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Could *Anisakis* be a valuable risk for Mediterranean consumers? Optimization of prevention procedures

Gaetano Cammilleri

Tutor: Prof. Andrea Pulvirenti

Ph.D Workshop Doctorate in Agri-Food Sciences, Technologies and Bio-Technologies 22 November 2019

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How good is sushi.....

But watch out!
Something can be inside.....

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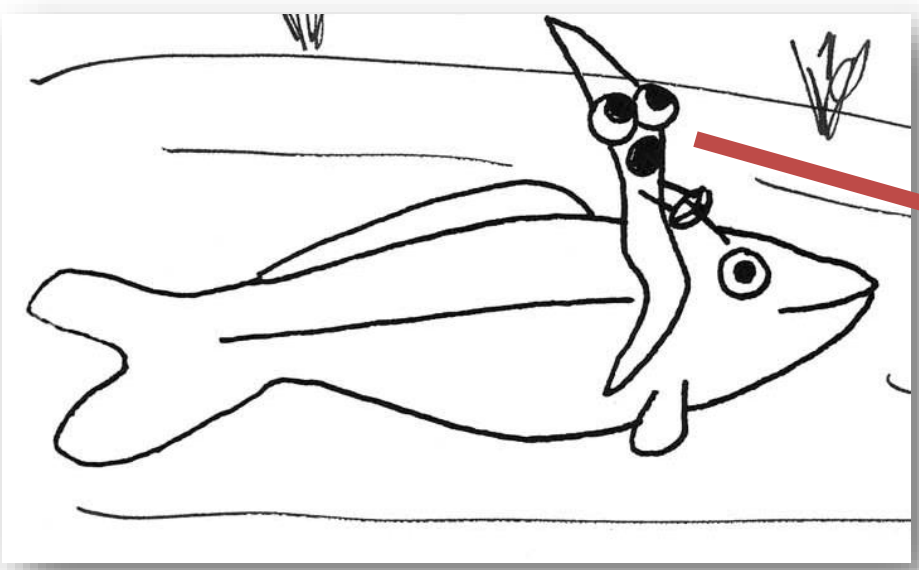
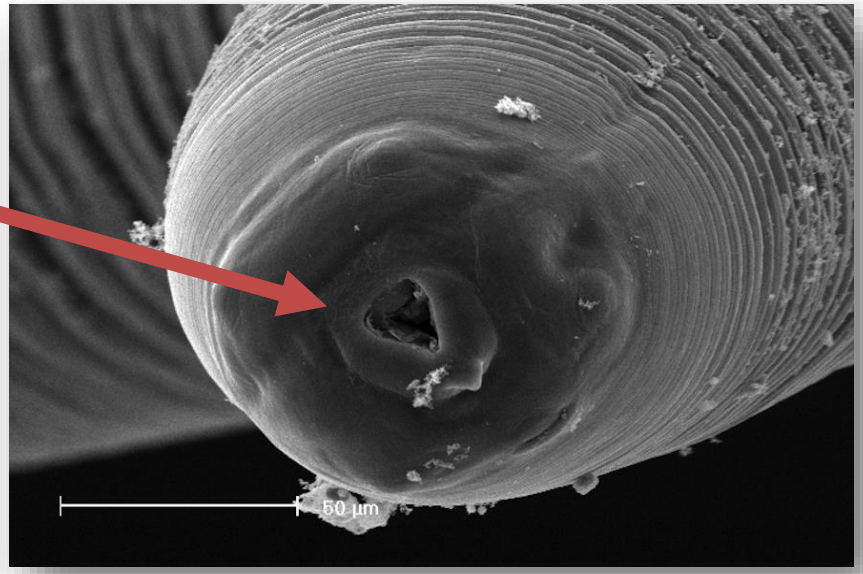
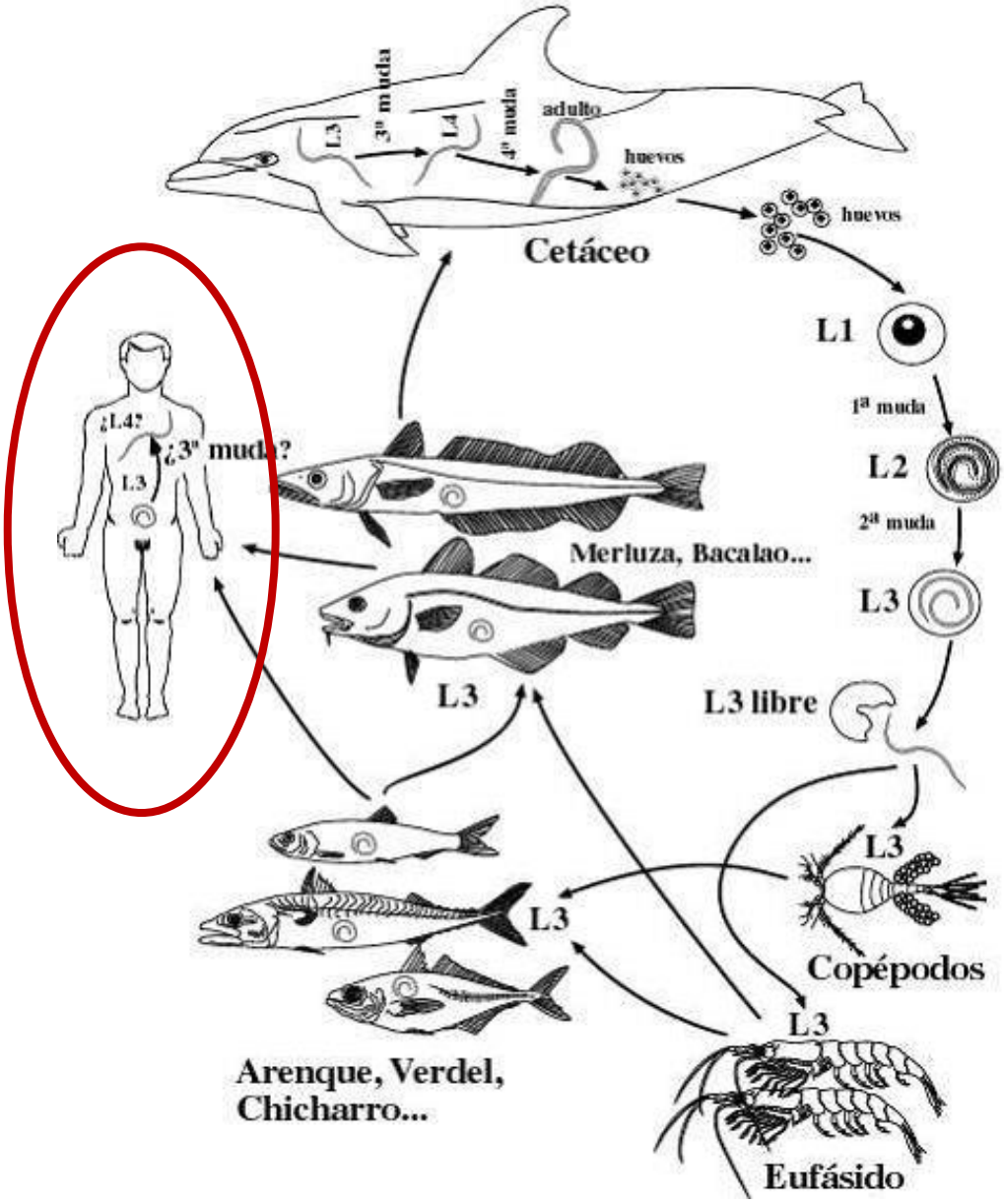
Have you ever seen these on fish products?

