

**Fish health in a changing sea:
A multidisciplinary One Health assessment of *Merluccius merluccius*
(Linnaeus, 1758) and *Sardinella aurita* Valenciennes,
1847 in the NW Mediterranean Sea**

Laura Muns Pujadas

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Departament de Biologia Animal, Biologia Vegetal i Ecologia

PhD thesis

Fish health in a changing sea:

A multidisciplinary One Health assessment of *Merluccius merluccius* (Linnaeus, 1758) and *Sardinella aurita* Valenciennes, 1847 in the NW Mediterranean Sea

A dissertation submitted by Laura Muns Pujadas in fulfilment of the requirements for the degree of Doctor of Philosophy granted by the International Doctorate in Aquaculture in June 2026

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“All we have to decide is what to do with the time that is given us”

— J.R.R. Tolkien —

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Abstract | Resum

Executive summary

The Mediterranean Sea is a global marine biodiversity hotspot that supports fisheries essential for regional economies and food security. However, increasing anthropogenic pressures may compromise the resilience and sustainability of exploited fish populations. This thesis applies a multidisciplinary One Health approach to evaluate the health status, exposure to environmental stressors, and food safety implications of two commercially relevant fish species from the Catalan coast (NW Mediterranean): the European hake (*Merluccius merluccius*) and the round sardinella (*Sardinella aurita*).

The first results section (**Chapter 4**) focuses on fish health and parasite-host interactions. Spatial and temporal analyses in hake and seasonal analyses in round sardinella revealed variations in condition, parasite communities, and histopathological alterations associated with environmental and biological factors. Although most lesions were mild and mainly linked to parasitic infections, the results highlight the value of integrative health assessments as indicators of environmental stress in wild fish populations. Additional studies provided the first comprehensive characterization of the cestode *Cleistobothrium crassiceps* and the first genetic and histopathological assessment of the blood fluke *Aporocotyle spinosicanalis* in Mediterranean hake populations (**Chapter 4.1 and 4.2**)

The second section (**Chapter 5**) evaluates the effects of anthropogenic items and heavy metals. Both species ingested anthropogenic items, mainly cellulosic fibres, with spatial and seasonal differences related to feeding behaviour and environmental conditions. Heavy metal concentrations generally remained below harmful thresholds, although mercury accumulation increased with fish size in hake. No consistent evidence linked contaminant exposure with significant health impairment in either species.

The final section (**Chapter 6**) addresses nutritional quality and food safety. Round sardinella showed high nutritional value, especially in autumn, when omega-3 fatty acid levels reached recommended dietary intake values, supporting its potential as an alternative fishery resource under the ongoing decline of traditional pelagic species. Heavy metal concentrations remained below European regulatory limits, although frequent consumption may represent a methylmercury exposure risk for infants. In contrast, the absence of zoonotic anisakid nematodes in both species from the Catalan coast suggests a low anisakiasis risk for local consumers.

Overall, this thesis highlights the importance of integrative health studies to assess the condition and resilience of exploited fish populations under global change, contributing to fisheries monitoring and more sustainable management strategies within a One Health framework.

Abstract

The Mediterranean Sea is one of the world's most important marine biodiversity hotspots that supports fisheries essential for regional economies and food security. However, this semi-enclosed basin is increasingly threatened by anthropogenic pressures including fishing pressure, pollution, habitat degradation, pathogens and global change. In the Balearic Sea (north-western Mediterranean), important commercial species such as the European hake, *Merluccius merluccius* (Linnaeus, 1758), have shown declining landings in recent decades. At the same time, global change is altering species distributions, favouring thermophilic species over traditional small pelagic fish. This is the case of round sardinella, *Sardinella aurita* Valenciennes, 1847, whose catches have increased in abundance in recent years, suggesting their potential as emerging fishery resources. However, they remain economically undervalued due to limited consumer acceptance and scarce information on their nutritional quality. In this context, assessing fish responses to multiple stressors is essential to evaluate stock condition, seafood safety and nutritional quality. A One Health perspective provides an integrative framework for this purpose, recognizing fish as both bioindicators of ecosystem quality and potential pathways for contaminants and zoonotic pathogens to humans.

This thesis applies a multidisciplinary One health approach to evaluate the health status, exposure to anthropogenic stressors and food safety implications of two commercial fish species from the Catalan coast (NW Mediterranean Sea): the European hake and the round sardinella. This study integrates the evaluation of fish condition, pathology, histopathology and parasite communities, anthropogenic items ingestion and accumulation of heavy metals. In addition, the nutritional quality of *S. aurita* is evaluated to determine its commercial viability. For the European hake, a widely distributed and commercially valuable species along the Catalan coast, a spatial comparison was conducted among three localities with different environmental conditions and anthropogenic pressures (Blanes, Barcelona and Ebro Delta), to identify potentially vulnerable or healthier populations. For round sardinella, a seasonal sampling strategy was performed at a local scale off Barcelona to investigate temporal variations in health, contaminant exposure and nutritional quality, considering its potential as an emerging fish resource. The results of this thesis are structured into three chapters (Chapters 4, 5, 6), each addressing a different aspect of the One Health perspective (fish health, anthropogenic stressors and food safety):

The first chapter of results (**Chapter 4**) provides an assessment of the overall health status of *M. merluccius* and *S. aurita* through the evaluation of condition indices, the identification of gross pathology and histopathological alterations and the description of the parasite community and

their potential impact as stressors. Spatial and seasonal differences for hakes and sardinellas, respectively, on condition indices, parasite descriptors and histopathological alterations were found and discussed. The European hake harboured a richer parasite community but showed lower intensities compared to round sardinella. In general, histopathological alterations were mild and commonly associated with parasitic infections and limited tissue damage. Overall, no clear detrimental effects on fish condition or organ functionality were detected, suggesting a host–parasite balance. However, specific pathologies, such as blood fluke infections, epitheliocystis and microsporidian infections highlight the need for multidisciplinary approaches to detect early signals of environmental or biological stress in wild fish populations.

Moreover, the identification of two parasite species infecting *M. merluccius* and potentially affecting their condition motivated two additional studies due to the limited knowledge regarding their morphology, genetic characterization and histopathological effects. In **Chapter 4.1**, the cestode *Cleistobothrium crassiceps* was comprehensively described for the first time in its type-host from the NW Mediterranean using morphological, molecular, histological and epidemiological approaches. The study identified key diagnostic traits, described its attachment mode and supported the synonymization of *C. cristinae* and *C. splendidum*. In **Chapter 4.2**, the histopathological effects, epidemiology and first genetic characterization in the NW Mediterranean of the blood fluke *Aporocotyle spinosicanalis* were investigated. Eggs mainly caused mild inflammatory reactions in gill filaments, whereas adult flukes induced more severe cardiac lesions, including fibrinoid necrosis and lymphocytic myocarditis. Larger hakes showed higher infection levels, likely linked to ontogenetic dietary shifts.

The second chapter of results (**Chapter 5**) addresses anthropogenic pollutants, including anthropogenic items (AIs; microplastics and non-synthetic items) and heavy metals in fish from the Catalan coast (NW Mediterranean Sea). In European hake, AIs ingestion was characterized and compared with surrounding water samples across three localities with different anthropogenic pressures, including a temporal comparison in Barcelona (2007 vs 2019). Fish ingested a mean of 1.39 (SD = 1.39) items/individual, while surrounding waters contained 0.85 fibres/m³ on average, with cellulosic fibres being dominant. Only small environmental fibres (<6 mm) matched those ingested, likely reflecting hake feeding behaviour. Heavy metal concentrations showed no spatial differences, although mercury increased with fish size. In round sardinella, seasonal variations in AIs ingestion were evaluated off Barcelona. Fish ingested a mean of 2.27 (SD = 2.13) items/individual, with highest levels in winter and lowest in summer, again dominated by cellulosic fibres. Seasonal patterns were consistent with environmental factors (e.g. rainfall and water stratification) and biological factors (e.g. feeding behaviour and reproduction). Higher AIs

ingestion in winter coincided with increased feeding intensity and filter-feeding behaviour, whereas lower summer ingestion was associated with reduced feeding activity and lower rainfall rates and riverine inputs. Overall, no consistent effects of AIs ingestion or metal exposure on fish health or parasite aggregation patterns were detected in either species.

The last chapter of results (**Chapter 6**) focuses on the nutritional benefits and potential health risks associated with fish consumption. The nutritional quality of *S. aurita* (proximate and fatty acid composition) was seasonally evaluated off Barcelona to assess its viability as an alternative pelagic resource and identify periods with the highest omega-3 levels. Potential risks associated with fish consumption were assessed through heavy metal concentrations and zoonotic parasite exposure in both *S. aurita* and *M. merluccius*. In addition, the distribution of zoonotic nematodes was compared across several Mediterranean regions. Round sardinella showed seasonal variability in lipid and fatty acid composition, with eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) levels in autumn meeting recommended daily intake values for a 100 g portion. Promoting its consumption during this period could also support more sustainable fisheries by redistributing fishing pressure among pelagic species and seasons. Heavy metal concentrations in both species remained below European Commission limits, although frequent consumption of both species may represent a potential methylmercury risk for infants. No zoonotic nematodes were detected in either species from the Catalan coast, reducing anisakiasis risk for consumers. In contrast, hakes from the Adriatic Sea showed high prevalence of *Anisakis pegreffii*, particularly in larger fish, highlighting the importance of fish size and capture area in anisakid distribution.

Overall, this thesis highlights the value of integrative fish health assessments to better understand the condition and resilience of exploited fish populations. This approach may support fisheries management by improving local monitoring programmes, identifying vulnerable populations or periods, and highlighting potential emerging fishery resources, thereby promoting more sustainable exploitation under ongoing global change.

Resum executiu

La mar Mediterrània és una de les regions amb més biodiversitat marina que sosté pesqueries essencials per a les economies regionals. Tanmateix, l'augment de les pressions antropogèniques pot comprometre la resiliència i sostenibilitat de les poblacions pesqueres. Aquesta tesi aplica un enfocament multidisciplinari basat en el concepte *One Health* per avaluar l'estat de salut, l'exposició a factors d'estrès ambientals i les implicacions per a la seguretat alimentària de dues espècies pesqueres comercials de la costa catalana (Mediterrani nord-occidental): el lluç (*Merluccius merluccius*) i l'alatxa (*Sardinella aurita*).

La primera secció de resultats (**Capítol 4**) es centra en la salut dels peixos i les interaccions hoste-paràsit. La comparativa espacial i temporal en lluç i l'anàlisi estacional en l'alatxa van revelar variacions en la condició, les comunitats parasitàries i les alteracions histopatològiques associades a factors ambientals i biològics. Tot i que la majoria de lesions eren lleus i relacionades amb infeccions parasitàries, les avaluacions integratives de salut destaquen com a indicadors d'estrès ambiental en poblacions de peixos salvatges. Estudis addicionals van proporcionar la primera caracterització completa del cestode *Cleistobothrium crassiceps* i l'avaluació genètica i histopatològica del trematode sanguini *Aporocotyle spinosicanalis* en poblacions mediterrànies de lluç (**Capítols 4.1 i 4.2**).

La segona secció (**Capítol 5**) avalua els efectes dels elements antropogènics i metalls pesants. Ambdues espècies van ingerir elements antropogènics, principalment fibres cel·lulòsiques, amb diferències espacials i estacionals relacionades amb el comportament alimentari i les condicions ambientals. Les concentracions de metalls es van trobar per sota dels líndars perjudicials, tot i que l'acumulació de mercuri augmentava amb la mida dels lluços. No es va trobar cap evidència que relacionés l'exposició als contaminants amb un deteriorament significatiu de la salut en cap de les dues espècies.

La secció final (**Capítol 6**) aborda la qualitat nutricional i la seguretat alimentària. L'alatxa va mostrar un alt valor nutricional, especialment a la tardor, quan els nivells d'àcids grassos omega-3 van assolir els valors recomanats, reforçant el seu potencial com a recurs pesquer alternatiu davant el declivi continuat de les espècies pelàgiques tradicionals. Les concentracions de metalls pesants es van mantenir per sota dels límits reguladors europeus, tot i que un consum freqüent podria representar un risc d'exposició al metilmercuri en infants. En canvi, l'absència de nematodes anisakids zoonòtics en ambdues espècies de la costa catalana suggereix un baix risc d'anisakiosi per als consumidors locals.

Resum

La mar Mediterrània és una de les regions amb més biodiversitat marina del món alhora que sosté pesqueries essencials per a les economies regionals i la seguretat alimentària. Tanmateix, aquesta conca semi-tancada està cada vegada més amenaçada per pressions antropogèniques com la sobrepesca, la contaminació, la degradació dels hàbitats, els patògens i el canvi global. Al mar Balear (Mediterrani nord-occidental), espècies comercials importants com el lluç, *Merluccius merluccius* (Linnaeus, 1758), han mostrat una disminució de desembarcaments durant les darreres dècades. Paral·lelament, el canvi global està alterant la distribució de les espècies, afavorint espècies termòfiles en detriment dels petits pelàgics tradicionals. Aquest és el cas de l'alatxa, *Sardinella aurita* Valenciennes, 1847, que les seves captures han augmentat en abundància en els darrers anys, suggerint el seu potencial com a nou recurs pesquer. Tot i això, continua tenint un baix valor comercial a causa del poc coneixement i de la manca d'informació sobre les seves propietats nutricionals. En aquest context, avaluar la resposta dels peixos a múltiples estressors és essencial per determinar l'estat de les poblacions, la seguretat alimentària i la qualitat nutricional del peix. La perspectiva *One Health* proporciona un marc integrador per abordar aquesta problemàtica, considerant els peixos tant com a bioindicadors de la qualitat dels ecosistemes com a possibles vies de transmissió de contaminants i patògens zoonòtics cap als humans.

Aquesta tesi aplica una aproximació multidisciplinària basada en el concepte *One Health* per avaluar l'estat de salut, l'exposició a estressors antropogènics i les implicacions en seguretat alimentària de dues espècies comercials de la costa catalana (Mediterrani nord-occidental): el lluç europeu i l'alatxa. L'estudi integra l'avaluació de la condició dels peixos, la patologia, les alteracions histopatològiques i les comunitats parasitàries, així com la ingesta d'elements antropogènics i l'acumulació de metalls pesants. A més, també s'avalua la qualitat nutricional de *S. aurita* per determinar-ne la viabilitat comercial. En el cas del lluç, una espècie àmpliament distribuïda i d'elevat valor comercial a la costa catalana, s'ha dut a terme una comparació entre tres localitats amb diferents condicions ambientals i pressions antropogèniques (Blanes, Barcelona i delta de l'Ebre) per identificar poblacions potencialment més vulnerables o saludables. Pel que fa a l'alatxa, s'ha dut a terme un mostreig estacional a escala local davant de Barcelona per investigar les variacions temporals en la salut, l'exposició a contaminants i la qualitat nutricional, tenint en compte el seu potencial com a recurs pesquer emergent. Els resultats d'aquesta tesi s'estructuren en tres capítols (Capítols 4, 5 i 6), cadascun centrat en un aspecte diferent de la perspectiva *One Health* (salut dels peixos, estressors antropogènics i seguretat alimentària).

El primer capítol de resultats (**Capítol 4**) avalua l'estat general de salut de *M. merluccius* i *S. aurita* mitjançant l'anàlisi d'índexs de condició, la identificació de lesions macroscòpiques i alteracions histopatològiques, i la descripció de les comunitats parasitàries i el seu possible paper com a estressors. Es van detectar diferències entre localitats en els lluços i diferències estacionals en les alatxes pel que fa als índexs de condició, descriptors parasitaris i alteracions histopatològiques. El lluç presentava una comunitat parasitària més rica en espècies, però amb intensitats d'infecció menors que les observades en l'alatxa. En general, les alteracions histopatològiques van ser lleus i principalment associades a infeccions parasitàries, causant un dany limitat en els teixits. Globalment, no es van detectar efectes clars sobre la condició dels peixos ni sobre la funcionalitat dels òrgans, fet que suggereix una situació d'equilibri hoste-paràsit. Tot i això, algunes patologies específiques, com les infeccions per trematodes sanguinis, l'epiteliocistis i les infeccions per microsporidis, evidencien la necessitat d'aplicar aproximacions multidisciplinàries per detectar senyals primerenques d'estrès ambiental o biològic en poblacions de peixos salvatges.

A més, la identificació de dues espècies de paràsits que infecten *M. merluccius* i que podrien afectar la seva condició va motivar dos estudis addicionals, degut al limitat coneixement sobre la seva morfologia, caracterització genètica i impacte histopatològic. Al **Capítol 4.1** es descriu de manera integral, per primera vegada al Mediterrani nord-occidental i en el seu hoste tipus, el cestode *Cleistobothrium crassiceps* mitjançant aproximacions morfològiques, moleculars, histològiques i epidemiològiques. L'estudi va permetre identificar trets diagnòstics clau, descriure el mecanisme de fixació del paràsit i donar suport a la sinonimització de *C. cristinae* i *C. splendidum*. Al **Capítol 4.2** es van investigar els efectes histopatològics, l'epidemiologia i la primera caracterització genètica al Mediterrani nord-occidental del trematode sanguini *Aporocotyle spinosicanalis*. Els ous causaven principalment reaccions inflamatòries lleus als filaments branquials, mentre que els adults provocaven lesions cardíques més severes, incloent necrosi fibrinoide i miocarditis limfocítica. Els lluços de major talla mostraven nivells d'infecció més elevats, probablement relacionats amb canvis ontogenètics en la dieta.

El segon capítol de resultats (**Capítol 5**) aborda els contaminants antropogènics, incloent-hi els elements antropogènics (AIs; microplàstics i elements no sintètics) i els metalls pesants en peixos de la costa catalana. En el lluç, es va caracteritzar la ingesta d'AIs i es va comparar amb les mostres d'aigua de l'entorn en tres localitats amb diferents pressions antropogèniques, incloent una comparació temporal a Barcelona (2007 vs. 2019). Els peixos van ingerir una mitjana de 1,39 (DE = 1,39) elements/individu, mentre que les mostres d'aigua contenien una mitjana de 0,85 fibres/m³, predominant-hi les fibres cel·lulòsiques. Només les fibres ambientals petites (<6 mm) coincidien amb les ingerides, probablement degut al comportament alimentari del lluç. Les

concentracions de metalls pesants no van mostrar diferències espacials, tot i que el mercuri augmentava amb la talla dels peixos. En l'alatxa, es van avaluar les variacions estacionals en la ingesta d'Als davant de Barcelona. Els peixos van ingerir una mitjana de 2,27 (DE = 2,13) elements/individu, amb els nivells més elevats a l'hivern i els més baixos a l'estiu, predominant novament les fibres cel·lulòsiques. Els patrons estacionals van ser coherents amb factors ambientals (com la pluja i l'estratificació de la columna d'aigua) i biològics (com el comportament alimentari i la reproducció). La major ingesta d'Als a l'hivern coincidia amb una major intensitat d'alimentació i comportaments de filtració, mentre que els baixos nivells d'ingesta a l'estiu es van associar amb una menor activitat alimentària, un comportament d'alimentació més selectiu i una reducció de les precipitacions i dels aportaments fluvials. En conjunt, no es van detectar efectes consistents de la ingesta d'Als ni de l'exposició a metalls sobre la salut dels peixos o l'acumulació de paràsits.

El darrer capítol de resultats (**Capítol 6**) es centra en els beneficis nutricionals i els possibles riscos per a la salut associats al consum de peix. La qualitat nutricional de *S. aurita* (composició proximal i perfil d'àcids grassos) es va avaluar estacionalment per determinar la seva viabilitat com a recurs pelàgic alternatiu i identificar els períodes amb més omega-3. Els riscos potencials associats al consum es van analitzar mitjançant l'estudi de metalls pesants i l'exposició a paràsits zoonòtics tant en *S. aurita* com en *M. merluccius*. A més, es va comparar la distribució de nematodes zoonòtics en diverses regions del Mediterrani. L'alatxa va mostrar variabilitat estacional en el contingut lipídic i la composició d'àcids grassos, amb nivells d'àcid eicosapentaenoic (EPA) + docosahexaenoic (DHA) a la tardor que complien les recomanacions diàries per a una ració de 100g. Promoure'n el consum durant aquest període podria afavorir pesqueries més sostenibles redistribuint la pressió pesquera entre espècies i estacions de l'any. Les concentracions de metalls en ambdues espècies es van mantenir per sota dels límits establerts per la Comissió Europea, tot i que el seu consum freqüent podria representar un risc potencial d'exposició a metilmercuri en infants. No es van detectar nematodes zoonòtics en cap de les dues espècies a la costa catalana, fet que redueix el risc d'anisakiosi per als consumidors. En canvi, els lluços de l'Adriàtic van mostrar una elevada prevalença d'*Anisakis pegreffii*, especialment en talles majors, destacant la importància de la talla i la zona de captura en la distribució de les espècies d'*Anisakis*.

CHAPTER 1

General introduction

1. Relevance of fisheries in the Mediterranean Sea

Throughout history, human societies have depended on marine ecosystems as vital sources of food but also as the foundation for trade, culture and coastal settlements (Bax et al., 2022; Holman et al., 2025). Despite environmental and socio-economic changes, fisheries continue to reflect this ancient connection, playing a crucial role in global economies and coastal communities worldwide (Bax et al., 2022; FAO, 2022).

The Mediterranean Sea is recognized as a global biodiversity hotspot, comprising over 17,000 marine species (Coll et al., 2010; MedECC, 2020) and supporting socio-economically important fisheries. These are mainly composed of small-scale vessels, bottom trawlers and purse seiners distributed along its coasts, providing employment, ensuring food security and generating valuable income for local communities (FAO, 2022, 2023) (Fig. 1). In 2023, catches in Mediterranean and Black Sea fisheries reached around 1.11 million tonnes, generating revenues of about 8.05 billion euros and giving employment over 197 000 people (FAO, 2025).

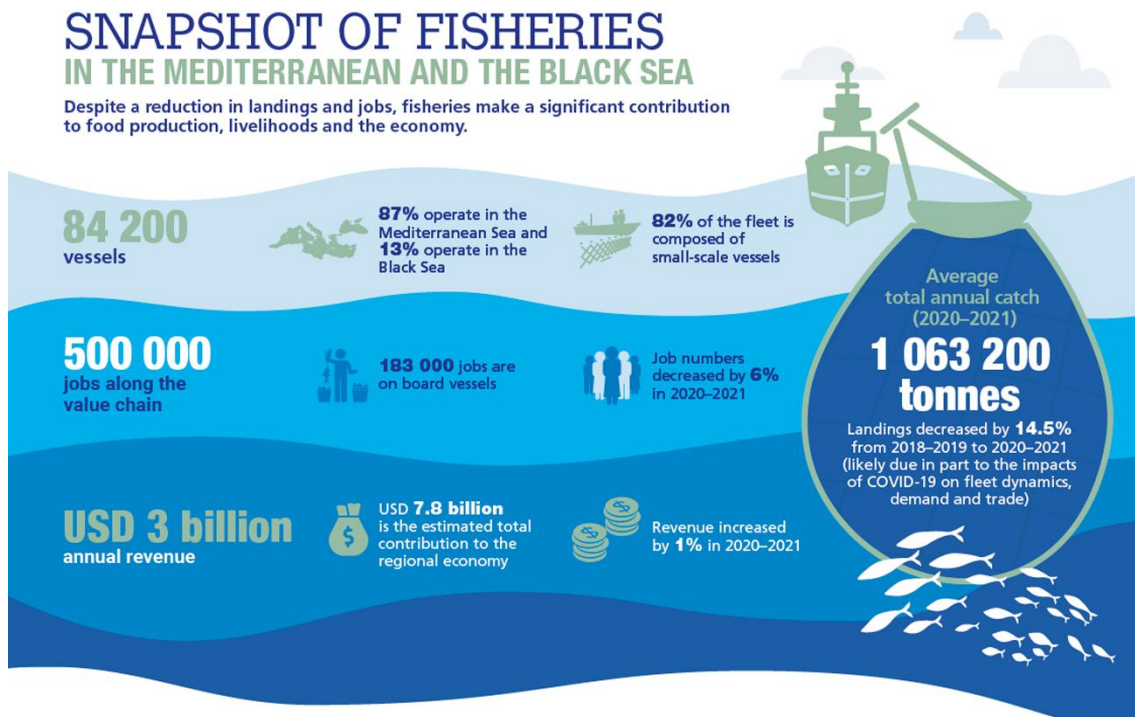


Fig. 1. Snapshot of the state of fisheries in the Mediterranean and Black Sea in 2021. Source: FAO, 2023.

