1982; Boily and Marcogliese, 1995; Rello et al., 2009). One reason suggested for the low abundance of *Anisakis* larvae in anchovies and sardines is the food they eat. The first intermediate hosts of *Anisakis* are small crustaceans (mainly euphausids and copepods) and other intermediates of zooplankton. Since anchovies and sardines live and feed in the open water column, the euphausids from the upper layers could be less parasitized by *Anisakis* than those from deep waters, resulting in less infection of pelagic fishes (Landry et al., 1992; Osanz 2001; Rello et al., 2008). This hypothesis could explain our results, together with the possibility that in the Monterosso fishing grounds the invertebrate hosts of *Anisakis* are not part of the habitual diet of sardines and anchovies or are preferred to other species. The composition of the zooplankton communities in these fishing grounds and examining the stomach contents of sardines and anchovies could be an area of future focus. No parasites were isolated from the anchovy musculature, this contrasts with previous findings in the anchovy from the Atlantic and Mediterranean (Rello et al., 2009; Angelucci et al., 2011; Mladineo et al., 2012; De Liberato et al., 2013; Mladineo and Poljak, 2013). Postmortem larval migration is highly influenced by the freshness of the sample, being accelerated with the time lapsed from the moment of fish catch (Mladineo et al., 2001; Suzuki et al., 2010). In our study, the anchovies were brought to the laboratory for nematode isolation within a few hours after being caught, precluding the possibility

of postmortem larval migration. Although the consumption of raw fish preparations such as marinated or salted anchovies and sardine is popular in Liguria, only one case has occurred there so far (Caramello et al., 2003). The majority of clinical anisakiasis cases have been reported in the central and southern regions of Italy. Similarly, a study investigating *Anisakis* hypersensitivity in Italy showed that the prevalence of *Anisakis* sensitization and allergic reactions after seafood ingestion was lower along the Ligurian coast than in Adriatic or Tyrrhenian coastal areas (AAITO-IFIACI, 2011). Besides the fact that anisakiasis symptoms are non-specific and the disease is often misdiagnosed (EFSA, 2010), the low abundance of larvae found in our study may be one of the explanations of the low number of anisakiasis cases occurred along Ligurian coast. Conclusively, the results of the present study suggest a low risk of anisakidosis from the consumption of marinated, salted or pickled anchovies and sardines from the Monterosso fishing grounds of the Ligurian Sea.

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**Table and figure captions**

**Table 1**

Characteristics of anchovies (*E. encrasicolus*) and sardines (*S. pilchardus*) analyzed for *Anisakis pegreffii*. [a]SD: Standard deviation

**Fig. 1.**

Sampling area map.. Samples of anchovies and sardines were collected from Punta Mesco (A) to Punta cavo (B) along the Ligurian Sea coast (NW Mediterranean Sea, Italy).

Figure 1

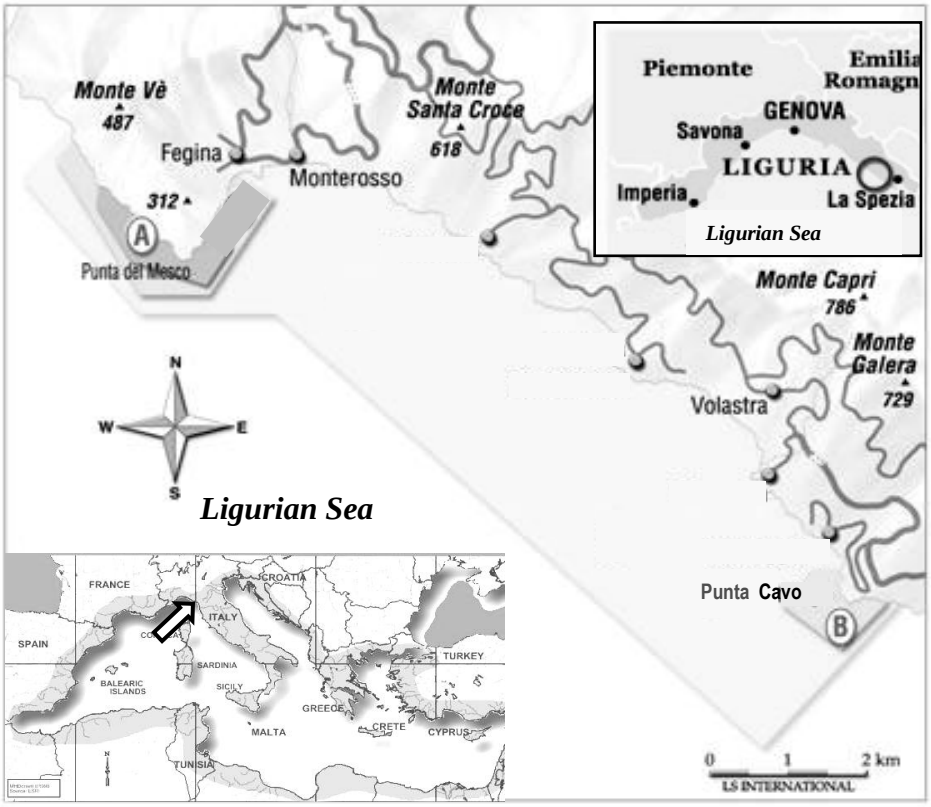

Table 1

| Fish species | Sampling date | Mean (SD[a]) fish length (cm) | Mean (SD) fish weight (*g*) | Number of infected fishes/tested | Number of *A. pegreffii* larvae | Prevalence (%) |
|---|---|---|---|---|---|---|
| *Engraulis encrasicolus* | April 2012 | 10.5 (1.5) | 7 (2) | 0/150 | 0 | 0 |
| | May 2012 | 12.5 (1.5) | 13.9 (4.8) | 1/150 | 1 | 0.7 |
| | June 2012 | 11 (3) | 11.9 (7.6) | 5/450 | 5 | 1.1 |
| | August 2012 | 12 (2) | 15 (3) | 0/150 | 0 | 0 |
| | September 2012 | 11 (3) | 10 (6) | 2/150 | 2 | 1.3 |
| | Total | 11 (3) | 11.7 (7.7) | 8/1050 | 8 | 0.8 |
| *Sardina pilchardus* | May 2012 | 12.2 (1.7) | 19.8 (5.8) | 0/150 | 0 | 0 |
| | July 2012 | 14.5 (2.5) | 27.1 (12.9) | 0/150 | 0 | 0 |
| | September 2012 | 13.5 (2.5) | 24.6 (11.1) | 0/150 | 0 | 0 |
| | October 2012 | 15.5 (2.5) | 27.4 (11.6) | 0/150 | 0 | 0 |
| | November 2012 | 13 (2) | 17.2 (6.8) | 0/150 | 0 | 0 |
| | Total | 14.3 (3.7) | 25.2 (14.8) | 0/750 | 0 | 0 |

**Highlights**

0.8% of anchovies were infected with *Anisakis* larvae.

Larvae were identified by PCR-RFLP as *A. pegreffii*.

All *Anisakis* larvae were detected in the visceral mass and none in the musculature.

No *Anisakis* larvae were found in *Sardina pilchardus*