Anisakis

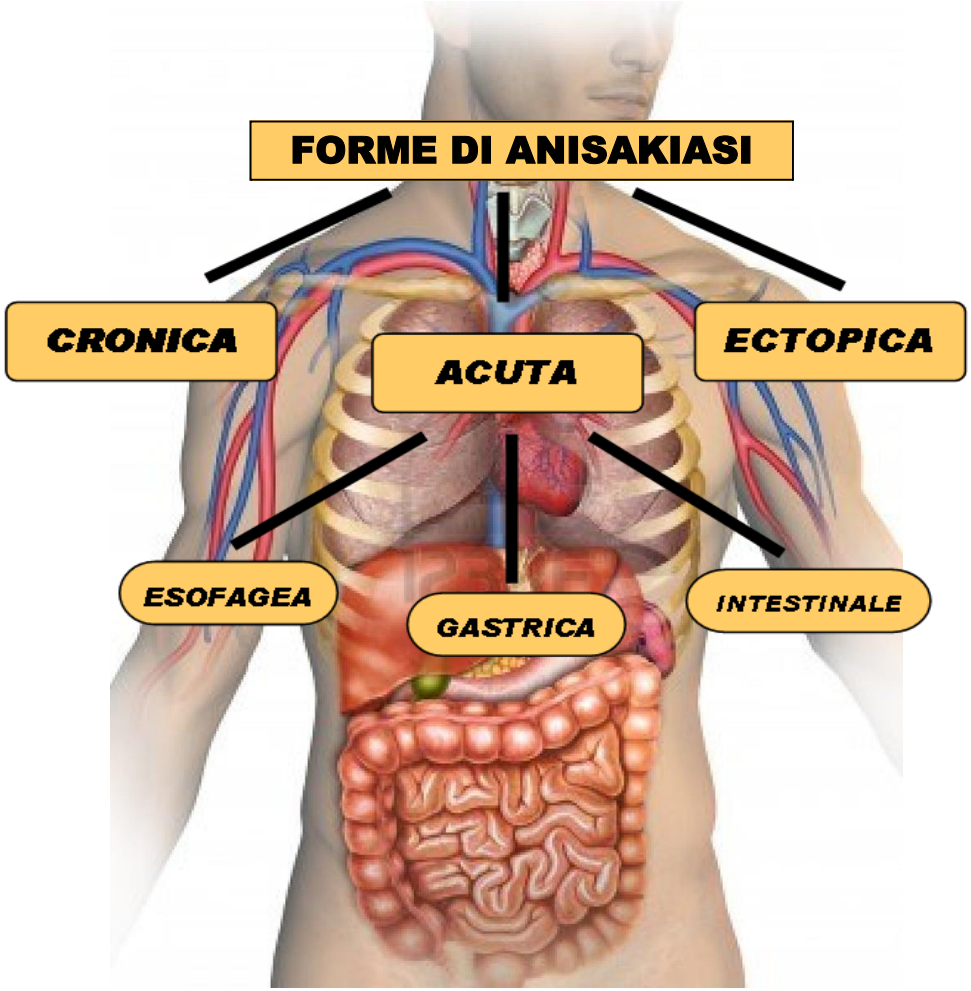




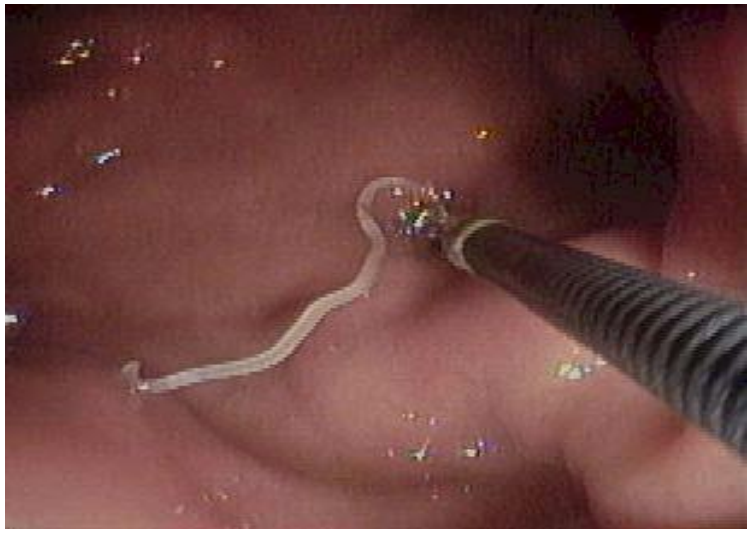
Life Cicle

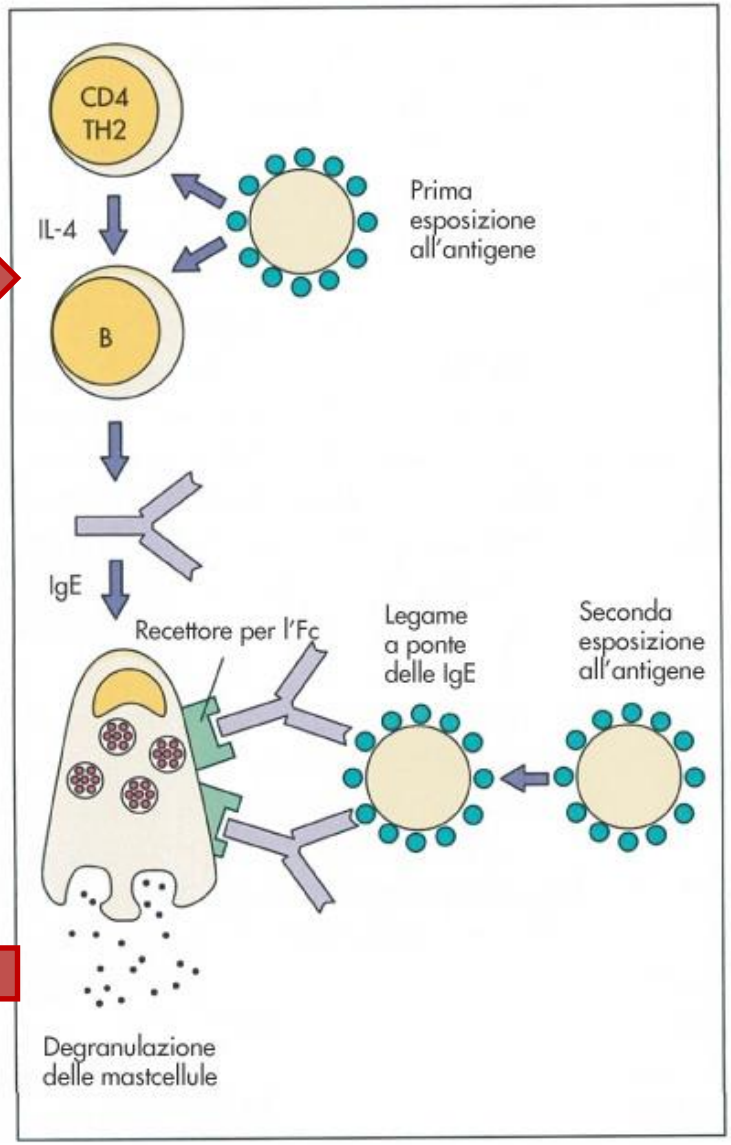

L3
3ª muda
L4
4ª muda
adulto
huevos
Cetáceo
huevos
L1
1ª muda
L2
2ª muda
L3
¿L4?
¿3ª muda?
L3
Merluza, Bacalao...
L3
L3 libre
L3
L3
Copépodos
L3
Arenque, Verdel,
Chicharro...
Eufásido

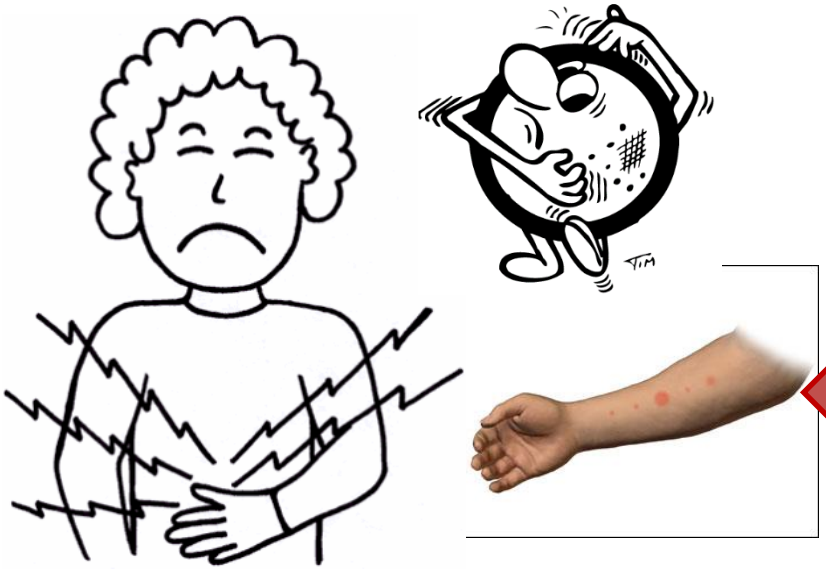
Anisakiasis



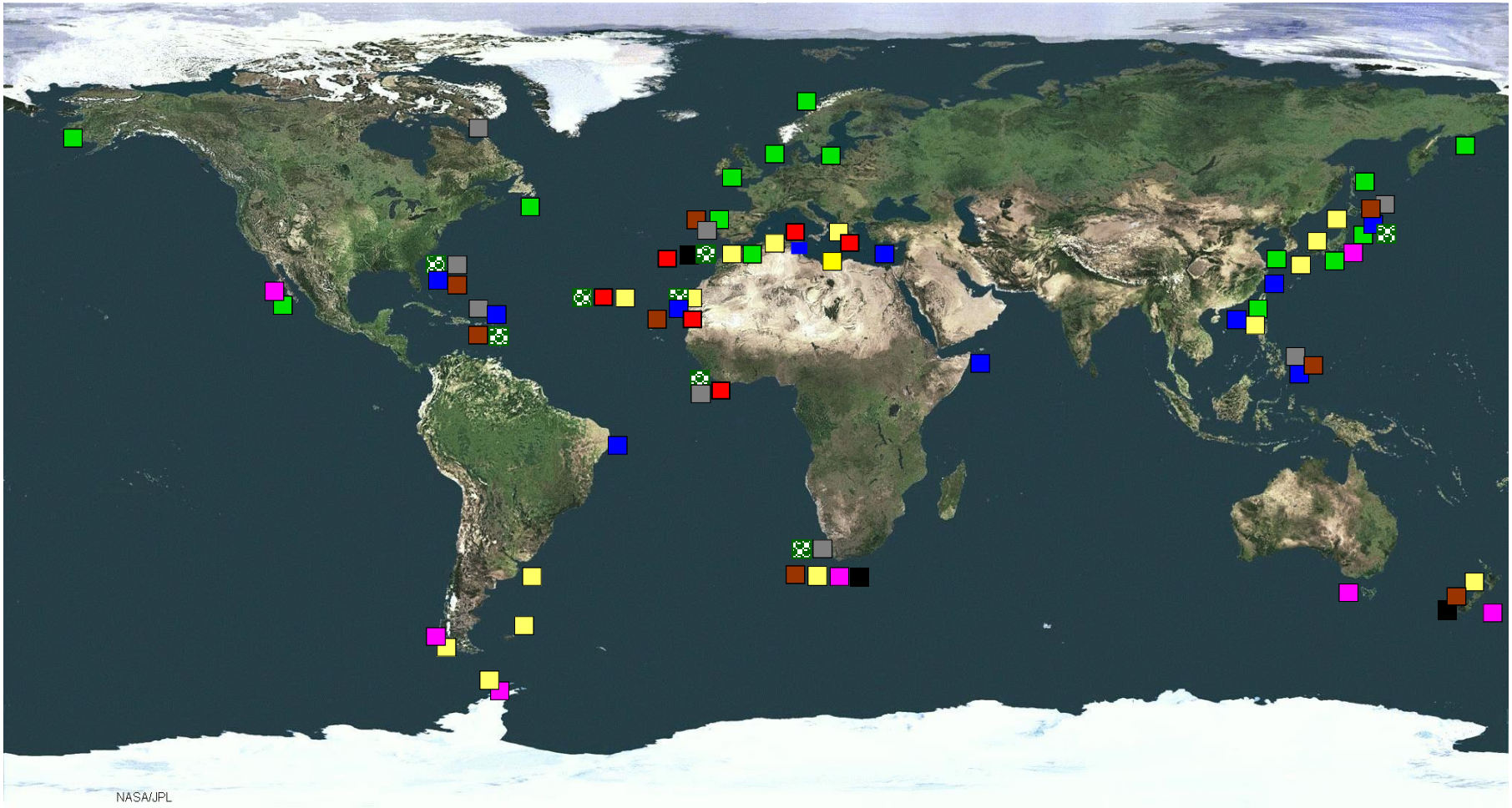
Gastro-allergic Anisakiasis and Anisakis allergy







Anisakis species in the world



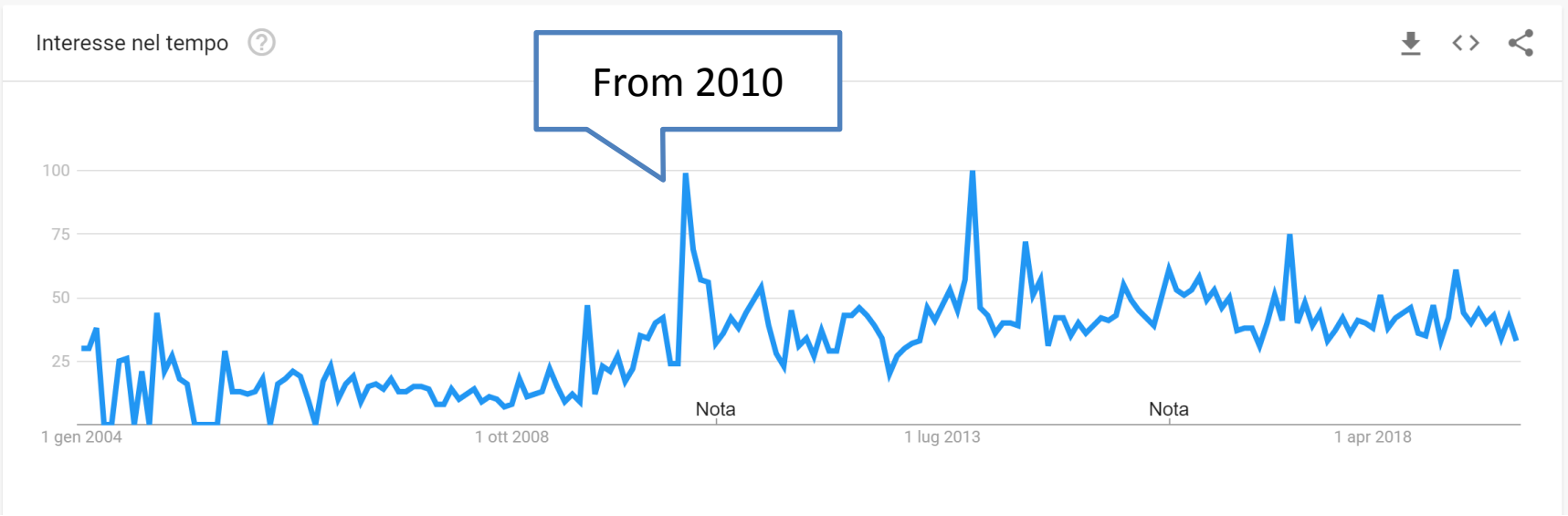
A. pegreffii *A. simplex* (*s.s.*) *A. berlandi* (=*A. simplex* C) *A. typica* *A. paggiae*