The semi-enclosed nature of the Mediterranean Sea and its particular hydrodynamics, combined with intense anthropogenic pressures (e.g., urban, industrial and agriculture activities and their associated pollutants, habitat degradation, eutrophication, invasive species and also fishing impacts), makes the basin particularly vulnerable to environmental changes (Coll et al., 2010;

MedECC, 2020). Around 52% of commercial fish stocks are overexploited below biological sustainable limits (FAO, 2025). This long-term pressure has likely altered population age structure and reduced their resilience to environmental fluctuations (Colloca et al., 2013, Albo-Puigserver et al., 2021), which are further exacerbated by ongoing stressors, such as seawater warming, acidification and restricted migration towards cooler areas (MedECC, 2020; Bindoff et al., 2019). Indeed, seawater temperature is increasing at an estimated rate of 0.35°C per decade in this region, making the Mediterranean Sea a particular hotspot for climate change (Pastor et al., 2020).

Among the wide variety of species exploited in the Mediterranean, European anchovy, *Engraulis encrasicolus* (Linnaeus, 1758) and European sardine, *Sardina pilchardus* (Walbaum, 1792) were the most caught species over the period 2022-2023, accounting for 32.1% of the total annual landings (FAO, 2025). In terms of economic value, these small pelagic species together with the European hake, *Merluccius merluccius* (Linnaeus, 1758), the deep-water rose shrimp, *Parapenaeus longirostris* (Lucas, 1846) and common octopus, *Octopus vulgaris* Cuvier, 1797, represented the largest contribution to total fisheries revenue (40% of total landings value) (FAO, 2025). Since the 1980s, total landings of cold-water fish species including *S. pilchardus*, *M. merluccius*, *Micromessistius poutassou* (Risso, 1827), *Scomber scombrus* Linnaeus, 1758 and *Nephrops norvegicus* (Linnaeus, 1758), have shown a marked decline (FAO, 2023), along with reductions in mean size and body condition of sardines and anchovies observed since the last decade (Saraux et al., 2019). Within this context, the implementation of management units (Geographical Subareas (GSAs)) defined by the General Fisheries Commission for the Mediterranean (GFCM) contributes to the promotion of more sustainable exploitation practices and limiting fishing efforts in some GSAs. Since 2013, specific regulations have been implemented by the European Union under different Multi-Annual management plans (MAP) in order to achieve the sustainable maximum yield of fish stocks (European Commission [EU], 2013), through the limitation of annual fishing effort, the adoption of selective gear and the temporary and permanent closure of specific fishing grounds. Since then, overall fishing pressure in the Mediterranean has decreased by nearly 50%, driven by a reduction in fleet capacity and fishing days (European Commission (STECF), 2025). While some regions, such as the Adriatic Sea and Strait of Sicily, have shown biomass increases of approximately 25% for certain key commercial species (FAO, 2025), this positive trend towards a more sustainable exploitation (in which fishing mortality does not exceed the level that allows the stock to produce its maximum sustainable yield), is not uniform (FAO, 2025). Biomass recovery appeared to be species and region specific, influenced by species life-history traits and their response to reduced fishing pressure. For

instance, the short-lived European anchovy showed a rapid biomass recovery under low exploitation levels, whereas the medium-lived European hake showed a delayed response to the 2019 multiannual plan and a more gradual biomass recovery two years later (FAO, 2025).

In parallel with the decline of small pelagic fish, thermophilic pelagic species have gradually expanded towards colder areas of the Mediterranean over the last decades due to global warming. It is the case of the round sardinella, *Sardinella aurita* Valenciennes, 1847 (Sabatés et al., 2006; Tsikliras, 2008), as well as the horse mackerel, *Trachurus trachurus* (Linnaeus, 1758), the Atlantic chub mackerel, *Scomber colias* Gmelin, 1789, and the European sprat, *Sprattus sprattus* (Linnaeus, 1758), which together contributed about 16% of total annual landings in the Mediterranean Sea during 2022-2023 (FAO, 2025). Consequently, global change is altering not only the structure and composition of marine species but also their distribution. This trend suggests that, as sardines and anchovies decline, these thermophilic species may become more important in Mediterranean fisheries. Hence, understanding how fish stock populations respond to both anthropogenic and climatic stressors is key to ensure the long-term sustainability of regional fisheries.

Considering that primary production and species richness are particularly higher in the western basin of the Mediterranean compared to the east (Coll et al., 2010; Veloy et al., 2022), the northwestern Mediterranean, and particularly the Balearic Sea, stands out as a biodiversity hotspot and one of the most productive and exploited regions of the basin, where environmental gradients, anthropogenic pressures and diverse fisheries coexist.

2. The targeted fishing ground: the Catalan coast (NW Mediterranean)

The Catalan coast (580 km in length), located in the northwestern part of the Balearic Sea (NW Mediterranean Sea), is one of the most productive regions in the basin (Bosc et al., 2004) (**Fig. 2**). Primary production on the continental shelf is enhanced by river runoff, local wind-driven upwelling, and shallow water mixing during winter, which increase nutrient availability to the photic zone (Agostini et al., 2002; Sabatés et al., 2024). For instance, freshwater inputs from important rivers such as the Ebro River (910 km length, 14000 hm³/y) fertilize the coastal zone with over 10,000 metric tons of nitrogen per year (Cruzado et al., 2002). The continental shelf along the Catalan coast is relatively narrow (<30 km) and morphologically heterogeneous, featuring canyon-channel systems, landslides, a mid-oceanic valley and deltaic formations, contributing to habitat diversity and assemblages of different marine communities (Demestre et al., 2000; Amblas et al., 2006) (**Fig. 2**). As a result, this area hosts important spawning and nursery grounds for both

pelagic and demersal species, making the Catalan coast an ecologically and economically significant fishing area (Sabatés et al., 2024).

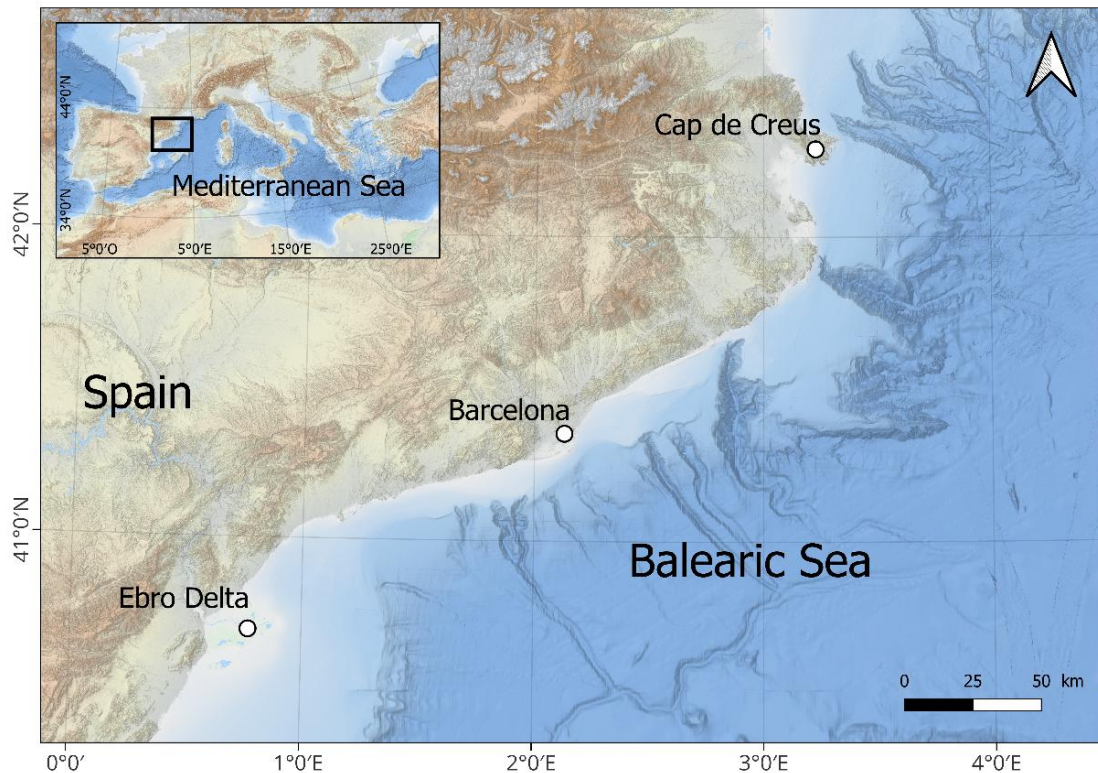


Fig. 2. Map of the Catalan coast in the Balearic Sea (north-western Mediterranean Sea). Map created using QGIS 3.34.3.

In 2023, the Catalan fishing fleet comprised 528 vessels and operated through 20 fish auction halls along the coast (ICATMAR, 2025a). The main fishing gears were bottom trawling, purse seine and small-scale fisheries (Carreton et al., 2025), with bottom-trawling generating the highest revenue (nearly 60%) despite purse seiner accounting for 54.13% of total landings (Generalitat de Catalunya, 2025). In 2024, total landings reached 19,272.03 tonnes, producing over 89 million euros in revenue. Among the approximately 200 commercially exploited species, ten of these species represented over 69% of total catches, predominantly sardines, anchovies and round sardinellas. The species contributing with the highest revenue included the red shrimp, sardines, anchovies and hakes (**Fig. 3**) (Generalitat de Catalunya, 2025).

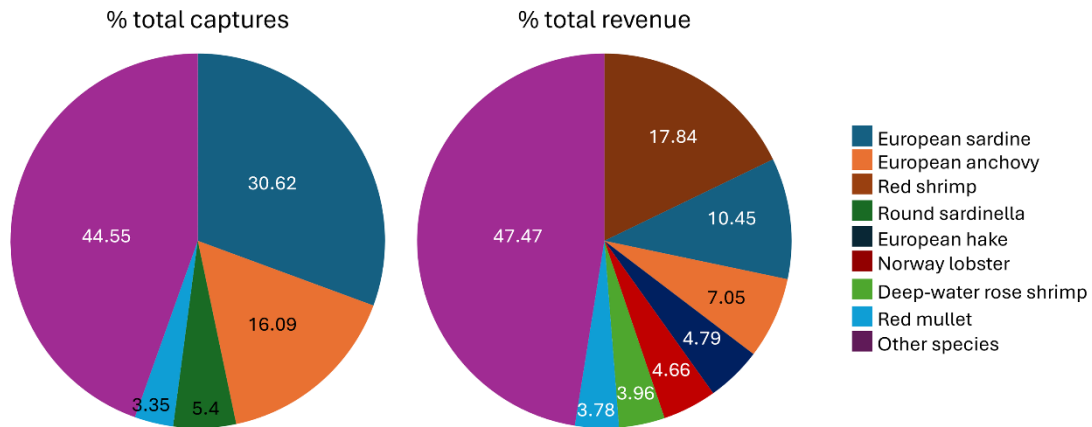


Fig. 3. Circular plots showing the percentage (%) of total captures and percentage (%) of total revenue of the commercialized species from the Catalan fish auction halls. Modified from Generalitat de Catalunya (2025).

Among these key species, the European hake represents one of the main demersal resources from bottom-trawling widely distributed on the Catalan continental margin (ICATMAR, 2025b). Most hake landings in this region are derived from bottom trawl fisheries (representing 95.50% of the total captures), and a considerable proportion of the individuals captured are juveniles below the minimum conservation reference size (20 cm total in length) (**Fig. 4a**). In contrast, adult individuals represent only a small fraction of total catches (approximately 5%), normally caught by set longliners and artisanal fisheries (ICATMAR, 2025c). In this region, annual hake landings have steadily decreased over the last two decades, reaching minimum levels in recent years, decreasing from around 2000 tonnes in 2002 to about 500 tonnes in 2024 (ICATMAR, 2025c) (**Fig. 4b**). Since 2019, this downward trend may be explained by the implementation of the Multi-Annual Plan (MAP) for demersal fisheries in the northwestern Mediterranean (European Commission [EU], 2019), which included *M. merluccius* as a target species and established a progressive reduction in fishing effort for the bottom trawling fleet (Carreton et al., 2025), and particularly a reduction for at least 20% of hake juveniles captures (Article 11: European Commission [EU], 2019). As a result, hake landings in 2024 have been reduced by 50% compared to 2015-2017 (ICATMAR, 2025c).

Although catches from this region represent a relatively small proportion of the total annual landings of European hake in the Mediterranean Sea (18791 tonnes in 2022-2023) (FAO, 2025) and are considerably lower than those reported in northeastern Atlantic fisheries (69656 tonnes in 2023) (FAO, 2024a), the predominance of juvenile captures highlights the vulnerability of local populations. Moreover, interannual variability in the abundance of small size classes suggests that recruitment may vary in response to environmental conditions. In particular, enhanced primary

production and lower water temperatures are known to favour hake recruitment but also food availability, hydrographic conditions and fishing pressure may influence recruits' abundance (Bartolino et al., 2008; Carreton et al., 2025; ICATMAR, 2025c).

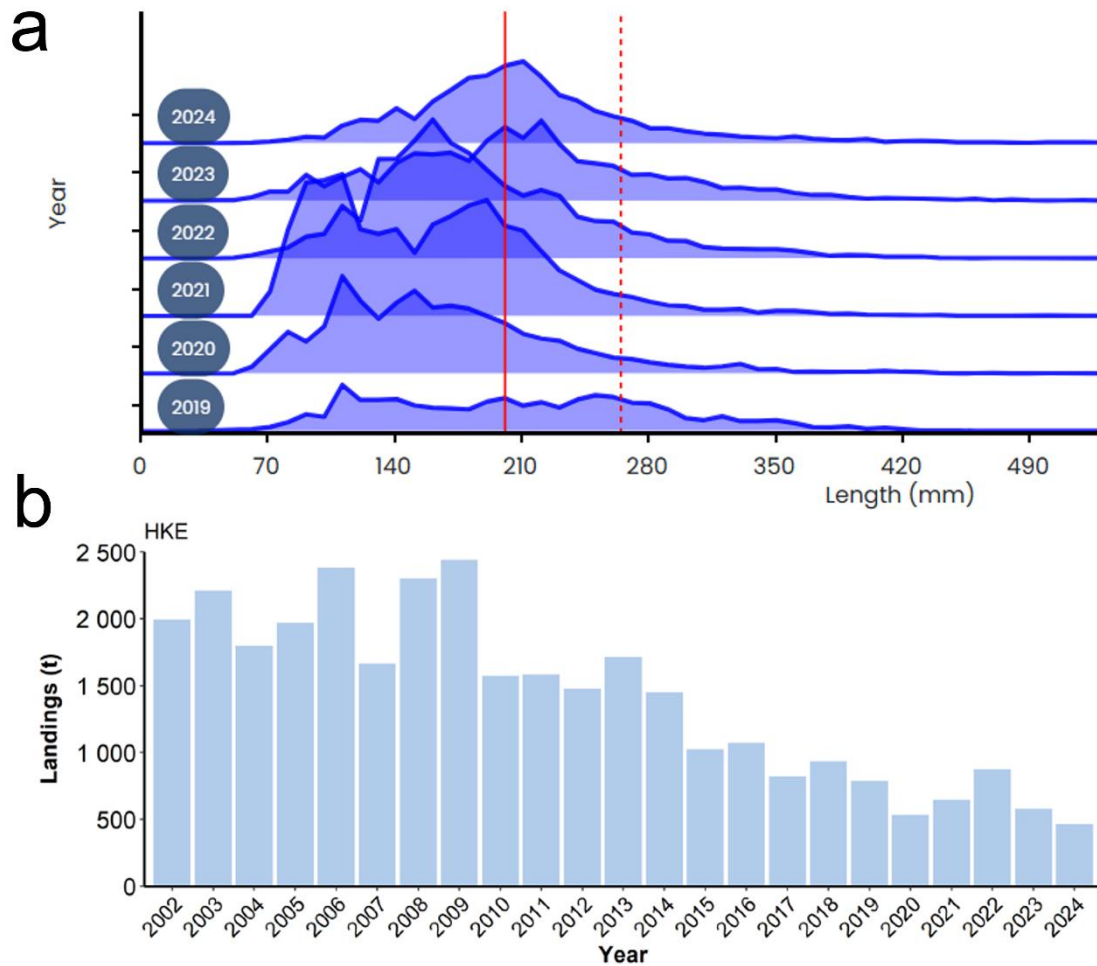


Fig. 4. a) Length distribution (mm) of European hake (*Merluccius merluccius*) from 2019 to 2024. Red line: minimum conservation reference size. Dashed line: maturity length (ML50%). **b)** Annual series of landings (t) from 2002 to 2024 for European hake at Catalan fish auction halls. Source: ICATMAR visor pesquer and ICATMAR 2025c.

In contrast to hake, the round sardinella, captured by purse seiner, is currently a non-target commercial species whose catches have increased notably in recent years, representing a substantial proportion of pelagic landings along the Catalan coast (ICATMAR, 2025b). Despite this increasing abundance, its economic relevance is still limited, as this species is unfamiliar to consumers and undervalued, resulting in a much lower commercial value compared to sardines and anchovies (ICATMAR, 2020). Nevertheless, the sustained increase of its population abundance suggests that *S. aurita* could represent a promising potential target species (Vila-Belmonte et al., 2024; Ollé-Vilanova et al., 2026), particularly in the current context in which other

small pelagic stocks (i.e. sardines and anchovies) are experiencing declining trends due to their sensitivity to environmental conditions such as increased sea surface temperature, low primary production and shifts in plankton composition and salinity (Saraux et al., 2019; Fernández-Corredor et al., 2021; Frigola-Tepe et al., 2025). Indeed, round sardinella landings have increased considerably along the Catalan coast (**Fig. 5**), particularly in the northern and central part. In six of the ten auction halls in these north and central regions (i.e. l'Escala, Palamós, Sant Feliu de Guíxols, Blanes, Arenys de Mar and Barcelona), *S. aurita* ranks among the four most abundant species landed in terms of tonnes. For example, at Barcelona its landings accounted for 2699.99 tonnes in 2022 (ICATMAR, 2025a) and at l'Escala (northern Catalan coast), its landings increased by around 167% in 2024 reaching 537 tonnes compared to the 2021-2023 period (ICATMAR, 2025a). Overall, total landings of this species along the Catalan coast reached around 1040 tonnes in 2024, but generated a low income of 0.54 million euros (less than 0.54 euros per kg) (**Fig. 5**), due to its low commercial value, compared to the income produced by sardines (with 5849 tonnes generating 9.3 million euros and 1.59 euros/kg) and anchovies (3048 tonnes generating 6.2 million euros and 2.05 euros/kg) (ICATMAR, 2025a). Likewise, although a minimum reference size for capture is established for sardines (11 cm) and anchovies (9 cm), for *S. aurita* it has not been established yet (Real Decreto 560-1995).

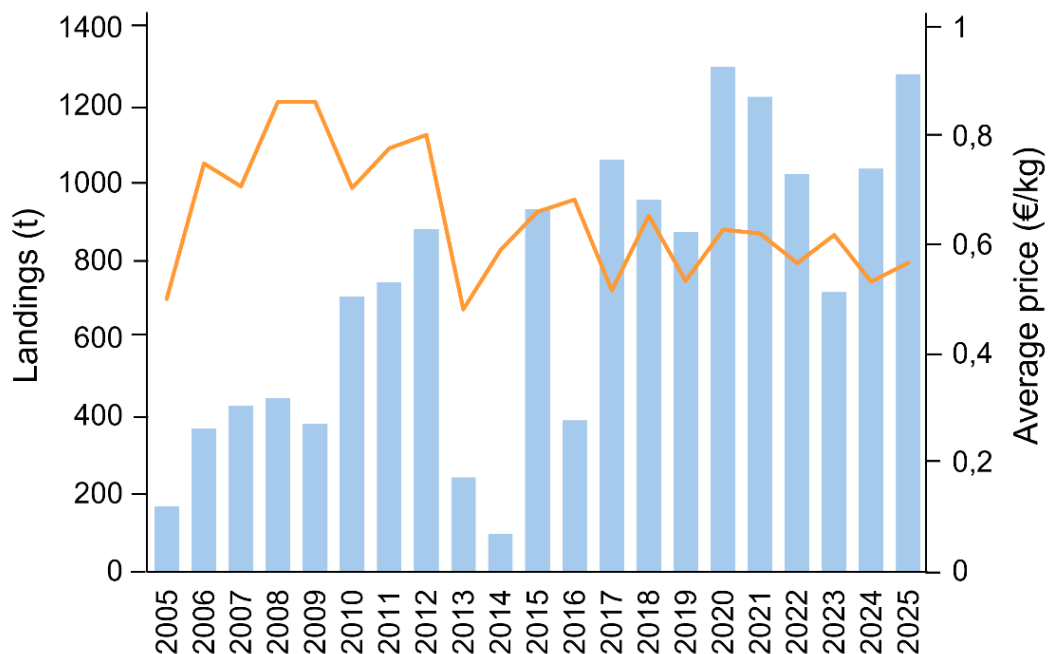


Fig. 5. Annual series of landings (tonnes) and average sale price (€/kg) for *Sardinella aurita* at Catalan fish auction hall. Source: ICATMAR (2026). Institut Català de Recerca per a la Governança del Mar dataset accessed on 12/05/2026. www.icatmar.cat

3. Biological and ecological overview of the studied fish species

3.1. *Merluccius merluccius* (Linnaeus, 1758)

The European hake (henceforth: hake) (**Fig. 6**) is a demersal and benthopelagic fish species widely distributed in the eastern North Atlantic Ocean and the Mediterranean Sea (Alheit & Pitcher, 1995). In both regions, this species occupies a wide bathymetric distribution range (from 30 to 1000 meters), although it mostly lives between 50 and 370 meters (Oliver & Massutí, 1995; Lloris et al., 2003; Korta et al., 2015). *M. merluccius* depth distribution varies throughout its life stages. In the Atlantic, eggs are distributed between 150-200 m depth, and larvae concentrate in depths from 50 to 150 meters. Juveniles inhabit muddy seafloors from shallow waters (25-125 meters) whereas older individuals are distributed on the slope at 200-500 meters depth associated with canyons and cliffs (Korta et al., 2015). In the Mediterranean, hakes display a similar spatial behaviour as juveniles mainly inhabit the continental shelf (<150 meters) while larger specimens live on the upper slope (150-350 meters) (Maynou et al., 2003). Moreover, juveniles exhibit seasonal bathymetric migrations, inhabiting shallow waters (60 – 160 meters) during autumn and winter, and extending their range to deeper areas of up to 300 meters in spring and summer (Maynou et al., 2003).

This fish species prefers bottom temperatures between 14°C and 18°C. It has a rapid growth rate during the first year, reaching between 16 and 18 cm (sexes combined) or 13-15 cm for males and 15.5-21.6 for females from the Mediterranean Sea (Mellon-Duval et al., 2009 and references therein), whereas the Atlantic juveniles reach 24 cm at the end of the first year (Kacher & Amara, 2005). Growth rate depends on sex, since from the second year (20-30 cm) onwards males grow slower than females, which is consistent with the sexual dimorphism observed in the size at first maturity (Mellon-Duval et al., 2009). Current estimates for hakes from the Catalan coast indicate that females reach sexual maturity at 31–33 cm and males at 24–26 cm (ICATMAR, 2025c). These sizes are lower than those reported for Atlantic populations (38.8 cm for females and 23.2 cm for males), although a reduction in size in the Atlantic Ocean (10.9 cm in females and 12.9 cm in males) has also been documented since the 1980s (Lojo et al., 2022). The comparison with previous Mediterranean studies shows a similar downward trend, suggesting that the current lower sizes at maturity may be attributed to environmental variations, different stock biomass levels, fishing mortality or evolutionary responses to sustained fishing pressure (see Table 6 by Carbonara et al., 2019; Korta et al., 2015).

In both regions, this species is a multiple batch-spawner with indeterminate fecundity strategy and asynchronous oocyte development, and spawning occurs throughout the year but with

specific peaks (Korta et al., 2015; Carbonara et al., 2019). In the Atlantic, spawning occurs from December to July with peaks that differ depending on the latitude and which hakes follow through migrations. Thus, in southern regions (i.e., Bay of Biscay and Galician coast) reproduction takes place from January to March whereas in northern areas such as the Celtic Sea, spawning season occurs from April to June and in the west coast of Norway is in August (ICES, 2008; Korta et al., 2015). In the Mediterranean, spawning season also varies among regions probably linked to environmental (i.e., temperature, currents, food availability) and fish-related factors (i.e., condition, life stage and parasitism) (Recasens et al., 2008; Ferrer-Maza et al., 2014; Carbonara et al., 2019). On the Catalan coast, reproduction reaches its maximum between September and December, while spawning activity decreases during winter, probably because of the unfavourable conditions associated with dense water cascading events and strong currents originating in the Gulf of Lion. A secondary, shorter reproductive peak also occurs in summer (Recasens et al., 2008; Ferrer-Maza et al., 2014). In contrast, in other regions from the Central Mediterranean (e.g. Tyrrhenian and Adriatic Seas), spawning peaks occur in winter and spring (Arneri & Morales-Nin, 2000; Recasens et al., 2008). In the Atlantic, the main spawning ground is the shelf break, concentrating over the Bay of Biscay, the southern Celtic Sea and in the NW coast of the Iberian Peninsula (Murua et al., 2010 and references therein), whereas in the Mediterranean, reproduction occur between the shelf break and the upper slope (150 – 350 m) (Recasens et al., 2008). In contrast, nursery areas are restricted to the continental shelf in both regions, where recruits develop and grow (Recasens et al., 2008; Sánchez & Gil, 2000).

In both regions, the hake has an opportunistic and benthopelagic feeding behaviour (Lloris et al., 2003; Cartes et al., 2004; Murua et al., 2010) mainly based on euphausiids, mysids, decapods crustaceans and small fish (Lloris et al., 2003; ICES, 2008). Its diet shows great variations according to the temporal and spatial scale, environmental conditions (i.e. temperature and phytoplankton concentrations) and prey availability in the environment as reported by diel vertical migrations (Cartes et al., 2004; Lloret-Lloret et al., 2020; Murua et al., 2010). Seasonality also influences prey availability and hake's diet, showing a dominance of crustaceans in summer whereas small fish are more ingested during winter (Lloret-Lloret et al., 2023; Stagioni et al., 2011). In addition, ontogenetic changes in feeding habits and bathymetrical migrations occur in juvenile hakes (20 – 25 cm) in response to an increase in their sensorial capabilities (Bozzano & Catalán, 2002; Murua et al., 2010), allowing them to widen their trophic spectrum to primarily fishes such as sardines, anchovies, horse mackerel and blue whiting (Murua et al., 2010; Lloret-Lloret et al., 2020).

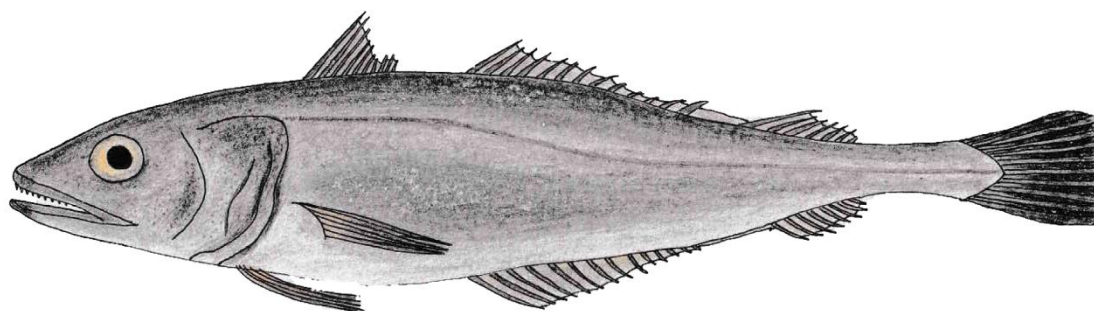


Fig. 6. Illustration of *Merluccius merluccius* (Linnaeus, 1758). Author: Laura Muns Pujadas.

3.2. *Sardinella aurita* Valenciennes, 1847

The round sardinella (*Sardinella aurita* Valenciennes, 1847) (**Fig. 7**) is a thermophilic small pelagic fish species widely distributed throughout the continental shelves of tropical and subtropical waters of the Atlantic and Pacific Oceans, as well as the Mediterranean and Black Seas (Froese & Pauly, 2025). It inhabits coastal and surface waters (from 0 to 350 meters) (Whitehead, 1985), showing a seasonal migratory pattern, approaching to coastal areas during summer for reproduction and moving to deeper waters in winter. This species prefers higher salinity values (34.0 – 36.5) and warm waters above 23°C (Palomera et al., 2007; Baldé et al., 2019), which have facilitated the expansion of its northern distribution limit across the central and northern Mediterranean such as the Balearic and Ligurian Seas, coinciding with the progressive warming of surface waters (Sabatés et al., 2006). There, it co-occurs with other pelagic clupeiform fish species, the sardine and anchovy, competing for similar trophic resources, particularly at larval stages (Palomera et al., 2007; Bachiller et al., 2021).

This species exhibits a rapid growth and a short life cycle, typical in small pelagic fish. Growth and lifespan show considerable spatial variability among regions: round sardinellas from the Mediterranean stocks (except for those from Tunisian waters; Gaamour et al., 2001) tend to have a shorter lifespan, typically to four or five years old, and lower lengths compared to populations from the northwestern African waters (Atlantic Ocean), which growth parameters are higher and can reach until eight years (Tsikliras et al., 2005). These differences among regions seem to be mainly related to variations in water temperature and food availability, but also in upwelling intensity, and concentration of chlorophyll-a, all of which influence round sardinella recruitment success and population abundance (Tsikliras et al., 2005; Baldé et al., 2019; Samba et al., 2021). Indeed, the northwestern African waters, such as the Senegalese coast, is richer in nutrients due

to constant coastal upwelling compared to the Mediterranean. However, a decline in fish length has been observed in the north-west Africa probably due to the fishing pressure as this species has been overexploited for the last two decades (Baldé et al., 2019; Samba et al., 2021).

Reproduction in *S. aurita* varies across its range. In Atlantic waters, spawning is closely linked to upwelling systems and occurs almost throughout the year, with peaks that shift according to local conditions, matching with high zooplankton productivity (Baldé et al., 2019). In the Mediterranean, spawning is highly temperature-dependent and synchronized with periods of high food availability (Tsikliras & Antonopoulou, 2006). Gonad maturation initiates once sea temperature exceed 20°C and spawning usually occurs during summer, peaking between July and August, in coastal areas shallower than 100 m, where temperatures range from 22 to 25°C (Sabatés et al., 2009; Hofstede et al., 2007). Sexual maturity is typically reached at the age of one or two years old, at approximately 14-15.8 cm in males and 15-16.8 cm in females in the Mediterranean (Mustać & Sinovčić, 2012), but at larger sizes in the Atlantic stocks (e.g., 24.8 cm in males and 25.7 cm in females along the Senegal coast; Baldé et al., 2019). In the southern Mediterranean, the spawning period tends to last longer, probably due to persistently high-water temperatures and prolonged stratification compared to northern areas such as the Aegean and Adriatic Seas (Tsikliras & Antonopoulou, 2006; Mustać & Sinovčić, 2012). Eggs and larvae are pelagic and tend to concentrate in low-salinity, nutrient-enriched coastal areas above the thermocline, environments typically influenced by upwelling and riverine runoff that stimulate phytoplankton production (Sabatés et al., 2006, 2009). Larvae may also extend offshore to around 150 meters. These coastal areas represent optimal nursery grounds that support high survival during early life stages. *S. aurita* shows a remarkable plasticity since it can adjust its reproductive timing to local environmental conditions (Baldé et al., 2019; Samba et al., 2021).

S. aurita exhibits a generalist and opportunistic feeding behaviour mainly based on zooplankton (Bachiller et al., 2021). Although copepods are their main prey, its diet shifts with seasonal variations according to food availability, switching from particulate to filter feeding, being able to feed on a variety of prey sizes groups: from microplankton during winter to decapods and euphausiids in summer and gelatinous zooplankton such as salps in spring (Albo-Puigserver et al., 2019; Bachiller et al., 2021). This trophic plasticity allows round sardinella to exploit a wider niche and occupy a higher trophic position than its sympatric pelagic counterparts (i.e., sardine and anchovy), potentially providing an advantage under fluctuating environmental conditions (Albo-Puigserver et al., 2016, 2019; Bachiller et al., 2021). This may be particularly relevant under warming seawater conditions, which favour the proliferation of gelatinous zooplankton species (e.g., jellyfish, salpida, siphonophores) (Calvo et al., 2011).

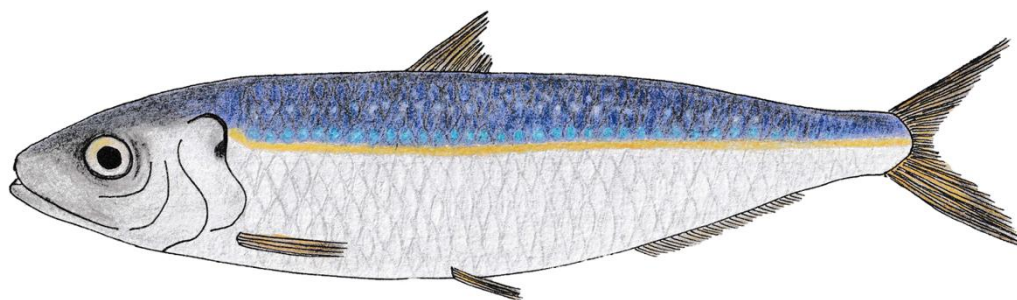


Fig. 7. Illustration of *Sardinella aurita* Valenciennes, 1847. Author: Laura Muns Pujadas

4. The One Health approach: fish, marine ecosystems and human health

The use of bioindicators, defined as biological processes, species or communities that provide information on environmental quality, are essential to assess ecosystem health over time (van der Oost et al., 2003). Because fish populations are continuously exposed to multiple environmental and anthropogenic stressors (e.g., pollution, pathogens, climate change and fishing pressure) that may act simultaneously in complex ways across time and space (Schull et al., 2023), and may respond to them at different biological levels, fish are widely recognized as effective bioindicators of marine ecosystems (Holt & Miller, 2011; Rudneva et al., 2011). Moreover, from an ecotoxicological point of view, fish are considered sentinel species due to their ability to accumulate pollutants, because they exhibit measurable physiological responses to toxic stress, and they are exposed to contaminants through multiple pathways, including direct uptake from their surrounding environment (water and/or sediments) and dietary intake via contaminated prey, allowing the assessment of contaminant transfer across trophic levels (Yancheva et al., 2015 and references therein). To assess the biological effects of these stressors, biomarkers (i.e. measurable molecular, cellular, histological, physiological or behavioural responses) are commonly used to reflect organism responses to chemical, physical or biological hazards at the sub-individual level (van der Oost et al., 2003; Janz, 2013). Some biomarkers respond to non-specific stressors (e.g., haematological, physiological and histological parameters), whereas others are more specific to certain toxics (e.g., biotransformation enzymes and metalloproteins) (van der Oost et al., 2003).

Accordingly, the evaluation of fish health status is internationally recognized under the Marine Strategy Framework Directive (MSFD) to achieve or maintain Good Environmental Status of marine ecosystems (MSFD, Directive 2008/56/EC). For commercial fish species, it is essential to understand how individuals cope with these stressors and how such responses could scale up to

affect populations and ecosystems. Indeed, impaired fitness has been identified as a potential driver of the reduction of population biomass and their catches in several fish species, with direct consequences to fisheries and ultimately to consumers (Lloret et al., 2012).

However, assessing the health of fish species in natural conditions is challenging because their responses are not only influenced by external pressures but are also modulated by intrinsic factors including age, reproductive stage, nutritional status and genetics (Schull et al., 2023). To accurately understand and evaluate these interactions, health assessments must rely on a holistic framework integrating diverse indicators and methodologies across multiple biological levels (e.g. OSPAR, 2013): from molecular, cellular and tissular levels (i.e. using biochemical markers and assessing tissue alterations with histopathology), individual (i.e. condition indices) to a community level (i.e. parasites), as implemented in several studies (e.g., Lyons et al., 2010; Dabrowska et al., 2012; Constenla et al., 2022; Rodríguez-Romeu et al., 2020, 2022; Higuieruelo et al., 2024). This multidisciplinary perspective allows a more accurately evaluation of individual condition and population health, providing essential guidelines for effective conservation and sustainable fisheries management.

Within this context, the One Health concept recognizes the interconnectedness of ecosystem, animal and human health (World Organisation for Animal Health 2020; WHO, 2022a) (**Fig. 8**). As defined by the One Health Institute of the University of California at Davis, One Health is an approach to ensure the well-being of people, animals and the environment through collaborative problem-solving at local, national and global scales. Although its roots traced back to ancient medical traditions, this concept has gained strong relevance in the 21st century as growing concerns about zoonotic diseases, environmental degradation and food security have highlighted the need for integrated health solutions (Roy et al., 2025).

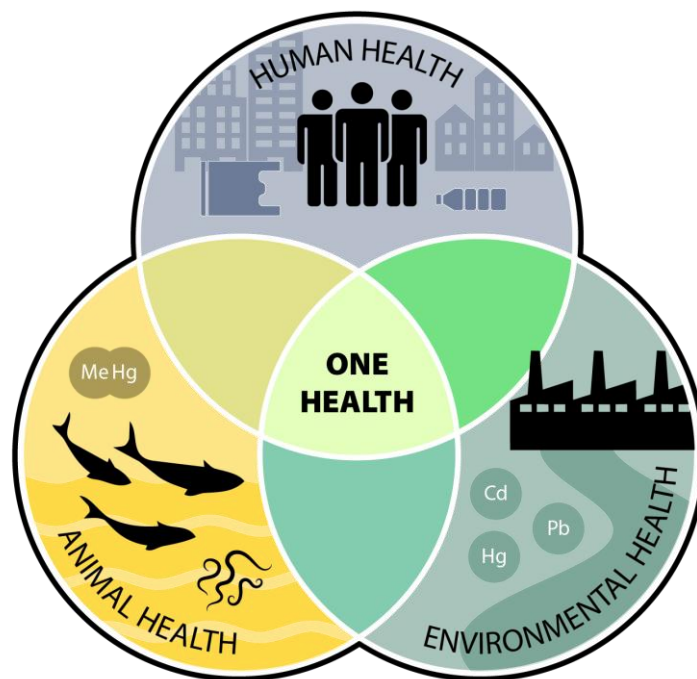


Fig. 8. Venn diagram of the One Health approach viewing environmental, animal and human health interconnected, with anthropogenic and biological stressors (e.g., plastics, heavy metals, parasites) affecting all levels. Author: Laura Muns Pujadas

4.1. Fish health indicators

4.1.1. Pathology and histopathology

Among fish health indicators, pathology and histopathology are effective broad-scope diagnostic techniques to detect changes or alterations in the normal morphological structure of cells, tissues, organs and systems in organisms (Wolf et al., 2015; Costa, 2018). Histopathological alterations can result from environmental and anthropogenic pollution or infectious agents such as parasites (Feist et al., 2015; Feist & Longshaw, 2008), as well as other biotic factors, providing insights into individual fitness and their potential population impacts. Indeed, histological alterations are sensitive biomarkers in ecotoxicological studies for monitoring acute and chronic exposure to pollutants, due to the ease of sample collection and storage, the possibility to examine multiple organs within the same individual, and their ability to identify lesions that may represent biologically relevant endpoints of exposure (Hinton et al., 1992; Bernet et al., 1999; Au, 2004, Dabrowska et al., 2012, Yancheva et al., 2015). However, it is not possible to match histological alterations to specific pollutants and some of them are mild alterations that may reflect normal conditions (Au, 2004; Costa et al., 2009; Dabrowska et al., 2012; van Dyk et al., 2012; Yancheva et al., 2015). Although biochemical parameters are more specific, sensitive and rapid, their biological

and toxicological relevance is limited due to the difficulty to extrapolate it to ecosystem level effects (Au, 2004; Costa et al., 2009). Biomonitoring pollution studies (e.g. the International Council for the Exploration of the Sea, ICES; Marine Strategy Framework Directive, MSFD; the United Nations Environment Program, UNEP; OSPAR Coordinated Environmental Monitoring Programme (CEMP) for the northeast Atlantic and NOAA's National Status and Trends Program) have established several histopathological alterations as indicators of toxic or carcinogenic effects resulting from exposure to contaminants in marine environments (Au, 2004). Key target organs include the gills, which are directly exposed to the surrounding environment and are responsible for vital functions including respiration and osmoregulation, and the liver, for its role in the biotransformation and elimination of noxious substances (Bernet et al., 1999). Considering that several studies have demonstrated that environmental pollutants may cause pathogenic lesions in fish liver (e.g., Stentiford et al., 2003; Feist et al., 2004; Lang et al., 2006), specific technical guidelines and quality assurance for monitoring liver histopathology have been described by the Program for Quality Assurance of Biological Effects in Monitoring (BEQUALM) to correctly evaluate cellular and tissue responses as biomarkers of exposure to stressors (BEQUALM, 2005). Other organs such as the digestive tract, spleen, gonads, kidney and heart are also informative due to their exposure to different infectious agents (Wolf et al., 2015). Histopathological screening is widely recognised as an efficient early-warning tool capable of detecting a broad range of changes before they manifest at higher biological levels (Ruiz-Picos et al., 2015).

4.1.2. Condition indices

At the individual level, condition indices provide complementary information on the nutritional status and fitness of fish individuals as they respond to an array of intrinsic and/or extrinsic factors including food availability, environmental variables, parasitism and anthropogenic factors (Lloret et al., 2014). Particularly, Le Cren's relative body condition index (Kn) measures body mass relative to length, reflecting individual's fattening and overall nutritional condition (Le Cren, 1951). Kn can be affected in response to pollutant exposure, suboptimal environmental conditions or parasitic infections (Dragun et al., 2013; Hasegawa & Poulin, 2025). The stomach fullness index is also used as it reflects changes in feeding activity and intensity (Hyslop, 1980). The hepatosomatic index (HSI) and gonadosomatic index (GSI) assess liver and gonad weight relative to body mass, providing insight into energy storage and reproductive potential, respectively (Wootton, 1989). Moreover, levels of HSI and GSI may also increase or decrease in exposure to pollutants due to enhanced detoxification enzymatic activities, that at the histological level may result into several alterations such as hepatocellular and nuclear hypertrophy and foci of hepatocellular vacuolation associated with increased cell size (Khan et al., 2010; van Dyk et al., 2012).

4.1.3. Parasites

Ultimately, parasites are integral components of marine ecosystems, and their community composition reflects both host biology and environmental health (Marcogliese, 2005; Nachev & Sures, 2016). Parasites have been widely used as bioindicators of host feeding habits and trophic interactions, particularly trophically-transmitted endoparasites with complex life cycles, which can be used to infer host preys, dietary shifts and trophic level (Locke, 2014; Isbert et al., 2014). In addition, parasite communities have been applied as biological indicators of host phylogenetic relationships and population structure, since closely related fish hosts tend to share more parasite species, as do host populations that are geographically closer to each other (Timi et al., 2010; Locke et al., 2013). Finally, they can also act as sentinels of ecological disturbance, as changes or reductions in abundance, richness or diversity often indicate environmental perturbations, pollutant exposure or alterations in food web structure (Palomba et al., 2023; Sures & Nachev, 2022). Particularly, heteroxenous parasites such as digeneans tend to be more sensitive to environmental stressors because their life cycles involve two or more hosts. As a result, their transmission depends on the availability of these intermediate hosts, which their abundance can be directly affected by environmental disturbances, as well as their fragile free-living larval stages. In contrast, monoxenous (e.g. monogeneans) with direct life cycle requires a single host and show a stronger resistance to natural changes (MacKenzie, 1999; Pérez-del-Olmo et al., 2007).

4.2. Environmental health: Fish stressors and contaminants

4.2.1. Parasites

Although parasites have been used as biological indicators, they can also act as pathogens and stressors for fish. Typically, host-parasite interactions are balanced, but high parasite loads or the presence of particularly pathogenic taxa in an individual host may cause pathological (e.g., haemorrhages, hyperplasia, inflammation, epithelial erosion), immunological and reproductive damage (Sitjá-Bobadilla et al., 2008; Iwanowicz, 2011 and references therein, Sures & Nachev, 2022 and references therein), ultimately impairing host growth, fecundity and survival, with effects that may extent to population and community levels (Lloret et al., 2014; Sures & Nachev, 2022 and references therein). Moreover, parasite infections may increase host susceptibility to other environmental or anthropogenic stressors, weakening its immune system by reducing their energy reserves and reproductive potential (Lloret et al., 2014; Sures & Nachev, 2022). Parallely, environmental stress such as increased temperature, toxic chemicals or malnutrition may also increase fish susceptibility to parasitic infections and diseases (Marcogliese, 2025). Parasitic infections are mostly reported in cultured fish species whereas the effects of parasites on wild fish

species are still scarcely known (Lloret et al., 2014). Indeed, parasites and other pathogens (i.e. bacteria, viruses) can cause important economic losses due to disease outbreaks and mortality events in commercially exploited species, particularly within the aquaculture industry, where high host densities may favour pathogen proliferation and subsequent spillover into the surrounding environment, with potential consequences for wild fish populations (Padrós et al., 2025). In general, negative relationships between fish condition and high parasitic infections have been reported in wild species, particularly due to their impact on fish energy reserves (Ferrer-Maza et al., 2014, 2016; Timi & Poulin, 2020). For instance, parasites have been linked to lower levels of lipids from anchovies in the Black Sea, reducing their energy available for reproductive migrations (Akkuş & Gücü, 2021). However, other studies have failed to demonstrate a clear relationship between fish condition and parasitism, indicating that parasite effects are not uniform but may vary among parasite taxa and level of virulence, and also depending on host species' susceptibility and tolerance to infection (Lloret et al., 2014; Mouritsen, 2025).