A. physeteris *A. brevispiculata* *A. ziphidarum* *A. nascettii*

Anisakis and Anisakiasis

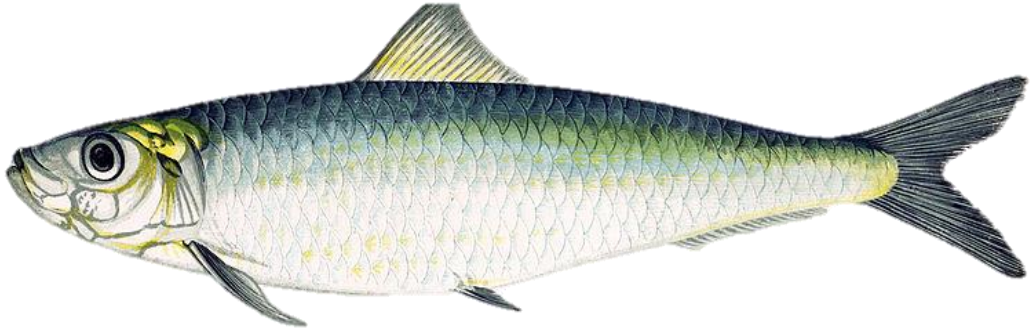
- First peak of interest on 1996: first case of Anisakiasis in Italy (Stallone et al. 1996)

- Introduction of Japanese traditional food



Aim of my study

Assess the risk level related to *Anisakis spp.* in Mediterranean basin and develop prevention strategies and techniques to reduce it



Work package 1

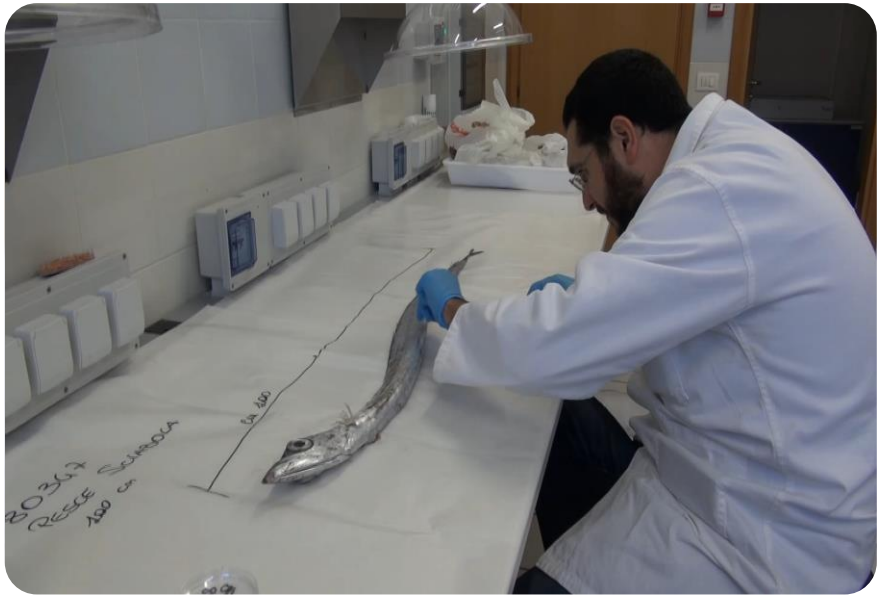
Assess the presence of Anisakidae in Mediterranean fish and fish products and investigate the seasonal infestation trend to have a chronological risk assessment regarding their consumption and adopt targeted fishing strategies

Materials and methods

7543 commercial fish samples

- *Engraulis encrasicolus*
- *Sardina pilchardus*
- *Scomber scombrus*
- *Lepidopus caudatus*
- *Trachurus trachurus*

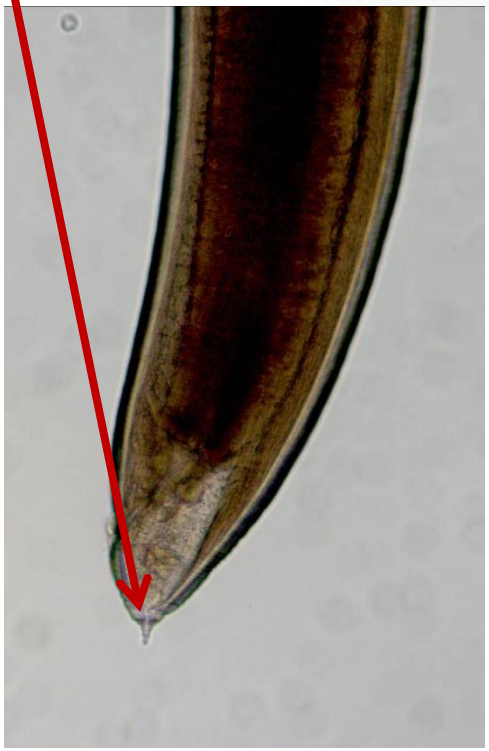
Visual inspection and chloro-peptic digestion



Infestation parameters assessment

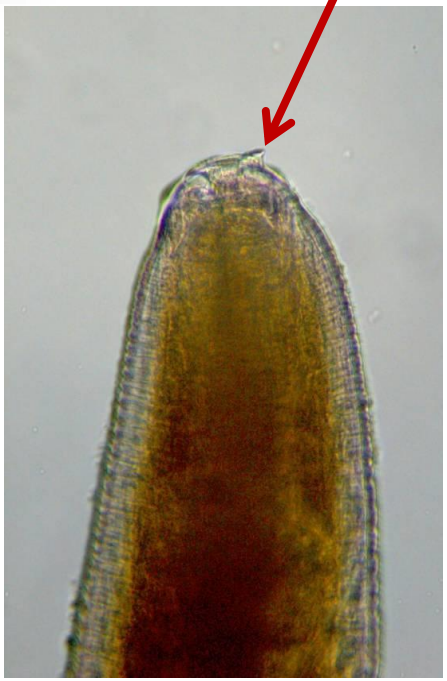
Morphological identification

Mucron



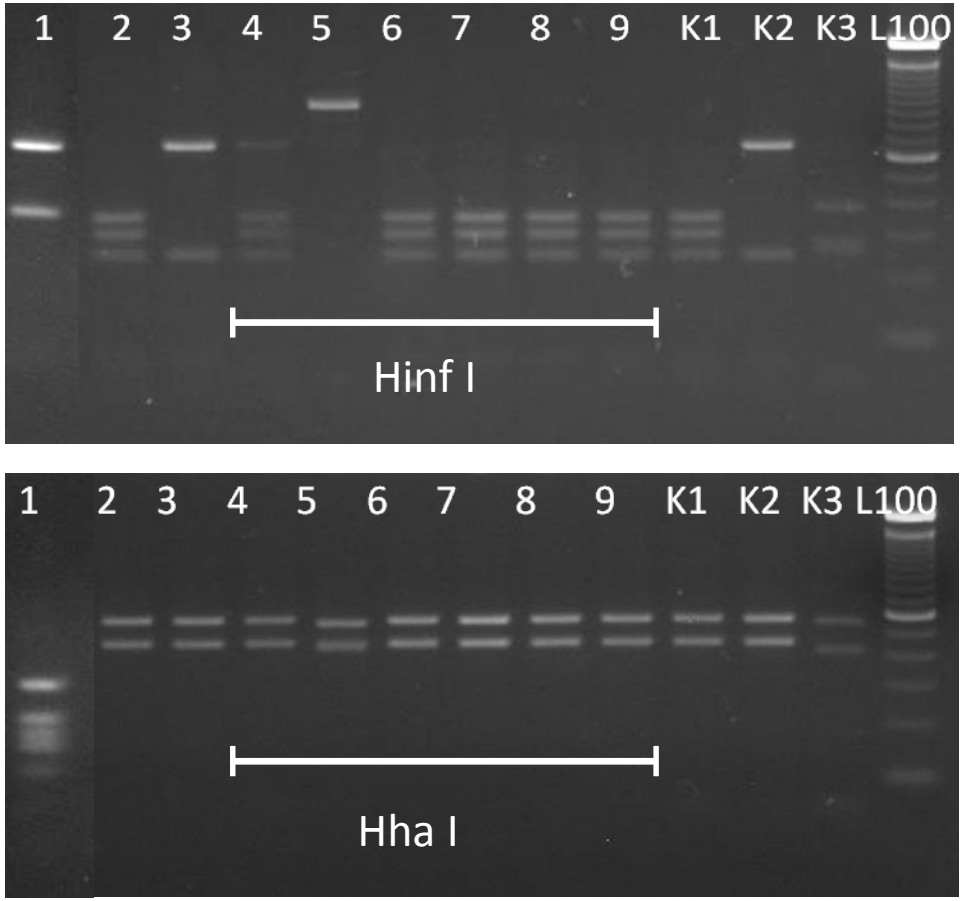
Morphotype I

Boring tooth



Species identification

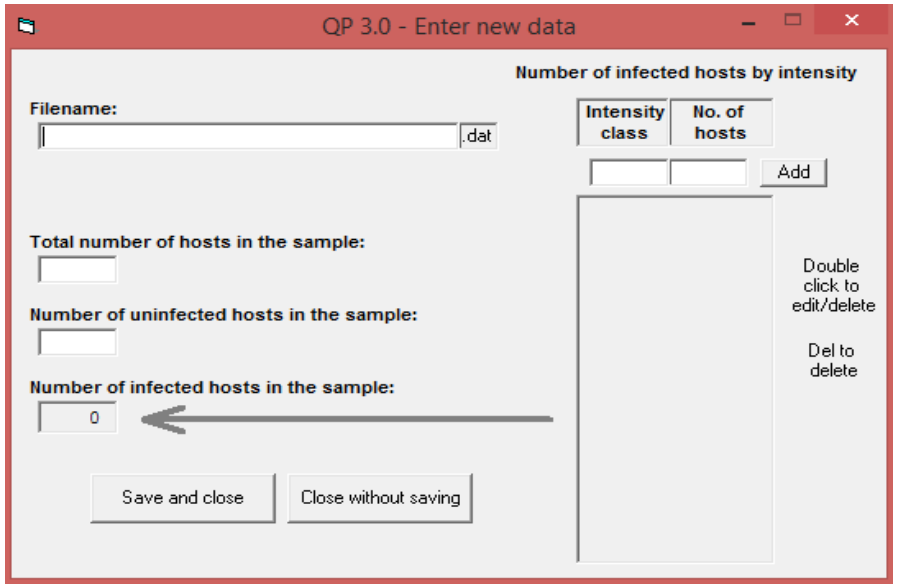
PCR-RFLP: Identification of *Anisakis* species (Zhu et al. 2000)



1 Anisakis typica
2 Anisakis pegreffii
3 Anisakis simplex
4 Genotipo Ibrido
5 Anisakis paggiae
6-9 Anisakis pegreffii
K1 Anisakis pegreffii
K2 Anisakis simplex
K3 Anisakis physeteris