4.2.2. Anthropogenic stressors: anthropogenic items and heavy metals

Understanding how fish respond to their surrounding environment is essential in the current period marked by intensifying human activities and rapidly changing marine conditions (Bindoff et al., 2019). Marine pollution originates predominantly from land-based sources and encompasses a wide range of contaminants, some of which have been extensively studied and are internationally regulated due to their recognized environmental and health risks, including heavy metals (e.g., mercury, lead, cadmium), persistent organic compounds (e.g., polychlorinated biphenyls (PCBs)) and pesticides (Grobelak & Kowalska, 2022). Owing to their persistence, bioaccumulative potential and toxicity, these contaminants pose significant risks to marine organisms by inducing endocrine disruption, immunotoxicity and reproductive impairment, amongst others, as well as to human consumers (Impellitteri et al., 2023). In addition to these traditional pollutants, marine environments face a broad spectrum of contaminants of emerging concern (CECs) including pharmaceuticals, antibiotics, personal care products, flame retardants, household cleaning products and nano- and micro-particles, representing significant components for assessing environmental quality and their potential biological effects on marine organisms due to the lack of regulation (Grobelak & Kowalska, 2022; Reichelt-Brushett, 2023). The Mediterranean Sea is particularly vulnerable to these pollutants (Impellitteri et al., 2023). Its semi-enclosed nature with limited water exchange, dense coastal populations, high industrial and touristic activities, intense maritime traffic and the inputs of major rivers that are also strongly affected by human activities (e.g. Nile River, Po River) (Laubier, 2005), make the Mediterranean

Sea as one of the most polluted areas in the world, and particularly, a hotspot for microplastics and heavy metals (Copat et al., 2015; Sharma et al., 2021).

4.2.2.1. Anthropogenic items (AIs)

Plastic pollution is one of the most significant global threats to the marine ecosystem, comprising 50-80% of the litter in the oceans (Cressey, 2016). Since plastic debris was first reported in the 1970s (Carpenter et al., 1972), its presence has been documented throughout the marine ecosystem (Cole et al., 2011; Eriksen et al., 2014; Thompson, 2015; Suaria et al., 2020). However, even with ongoing efforts to reduce plastic use, around eight to ten million metric tons of plastic waste are discharged every year into the oceans (Pilapitiya & Ratnayake, 2024), and it is estimated that almost 60% of them could reach the natural environment (Geyer et al., 2017). Once released, due to their long environmental residence time and density, plastics can be vertically transferred down to the seafloor (Napper & Thompson, 2020) or horizontally transported by wind-driven circulation until being concentrated in ocean gyres (van Sebille et al., 2020). Their origin is mainly linked to human activities, from both land- and sea-based sources (Browne et al., 2011) (**Fig. 9**).

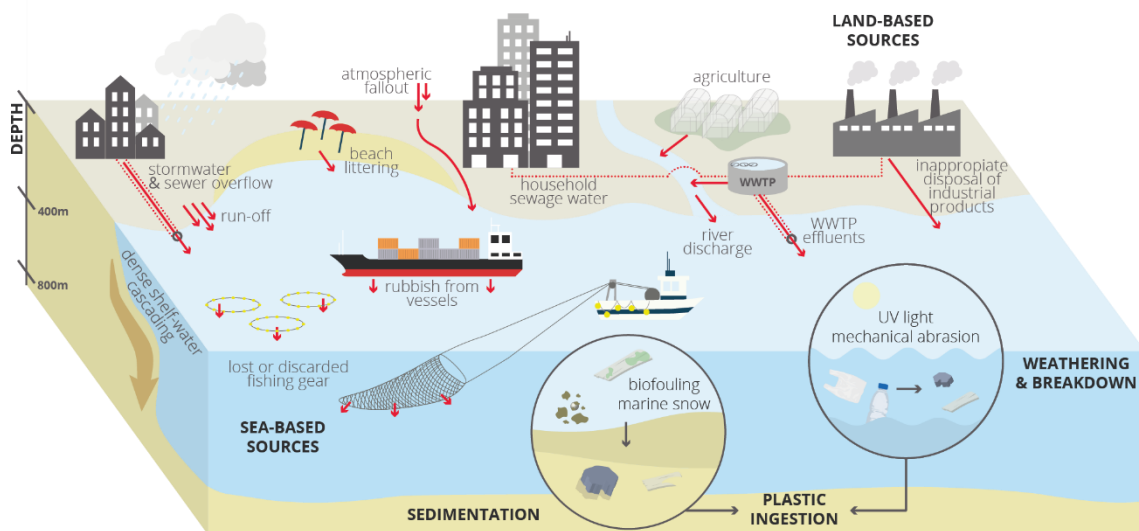


Fig. 9. Main sources and pathways of plastic and microplastic litter into the environment. Source: Carreras-Colom, 2021 PhD thesis.

Their fragmentation into smaller items, known as microplastics (MPs, <5 mm; Hartmann et al., 2019), has accelerated their ubiquity in the marine environment. Moreover, fibres and specifically non-synthetic fibres composed of processed polymers from natural materials (e.g., regenerated cellulose and cotton), mostly coming from wastewater discharges from textile industries and domestic laundering (Santonicola et al., 2023), appear to be the predominant type of item found throughout the world's oceans, accounting for more than 80% of all anthropogenic items (AIs,

including synthetic and non-synthetic elements) (Barrows et al., 2018; Suaria et al., 2020). The Mediterranean Sea is considered the sixth major accumulation area for marine litter worldwide, with the highest concentrations occurring near densely populated coastal regions such as the Catalan Sea, the NW Adriatic Sea, Sicilian Sea and Gulf of Lion (Cózar et al., 2015; Mancuso et al., 2023). Thus, AIs abundance and distribution show high spatial heterogeneity along the Mediterranean and their presence has been reported in different environments, from surface waters to the seafloor sediments (Suaria et al., 2020; Simon-Sánchez et al., 2022) and within the digestive tract of multiple organisms (Fossi et al., 2018).

A key concern regarding the ingestion of these AIs is the potential negative impact on fish health. Experimental studies have documented various physical effects (e.g. mechanical abrasion, obstruction), and physiological responses (e.g. oxidative stress, cellular damage, inflammation, impaired metabolism and altered neurotransmission) associated with MPs and their additive chemicals (e.g. plasticizers, dyes, flame retardants, biocides) (Espinosa et al., 2017; Kögel et al., 2020; Morrison et al., 2022 and references therein). Moreover, MPs were stated to act as vectors for other adsorbed hydrophobic compounds present in the marine environment (e.g., persistent organic pollutants and heavy metals) potentially facilitating their uptake by organisms and their subsequent transfer and toxic impact through the food web (Cole et al., 2011; Rochman et al., 2013; Koelmans et al., 2016). However, attribute these findings directly to MPs ingestion in natural ecosystems is particularly challenging. Laboratory studies often rely on unrealistic high concentrations of MPs and standardized particle types (in terms of composition, shape and size), which do not reflect those found in the natural environment (Hossain & Olden, 2022). Marine ecosystems are vast, complex and highly variable, making it difficult to quantify and infer the specific effects and interactions of these contaminants on fish health. Accordingly, field studies have not consistently demonstrated clear detrimental effects, even in species exhibiting high prevalence and abundance of ingested AIs, highlighting the difficulty of inferring impacts under natural conditions (Carreras-Colom et al., 2020, 2022; Rodríguez-Romeu et al., 2020, 2022; Higuieruelo et al., 2024).

4.2.2.2. Heavy metals

On the other hand, heavy metals also contribute significantly to marine pollution. Although most of them naturally occur in the environment through volcanic activity and weathering of rocks (Shah, 2021), their concentrations have significantly increased due to industrial and urban wastewater discharges, agricultural runoff, mining and atmospheric emissions such as coal combustion (Reichelt-Brushett, 2023) (**Fig. 10**).

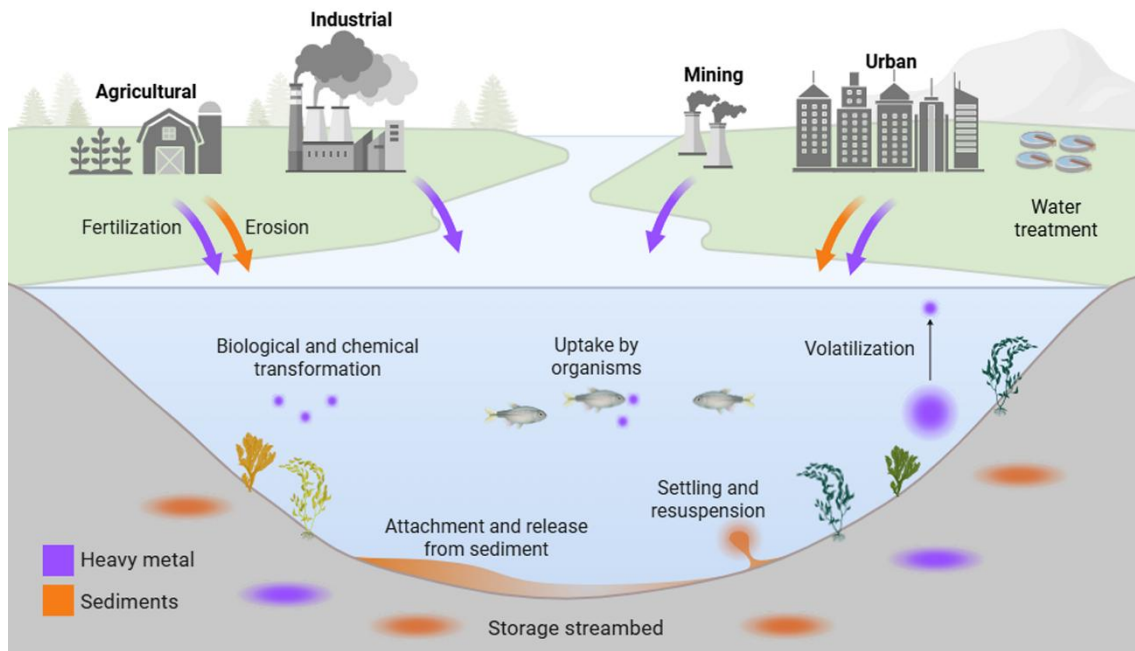


Fig. 10. Sources and pathways of heavy metals into the marine environment. Source: Maritan, 2025

Some metals, such as zinc (Zn), copper (Cu) and iron (Fe), are essential micronutrients required at trace concentrations whereas others, such as cadmium (Cd), lead (Pb) and mercury (Hg) have no biological role and are toxic even at low levels. These elements are transported to the marine environment via dust, river input and atmosphere deposition (Reichelt-Brushett, 2023). Once introduced, they may either remain dissolved or particulate in the water column, settle in sediments where they can act as long-term reservoirs or bioaccumulate in organisms and consequently biomagnify towards upper levels of food webs (Oros, 2025). Their fate depends on their bioavailability which is strongly influenced by environmental conditions and water physico-chemical parameters such as temperature, dissolved oxygen, pH, salinity, suspended organic matter, and primary production (Harmesa et al., 2024; Oros, 2025). Because of their toxicity, persistence and non-biodegradability, heavy metals can exert detrimental effects across marine food webs, becoming major environmental pollutants (Shah, 2021). In the Mediterranean, in addition to anthropogenic sources, natural geological processes such as geothermal fluxes and submarine volcanism contribute naturally to elevate concentrations of certain trace metals (e.g., nickel, arsenic and chromium), increasing the overall metal concentration across the basin (Copat et al., 2015). Moreover, particularly high methylmercury (MeHg) concentrations have been reported in many marine organisms from this basin, especially teleosts top predators and sharks (Cossa et al., 2012; Cresson et al., 2014; Higuieruelo et al., 2025), reflecting a well-documented “Mediterranean mercury anomaly”. This phenomenon is of particular concern given the high bioaccumulation potential of MeHg and its transfer through food webs (Cossa & Coquery, 2005).

Metal uptake and accumulation in marine organisms depend on both environmental and biological factors, including age, sex, size, depth, reproductive stage, feeding behaviour and geographic location (Noman et al., 2022). Moreover, each metal has a characteristic affinity to bioaccumulate in target tissues. For instance, methylmercury tend to accumulate in muscle, Cd in liver and kidney, and Pb in calcium-rich structures such as bones and gills (Oros, 2025). The exposure of fish to chemical contaminants such as metallic elements can induce different lesions to several organs, being gills, liver and kidney as the most important target organs (Au, 2004; Yancheva et al., 2015). Some of the most recurrent histopathological alterations in gills associated with the presence of metals include epithelial and lamellar hyperplasia and oedema, epithelial lifting, vascular congestion, aneurism and necrosis (Naz et al., 2021; Onita et al., 2021). In the liver, hepatocellular vacuolation or degeneration, pyknotic nuclei, necrosis, hepatocellular nuclear pleomorphism, inflammatory infiltrates and hyperaemia were commonly reported in several studies (Hermenean et al., 2015; Naz et al., 2021; Onita et al., 2021), and in kidney, tubular fibrosis, degeneration, inflammatory cell infiltration, glomerular contraction, reduction of renal hematopoietic system and necrosis were detected under the influence of metals (Hermenean et al., 2015; Yancheva et al., 2015 and references therein; Naz et al., 2021; Onita et al., 2021). Therefore, heavy metals are well-known to detrimentally affect fish physiological functions, increase susceptibility to disease, impair growth and reproduction success, which in some cases can result in mortality (Sharma et al., 2025).

4.3. Human health: fish consumption and food safety

Fish play a central role connecting marine ecosystems and human societies while also serving as important food sources worldwide. They constitute a major source of protein, minerals (e.g., calcium, iron, selenium, zinc), vitamins (e.g., A, D, B12), and long-chain polyunsaturated omega-3 fatty acids, particularly from fatty fish species, contributing to the cardiovascular and neurological health, growth, development, and offering prevention of chronic diseases such as diabetes and coronary heart diseases (Sidhu, 2003 and references therein). As the global population continues to grow, seafood consumption (20.7 kg per capita in 2022 globally) has increased steadily over the last decade, intensifying the reliance on marine resources as a key component of a healthy and sustainable diet (Bogard et al., 2019; FAO, 2024b). However, fish stocks capacity is limited and human pressures on the coastal environment have led to a greater contamination of the aquatic systems, raising concerns about the quality and safety of seafood (Marques et al., 2014). Thus, for fish to effectively support human health, it must be not only nutritious but also safe for consumption (Mozaffarian & Rimm, 2006). This requires ensuring the absence or adequate control of potentially pathogenic biological agents such as bacteria, viruses, and parasites, that

may pose risks to consumers. Sanitary public concerns have been raised due to the presence of zoonotic pathogens that can be transmitted to humans through the consumption of raw or undercooked fish, causing gastroallergic reactions (Mattiucci et al., 2013; Roy et al., 2025). Among these, nematodes of the genus *Anisakis* are particularly relevant in wild marine fish, due to the fish-borne zoonotic disease that may affect humans, known as anisakiasis (Mattiucci et al., 2022). Although proper cooking and freezing effectively eliminate these parasites, the growing popularity of raw preparations (e.g., sushi, pickled in vinegar) and the expansion of global seafood trade have increased the likelihood for exposure (Butt et al., 2004).

Likewise, attention must be given to the potential presence of anthropogenic contaminants, including heavy metals, persistent organic pollutants, or other substances, which can accumulate in tissues and represent health risks (Mozaffarian & Rimm, 2006). In particular, the most concerning heavy metals are cadmium (Cd), lead (Pb) and methylmercury (MeHg). Indeed, MeHg biomagnifies through marine food webs and can reach concentrations in predatory fish that exceed recommended safety thresholds for consumption (Campbell et al., 2006; Souza-Araujo et al., 2016; Higuero et al., 2025). Chronic exposure to toxic metals is associated with neurological, developmental and immunological impairments in humans, with children and pregnant women being especially vulnerable (WHO 2019, 2023, 2025). Specifically, MeHg primarily targets the central nervous system and is also linked to nephrotoxicity and gastrointestinal disorders, including ulcerations and haemorrhage (see Castro-González & Méndez-Armenta, 2008). Cd can induce renal tubular dysfunction, cardiovascular diseases and neurological disorders, while Pb interferes with the nervous system, haematological and cardiovascular system (see Castro-González & Méndez-Armenta, 2008). Because of these well-documented health risks, international regulations (European Commission [EU], 2023) and risk assessment guidelines from the European Food Safety Authority (EFSA 2014, 2020) and the joint FAO/WHO Expert committee on Food Additives (JECFA) have established maximum permitted concentrations and dietary intake limits for Hg, Pb and Cd in seafood products. On the other hand, seafood ingestion has also been related to exposure to other contaminants such as anthropogenic items which were initially considered the primary exposure pathway to humans (Galloway, 2015). However, growing evidence indicated that other routes, particularly inhalation or ingestion of indoor dust and other food items, airborne particles and drinking water represented more relevant sources of AIs exposure for humans (Morrison et al., 2022). In fish, AIs typically occur in low abundance in the gastrointestinal tract, which is commonly removed prior to consumption (Rodríguez-Romeu et al., 2020, 2022). Moreover, because their size (1-5 μm) prevents passage through the intestinal epithelium barrier, there is currently no robust evidence that these items

translocate into edible tissues such as the muscle (Burns & Boxall, 2018). Thus, consuming fish would represent a negligible route of AIs exposure to humans. Instead, health concerns increasingly focus on chemical additives and absorbed pollutants associated with microplastics, as well as the nanoplastic fraction ($< 1 \mu\text{m}$) capable of crossing the gastrointestinal barrier and reach other organs. Experimental studies in mammals and cell cultures have demonstrated that nanoplastics can induce oxidative stress, inflammation, alterations in lipid metabolism and cell membrane damage (WHO, 2022b and references therein). Likewise, widely used plastic additives such as bisphenol A and phthalates are recognised endocrine-disrupting chemicals associated with reproductive impairment, developmental abnormalities, metabolic disorders and increased cancer risk (Morrison et al., 2022 and references therein). Although direct evidence in humans remains still unknown, these findings highlight the need for further research on evaluating their implications for human health.

In this context, fish consumption should be understood as a balance between its nutritional benefits and the need to guarantee its safety. The implementation of good practices throughout the entire production chain, from capture to processing and distribution, are essential to maximize its benefits while minimizing associated risks.

Accordingly, the present thesis adopts a One Health perspective, using fish both as bioindicators of ecosystem quality and as a direct pathway through which contaminants and zoonotic pathogens can reach humans, thereby reflecting the interdependence among environmental, animal and human health, that will contribute to the development of more effective strategies for sustainable fisheries.

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CHAPTER 2

Objectives

Considering that wild fisheries are good examples of the close connection among people, fish and environment, knowledge of the health status of commercial fish stocks is essential within a One Health perspective. Consumer well-being depends on healthy fisheries resources, but marine ecosystems and their stocks can be affected by anthropogenic pollutants and other stressors posing potential risks to food safety and human health. Within this context, the European hake (*Merluccius merluccius*), a historically exploited and highly valued species along the Catalan coast is assessed across three localities (Blanes, Barcelona and Ebro Delta) in the Balearic Sea (north-western Mediterranean Sea), to identify spatial variability in their populations condition and potential stressors linked to different anthropogenic pressures. In contrast, the round sardinella (*Sardinella aurita*), an emergent thermophilic species, is investigated at a more local scale on the coast of Barcelona, focusing on the seasonal variability in its health condition and nutritional quality to evaluate its commercial suitability and determine its contribution to the diversification of fishing pressure under the current decline of important fish stocks.

For this reason, the main goal of this thesis is to assess the health status of the European hake and the round sardinella from the Catalan coast (NW Mediterranean Sea), within a One Health framework. This study is performed using a multidisciplinary approach, integrating their biology and ecology with an evaluation of their fitness, pathology and histological alterations, and examining their parasite composition community and exposure to anthropogenic stressors (i.e., anthropogenic items ingestion and the accumulation of heavy metals), to provide guidelines for sustainable fisheries management, enhance food safety and improve the commercial value of these species. To fulfil this general goal, the following specific objectives have been established:

1. To assess the health status of both fish species through the evaluation of their condition indices, the description of pathologies and histopathological alterations and the characterisation of their parasite community composition (Chapter 4).
 - 1.1. To provide novel insights into the current knowledge of parasite species infecting the European hake that could affect its health condition (Chapter 4.1, 4.2).
2. To quantify the levels of contaminants (anthropogenic items in the digestive tract and heavy metals in muscle tissue) and evaluate their potential health impacts in the target fish species (Chapter 5).
3. To evaluate the viability of both fish species as fishery resources in terms of nutritional benefits and food safety for human consumption (Chapter 6).
 - 3.1. To determine the nutritional status of *S. aurita*, including proximate composition and fatty acid profiles, to assess its potential commercial value (Chapter 6).
 - 3.2. To assess heavy metals intake from the consumption of both species (Chapter 6).

- 3.3. To analyse the presence of zoonotic parasites and their geographic distribution across the Mediterranean Sea in both species (Chapter 6).

To achieve these objectives, the present thesis has been structured into three chapters of results (Chapter 4 to 6), each of which include several contributions that have been published in international peer-reviewed journals or are currently in preparation for submission:

- Muns-Pujadas, L., Constenla, M., Dallarés, S., Padrós, F. (2024). Diving into the fish pathology of an important commercial fish species: the case of the European hake (*Merluccius merluccius* Linnaeus, 1758) in the northwest Mediterranean Sea. *Journal of Fish Biology*, 105, 1906–1918. <https://doi.org/10.1111/JFB.15940>.
- Muns-Pujadas, L., Constenla, M., Dallarés, S. (2025). A fresh look at *Cleistobothrium crassiceps* (Rudolphi 1819) Lühe, 1899 (Cestoda: Bothriocephalidea) two centuries later: first complete description and novel observations from its type-host in the NW Mediterranean Sea. *Parasitology Research*, 124, 138. <https://doi.org/10.1007/s00436-025-08571-4>.
- Muns-Pujadas, L., Constenla, M., Dallarés, S., Valmaseda-Angulo, M., Montero, F.E., Padrós, F. Blood intruders: *Aporocotyle spinosicanalis* (Williams, 1958) from its type-host *Merluccius merluccius* and its histopathological impacts in the NW Mediterranean Sea. In preparation.
- Muns-Pujadas, L., Dallarés, S., Constenla, M., Padrós, F., Carreras-Colom, E., Grelaud, M., Carrassón, M., Soler-Membrives, A. (2023). Revealing the capability of the European hake to cope with micro-litter environmental exposure and its inferred potential health impact in the NW Mediterranean Sea. *Marine Environmental Research*, 186, 105921. <https://doi.org/10.1016/j.marenvres.2023.105921>.
- Muns-Pujadas, L., Constenla, M., Dallarés, S., Carreras-Colom, E., Soler-Membrives, A., Padrós, F. Assessing the seasonal uptake of micro-litter by round sardinella (*Sardinella aurita* Valenciennes, 1847) and its potential health impact in the NW Mediterranean Sea. *Continental Shelf Research*, Under review.
- Muns-Pujadas, L., Constenla, M., Dallarés, S., Soler-Membrives, A., Padrós, F., Sala, R. (2025). The round sardinella (*Sardinella aurita* Valenciennes, 1847) from the NW Mediterranean: a healthy and safe choice for human consumption. *Mediterranean Marine Science*, 26 (1), 16–29. <https://doi.org/10.12681/mms.38787>.
- Muns-Pujadas, L., Constenla, M., Dallarés, S., Padrós, F., Fioravanti, M.L., Caffara, M., Gustinelli, A. Distribution of zoonotic anisakid nematodes in two commercial Mediterranean fish species. In preparation.

CHAPTER 3

Materials & Methods

1. Study area and sample collection

A total of 280 *Merluccius merluccius* and 348 *Sardinella aurita* specimens were examined between 2007 and 2025 for different purposes including analyses of anthropogenic items ingestion, parasitology, histopathology, nutritional status (proximate composition and fatty acids) and heavy metals (**Table 1**).

Specimens were captured on board commercial fishing trawlers (in the case of *M. merluccius*) and purse seiner fishing vessels (in the case of *S. aurita*) on the continental shelf of the Catalan coast (Balearic Sea, north-western Mediterranean Sea) (**Fig. 1**). Moreover, for the studies focused on specific *M. merluccius* parasite species (i.e. *Cleistobothrium crassiceps* and *Aporocotyle spinosicanalis*), additional fish were collected from the Catalan coast, as detailed in Chapters 4.1. and 4.2. Specimens from both species were also collected in various localities of the Tyrrhenian and Adriatic Seas (central Mediterranean Sea), only for parasitological analysis (**Table 1**; **Fig. 1**).

Immediately upon capture, individuals were either fixed *in toto* in 10% buffered formalin (after performing an abdominal incision to allow proper fixation of internal organs), preserved fresh or frozen according to subsequent analyses (**Table 1**). Selected individuals were of similar size whenever possible to enable data comparison. The subsequent dissection procedures are described in each Chapter (4-6) according to the specific purpose of each analysis.

M. merluccius specimens from 2007 and 2019 were collected within the framework of two national research projects: BIOMARE (Spanish Ministry of Science and Innovation) and PLASMAR (Spanish Ministry of Science, Innovation and Universities), respectively. Both projects were conducted along the Catalan coast, including the area of Barcelona, and amongst their specific objectives, both focused on evaluating the fish health in different fish species (including *M. merluccius*) in relation to anthropogenic pressures. Particularly, the BIOMARE project investigated the relationship between fish health responses at different biological levels and exposure to anthropogenic pollutants, considering natural ecosystem variability. In contrast, the PLASMAR project aimed to address the impact of microplastics on the populations of some species of fish and crustaceans of fishery interest in the NW Mediterranean, assessing their health status through condition indices, parasitological and histopathological analyses in three different sites (Barcelona, Blanes and Ebro Delta coasts).

Table 1. Summary of sampling data including location, coordinates, mean depth (m), date, number of individuals (n), preservation method and type of analysis performed for each studied fish species. Abbreviations: Als, anthropogenic items; PA, parasitology; HP, histopathology; PC, proximate composition; FA, fatty acids; HM, heavy metals.

Location		Latitude	Longitude	Depth	Date	n	Preservation	Analysis
<i>Merluccius merluccius</i>								
N Balearic Sea								
Blanes	BL	41°34'48.2"N	2°47'43.7"E	111.5	04/07/2019	22	Formalin	Als, PA, HP
						9	Frozen	HM
Barcelona	BCN0	41°25'22.05"N	2°20'55.29"E	62.4	18/07/2007	18	Formalin	PA, HP
						20	Formalin	Als
Barcelona	BCN1	41° 19'45.72"N	2° 17'8.279"E	87	17/07/2019	21	Formalin	Als, PA, HP
						10	Frozen	HM
Ebro Delta	ED	40°25'56.9"N	0°53'49.7"E	65	26/06/2019	19	Formalin	Als, PA, HP
						10	Frozen	HM
S Balearic Sea								
Valencia	VA	39°23'46"N	0°12'26"W	53	20/05/2025	15	Fresh/frozen	PA
N Adriatic Sea								
Rimini	RI	44° 26' 053"N	12° 51' 628"E	38	30/01/2024	89	Fresh/frozen	PA
N Tyrrhenian Sea								
Rosignano	RG	43°22'07"N	10°24'09"E	30	23/05/2024	20	Fresh/frozen	PA
C Tyrrhenian Sea								
Roma	RO	41°41'53"N	12°11'07"E	58	16/05/2024	20	Fresh/frozen	PA
S Tyrrhenian Sea								
Napoli	NA	40°42'13"N	14°11'40"E	120	02/05/2024	40	Fresh/frozen	PA
Salerno	SA	39°55'46"N	15°04'17"E	150	17/05/2024	14	Fresh/frozen	PA
<i>Sardinella aurita</i>								
N Balearic Sea								
Barcelona	BCN0	41°22'50.0"N	2°13'00.0"E	35.11	21/02/2022	10	Frozen	HM
	BCN1	41°25'08.9"N	2°16'14.7"E	33.43	26/07/2022	20	Formalin	Als, PA, HP
						20	Frozen	PC, FA
	BCN2	41°14'01.2"N	2°01'17.9"E	36.78	5/12/2022	20	Formalin	Als, PA, HP
						20	Frozen	PC, FA
	BCN3	41°27'26.3"N	2°26'05.5"E	53.26	21/03/2023	20	Formalin	Als, PA, HP
						20	Frozen	PC, FA
	BCN4	41°22'19.0"N	2°18'43.0"E	63.24	23/05/2023	20	Formalin	Als, PA, HP
						20	Frozen	PC, FA
	BCN5	41°14'49.3"N	2°05'50.8"E	52.98	28/07/2023	20	Formalin	Als, PA, HP
						20	Frozen	PC, FA
N Adriatic Sea								
Rimini	RI	44°19'33.1"N	12°32'27.8"E	38.74	12/02/2024	39	Fresh/frozen	PA
Scardovari	SC	44°50'49.0"N	12°34'15.0"E	33.51	03/04/2024	22	Fresh/frozen	PA
S Adriatic Sea								
Bari	BA	41°33'54.0"N	15°59'48.0"E	35.69	26/02/2024	77	Fresh/frozen	PA

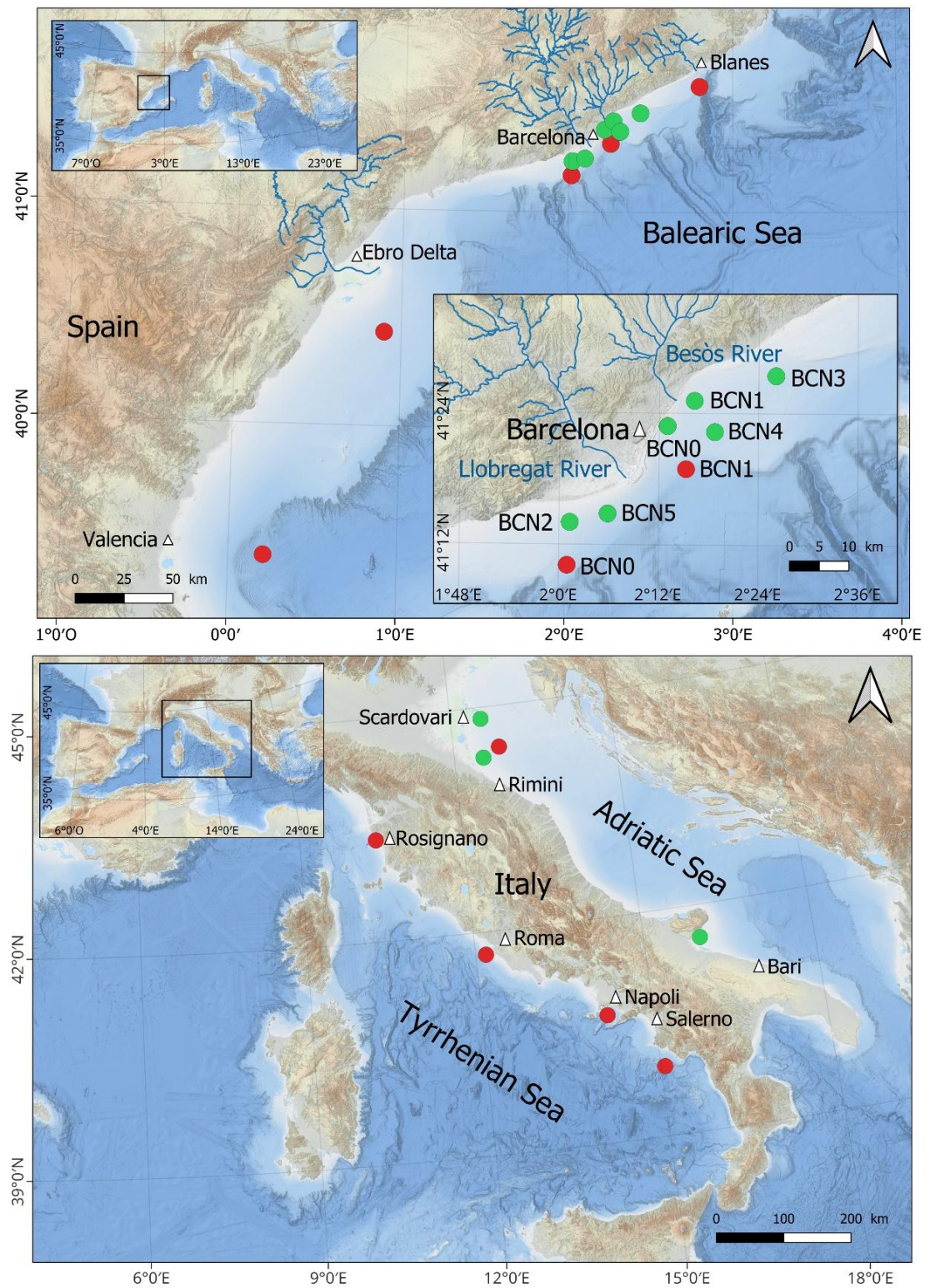


Fig. 1. Map of the study area showing the different sampling points from different areas off the Balearic Sea (NW Mediterranean Sea) and Tyrrhenian and Adriatic Seas (Central Mediterranean Sea) from *Merluccius merluccius* (red) and *Sardinella aurita* (green).

The Barcelona coast was selected as a highly impacted area due to its densely populated metropolitan area (over 1.7 million inhabitants; Idescat, 2025), along with intense urban and industrial activities, heavy maritime transport (over 8,383 vessels and 67 million tonnes of goods moved in 2024; Idescat, 2024a), tourism (receiving 5 million visitors in 2025 in the coastal area; Direcció General de Turisme, 2025) and significant fishing activity (2,221.96 tonnes of landings; ICATMAR, 2025). The area is also influenced by the Besòs River (58 km long and 87 hm³/y) and Llobregat River (170 km long and 172 hm³/y), which receive inputs from both urban and industrial sources (Idescat, 2024b). Blanes coast, in turn, represented a comparatively less impacted area owing to its lower industrial activity and sparse population. Finally, Ebro Delta coast was included for its particular characteristics, under the influence of the Ebro River (910 km length, 14000 hm³/y), which receives inputs from extensive agricultural and industrial activities along its basin (Galimany et al., 2019).

Within the PLASMAR project, ASTD152 CTD casts were performed at the same three locations at 5 m above the seafloor prior to fishing trawls to record environmental variables (temperature, salinity, oxygen concentration and turbidity) (**Table 2**).

Table 2. Depth range and abiotic parameters (temperature, salinity, turbidity and oxygen concentration) of the near-bottom water layer (5 m above the seafloor) measured in the three sampled localities.

	Blanes	Barcelona	Ebro Delta
Depth range (m)	68-73	37-42	34-39
Temperature (°C)	14.12	17.20	15.57
Salinity (g/L)	38.20	38.01	38.1
Turbidity (FTU)	0.47	0.25	0.12
Oxygen (mg/L)	8.41	9.12	9.6

For *S. aurita*, samplings were performed seasonally between 2022 and 2023 at the coast of Barcelona. This area is strongly influenced by seasonal variability, with the presence of a well-defined thermocline in summer and its destratification in winter promoting a vertical mixing of nutrients. More specifically, water column stratification begins in spring (March-April) because of increased solar heating, and becomes fully developed in summer, with a defined thermocline at 20-30 meters depth (Sabatés et al., 2007). This stratification limits vertical mixing and nutrient supply to surface waters, confining primary production to the deep chlorophyll maximum at 60-80 meters depth within the photic zone, while surface productivity is mainly sustained by riverine inputs (Sabatés et al., 2007). In autumn (October-November), decreasing surface temperatures

and wind-driven vertical mixing associated with storm episodes weaken the thermocline, leading to vertical homogenization in winter (December to February), and promoting the upward transport of nutrients (Sabatés et al., 2007; Grifoll et al., 2013; Guillén et al., 2018).

Due to the lack of recorded environmental conditions, environmental data of the study area for each season (i.e. sea surface temperature, chlorophyll-a concentrations, average volume of the Besòs and Llobregat rivers and rainfall trends) were provided from external databases (**Table 3**) for further interpretation of results from Chapter 5.

Sea surface temperature (SST, °C) and chlorophyll-a concentrations (chl-a, mg/m³) from each seasonal sampling were retrieved from the E.U. Copernicus Marine Service Information for the period 2022-2023. For SST, data was used from the reprocessed high-resolution satellite product MED-REP-L4 (<https://doi.org/10.48670/moi-00173>, accessed on 2 May 2026; Pisano et al., 2016; Embury et al., 2024) with a spatial resolution of 0.05° × 0.05° and at daily resolution. For chl-a, data was used from the OCEANCOLOUR_GLO_BGC_L3_NRT_009_101 product (<https://doi.org/10.48670/moi-00278>, accessed on 2 May 2026; Gohin et al., 2002) with a spatial resolution of 4 x 4 km and at daily resolution (**Table 3**). Moreover, the seasonal average volume of the Besòs and Llobregat rivers and the seasonal cumulative precipitation were also used from the Statistical Institute of Catalonia (Idescat, 2024c) and Servei Meteorològic de Catalunya (Meteocat, 2026), respectively (**Fig. 2**).

Table 3. Sea surface temperature (SST) and chlorophyll-a concentration (Chl-a) derived from E.U. Copernicus Marine Service Information satellite observations (<https://doi.org/10.48670/moi-00173> and <https://doi.org/10.48670/moi-00278>, accessed on 2 May 2026) for each sampling date are shown. The average volume of the Llobregat and Besòs Rivers from each sampling season are also displayed from the Statistical Institute of Catalonia database.

	Summer 22	Autumn 22	Winter 23	Spring 23	Summer 23
SST (°C)	27.63	16.77	14.67	17.61	27.06
Chl-a (mg/m ³)	0.045	0.11	0.24	0.09	0.05
Average volume (m ³ /s)					
Llobregat River	7.78	5.37	3.84	5.77	4.70
Besòs River	2.03	2.04	2.12	2.11	1.87

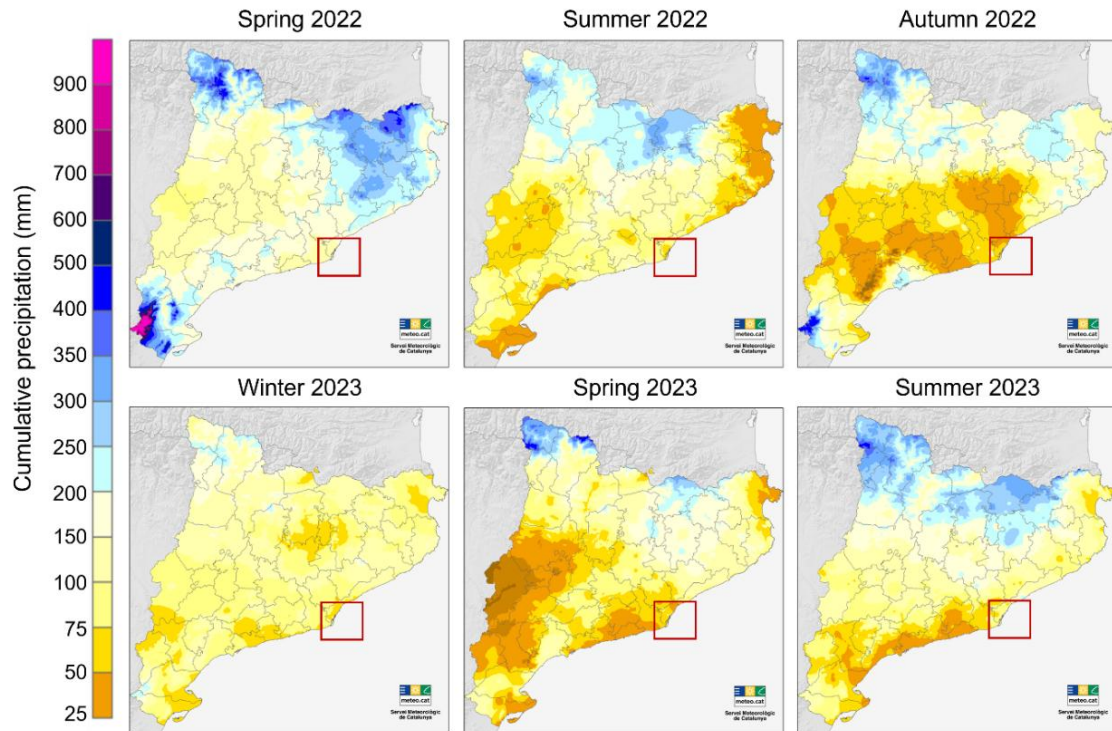


Fig. 2. Seasonal maps showing cumulative precipitation (mm) in Catalonia (Spain) during the sampling period 2022-2023. The red square indicates the study area off the coast of Barcelona (NW Mediterranean Sea). Maps were obtained from the Servei Meteorològic de Catalunya.

The additional specimens from the Central Mediterranean were collected to provide a broader geographical perspective on zoonotic parasites of both species across contrasting environmental gradients, anthropogenic pressures and habitat characteristics within key fishing grounds. In the Adriatic, sampling was conducted off Rimini (northern), a shallow and highly productive area influenced by riverine nutrient inputs being the most important the Po River, and off Bari (southern), which is characterized by a narrower shelf and deeper oceanic waters (Danovaro & Boero, 2019). In the Tyrrhenian Sea, sampling sites followed a latitudinal gradient covering the northern (off Rosignano), central (Rome) and southern (Naples and Salerno) areas. These sites represent a transition from cooler, shallower shelves influenced by riverine inputs in the north to narrow oceanic coasts with lower primary productivity dominated by canyon-driven upwellings in the south (i.e. Gulf of Naples) (Cognetti et al., 2000; Danovaro & Boero, 2019).

2. Health condition analysis

2.1. Condition indices

For each fish, the liver (LW), gonads (GW) and stomach (SW) were weighed individually to the nearest mg. Then, the eviscerated weight (EW) of each specimen was recorded to the nearest g. Fish condition was assessed by Le Cren's relative body condition index ($Kn = EW / (\alpha \times SL^\beta)$) (Le Cren, 1951), where Kn is the relative body condition, α and β are the slope and the intercept of the weight-length relationship representing the entire dataset of sampled fish, the hepatosomatic index ($HSI = (LW/EW) \times 100$) and the gonadosomatic index ($GSI = (GW/EW) \times 100$). Feeding intensity was calculated by the stomach-fullness index ($FULL = (CW/EW) \times 100$), using the stomach content weight (CW) recorded after AI's inspection.

2.2. Parasitological analysis

Fish external surfaces and excised organs (stomach, intestine, pyloric caeca, liver, gonads, spleen, heart, kidney, brain and gills) were examined under a stereomicroscope (5–40x of magnification) for the presence of macroscopic alterations and/or parasites. The whole fillet muscle of each specimen was either individually placed in-between glass compressors (used for trichinosis diagnostic) for or in plastic bags and subjected to the UV-press method for the detection of nematodes (Gómez-Morales et al., 2018; ISO 23036-1:2021 Microbiology of the food chain—Methods for the detection of Anisakidae L3 larvae in fish and fishery products Part 1: UV-press method). Samples were compressed to a 2 mm thickness and frozen at -20°C for a minimum of 24 hours prior to examination under 366 nm UV light in a dark room.

All parasites recovered were collected, counted, stored in 70% ethanol for morphological identification or in 95% ethanol for molecular analysis, and identified to the lowest taxonomic level whenever possible (Kabata, 1979; Moravec, 1994; Gibson et al., 2002). For morphological identification, platyhelminths and acanthocephalans were stained with iron acetocarmine, dehydrated through a graded ethanol series, cleared in clove oil and examined as permanent mounts in Canada balsam. Arthropods were cleared and examined as semi-permanent mounts in glycerine. Nematodes recovered from fixed fish were cleared and examined as semi-permanent mounts in glycerine, whereas those from fresh or frozen fish were preserved in 95% ethanol for molecular analysis.

For nematodes molecular identification, the DNA was extracted from the central part of the nematode body using the PureLink® Genomic DNA Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. DNA amplification was performed on the ITS rDNA regions with the primers NC5 (forward, 5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (reverse, 5'-

TTAGTTTCTTCCTCCGCT-3') (Zhu et al., 1998). The resulting PCR products were electrophoresed on 1% agarose gels stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA) in 0.5X TBE. For the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), 10 µl of the amplified DNA was digested with 1.5 µl of restriction enzymes HinfI, HaeIII, and AluI (D'Amelio et al., 2000) in a total reaction volume of 20 µl at 37 °C for 2 hours (Abollo et al., 2003). Restriction fragments were separated on 2% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA) in 0.5X TBE. The resulting species banding for each restriction enzyme was compared with existing literature (Tedesco et al., 2018; Macchioni et al., 2021; Caffara et al., 2025). Each reaction included sequenced *Anisakis pegreffii* and *Hysterothylacium aduncum* as positive controls.

2.3. Histopathological analysis

A portion of organs fixed in 10% buffered formalin was embedded in paraffin and processed by routine paraffin histology techniques. Samples were sectioned at 4–5 µm and stained with Haematoxylin and Eosin (H&E). Some of these sections were additionally stained with Periodic Acid Schiff (PAS). Stained slides were thoroughly screened and qualitatively examined under the microscope for the detection and identification of specific disorders. Images were taken with a Leica camera model CTR5000 connected to a Leica DM500B microscope.

3. Anthropogenic pollutants analyses

3.1. Anthropogenic items (AIs)

3.1.1. Visual inspection and collection of AIs

The screening of the gastrointestinal content was performed by visual inspection under a stereomicroscope (5-40x magnification). For this, the content of the stomach, intestine and pyloric caeca (if present) for each fish was emptied and individually placed in separate glass Petri dishes. The gastrointestinal content was dispersed with ethanol 70% and distributed in more than one Petri dish when necessary and thus surveyed by aliquots to isolate all the AIs present in the samples. Each AI found was carefully cleared of organic debris or digestive tract contents using a fine dissecting needle. Then, they were counted, individually mounted between glass slides in filtered distilled water and observed under a light microscope for their characterization. A selection criterion adapted from Hidalgo-Ruz et al. (2012) for fibres was followed to prevent misidentification of AIs with organic material (e.g. seagrass or seaweed).

3.1.2. Environmental water samples

In order to analyse the AIs from near-bottom water samples collected at the different sampling points in 2019, an aliquot of 100 mL of the homogenised mixture of fixed samples (0.5L from samples off Blanes and Barcelona and 0.8L from samples off Ebro Delta) was filtered through a 1 mm mesh size stainless sieve into two fractions (>1mm and <1mm). Due to the huge amount of organic material, the <1mm fraction was split into ten aliquots with a McLane rotary splitter (splitting error <4%). In order to prevent the degradation of cellulosic fibres (as explained by Carreras-Colom et al., 2022), three of the ten aliquots were put together and exposed to a soft alkaline digestion (Cole et al., 2014): 20 mL of NaOH (1M) were added to the subsamples and left to react for 24h at room temperature. Subsamples were split again and two sub-aliquots were vacuum-filtered onto polycarbonate membranes (Millipore, Ø47mm, 0.45µm mesh size). The filters were dried overnight at 40°C and visually inspected for the presence of AIs. For the >1mm fraction, AIs were directly picked from the stainless sieve under a stereomicroscope. After AIs extraction, all the concentrations obtained for the <1mm fraction as for the >1mm fraction were corrected in order to take into account all the splitting steps. AIs from both fractions were collected, counted and mounted individually between glass slides in filtered distilled water for their characterization.