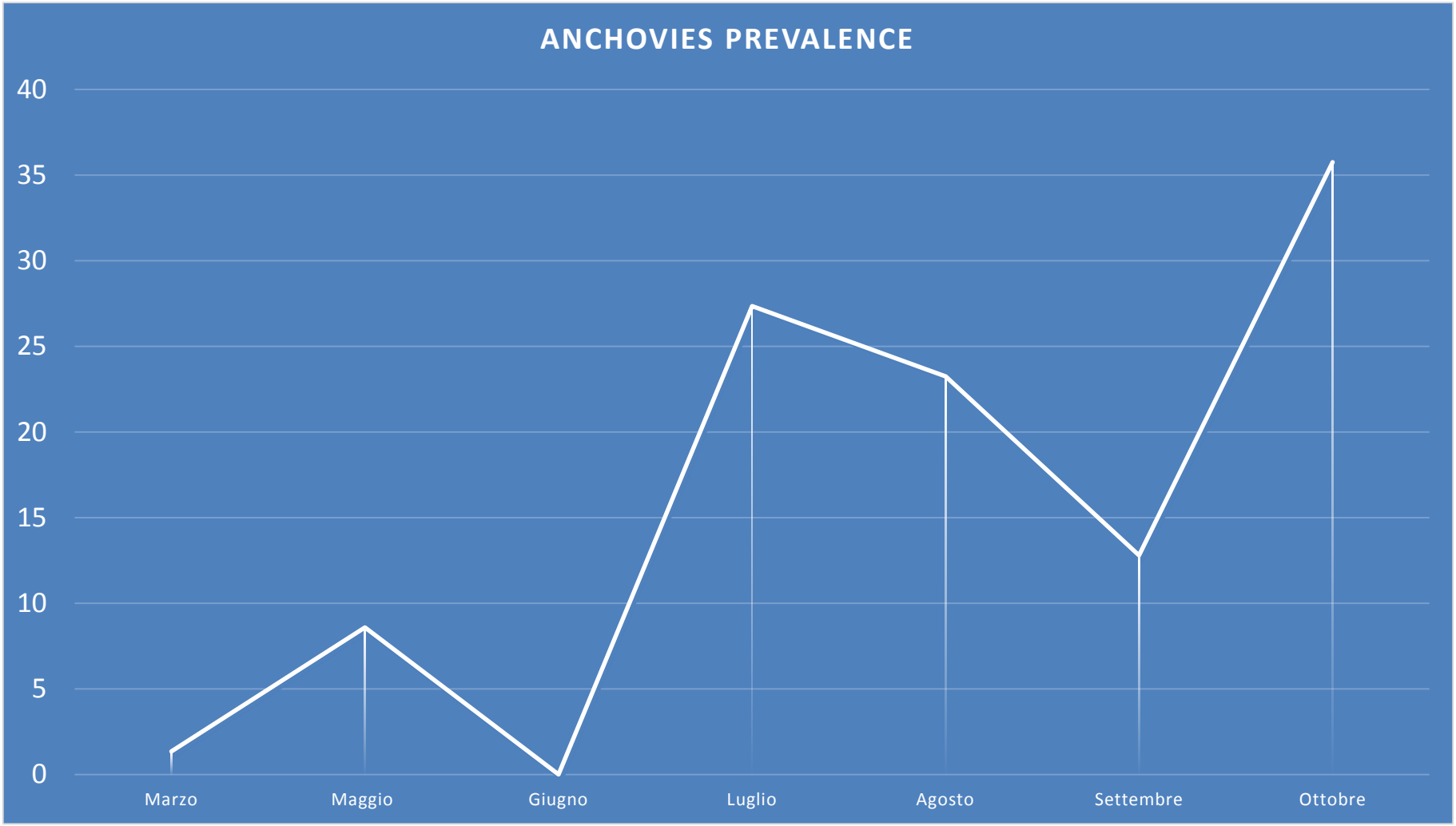
Materials and methods

- Epizootic parameters calculated by QP 3.0 software



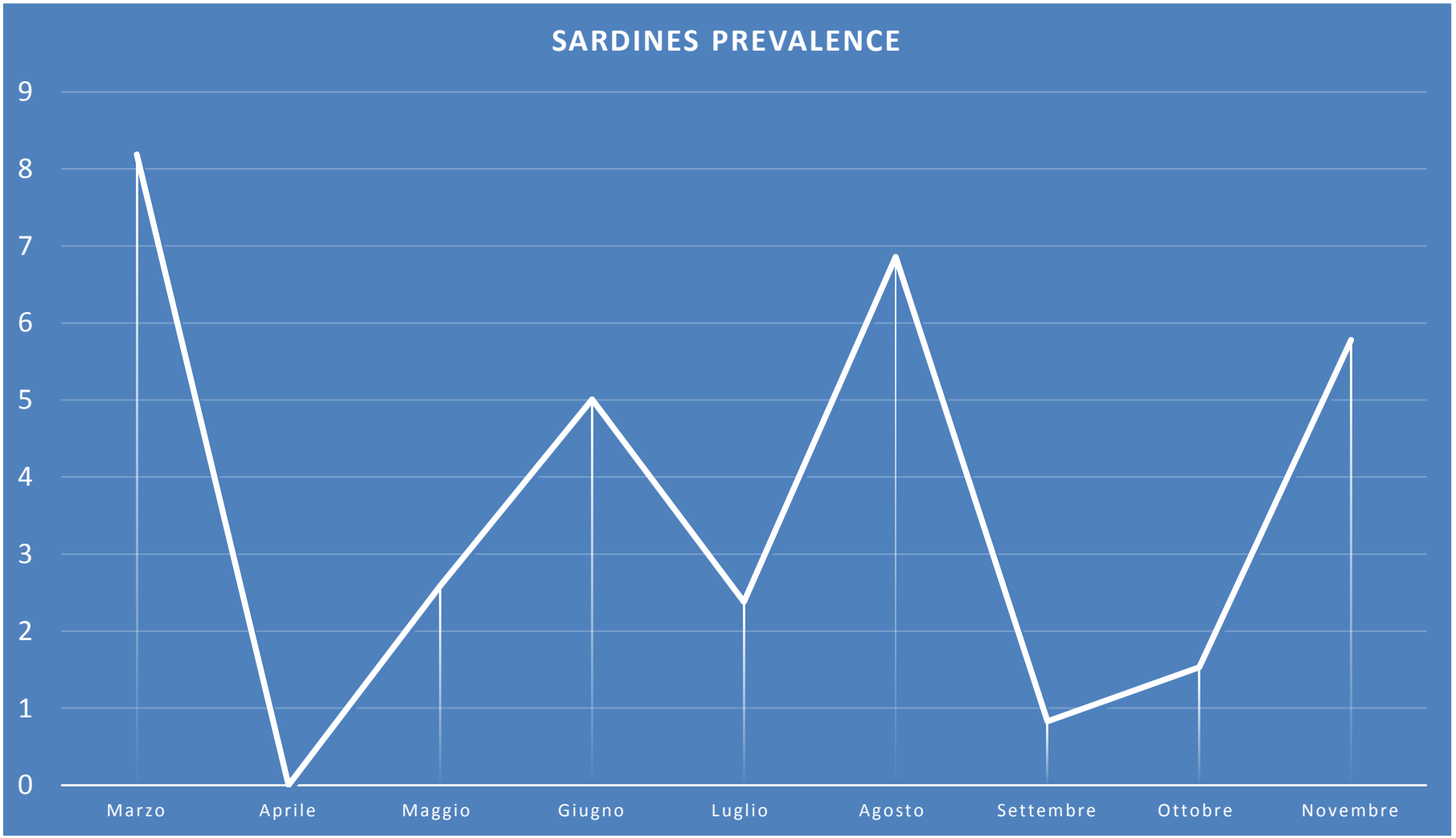
RESULTS

- 8362 larvae found in all the fish samples examined
- Prevalence: ↓ sardines (3.68%) ↑ silver scabbardfish (100%)
- Mean intensity: ↓ anchovies and sardines (1) ↑ silver scabbard fish (157,7)
- All the larvae collected belonged morphologically to *Anisakis spp.* morphotype I
- 10% of the larvae collected were subjected to molecular analysis
- 81,5% *A. pegreffii*
- 13,2% *A. simplex s.s.*
- 5,1% *A. simplex s.s./A.pegreffii* Hybrid form
- 0,2% *A. typica*



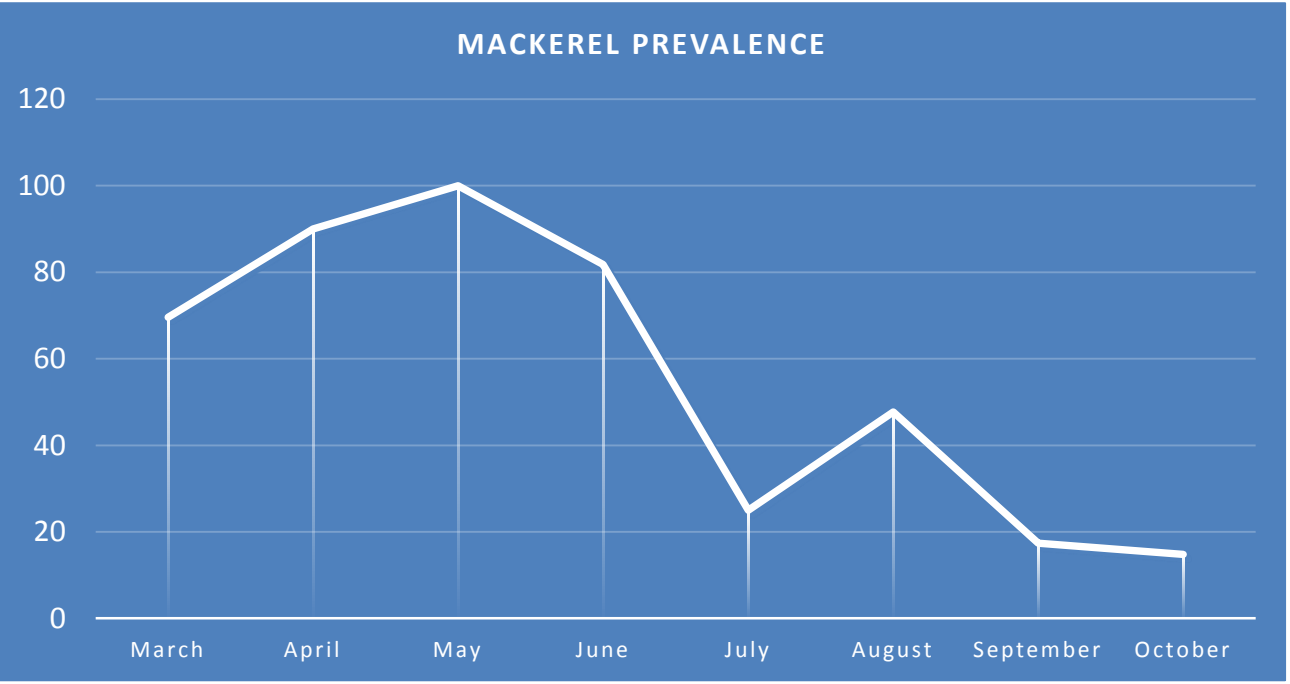
Y axis = prevalence of infestation (%)
X axis = Month of sampling