3.1.3. AIs classification and measurements

Images of each AI were taken at 50x to 400x magnification with a Leica camera model CTR5000 connected to a Leica DM500B microscope and an image-processing software (ProgRes® C3). AIs were first classified according to their shape (i.e. fibres and fragments). The area of fragments was recorded and in the case of fibres, measurements of total length and mean diameter (calculated as the mean of three diameter values taken from the centre and near the ends) of each fibre were taken. Fibres were visually characterized by light microscopy and classified into different typologies according to their morphological appearance (see **Table 4**). Diagnostic criteria included colour, uniformity of the diameter, surface texture, presence of granules, stretches or ends shape, and were established following previous studies also characterizing AIs in the study area (Zhu et al., 2019; Carreras-Colom et al., 2020; Rodríguez-Romeu et al., 2020).

Table 4. Visual characterisation of the AIs according to their morphological appearance. Each category is represented in Figures 2 and 7 from Chapter 5.

Category	Visual description
A	Cellulosic-like fibre of non-constant diameter, flexible, frayed or broken ends and sometimes worn backbone surface. Often, colour-degraded in dyed fibres. Two types of morphologies were identified: - Flat cross-sectional shape with ribbon-like twists or convolutions and angular edges when folded. Sometimes refringent. Generally transparent, dark or blue. - Thick and braided surface with round cross-section and no flattened. Mainly transparent or dark coloured and opaque.
B	Rigid and smooth cylindrical surface of uniform diameter with delustrant granules (typically black) disrupted by eventually molten areas. Round cross-sectional shape and splintered or circular broken ends. Sometimes refringent. Without signs of fraying surface. Transparent or bright coloured.
C	Smooth and cylindrical surface with one dark prominent streak running parallel to backbone length. Often refringent. Mainly round cross-sectional shape. Mostly bright coloured.
D	Fibre of smooth textured surface of uniform diameter with round cross-sectional shape. Multiple transparent bubbles along the backbone surface, giving a granular appearance. Sometimes with frayed ends. Generally transparent and opaque.
E	Two types of morphologies were detected: - Fibre-shaped: Smooth textured surface of uniform diameter with round cross-sectional shape. Fraying surface with multiple cracks and marks and occasionally with delustrant granules. Generally opaque. - Fragment: Irregular surface with multiple cracks and marks and occasionally with delustrant granules. Mostly bright coloured.

3.1.4. AIs polymer composition identification

Polymer composition of AIs >1 cm was identified by FTIR (Fourier-Transformed Infrared Spectrometry) using a Tensor 27 FTIR spectrometer (Bruker Optik GmbH, Germany) operating in Attenuated Total Reflectance (ATR) mode. Spectra were recorded as 16 scans in the spectral range of 600-4000 cm^{-1} (Servei d'Anàlisi Química, Universitat Autònoma de Barcelona). AIs <1 cm were chemically analysed by micro-FTIR using a Thermo Scientific™ Nicolet™ iN10 MX Infrared Imaging Microscope, and spectra were recorded as four scans in the spectral range of 800-4000 cm^{-1} (CCitUB, Universitat de Barcelona). Specifications of the selection for the subsamples of AIs

recovered from each fish species are explained in Chapter 5. Spectra were treated with Spectragryph 1.2.11 (Menges, 2021) to select the spectral range of 800-3600 cm^{-1} and to clean and standardize spectra (i.e. baseline correction and normalization of peaks). The resulting corrected spectra were compared with reference spectra (Primpke et al., 2018) and similarity correlation values higher than 70% were accepted as a reliable identification. Correlation values ranging between 60 and 70% were also accepted only when their spectra matched visually with the reference and the visual appearance of the AI fitted with the description of the typology. Since the success rate in polymer visual identification exceeded 70%, AIs that were only visually characterized were assigned to the expected polymer type for their respective typology.

3.1.5. Quality assurance and quality control

In order to avoid airborne contamination, several preventive measures were taken during fish dissection procedures and the inspection of AIs. The dissection of all fixed fish was carried out in a laminar flow cabinet (class II). The working surface was periodically wiped with a lint-free cloth to keep it clean during sample dissection procedures. Dissection material and glass vials for keeping the different organs devoted to AIs analysis were rinsed three times with filtered distilled water (50 μm metal sieve) before use, and nitrile gloves and cotton lab coat were always used.

Screening of digestive contents was carried out inside an isolation device similar to the one proposed by Torre et al. (2016), consisting of a plastic cover that isolated the stereomicroscope and the working area from the surrounding environment. Before inspecting each organ, dissection tools and glass Petri dishes were rinsed with filtered distilled water. Procedural controls, consisting of two Petri dishes filled with filtered distilled water, were placed outside and inside the isolation device, respectively, before each examination and checked at the end to assess background airborne contamination during screenings. As an outcome, the quantity of fibres found in the outside controls was on average 5 times higher than in the inside controls, thus demonstrating the effectiveness of the isolation device. Only fibre-shaped items were found in controls, and they appeared on the water surface (indicating that they were deposited from air). Concordantly, during screenings of gastrointestinal contents, fibres that were observed floating on the surface of the water containing the sample were not counted, as they were considered airborne contamination. No other correction was applied to the final values of AIs reported.

Water samples processing was performed in a clean laboratory, consisting in an 8 m^2 hardwall laminar flow chamber ventilated through 4 laminar flow HEPA filters (H14). All material was previously rinsed and distinctive orange lab coats were used at all times. Procedural blanks were

performed to check for potential contamination, and a mean of 0.63 fibres/blank were found. No fibres matching with the coloured coat were detected attributable to airborne contamination.

3.2. Heavy metals analysis

Analyses of concentrations of cadmium (Cd), lead (Pb), zinc (Zn), copper (Cu), nickel (Ni), and arsenic (As) from *M. merluccius* were carried out at the Servei d'Anàlisi Química (SAQ, Chemistry Analysis Service) from the Universitat Autònoma de Barcelona (UAB), whereas the analyses of these metals in *S. aurita*, as well as the determination of mercury (Hg) in both species were performed in collaboration with the Instituto Español de Oceanografía (IEO-CSIC, Vigo, Spain).

Abdominal muscle samples of *M. merluccius* previously frozen (about 3 g of mean weight) analysed in SAQ were lyophilised at 60°C for 72 hours. Samples were weighed prior to and after lyophilization to determine their moisture content. Then, each sample was homogenized and stored in a 1.5 ml Eppendorf at 5 °C for further metal analysis. From each sample, 0.2 g went through a digestion (5 mL of HNO₃ and 0.5 mL of HF) in an automated microwave (Milestone, Ultrawave model) at 240°C for 15 minutes. Afterwards, concentrations of Cd, Pb, Zn, Cu, Ni and As were determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent, 7500CE model). Certified reference materials (ERM-BB422 Fish Muscle, Joint Research Centre) and laboratory blanks were included. No corrections were applied as the percentage recovery from certified reference materials was acceptable (ranging between 85 and 115%).

Regarding samples analysed in the IEO-CSIC, *S. aurita* muscle samples previously frozen (about 5 g of mean weight) were lyophilised at 60°C for 72 hours. Samples were weighed prior to and after lyophilization to determine their moisture content. Briefly, 300 mg of homogenised samples were subjected to an acid digestion using concentrated HNO₃ (PlasmaPURE, SCP Sciences) on Teflon reactors in a microwave digestion system (MARS6, CEM) according to Besada et al. (2014). Given the fact that some metals were near the limit of quantification (LOQ), the analyses were repeated using a hotplate digestion method as described in Sánchez-Marín et al. (2023). The results obtained from both digestion methods were very similar, so results are provided as the average of both methodological replicates where possible. Concentrations of Cd, Pb, Zn, Cu, Ni and As were determined by inductively coupled plasma-mass spectrometry (Agilent 8900 ICP-MS) as outlined by Sánchez-Marín et al. (2023). At IEO-CSIC, total mercury (Hg) concentration was also determined in the solid samples from both fish species (*M. merluccius* and *S. aurita*) by pyrolysis atomic absorption spectrometry with gold amalgamation (employing an AMA254 Advanced Mercury Analyser (LECO Instruments), as described in Belmonte et al. (2021). This method

determines total Hg but not methylmercury (MeHg) content. However, considering that MeHg represents 70 to 100% of Hg in teleosts, total Hg is a good indicator of MeHg (Cresson et al., 2014). Certified reference materials (ERM®-BB422 Fish Muscle, Joint Research Centre; DORM-2 from the National Research Council of Canada) and laboratory blanks were included. Quality assurance was also endorsed through participation in QUASIMEME (Quality Assurance of Information in Marine Environmental Monitoring in Europe) with satisfactory Z-scores for each metal reported. No corrections were applied as the percentage recovery from certified reference materials was acceptable (ranging between 88 and 114%). Values of heavy metals concentrations that were not detected in the determination were considered values below the limit of detection (LOD) (**Table 5**). In order to include these values into the statistical analysis, LOD values were substituted with a value equivalent to half of the LOD (LOD/2), as this is the most commonly approach used in these cases (Wood et al., 2011).

Table 5. Limits of detection (LOD) determined for each analytical method established by the Servei d'Anàlisi Química (SAQ) from the Universitat Autònoma de Barcelona (UAB) and the Instituto Español de Oceanografía (IEO-CSIC). Data are expressed in micrograms per gram of dry weight ($\mu\text{g/g dw}$). Modified from Higuero et al. (2025).

	Ni	Cu	Zn	As	Cd	Pb	Hg
SAQ (UAB)	< 0.019	< 0.093	< 0.93	< 0.019	< 0.0009	< 0.009	-
IEO MARS 6	< 0.024	< 0.012	< 3.77	< 0.014	< 0.0007	< 0.008	< 0.003
IEO Hot plate	0.003	0.0005	0.005	0.0003	0.0003	0.0008	0.0008

4. Nutritional status analyses

Analytical determinations of chemical composition were performed according to AOAC (Association of Official Agricultural Chemists) International methods. Crude protein (method 954.01) was determined according to the Kjeldahl method using a Kjeltec™ 8400 Analyzer Unit (Foss España S.A.) after acid digestion. Total lipid was determined as ether extract through Soxhlet analysis (920.39) with a SoxTec™ 2055 auto-extraction unit after hydrolysis with a SoxCap™ 2047. Fatty acid composition was determined by direct transesterification following the methodology used by Carrapiso et al. (2000). Nonadecanoic acid (C19:0; Sigma-Aldrich Chemical Co.; St. Louis, MO, USA) was added as an internal standard. The methyl esters obtained were injected into a gas chromatographer coupled with a flame ionization detector (HP6890, Agilent Technologies). Chromatographic fatty acid peaks were identified using standard samples (Supelco 37 Component FAME Mix, Sigma Aldrich). The content of each fatty acid was expressed as the percentage of the total fatty acid content.

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CHAPTER 4

Fish health status

Muns-Pujadas, L., Constenla, M., Dallarés, S., Padrós, F. (2024). Diving into the fish pathology of an important commercial fish species: the case of the European hake (*Merluccius merluccius* Linnaeus, 1758) in the northwest Mediterranean Sea. *Journal of Fish Biology*, 105, 1906–1918. <https://doi.org/10.1111/JFB.15940>.

Abstract

Monitoring the health condition of commercial species is essential for improving fisheries management, as it provides key information on population fitness and resilience to a wide variety of stressors. However, integrative studies combining health indicators at different biological levels remain scarce. This chapter assesses the health status of the European hake (*Merluccius merluccius*) and the round sardinella (*Sardinella aurita*) from the Catalan coast (NW Mediterranean Sea), through the analysis of condition indices, gross pathology, histopathological alterations and parasite communities. For *M. merluccius*, specimens collected in 2019 from three localities with contrasting environmental conditions and anthropogenic pressures (off Blanes, Barcelona and Ebro Delta) were compared to identify spatial health differences. Additionally, specimens collected off Barcelona in 2007 were included to evaluate potential changes within a 12 year-gap. For *S. aurita*, a seasonal sampling between 2022 and 2023 off Barcelona coast was performed to delve into potential seasonal changes in their condition, since it is a less studied and less well-known species than European hake. No major lesions were found in any organ, but several parasites were detected, mostly in gills and within the digestive tract. Parasite communities differed significantly between species, reflecting their distinct trophic habitats: the benthopelagic hake presented a richer parasite community (22 species) dominated by the nematode *Hysterothylacium aduncum* and the cestode *Cleistobothrium crassiceps*. In contrast, the pelagic sardinella showed a less diverse parasite community (8 species), but with higher infection intensities, composed of digeneans (e.g. *Aphanurus* sp. and *Parahemiurus merus*) and Tetraphyllidean cestode larvae. Prevalence and intensity of some histopathological alterations differed spatially in hake and seasonally in sardinella. In hake gills, lesions included foci and extensive inflammation and hyperplasia (73.53%), potentially related to monogeneans and copepods, cysts of unknown aetiology (58.17%), and lamellar inflammation associated with *Aporocotyle spinosicanalis* eggs (24.12%). In sardinellas, gills alterations included foci of inflammation and hyperplasia (52%), degenerative material (47%) and epitheliocystis (EPC) (35%). Granulomas and inflammatory focus were mainly detected in hake's liver, spleen and stomach apparently associated with nematodes, but scarce in sardinella. Lesions were likely associated with parasites, with limited extension and intensity despite the high parasitic infections prevalences, suggesting stable host-parasite relationships. Overall, no clear detrimental effects on individual health or organ functionality was observed. However, some pathologies such as *A. spinosicanalis* egg infestation in hake gills, EPC, and microsporidian infections in sardinellas, deserve special attention due to their potential impact on fish health and fisheries, highlighting the importance of multidisciplinary health assessments in wild fish populations.

1. Introduction

Fish health is a key component of sustainable fisheries management, as individuals' fitness directly affects growth, reproduction, survival and ultimately stock productivity (Lloret et al., 2012). Within aquatic ecosystems, diseases are natural processes that may act as regulators of population abundance and therefore affect fish stocks (Hedrick, 1998). Broadly, diseases can be defined as any impairment that interferes with or modifies the performance of normal functions, including pathogens, environmental factors, nutrition or genetic defects (Wobeser, 1981) which may compromise the health of fish communities (Johansen et al., 2011). Their severity and impact often increase under certain environmental conditions, such as pollution, temperature anomalies or habitat degradation, evidencing the complex interactions among the host, pathogens and environment (Hedrick et al., 1998; Mallett et al., 2024). At the individual level, diseases can induce different types of alterations and impairment of organs and systems functionality, compromising the viability of the organisms. As a response, these organs and systems can manage to react with a characteristic pattern and usually with the involvement of the immune system, to compensate, repair and recover, but sometimes the organism is unable to cope with these changes. When this happens, there is a progressive deterioration of the condition that can even cause the death of fish (Roberts, 2012).

Assessing fish health can be approached using a variety of techniques including biochemical indicators such as enzymatic activities, protein, lipid, glucose, glycogen and lactate content, physiological indicators including stress hormones and hemoglobin levels and morphophysiological indices such as the hepatosomatic and gonadosomatic index (Lloret et al., 2014). In addition, morphological assessment techniques such as gross pathology and histopathology are very useful and wide-cope techniques to detect and evaluate diseases and pathological conditions in fish populations (Wolf et al., 2015). These morphological techniques provide different levels of precision in the evaluation and particularly histopathological techniques can reveal subtle changes or alterations in cells or tissues that may not be apparent during gross pathology examination (Eiras et al., 2008; Chapman et al., 2021), as well as the detection of the potential causative agents (Costa, 2018a). In fish histopathological studies, gills and liver are usually selected as main target organs. In the case of gills due to their direct exposure with the water as well as its relevant role in respiration and osmoregulation, and as responsible for the biotransformation and elimination of noxious substances in the case of the liver (Bernet et al., 1999; Feist et al., 2004; Au, 2004; Costa et al., 2018a; Wolf et al., 2015), as well as for target organs for many infectious agents and parasites. Other organs such as the digestive tract, gonads, spleen and kidney are also frequently selected by their relevance for different physiological functions and

as they are organs for many pathogens, including parasites. A systematic and precise evaluation of all these organs are particularly useful for assessing the individual or stock global health status, although accurate histopathological diagnosis requires proper sample handling and expert interpretation of histologic slides to prevent misdiagnosis and misinterpretation (Wolf et al., 2015).

Parasitic infections are particularly widespread among wild fish (Feist & Longshaw, 2008). Although most parasites in normal conditions have low or negligible impact on fish, in some cases their significance may be enhanced depending on the infection intensity and severity, the host response and other co-existing factors, such as the condition of the fish host or environmental effects (Feist & Longshaw, 2008; Sures & Nachev, 2022). The combined use of parasitological and histopathological techniques is especially useful to assess their presence as well as the potential damage to the host. As examples of responses, most endoparasites are typically encapsulated by host reaction in internal organs while ectoparasites usually elicit severe damage due to the mechanical damage caused by their attachment (i.e. in the gills) (Feist & Longshaw, 2008). Hence, infected fish may experience reduced growth, impaired organ function, reduced reproduction success, increased susceptibility to other diseases, or increased mortality, which can lead to population declines and ecosystem-level effects (Ryberg et al., 2020).

The study of causative pathogens and diseases in marine fish has been in the spotlight mainly on farmed fish species, such as the Atlantic salmon, *Salmo salar* Linnaeus, 1758 and the Atlantic cod, *Gadus morhua* Linnaeus, 1758 in the Atlantic ocean, and gilthead seabream, *Sparus aurata* Linnaeus, 1758 and European seabass, *Dicentrarchus labrax* (Linnaeus, 1758) in the Mediterranean sea, due to their potential negative impacts in aquaculture (Hemmingsen & Mackenzie, 2001; Bricknell et al., 2006; Johansen et al., 2011; Cascarano et al., 2021; Rigos et al., 2025). This difference in the level of knowledge regarding diseases is mainly due to the greater impact of diseases in fish farming facilities, as a result of higher population densities within the same area, the presence of increased stress factors, and the greater ease with which many pathogenic agents can establish and develop under these conditions, as well as the greater ease of disease monitoring, sample collection, and, ultimately, the relevance that disease burden represents for aquaculture production companies (Maezono et al., 2025; Padrós et al., 2025).

In contrast, research on diseases naturally occurring in wild fish populations remains scarce, even for species of high commercial interest such as the European hake, *Merluccius merluccius* (Linnaeus, 1758). Most studies conducted in wild fish have been mainly focused to explore the effects of environmental contaminants (i.e. heavy metals and organic compounds), using external

diseases and histopathological lesions as bioindicators of environmental quality (e.g., skin lesions, liver pathologies) in flatfish species such as common dab, *Limanda limanda* (Linnaeus, 1758) and flounder *Platichthys flesus* (Linnaeus, 1758) from the North Sea (Myers et al., 1994; Feist et al., 2004; Lang et al., 2017). However, histopathological alterations found in wild condition such as inflammatory changes, atrophy, necrosis, apoptosis, neoplasms, foci of cellular alteration, and melanomacrophage centres (MMCs) cannot always be attributed only to pollutants' effects, as reported in hakes and European anchovy, *Engraulis encrasicolus* (Linnaeus, 1758) from the Bay of Biscay (northern Iberian Peninsula) (Marigómez et al., 2006; Cuevas et al., 2015). They may also result from natural biological variability related to confounding factors such as age, sex or reproductive stage, genetics of the stocks, and particularly with the effects of parasites and other pathogens such as virus, bacteria, fungi and other microorganisms, which can complicate the interpretation of histopathological changes (Bucke, 1997; Au, 2004; Feist & Longshaw, 2008; Cuevas et al., 2015).

Therefore, establishing a baseline to describe and define the normal morphology and characteristics of organs, systems and tissues and potential alterations occurring naturally in wild populations is essential to distinguish between natural variability and changes induced by environmental stressors or pathogens. In some cases, only some few not relevant changes or even no relevant changes at all are found. For instance, hepatic lipid-like vacuolation is reported as a common and non-pathologic histological change in gadoids such as the Atlantic cod, related to dietary factors and their nature to accumulate glycogen and lipids as hepatic energetic reserves (Wolf et al., 2015). Other alterations including MMCs, mild inflammatory changes and cysts of unknown etiology have been described in different species (e.g., *Phycis blennoides* (Brünnich, 1768), *Mora moro* (Risso, 1810), *Micromesistius poutassou* (Risso, 1827) and *E. encrasicolus*) and regions but have not been clearly associated with anthropogenic impacts or pathogens (Dallarés et al., 2014, 2016; Sayyaf Dezfuli et al., 2021; Rodríguez-Romeu et al., 2022), illustrating different alterations that can occur under natural conditions. Histological studies have also been applied to other fields such as to support the assessment of the reproduction of many fish species (Blazer, 2002). Some of these studies particularly investigate the gonadal development and the reproductive biology of commercially important fish species such as hakes (Ferrer-Maza et al., 2014; Carbonara et al., 2019; Candelma et al., 2021; Mascoli et al., 2023), sardines, *Sardina pilchardus* (Walbaum, 1792) (Nunes et al., 2011) and anchovies (Ferrerri et al., 2009; Ferrer-Maza et al., 2016), but in general, histopathological assessment of other organs are still scarce. For many wild fish species of low commercial value, this information remains even more limited. This is particularly evident in the case of the round sardinella, *Sardinella aurita* Valenciennes, 1847, for

which histopathological information is mainly available from regions where this species is commercially exploited. Indeed, recent studies have also attempted to study the gonad development of this species (Baali et al., 2021; Mustač & Sinovčić, 2021) and also to the pathological effects derived from heavily infections of microsporidian xenomas (López-Verdejo et al., 2024). Although this species has a relatively low economic value in European countries of the Mediterranean, it plays a significant role in pelagic coastal ecosystems as a trophic competitor to closely related Clupeiformes (sardines and anchovies), especially as its range expanded northward in the Mediterranean Sea for the last decades (Albo-Puigserver et al., 2019).

Thus, the present study aims to assess the health condition of *M. merluccius* and *S. aurita* through the evaluation of condition indices, the description of their parasite communities, the gross pathology and histopathological alterations (through a qualitative and semi-quantitative histological approach) found in different target organs. For *M. merluccius*, a spatial and temporal comparison was performed in 2019 along three different localities off the Catalan coast (NW Mediterranean Sea) with different environmental conditions, as well as a temporal comparison with specimens collected in 2007 off Barcelona to detect potential health variations. In the case of *S. aurita*, a seasonal comparison between 2022 and 2023 was conducted on the coast of Barcelona.

2. Material and methods

2.1. Study area and data collection

A total of 82 *M. merluccius* specimens were collected on board of commercial fishing trawlers on the continental shelf of three localities (Blanes, Barcelona and Ebro Delta) across the Catalan coast (NW Mediterranean) in summer 2019 (n=62) (see Table 1 and Fig. 1 from Chapter 3.1 M&M). ASTD152 CTD casts were performed at the same locations at 5 m above the seafloor prior to fishing trawls to record environmental variables (temperature, salinity, oxygen concentration and turbidity) (Table 2, Chapter 3.1 M&M). An additional sampling of *M. merluccius* from Barcelona collected in 2007 (n=18) was also included (see Table 1 and Fig. 1 from Chapter 3.1 M&M).

Moreover, a total of 100 *S. aurita* specimens were seasonally captured between summer of 2022 and summer of 2023 off Barcelona coast (n=20, each season) (see Table 1 and Fig. 1 from Chapter 3.1 M&M).

2.2. Health condition analysis

After capture, fresh fish were immediately fixed in toto in 10% buffered formalin for further analysis. Fixed fish were measured (standard length, SL), weighted (total weight, TW), externally examined and dissected. Fish health condition was assessed through condition indices (Le Cren relative condition index (Kn), hepatosomatic index (HSI) and gonadosomatic index (GSI)) and stomach fullness (FULL) (see Chapter 3.2.1 M&M). The macroscopic gonadal stages were identified following the description provided by ICES (2007) and by Mustač & Sinovčić (2021) for *M. merluccius* and *S. aurita*, respectively.