Friedman chi-squared = 6.9333,
df = 2, p-value = 0.03122

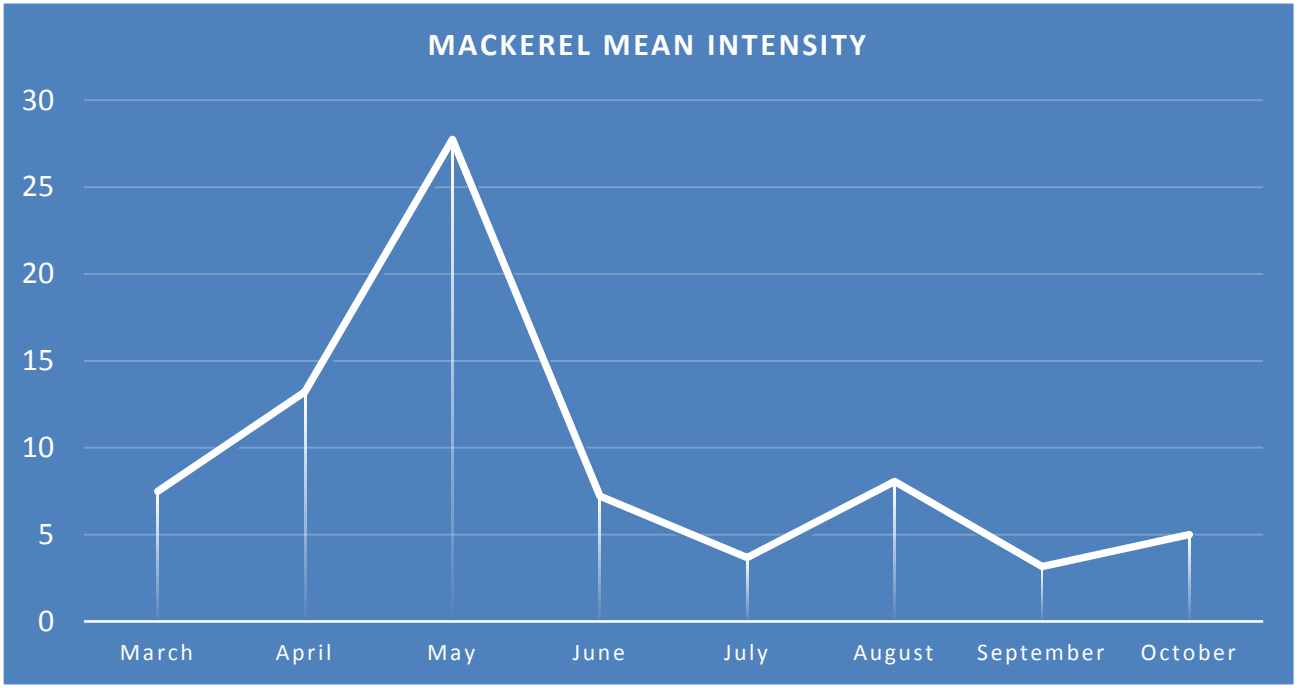


Y axis = prevalence of infestation (%)
X axis = Month of sampling

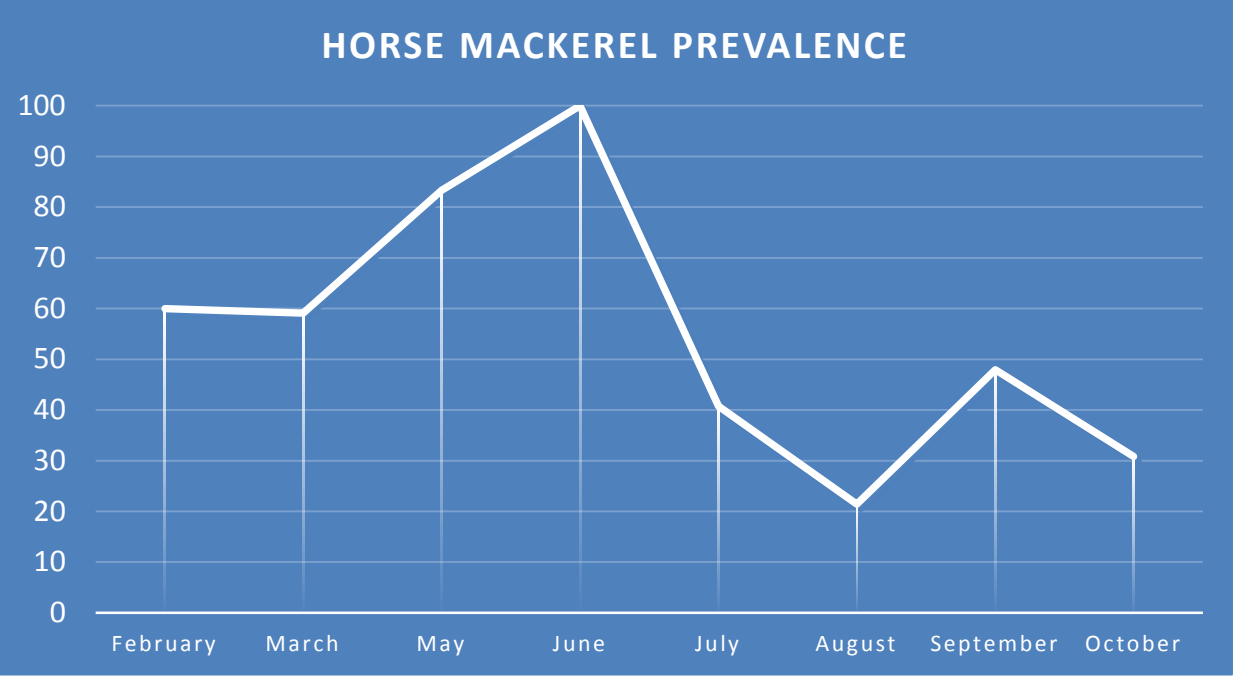
Friedman chi-squared = 5.9111,
df = 2, p-value = 0.05205



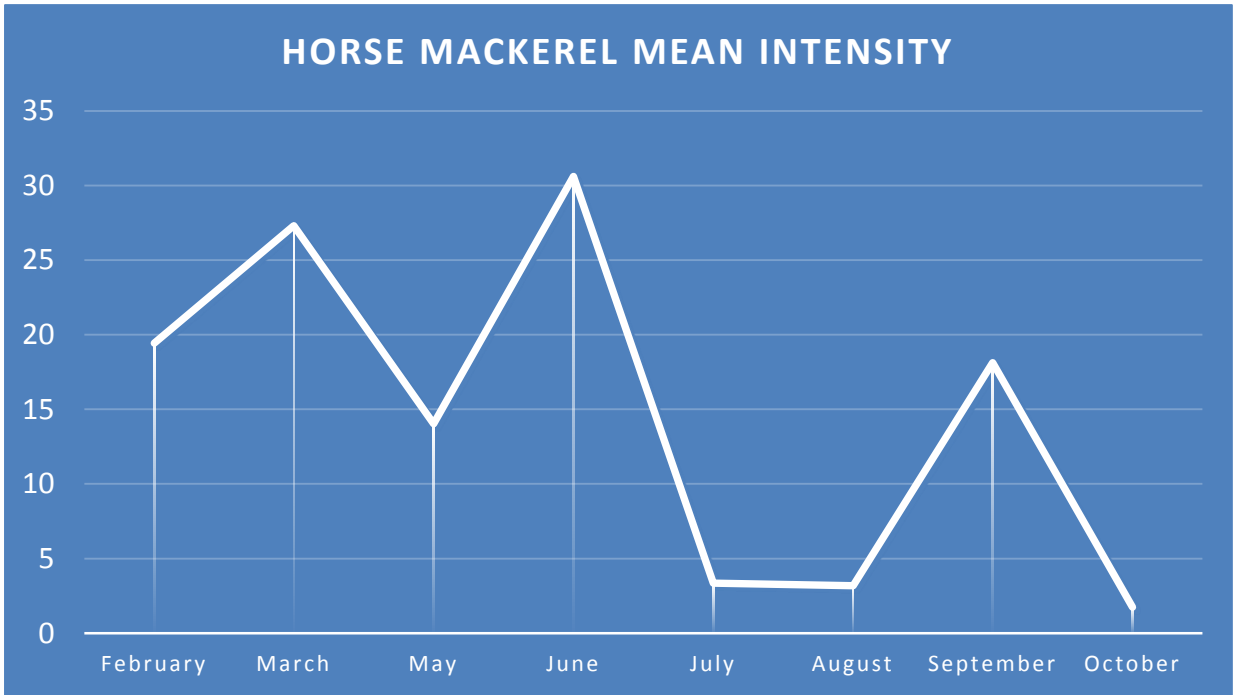
Friedman chi-squared = 3, df = 2, p-value = 0.2231



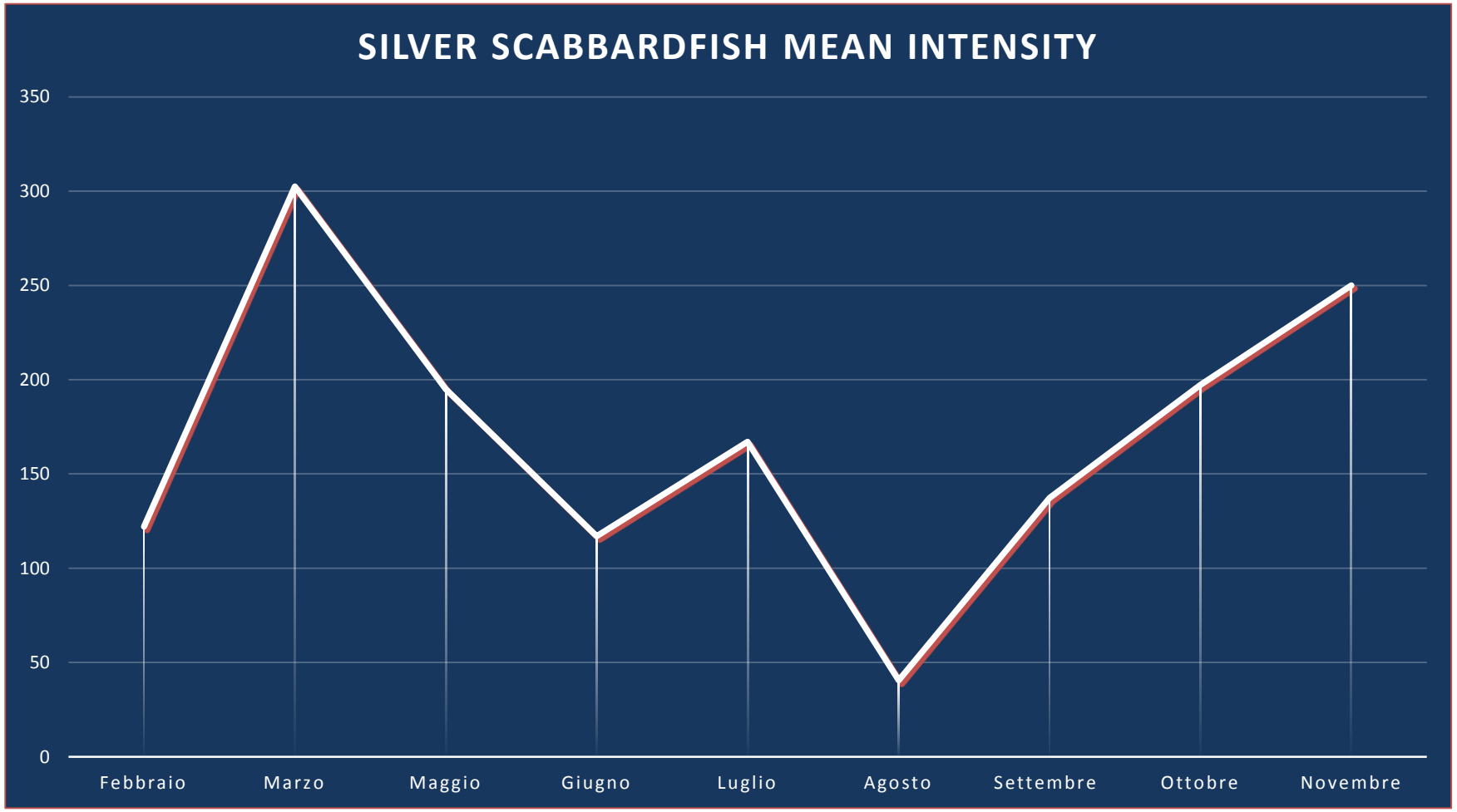
Friedman chi-squared = 25.84, df = 2, p-value = 0.000002439



Friedman chi-squared = 6,
df = 3, p-value = 0.1116



Friedman chi-squared = 24.679,
df = 3, p-value = 0.00001802



Y axis = Mean intensity X axis = Month of sampling

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Natural Product Research

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Seasonal trend of Anisakidae infestation in South Mediterranean bluefish

Gaetano Cammilleri, Andrea Pulvirenti, Antonella Costa, Stefania Graci, Rosaria Collura, Maria Drussilla Buscemi, Sonia Sciortino, Valeria Vitale Badaco, Mirella Vazzana, Mariagrazia Brunone, Antonio Vella, Calogero Di Bella & Vincenzo Ferrantelli

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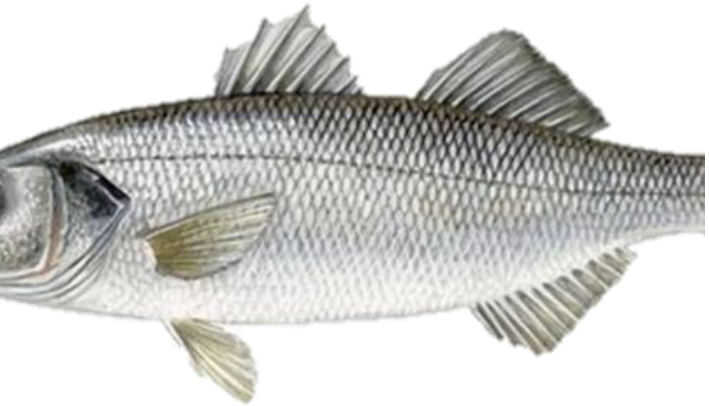


Work package 2

Assess the presence of *Anisakis* spp. in farmed fish of the Mediterranean

European Food Safety Authority

EFSA Journal 2010; 8(4):1543

SCIENTIFIC OPINION

Scientific Opinion on risk assessment of parasites in fishery products[1]

EFSA Panel on Biological Hazards (BIOHAZ)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

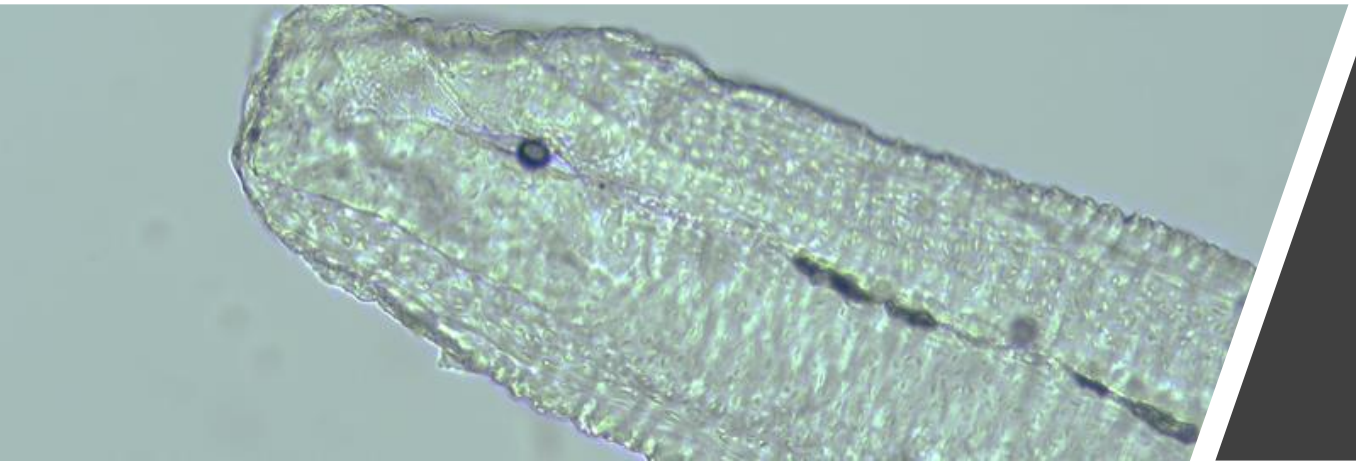
Human fishery product-borne parasitic diseases are caused by cestodes, trematodes and nematodes and are caused by infection following ingestion of viable parasites, or as allergic (hypersensitivity) reactions against parasite antigens. For allergy, the only parasite in fishery products implicated is the nematode *Anisakis simplex*, and sensitisation occurs via infection by live larvae. Once sensitised, response to nematode allergens can be highly aggressive and generate severe disease. In a sensitised individual, infection can provoke a concurrent *A. simplex* allergic episode or can be elicited by exposure to allergen alone from killed parasite: the relative epidemiological impact for each is unknown. Allergy to *A. simplex* is relatively common in some regions in Spain but rarely reported in other parts of Europe. Prevention of sensitisation is most likely to be effective by control of *A. simplex* infection. There is more information on the resistance to physical and chemical treatments by *A. simplex* than for other fishery parasites, and the properties of other parasites are likely to be similar. Many traditional marinating and cold smoking methods are not sufficient to kill *A. simplex* and freezing or heat treatments remain the most effective processes guaranteeing killing. All wild caught seawater and freshwater fish are must be considered at risk of containing any viable parasites of human health concern if these products are to be eaten raw or almost raw. For wild-catch fish, no sea fishing grounds can be considered free of *A. simplex.* For farmed Atlantic salmon reared in floating cages or onshore tanks and fed on compound feedstuffs however, the current risk of infection with anisakids is negligible. Apart from farmed Atlantic salmon, sufficient monitoring data are not available for any other farmed fish therefore it is not possible to identify which fish species do not present a health hazard with respect to the presence of parasites.

Materials and methods

151 samples of European Sea bass (*Dicentrarchus labrax*) examined by visual inspection and artificial digestion

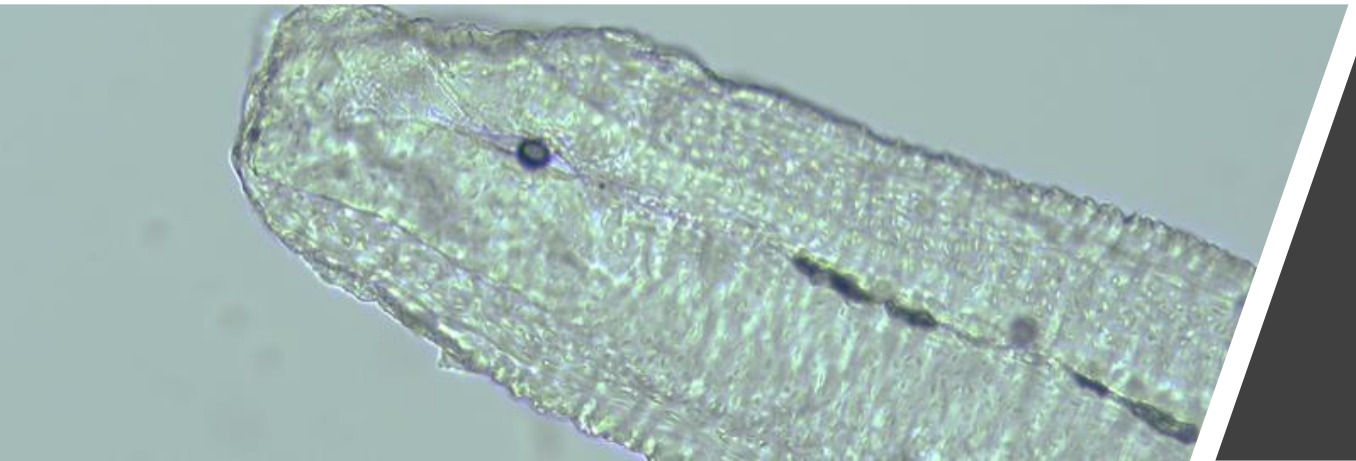
RESULTS

Presence of two *Anisakis pegreffii* nematodes in only one sample



The European sea bass sample infected by *Anisakis* showed a low Fulton's K index (0.83)

Particular stressed condition that lead the fish to feed on infected crustaceans and small fish entering the cage



Veterinary Parasitology 259 (2018) 13–16

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Veterinary Parasitology

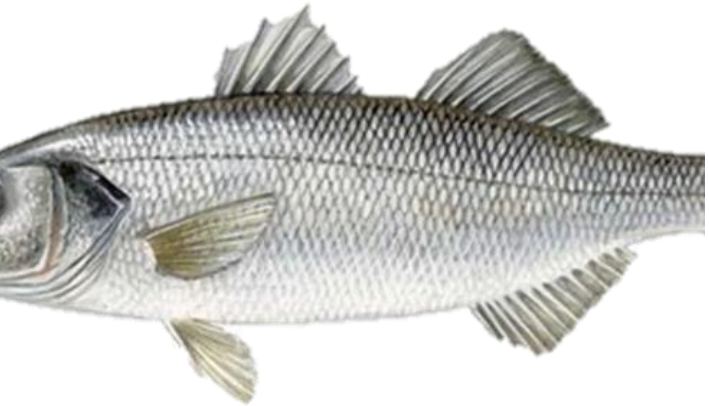
journal homepage: www.elsevier.com/locate/vetpar

Short communication

Presence of Anisakis pegreffii in farmed sea bass (*Dicentrarchus labrax* L.) commercialized in Southern Italy: A first report

Gaetano Cammilleri[a], Antonella Costa[a,*], Stefania Graci[a], Maria Drussilla Buscemi[a], Rosaria Collura[a], Antonio Vella[a], Andrea Pulvirenti[b], Antonello Cicero[a], Giuseppe Giangrosso[a], Pietro Schembri[c], Vincenzo Ferrantelli[a]

[a] *Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", via Gino Marinuzzi 3, 90129 Palermo, Italy*
[b] *Dipartimento di Scienze della Vita, Università degli studi di Modena e Reggio Emilia, Via Università 124, 41121 Modena, Italy*
[c] *Servizio 7 - Sicurezza Alimentare, Azienda Sanitaria Provinciale di Palermo, Piazza Ottavio Ziino, 24, 90145 Palermo, Italy*



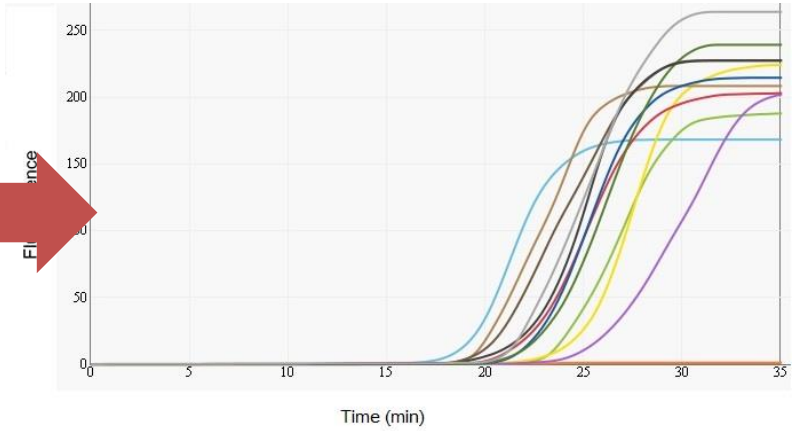
Work package 3

Carry out and validate a commercial Loop mediated isothermal amplification (LAMP) for the rapid detection of *Anisakis* parasites in processed fish products