The excised organs were examined under a stereomicroscope (5–40x of magnification) for the presence of macroscopic alterations and/or parasites (see Chapter 3.2.2 M&M). Parasite prevalence (P) and mean intensity (MI) were calculated according to Bush et al. (1997) for the total of parasite species recovered. During the dissection of each fixed individual from both species, the gills from the left side and a portion of liver, spleen, kidney, stomach, intestine and gonads were processed by routine paraffin histology, as described in Chapter 3.2.3 M&M.

2.3. Histopathological assessment procedure

For the evaluation of the different organs, a single histological section was generally prepared and examined in order to obtain a representative cross-sectional area of the organ or tissue. In some cases, however, additional sections were prepared and evaluated when necessary. For the gills, efforts were made to obtain one section from each gill arch, ensuring a relatively even representation of filaments along the entire arch. Prevalence (P) of histopathological alterations were calculated as the percentage of fish affected by a specific alteration for each organ. Mean intensity (MI) of each histological alteration found at each organ with a prevalence >15% was calculated as the average number of the alteration in each individual, excluding those fish without the lesion. Moreover, a semi-quantitative analysis was applied based on two main alteration categories (focal inflammatory response and epithelial hyperplasia with lamellar fusion). In these cases, from each gill section, the extension or the affected area was assessed by the number of lamellae affected by each alteration foci and was scored as: mild (1) (<4 lamellae per filament), moderate (2) (4–10 lamellae) and severe (3) (>10 lamellae). In addition, in order to evaluate the intensity of the infection by blood flukes, the total number of eggs within each affected filament was counted and scored as: mild (1) (<5 eggs per filament), moderate (2) (5–15 eggs per filament) and severe (3) (>15 eggs per filament). Mean intensity was calculated as the mean semi-quantitative score per affected individual for each locality. For other alterations detected in the rest of organs (i.e. liver, spleen, stomach and intestine), three fields of view at 100x or 200x of

magnification (0.91 mm²/screen and 0.24 mm²/screen, respectively), were randomly selected and counts of the number of each histopathological alteration were performed on each section. Mean densities (MD) in number of these alterations per square millimetre were calculated for each of the three fields and mean values were given for each specimen.

2.4. Data analysis

Calculations of the following data were performed separately for *M. merluccius* individuals collected in Barcelona for each year (2007 vs 2019), for hakes captured in 2019 across each locality (Blanes, Barcelona and Ebro Delta), and for *S. aurita* specimens for each season. All variables (SL, FULL, condition indices, P and MI of parasites and P, MI and MD of histopathological alterations) were tested for normality and homoscedasticity using the Shapiro-Wilk test and the Levene's test, respectively. Data distribution was also plotted for visual assessment. For each fish species, significant differences on biological data (SL and FULL) and condition indices (Kn, HSI and GSI) among years, localities and seasons were tested using ANOVA or Kruskal-Wallis tests with TukeyHSD or Dunn's post-hoc pairwise comparisons tests, for parametric or non-parametric data, respectively.

Different parasitological descriptors were calculated according to Bush et al. (1997). At the component population level (i.e. all individuals of all parasite species within a host population), total prevalence (TP), as the proportion of infected hosts within the host population and species richness (S), as the total number of parasite species within a host population, were calculated. Infracommunity parasite descriptors (i.e. all individuals of all parasite species in an individual fish host) were calculated including mean species richness (MSR), as the average number of parasite species within an individual host; Berger-Parker's dominance index (B-P; Berger and Parker, 1970), as the number of individuals belonging to the most abundant parasite species divided by the total number of parasites in each individual host and mean diversity index (H) estimated by Brillouin's index (PRIMER 6 software; Anderson et al., 2008), which considers both species richness and the distribution of individuals within parasite species. At the infrapopulation level (i.e. all individuals of a parasite species in an individual fish host), total mean intensity (TMI) was also provided as the average total number of parasite individuals from all parasite taxa per infected host. Parasite taxa with a prevalence >15 % in at least one locality or season were considered common and therefore were used for the following statistical calculations.

For each host species, fish size effect was first tested for P and MI of common parasites, using General Linear Models (GLM) (logistic model for P and negative binomial model for the rest of the variables) for parametric data or Generalised Linear Models (GZM) for non-parametric data.

Differences in P and MI of common parasites among years, localities or seasons were tested using GLM or GZM with SL and FULL as covariates. Post-hoc pairwise comparisons were conducted using estimated marginal means tests (EMM, emmeans function) using Holm adjustment. These differences were also tested for immature, male and female individuals. GZM were also used to detect potential differences in parasitological community descriptors among years, localities and seasons, with SL as a covariate, and using logistic models for TP, negative binomial models for TMI and MSR, beta regression with logit link model for B-P and gamma for H.

To further explore the spatial structure of parasite assemblages of *M. merluccius* among localities and *S. aurita* among seasons, a non-metric multidimensional scaling (nMDS; “vegan” package) ordination was performed based on a Bray-Curtis dissimilarity matrix calculated from log-transformed common species abundance data. The Indicator Value Index (IndVal) (Dufrêne & Legendre, 1997) was used to determine the most representative species of each locality or season. To test for potential differences in the composition and structure of parasite assemblages among the sampled localities or seasons, permutation analysis of variance (PERMANOVA, Anderson et al., 2008; “vegan” package), including SL as a covariable and “locality” or “season” and “sex” as a factor, were conducted using the Adonis2 function with 9999 unrestricted permutations of raw data. Analyses were based on Bray-Curtis and Jaccard dissimilarity matrices for parasite abundance and prevalence data, respectively. The same models were tested with PERMDISP tests (Anderson et al., 2008) to evaluate differences in dispersion to distinguish whether the observed effects reflect true differences in the parasite assemblages or variability within groups. Dispersions were measured as distances from each group (locality or season) to the centroid, and significance was tested using 9999 permutations.

Considering that fish size did not significantly affect the P, MI or MD of histopathological alterations, differences in the P of alterations in hakes among localities and years and from round sardinella among seasons, were tested using Chi-squared or Fisher-exact tests (when sample size was too small). Pairwise comparisons with *pairwiseNominalIndependence* function were subsequently performed. Likewise, differences in MI or MD of histopathological alterations among localities, seasons and years (in the case of hakes from Barcelona) were tested using T-Student (< 2 variables) and ANOVA tests (>2 variables), assuming non-equal variances, or Wilcoxon Rank Sum (<2 variables) and Kruskal-Wallis tests (>2 variables), when normality conditions were not accomplished. Post-hoc pairwise comparisons were performed using TukeyHSD and Dunn’s multiple comparison tests. Possible relationships among parasites (P and MI), histological alterations (P and MI or MD) and condition indices (Kn, HSI and GSI) were tested using GLM or GZM (binary logistic model for P and negative binomial model for MI and MD).

Moreover, Redundancy analyses (RDA; “vegan” package) were performed in both species to further visualize potential relationships of fish responses (i.e. histopathological alterations and condition indices) with parasites infection (abundance of parasite species), SL and environmental conditions such as temperature, salinity, turbidity and oxygen concentration (only in the case of *M. merluccius*) as explanatory variables. MI and MD of histopathological alterations and abundance of parasites were log-transformed. A backwards stepwise selection procedure based on a permutation test was performed to select the most significant explanatory variables. The statistical significance of the constrained ordination and explanatory variables was assessed using 999 permutations.

Data analysis was performed using RStudio software, version 4.1.2. Statistical significance was set at 0.05.

3. Results

3.1. *Merluccius merluccius*

3.1.1. Biometric data and condition indices

Specimens of *M. merluccius* ranged between 14.5 and 26.0 cm of SL, with hakes off Barcelona captured in 2007 larger than those of 2019 (K-W, $\chi^2 = 21.37$, $p < 0.001$) (**Table 1**). Most hakes collected in 2019 were immature, whereas more than half of those from 2007 were maturing. Sex ratio varied among localities although in general females were predominant. Fish off Ebro Delta presented significantly higher values of Kn (ANOVA, $F_{(79, 2)} = 10.575$, $p < 0.001$) and HSI (ANOVA, $F_{(59, 2)} = 4.915$, $p < 0.001$), whereas fish from Barcelona collected in 2019 presented significantly lower values of GSI (K-W, $\chi^2 = 12.52$, $p = 0.001$) compared to those from other localities and to fish from 2007 (ANOVA, $F_{(33, 1)} = 56.21$, $p < 0.001$). No significant differences among localities nor between years were detected regarding FULL ($p > 0.05$) (**Table 1**).

Table 1. Collection data, biological parameters and condition indices are displayed for *Merluccius merluccius* specimens from the Catalan coast (NW Mediterranean). The number (n) of fish for each fish species, sampling locality, season and year, mean followed by standard deviation (SD) of standard length (SL), Le Cren relative condition index (Kn), hepatosomatic index (HSI), gonadosomatic index (GSI) and stomach fullness (FULL), percentage of mature individuals and the sex ratio (M:F) are shown. Different superscript lowercase and capital letters indicate differences among localities and years, respectively.

Locality	Season	Year	n	SL (cm)	Kn	HSI	GSI	FULL	Immature (%)	Sex ratio (M:F)
Blanes	Summer	2019	22	19.40 (3.16)	1.01 (0.12) ^b	2.68 (0.88) ^b	0.16 (0.18) ^a	1.91 (3.74)	72.72%	2:11
Barcelona	Summer	2007	18	22.37 (3.10) ^A	0.97 (0.05)	2.22 (0.51)	0.24 (0.12) ^A	3.44 (3.87)	44.44%	1:9
Barcelona	Summer	2019	21	17.31 (1.31) ^B	0.97 (0.06) ^b	2.88 (0.74) ^b	0.04 (0.04) ^{bb}	4.80 (5.35)	80.95%	1:4
Ebro Delta	Summer	2019	19	17.82 (2.55)	1.09 (0.11) ^a	3.60 (1.12) ^a	0.28 (0.57) ^a	4.87 (3.80)	89.47%	4:5

3.1.2. Parasitological assessment

A total of 76.47% of the hakes sampled in 2007 and 93.56% of those sampled in 2019 were infected by at least one parasite. A total of 250 parasite specimens belonging to seven different metazoan taxa (**Table 2**) were recovered during the examination under the stereomicroscope. The gills and intestines were the most parasite-infested organs. Digeneans represented the main contributors to species richness in both years, whereas the nematode *Hysterothylacium aduncum* (Rudolphi, 1802) Deardorff & Overstreet, 1981 and the cestode *Cleistobothrium crassiceps* (Rudolphi, 1819) Lühe, 1899 were the predominant species, both occurring in the stomach and intestine.

Overall, TMI was 3.31 parasites/fish, although hakes from Barcelona displayed higher intensities than those of Ebro Delta (GZM, $z = 2.61$, $p = 0.009$). MSR and H index in hakes from Barcelona of 2019 were higher compared to those of 2007 (GZM, $z = 4.32$, $p < 0.001$ and $z = 2.72$, $p = 0.008$, respectively). Fish from Blanes-2019 and Barcelona-2007 displayed higher parasite dominance compared to those from other localities (GZM, $z = 2.48 - 2.95$, $p = 0.02$ and $z = 3.24$, $p = 0.007$, respectively) (**Table 2**).

For 2019 samples, larger individuals displayed higher MI of the digenean *Aporocotyle spinosicanalis* Williams, 1958, located in the gills and heart, and *C. crassiceps* (GZM, $z = 2.98$, $p = 0.003$ and $z = 3.17$, $p = 0.001$, respectively), as well as a higher P of *Hysterothylacium fabri* (Rudolphi, 1819) Deardorff & Overstreet, 1980 (GZM, $z = 2.64$, $p = 0.008$). The P of *C. crassiceps* was significantly higher in Barcelona compared to the Ebro Delta (GZM, $z = 2.74$, $p = 0.01$).

Additionally, fish size interacted with the locality of Blanes for the P of *H. aduncum*, showing a higher probability of infection in larger fish (GZM, $z = 2.26$, $p = 0.02$).

Significant temporal differences were also detected between the parasite communities of 2007 and 2019. Both *H. aduncum* and *C. crassiceps* were significantly more prevalent in 2019 (GZM, $z = 2.50$, $p = 0.01$ and $z = 2.92$, $p = 0.022$, respectively) (**Table 2**). In contrast, *A. spinosicanalis* was not present in hakes from Barcelona 2019, compared to those of 2007. No significant temporal differences were observed for mean intensity of any parasite ($p > 0.05$). No significant variations in parasite P or MI among immature, male, and female fish within each year or locality was detected ($p > 0.05$).

The nMDS ordination plot based on a Bray-Curtis dissimilarity matrix (stress = 0.11) revealed an overlap of parasite assemblages from hakes among the sampled localities, showing that the overall community structure was similar along the Catalan coast (**Fig. 1**). However, PERMANOVA analysis revealed significant effects of SL on both the structure and composition of hake's parasite community across the sampled localities, with a significant interaction between SL and locality (PERMANOVA, $F_{(57,2)} = 2.41$, $p = 0.01$ and $F_{(57,2)} = 2.53$, $p = 0.02$, respectively). The PERMDISP test did not show any significant differences in data dispersion. The Bray-Curtis similarity index was higher within localities (ranging between 22 – 42%) than among localities (ranging between 16 – 34%). The most homogeneous parasite assemblage was observed in hakes from Ebro Delta (42%) whereas specimens from Barcelona-2007 showed the highest intraspecific differentiation (22%). Assemblages from Barcelona-2019 and Ebro Delta were the most comparable (34%). The Indicator Value (IndVal) analysis identified *H. aduncum* moderately associated with hakes from Ebro Delta (IndVal = 0.42, $p = 0.003$) (**Fig. 1**).

Table 2. Prevalence (%) and mean intensity (MI) followed by range values (in brackets) of the parasites recovered from *Merluccius merluccius* in 2007 and 2019.

Parasitological descriptors with 95% confidence interval (in brackets) are also shown. Keys for developmental stages: A, adult; J, juvenile; L, larvae. Keys for locations within host: G, gills; H, heart; I, intestine; K, kidney; L, liver; Sp, spleen; S, stomach; PT, perivisceral tissues. Different superscript lowercase and capital letters indicate significant differences in the P% of parasite species among localities or years, respectively. Dashes indicate absence of the parasite or histopathological alteration. X: non-available data. ^aData from histological analyses.

PARASITE TAXA	Stage	Location	Blanes 2019		Barcelona 2007		Barcelona 2019		Ebro Delta 2019	
			P%	MI (min-max)	P%	MI (min-max)	P%	MI (min-max)	P%	MI (min-max)
Myxozoa										
<i>Gadimyxa</i> sp. ^a		K	45.45 ^b	X	X	X	66.66 ^{ab}	X	78.94 ^a	X
Polyopisthocotylea										
<i>Anthocotyle merluccii</i>	A	G	4.54	1.00 (1-1)	-	-	14.28	1.00 (1-1)	5.26	1.00 (1-1)
Digenea										
<i>Aporocotyle spinosicanalis</i>	A	H	22.72	2.60 (1-5)	22.22	1.25 (1-2)	-	-	10.52	4.00 (1-7)
<i>Aphanurus virgula</i>	A	S	-	-	5.55	1.00 (1-1)	-	-	-	-
Indeterminate sp.	J	S, I	9.09	3.50 (2-5)	-	-	-	-	5.26	1.00 (1-1)
<i>Allopoisthocleithum gibbosum</i>	A	I	-	-	-	-	19.04	2.00 (1-3)	10.52	1.00 (1-1)
<i>Lecithochirium musculus</i>	A	S	-	-	-	-	-	-	5.26	1.00 (1-1)
Subfam. Lethadeninae	A	S	-	-	-	-	-	-	5.26	1.00 (1-1)
<i>Lecithocladium</i> sp.	A	S	-	-	-	-	14.28	1.67 (1-3)	10.52	1.00 (1-1)
<i>Hemiurus</i> sp.	A	S	-	-	5.55	1.00 (1-1)	23.81	1.40 (1-2)	-	-
<i>Ectenurus lepidus</i>	A	S	-	-	-	-	9.52	2.00 (2-2)	-	-
<i>Parahemiurus merus</i>	A	I	-	-	-	-	4.76	1.00 (1-1)	-	-
<i>Prosorhynchus crucibulum</i>	A	S	-	-	5.55	1.00 (1-1)	-	-	-	-
Cestoda										
<i>Clestopbothrium crassiceps</i>	A, J, L	I	50.00 ^{ab}	1.82 (1-8)	22.22 ^B	1.75 (1-3)	71.43 ^{Aa}	1.40 (1-2)	26.31 ^b	1.00 (1-1)
<i>Scolex pleuronectis</i>	L	I	-	-	5.55	2.00 (2-2)	-	-	-	-

(Continued)

PARASITE TAXA	Stage	Location	Blanes 2019		Barcelona 2007		Barcelona 2019		Ebro Delta 2019	
			P%	MI (min-max)	P%	MI (min-max)	P%	MI (min-max)	P%	MI (min-max)
Acanthocephala										
<i>Acanthocephaloides</i> sp.	A	I	–	–	–	–	–	–	5.26	1.00 (1–1)
Nematoda										
<i>Hysterothylacium aduncum</i>	L3, L4, A	S, I, L	31.81	1.71 (1–3)	11.11 ^B	2.00 (1–3)	52.38 ^A	1.55 (1–2)	68.42	2.08 (1–5)
<i>Hysterothylacium fabri</i>	L3, L4, A	S, I, L, PT	50.00	1.30 (1–3)	22.22	1.00 (1–1)	19.04	2.00 (1–4)	5.26	2.00 (2–2)
<i>Raphidascaris</i> sp.	L3	I	4.54	1.00 (1–1)	–	–	–	–	–	–
Copepoda										
<i>Lernaeocera luscii</i>	A	G	9.09	2.00 (1–3)	11.11	2.50 (1–4)	14.28	1.00 (1–1)	–	–
<i>Neobrachiella merlucii</i>	A	G	–	–	–	–	9.52	1.00 (1–1)	–	–
Isopoda										
Fam. Cymothoidae	A	S	9.09	1.00 (1–1)	–	–	–	–	–	–
Total Prevalence										
Total Mean Intensity			86.36	3.52 (2.09 – 4.95) ^{ab}	76.47	2.41 (1.49 – 3.33)	100.00	4.36 (3.21 – 5.51) ^a	94.73	2.4 (1.68 – 3.11) ^b
Total Richness			10		8		12		12	
Mean Species Richness			2.23 (1.58 – 2.86)		1.06 (0.59 – 1.52) ^B		3.24 (2.66 – 3.81) ^A		2.58 (2.01 – 3.14)	
Brillouin Diversity Index			0.33 (0.16 – 0.49)		0.20 (0.06 – 0.33) ^B		0.56 (0.42 – 0.70) ^A		0.38 (0.27 – 0.50)	
Berger-Parker Dominance Index			0.75 (0.63 – 0.87) ^a		0.81 (0.67 – 0.94) ^A		0.54 (0.44 – 0.64) ^{bB}		0.55 (0.43 – 0.67) ^b	

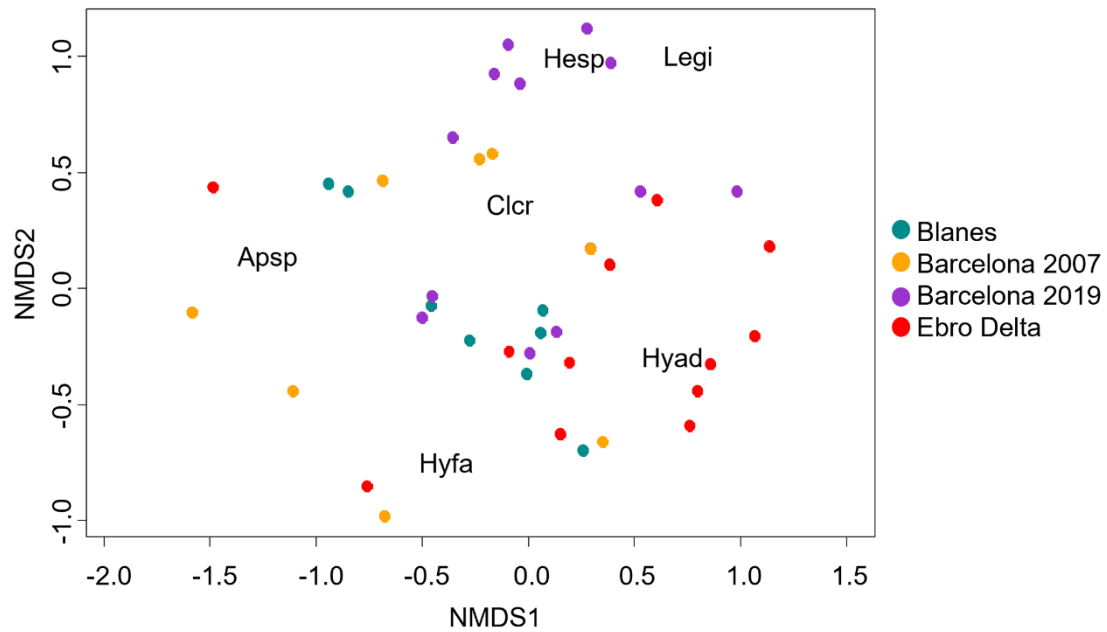


Fig. 1. Non-metric multidimensional scaling (nMDS) ordination plot based on a Bray-Curtis dissimilarity matrix calculated from log-transformed parasite abundance data of *Merluccius merluccius* across three localities from the Catalan coast (NW Mediterranean) and one from 2007. Each point represents an individual host coloured according to the region. Labels indicate parasite species significantly associated with specific regions according to the Indicator Value analysis (IndVal): Apsp, *Aporocotyle spinosicanalis*; Clcr, *Cleistobothrium crassiceps*; Hesp, *Hemiurus* sp.; Hyad, *Hysterothylacium aduncum*; Hyfa, *Hysterothylacium fabri*; Legi, *Allopoisthocythum gibbosum*.

3.1.3. Gross pathology observations

No major macroscopic alterations were found in any examined organ of *M. merluccius*, except for a dark-coloured liver in a single specimen. On the gills, the only alteration detected were small whitish spherical nodules of about 200-300 µm in diameter located within the gill filament that histologically corresponded to cysts of unknown etiology (CUEs). The copepod *Lernaeocera lusci* (Bassett-Smith, 1896) was also detected attached to the branchial gill arch in 6.48% of the hake specimens examined from both years.

3.1.4. Histological and histopathological findings

3.1.4.1. Gills

The normal histological aspect of hake gill filaments is shown in **Fig. 2** and typically showed the general structure observed in most teleost fish species. The gill filament was characterized by a cartilaginous matrix covered by a thin epithelium of chloride and mucous cells. The secondary lamellae were comprised by pillar cells, erythrocytes and epithelial cells (**Fig. 2**). About 89% of the filaments examined in hakes displayed a normal morphology, but the remaining 11% of the filaments presented at least one alteration, regardless the type, size or extension of the alteration (**Table 3**).



Fig. 2. Histological (H&E stain) gill sections in *Merluccius merluccius* of a normal gill filament cell structure, sagittal section through cartilaginous support. 1, primary filament; 2, secondary filament; 3, cartilaginous matrix; 4, chloride cells; 5, epithelial cells; 6, erythrocytes; 7, pillar cells; 8, mucous cells. The gill primary filaments were composed of a cartilaginous core covered by an epithelial layer and a few mucous and chloride cells. The internal part of the lamellae was alternated with erythrocytes and pillar cells, and by the presence of epithelial and mucous cells. Scale bar: 100 μ m.