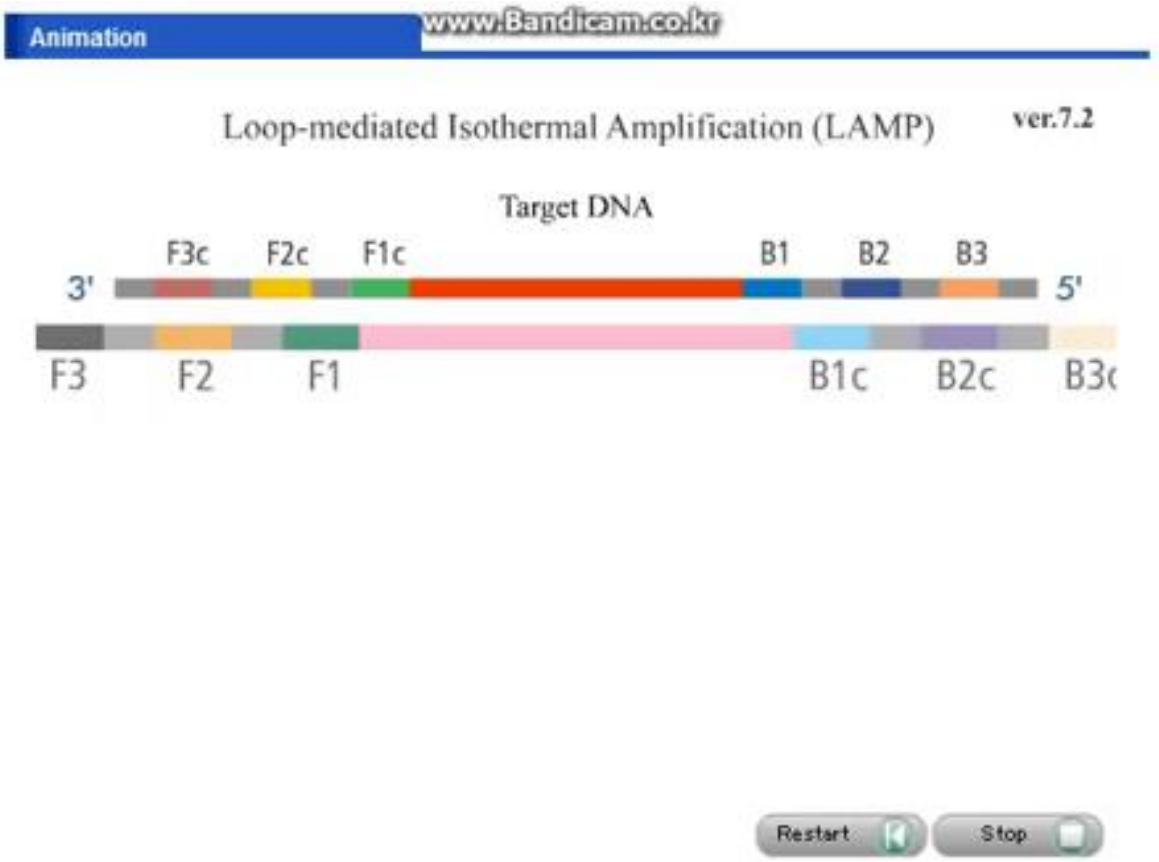
What can be done for the fishery sector to help manufacturers establish concepts for the hazard analysis (HACCPs)?

Need of a rapid, low cost and easy method to detect the presence of Anisakis in processed fish products.

COMMERCIAL LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR THE RAPID DETECTION OF ANISAKIS DNA IN PROCESSED FISH PRODUCTS



LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)



LAMP assay: Primer design

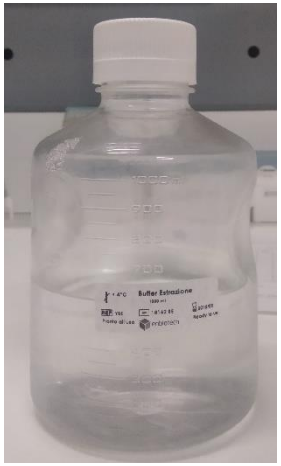
- 14 DNA sequences belonging to *Anisakis* spp. reported in GenBank™ were considered for primer design
- Several assumptions were considered (distances between primers, GC content, melting temperature, absence of complementarity/regions of secondary structures) taking into account the time to results parameter.
- A set of 6 primers was designed (two outer (F3 and B3), two inner (FIP and BIP) and two loop (LF and LB)) using the Primer Explorer software

PrimerExplorer V4 Software

LAMP assay: Extraction optimization

250±50 mg

Extraction buffer



4 ml

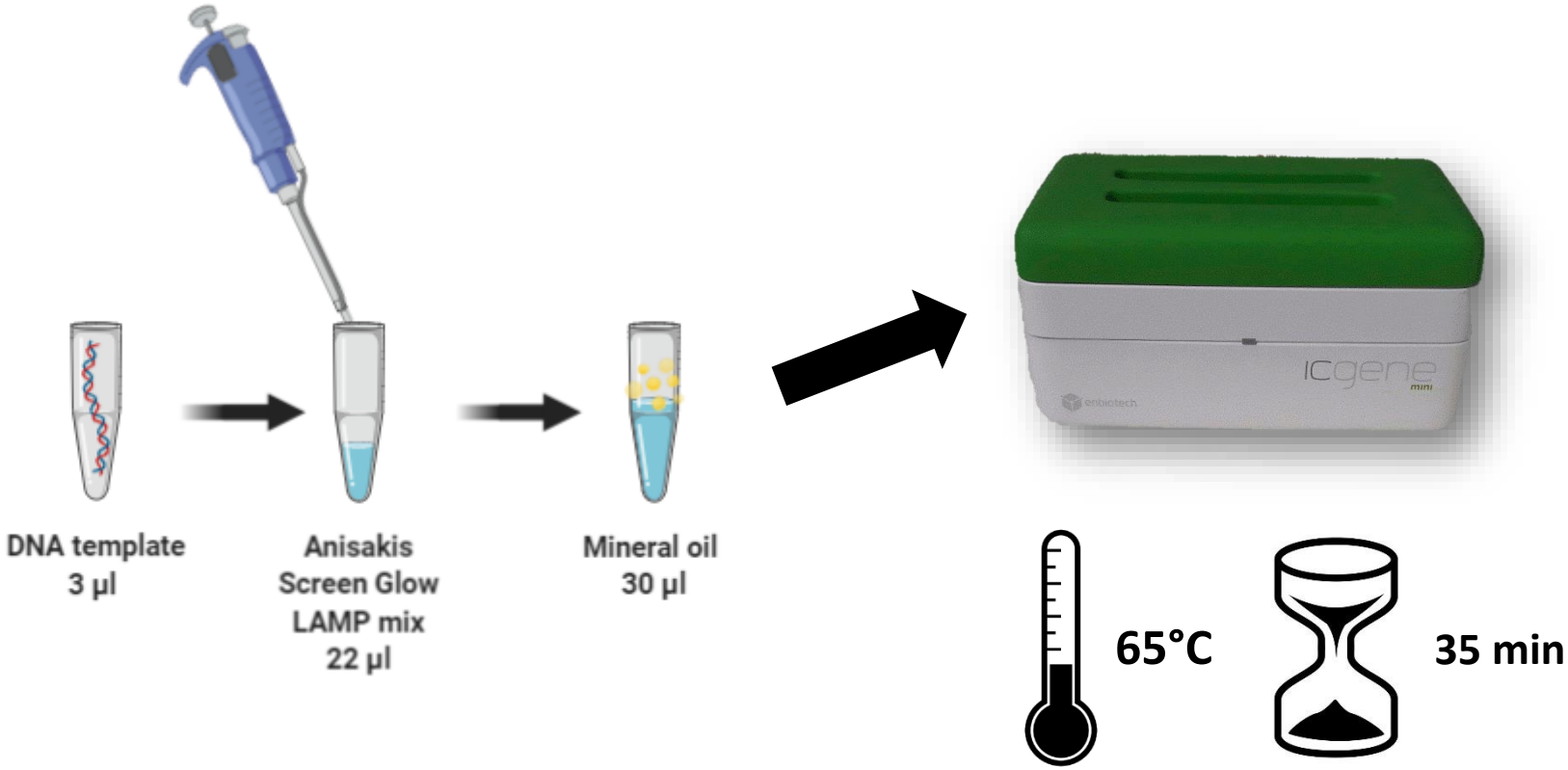
Incubation 40±5 min

1:5 dilution

Best conditions for effective real-time detection of DNA amplification by the LAMP method proposed

LAMP assay optimization

- **Real time monitoring of the time and temperature of reaction;**



Best conditions for effective real-time detection of DNA amplification by the LAMP method proposed

LAMP assay validation

Naturally negative (non-contaminated by Anisakis) samples:

- **n. 40 homogenised farmed trout**
- **n. 40 homogenised farmed sea bream**
- **n. 40 homogenised farmed salmon**

Naturally positive samples for matrix effects evaluation

- **n. 40 anchovy paste**
- **n. 40 anchovy in oil**
- **n. 40 salted sardines**

***Anisakis* larvae collected from *L. caudatus*, *C. harengus* and *M. merlucius* for artificial infestation**

LAMP assay validation

Specificity

Two different experiments

Artificial contamination with Genomic DNA:

- *P. decipiens s.s.*,
- *P. krabbei*,
- *P. cattani*,
- *P. azarasi*,
- *C. rudolphii A*
- *Hysterothylacium aduncum*

(tested in duplicate)

Artificial infestation with larvae:

- *Pseudoterranova sp.*
- *Contracaecum sp.*
- *Hysterothylacium* sp.

(subjected to DNA extraction and analysed in duplicate)

Twenty samples of each processed fish product type, of which ten artificially infested (5 with genomic DNA and 5 with larvae)

LAMP assay validation

Sensitivity

Two different experiments

Artificial contamination with Genomic DNA:

- *A. pegreffii*
- *A. simplex s.s.*
- *A. typical*
- *A.ziphidarum*
- *A. physeteris*

(tested in duplicate)

Artificial infestation with larvae:

- *Anisakis* spp. type I
- *Anisakis* spp. type II

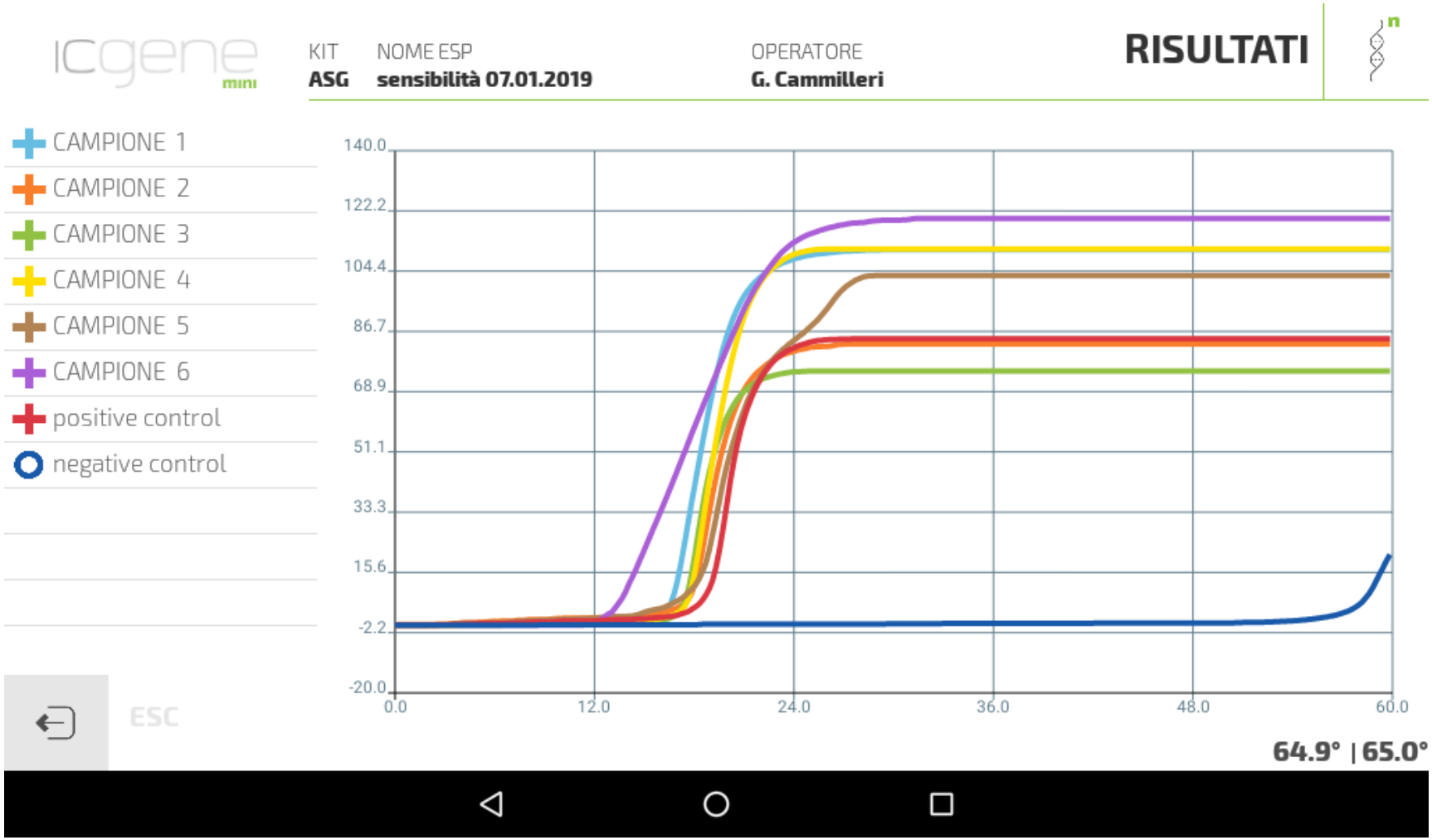
(subjected to DNA extraction and analysed in duplicate)

Twenty samples of each processed fish product type

LOD: Serial 10-fold dilution of the DNA extracted from *Anisakis* spp. larvae with nuclease-free water (10 replicates from each sample type independently)

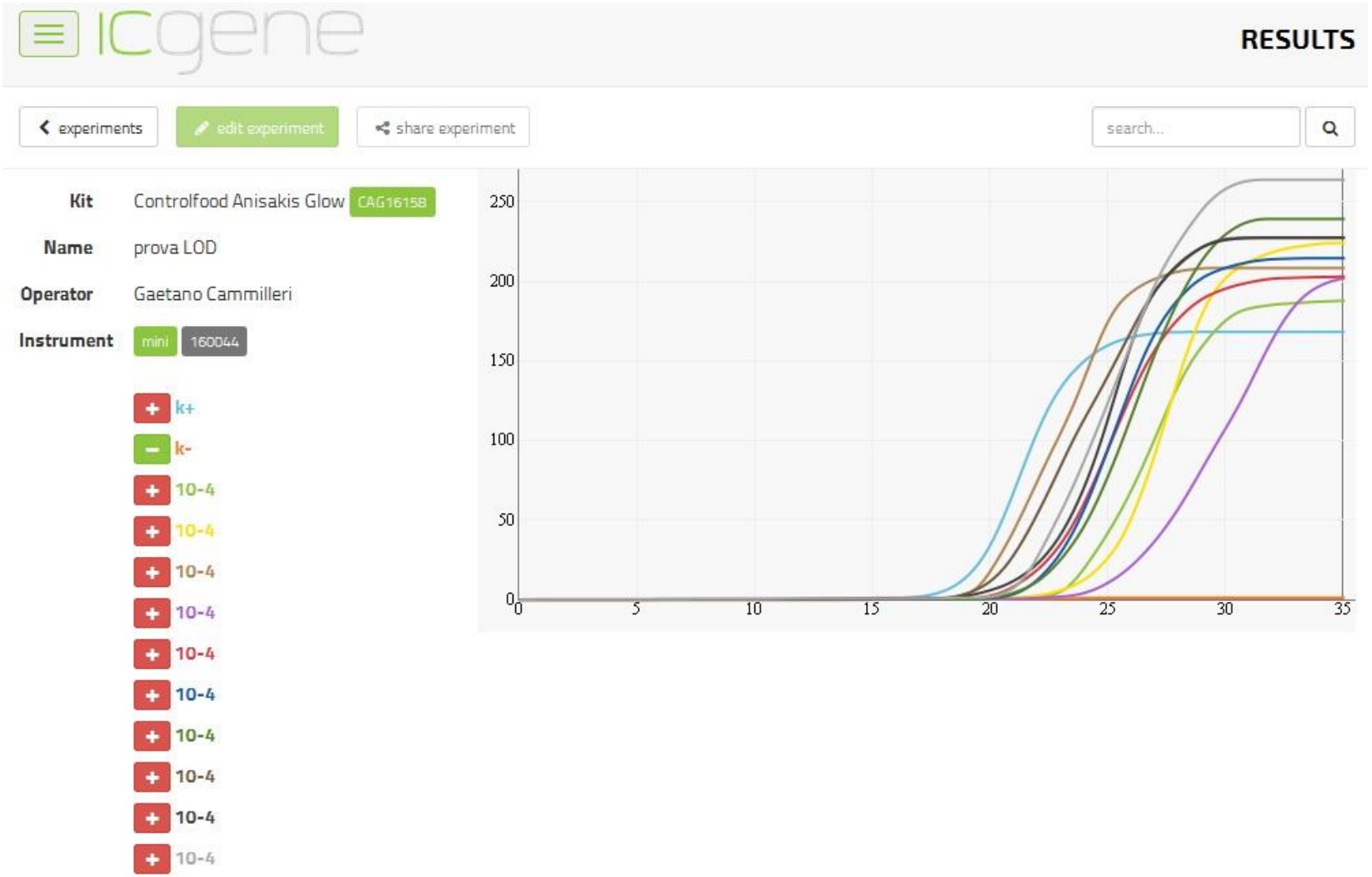
RESULTS

- Sensitivity of 100% for each sample type analysed - Able to detect each sample contaminated with *A. simplex* s.s., *A. pegreffii*, *A. physeteris*, *A. ziphidarum* and *A. typica* DNA.



RESULTS

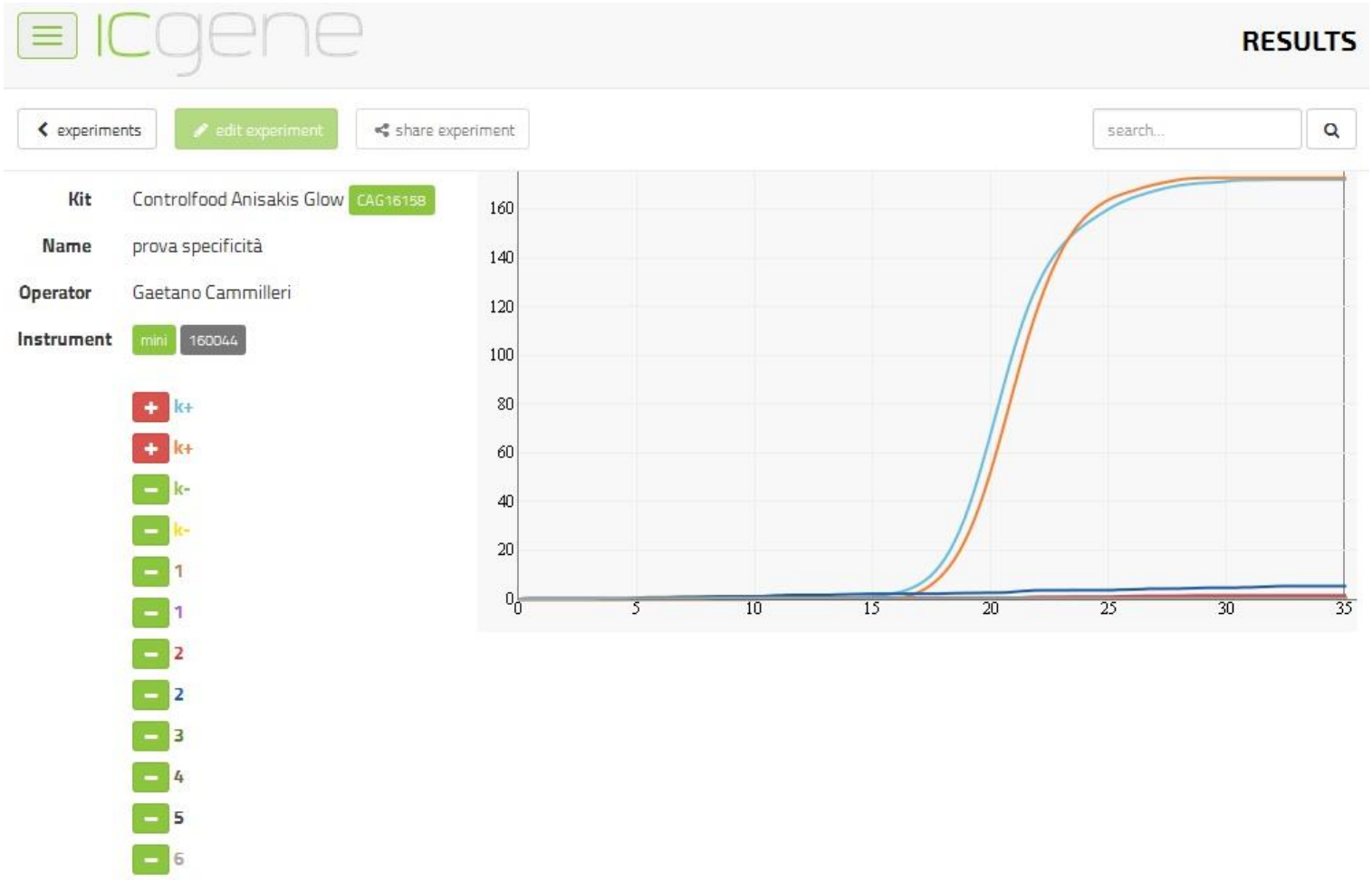
- The assay detected *Anisakis* spp. DNA to a dilution of 10^{-4} (0.00022 ng μl^{-1}), giving an amplification for all the replicates



RESULTS

- No amplification in uninfected samples specificity =100%.

No amplification was obtained on processed fish samples contaminated with *Contracaecum* sp. *Pseudoterranova* sp. and *Hysterothylacium* sp. larvae and DNA



foods

MDPI

Article

Validation of a Commercial Loop-Mediated Isothermal Amplification (LAMP) Assay for the Rapid Detection of *Anisakis* spp. DNA in Processed Fish Products

Gaetano Cammilleri [1,2,*], Vincenzo Ferrantelli [1], Andrea Pulvirenti [2], Chiara Drago [3], Giuseppe Stampone [3], Gema Del Rocio Quintero Macias [3], Sandro Drago [3], Giuseppe Arcoleo [3], Antonella Costa [1], Francesco Geraci [1] and Calogero Di Bella [1]

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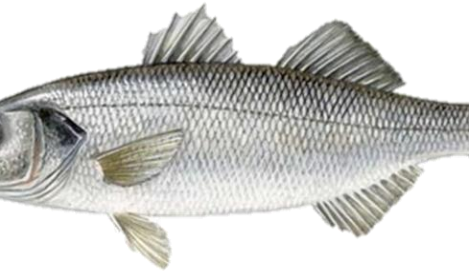
CONCLUSIONS



WP1: The data obtained are useful to plan a seasonal fishing strategy to reduce the risks related to the presence of *Anisakis* in fish products.

WP2: First report on the presence of Anisakidae nematodes in farmed European sea bass. However, due to the low prevalence found, the risk in farmed fish remain very low.

WP3: The rapidity, sensitivity, and ease of use suggest that LAMP assay can be a valid alternative for routine examination in the fishery sector to help manufacturers establish concepts for hazard analysis and critical control points (HACCPs) evaluation.

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