Table 3. Prevalence (%) and mean intensity (MI) or mean density (MD) in terms of number/mm² followed by the standard deviation (SD) of the histopathological alterations found in *M. merluccius*. Keys for histopathological alterations: CUEs, cysts of unknown etiology; MMCs, melanomacrophage centers; EPC, epitheliocystis. Keys for location within host: G, gills; L, liver; K, kidney; S, stomach; Sp, spleen. Different superscript capital and lowercase letters indicate significant differences in P% and MI or MD, respectively, among localities. Different superscript numbers indicate significant differences between years at Barcelona.

Histopathology	Location	Blanes 2019		Barcelona 2007		Barcelona 2019		Ebro Delta 2019	
		P%	MI (SD)	P%	MI (SD)	P%	MI (SD)	P%	MI (SD)
Alteration foci	G	86.36	1.20 (0.44)	83.33	1.16 (0.43)	71.43	1.33 (0.62)	52.63	1.28 (0.49)
Extensive hyperplasia	G	27.27	3.00 (2.53)	33.33	2.33 (1.21)	33.33	4.29 (2.36)	–	–
Extensive inflammation	G	13.63 ^B	2.00 (1.00)	–	–	33.33 ^A	1.14 (0.38)	15.79 ^B	2.00 (1.41)
CUEs	G	59.09	1.77 (1.42)	77.77 ¹	1.79 (0.80)	47.62 ²	1.50 (0.85)	31.57	2.33 (1.21)
EPC	G	4.45	1.00 (0.00)	–	–	–	–	4.45	1.00 (0.00)
<i>Aporocotyle spinosicanalis</i> eggs	G	22.72 ^A	1.75 (0.54)	16.66	1.63 (0.69)	–	–	5.26 ^B	1.65 (0.71)
		P%	MD (SD)	P%	MD (SD)	P%	MD (SD)	P%	MD (SD)
Granuloma	L	18.18	0.36 (0.00)	5.55 ²	X	19.05 ¹	0.27 (0.18)	21.05	0.27 (0.18)
	Sp	4.54	X	–	–	4.76	X	5.26	X
	S	22.72	0.22 (0.20)	X	X	23.81	0.22 (0.18)	31.57	0.24 (0.19)
MMCs	L	–	–	2.17	X	–	–	–	–
	Sp	18.18	12.82 (7.89) ^a	66.66 ¹	7.81 (2.91)	4.76 ²	X	26.31	3.04 (2.66) ^b
	K	–	–	–	–	4.76	X	–	–
Aggregated macrophages	L	4.54	X	66.66 ¹	5.30 (5.15)	4.76 ²	X	5.26	X
	Sp	22.72	27.94 (9.91) ^a	5.55 ²	X	52.38 ¹	18.6 (14.7) ^a	31.57	7.38 (3.97) ^b
Inflammatory focus	L	13.63	X	11.11	X	9.52	X	15.79	0.12 (0.21)
Rodlet cells	S	90.00	X	X	X	100.00	X	100.00	X

CUEs were one of the most frequent alterations observed in hake's gills, ranging from one to four per section of a single gill arch. CUEs consisted of a homogeneous core of acellular and eosinophilic material surrounded by a thin layer of basophilic material usually in central position in the filaments (**Fig. 3a-b**). Other tissular layers of variable thickness were observed surrounding them, composed by an inflammatory response of macrophages and connective tissue, capillary vessels and cartilage. The presence of CUEs was significantly higher in individuals from 2007 compared to 2019 (Chi-squared, $\chi^2 = 4.88$, $p = 0.03$) (**Table 3**).

Another recurrent finding detected in gill's filaments were i) telangiectasia, identified as blood expansions of lamellar capillaries and ii) inflammatory cell infiltration, which was generally composed of lymphocytes and granular cells. This inflammatory response was found as discrete foci (i.e. alteration foci) typically affecting the interlamellar spaces of 2 to 15 lamellae (**Fig. 3c-f**), but also within the filament epithelium. Moreover, more extensive areas of inflammation were

also detected affecting mostly the central core of the whole filament and to a lesser extent expanding to the interlamellar spaces. Epithelial lamellar hyperplasia with fusion of adjacent lamellae were also associated with discrete foci (affecting from 2 to more than 10 lamellae) (**Fig. 3c-f**) or even extensive (affecting one or more entire lamellar epithelial cells), leading to an increase in the total thickness of the gill epithelia.

In 2019, hake specimens from Barcelona presented higher P of extensive inflammation compared to the other localities and from hakes of 2007 (Fisher test, $p = 0.009$ and $p = 0.009$). No significant differences between years were detected in MI of alteration foci and extensive inflammation and hyperplasia ($p > 0.05$) (**Table 3**). These lesions were often observed closely associated to the attachment of *L. lusci* which also caused extensive inflammation and hyperplasia in the adjacent filaments (**Fig. 3g**). In some cases, areas with strong inflammatory reaction and necrosis were identified in the gill arch. Lamellar fusion and inflammation were also detected, but not always, associated with the presence of embolised eggs with different degree of development (embryos, oncomiracidia) of blood flukes (i.e. *A. spinosicanalis*) in the afferent arteries of gill filaments (**Fig. 3h**). In fact, a significant association was detected between the P of eggs and P alteration foci (GZM, $z = 2.21$, $p = 0.02$), but no significant associations were found for MI. A significantly higher P of eggs was detected in Blanes compared to other localities (Fisher test, $p = 0.036$) and in 2007 compared to 2019 (Chi-squared, $\chi^2 = 12.20$, $p = 0.00047$), but, in general, the overall mean intensity of eggs was mild or moderate (MI=1.68 eggs/filament) and no significant differences were found among localities nor between years (**Table 3**). Moreover, microsporidian cysts were detected in 3.22% of the hakes from 2019 and were identified as aggregates of small spores surrounded by a thin epithelium located between the interlamellar spaces but with no host reaction associated to them. Finally, a single case of epitheliocystis was also identified in one individual from 2019. Hypertrophied infected epithelial cells resulted in the formation of a basophilic oval cyst encapsulated by a membrane.

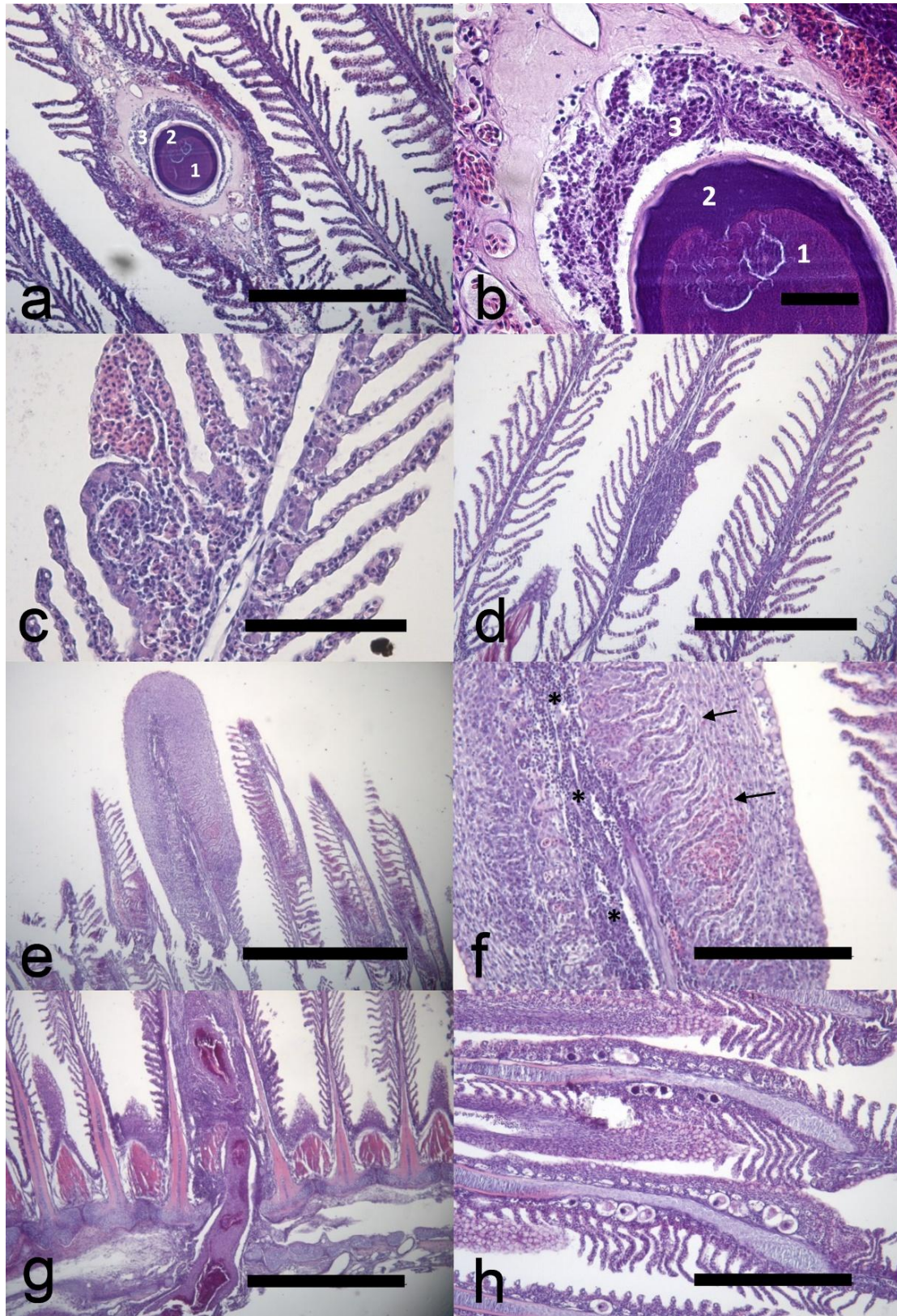


Fig. 3. Histological (H&E stain) gill sections in *Merluccius merluccius*. **a)** Cyst of unknown aetiology in a gill filament. An eosinophilic central core (1) surrounded by acellular basophilic material of varying thickness (2) is recognized. An inflammatory response of macrophages and connective tissue is observed surrounding the cyst (3). Scale bar = 400 μ m. **b)** Higher magnification of (a). Scale bar = 50 μ m. **c)** Mild, **d)** moderate,

and **e)** severe alteration foci. Note the presence of aggregation of immune cells infiltration in the gill tissue. Scale bar = 100, 400, and 800 μm , respectively. **f)** Higher magnification of (e). Note the presence of inflammatory cell infiltration of lymphocytes inside the filament with hyperplasia (asterisks) and a proliferation of cell activity leading to an increase in the thickness of the lamellar epithelium (arrows). Scale bar = 200 μm . **g)** Possible site fixation of a copepod infecting the gill causing an inflammatory response and extended hyperplasia involving two filaments. Scale bar = 800 μm . **h)** Gill filaments with some *Aporocotyle spinosicanalis* eggs with different degree of development. Scale bar = 400 μm .

3.1.4.2. Liver

A wide range of diverse hepatic parenchyma morphologies were identified in different hake specimens. These different morphologies were clearly associated with the amount of reserve substances (i.e. glycogen or lipid) stored within the hepatocyte cytoplasm (**Fig. 4a-d**). The round-white droplets most probably represented areas previously occupied by lipids that were removed during tissue processing. According to the degree and pattern of lipid droplets accumulated within the hepatocytes (termed as lipidic metamorphosis), the observations of the hepatic parenchyma were classified into three different categories: lipidic metamorphosis I, consisted of simultaneous areas of hepatocytes without reserve substances and hepatocytes displaying scarce lipid droplets within the cytoplasm (**Fig. 4b**); lipidic metamorphosis II, where lipid droplets occupied more than 50% of the cytoplasm (**Fig. 4c**); and lipidic metamorphosis III, where most of the cytoplasm was occupied by lipid droplets (**Fig. 4d**).

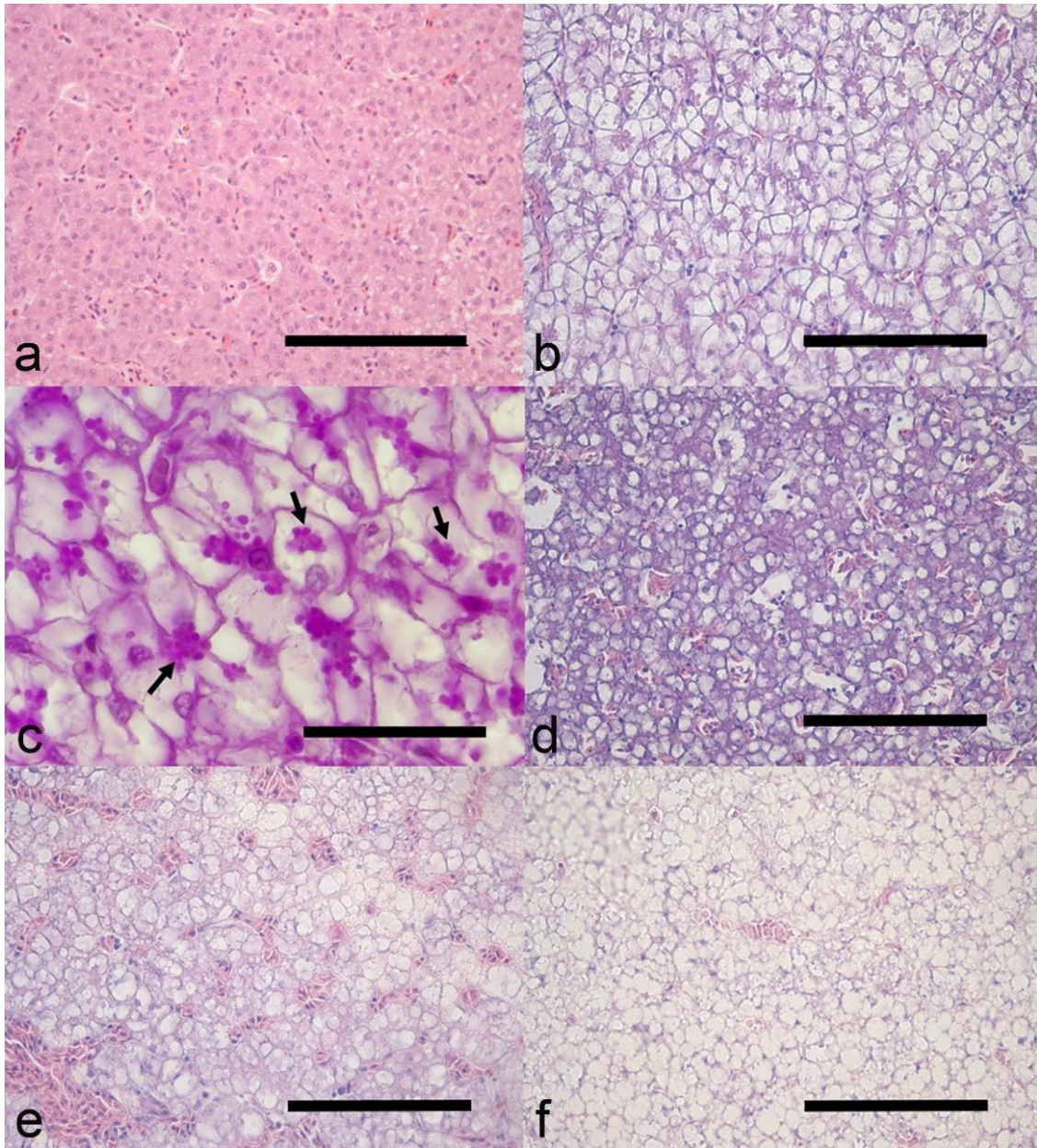


Fig. 4. Histological (H&E stain) sections of hepatic parenchyma morphologies of *Merluccius merluccius*. Scale bar = 100 μ m. **a)** Cord-like structure with hepatocytes completely devoid of reserve substances. **b)** Hepatocytes with glycogen-like spaces identified as granular material occupying almost all the cell volume and displacing the nucleus to the periphery of the cell. **c)** Hepatocytes with glycogen stained by Periodic Acid-Schiff (PAS) stain (arrows). Scale bar = 30 μ m. **d)** Lipidic metamorphosis stage I with small and non-aggregated lipid droplets within the hepatocytes, barely displacing the nucleus and cytoplasm towards the periphery of the cell. **e)** Lipidic metamorphosis stage II with different lipid droplet sizes, some of them aggregated in larger droplets, displacing the nucleus and the basophilic cytoplasm to the periphery of the cell, making it difficult to discern the limits of the cell. **f)** Lipidic metamorphosis stage III with large and abundant lipid droplets merging together within the hepatocytes totally displacing the nucleus to the periphery of the cell. Basophilic cytoplasm totally absent and cellular limits diffused and completely undistinguished.

Regarding the histopathological changes detected in hake's liver, different alterations found in the hepatic sections consisted of mild focal inflammatory reactions mainly composed of lymphocytes sometimes identified in the vicinity of the sinusoids (**Fig. 5a**), and granulomatous inflammatory responses of the host in the hepatic parenchyma and serosa. These granulomatous inflammations were present as discrete spherical granulomas consisting primarily of a concentrically arranged mixture of mononuclear cells (mainly probably macrophages and lymphocytes), and/or fibroblasts surrounding a necrotic centre sometimes associated to melanin, which could either correspond to the lesion caused by a pathogen (i.e. a nematode or other parasites) and most probably, the degraded form of the pathogen itself (**Fig. 5b**). Some apparently viable nematode larvae were mostly seen in the hepatic serosa but also in the parenchyma (**Fig. 5c**). Also, isolated or aggregated macrophages, identified as simple units of two or three cells containing brownish (lipofuscins or ceroid) or blackish (melanin) pigments were observed (**Fig. 5d**). Moreover, one individual from 2007 contained larger melanomacrophage centres (MMCs) with dark-brown pigment were detected as a distinctive grouping of more than five melanomacrophages displaying an organized structure (**Fig. 5e**). In two specimens from 2019, some areas of the hepatic tissue were found to be almost atrophic in which hepatocytes were reduced in size and in the process of apoptosis, leading to dilation of the sinusoids. Moreover, in these sections, granulomas and some macrophage aggregates of different sizes containing ceroid pigment were mostly concentrated near the sinusoids and serosa and to a lesser extent in the hepatic parenchyma (**Fig. 5f**). No significant differences among localities were found in the presence of granulomas, macrophage aggregates or MMCs in liver. However, significantly differences between years were observed, with higher P of granulomas in fish from 2019 and a higher P of macrophage aggregates in specimens from 2007 (Chi-squared, $\chi^2 = 7.07$, $p = 0.008$ and $\chi^2 = 16.71$, $p < 0.001$, respectively) (**Table 3**).

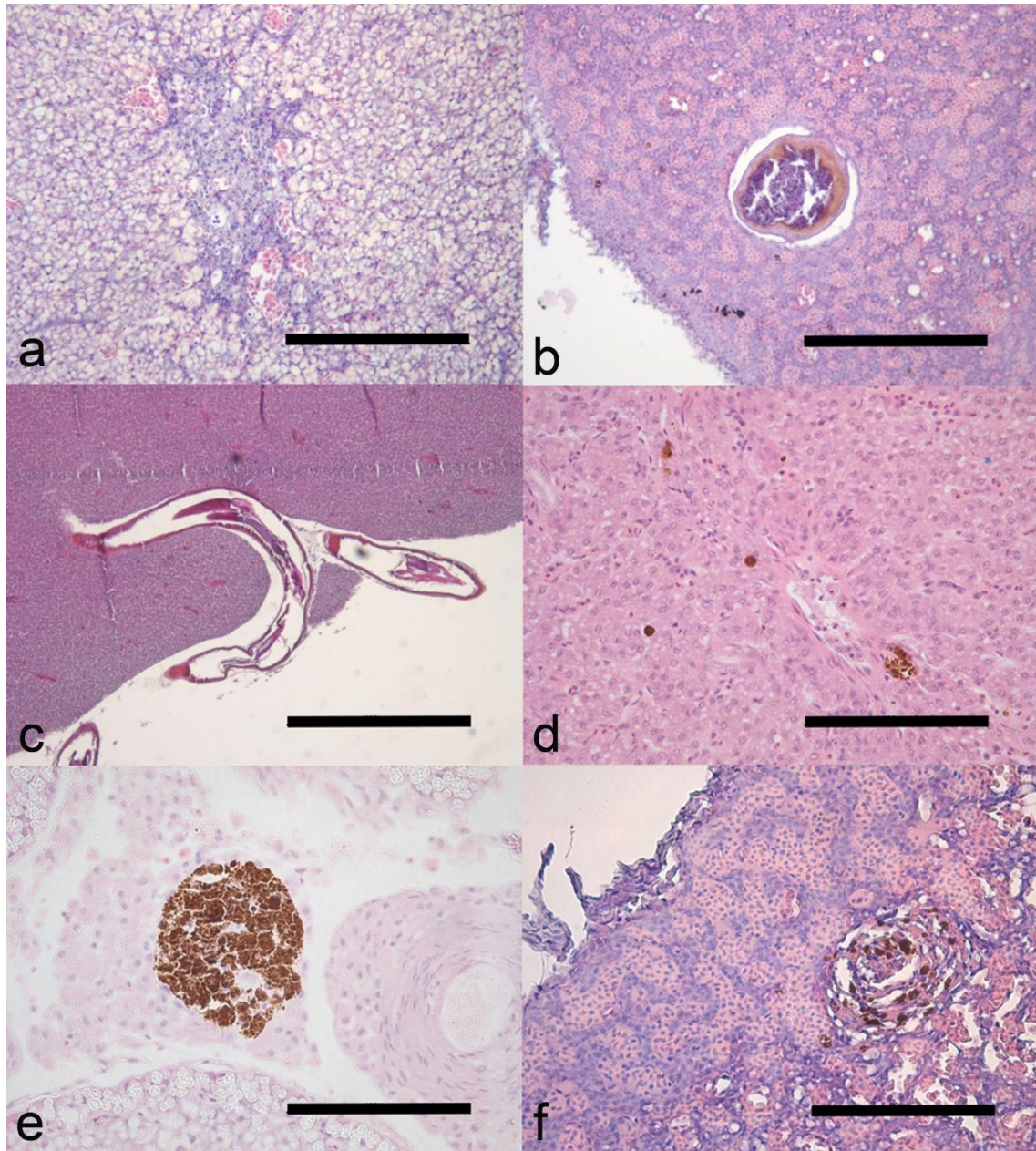


Fig. 5. Histological (H&E stain) sections of alterations identified in the hepatic tissue of *Merluccius merluccius*. **a)** Inflammatory focus in the hepatic parenchyma. Scale bar = 200µm. **b)** A granuloma in the hepatic parenchyma. Scale bar = 200µm. **c)** Nematode larvae in the hepatic parenchyma. Scale bar = 800µm. **d)** A few macrophages aggregates in the hepatic parenchyma. Scale bar = 100µm. **e)** Melanomacrophage centre (MMCs) within the hepatic tissue. Scale bar = 100µm. **f)** Hepatocytes in apoptosis and macrophages aggregates containing ceroid pigment within the hepatic parenchyma. Scale bar = 100µm.

3.1.4.3. Spleen

Hake's splenic tissue was characterized by the presence of scattered aggregations of macrophages (Fig. 6a) and MMCs containing melanin or lipofuscin pigment. These MMCs were in general small-sized (about 66 μm in diameter) and with an irregular shape contained a dark-brown pigment and were detected as a distinctive grouping of more than five melanomacrophages displaying an organized structure homogeneously distributed throughout the splenic tissue (Fig. 6b). A few cases of granulomas with the same structure as found in liver were also detected in 4.76% of the specimens. No significant differences in P of these alterations were detected among localities although hakes from Blanes presented a higher density of MMCs compared to those of Ebro Delta (ANOVA, $F_{(7,1)} = 8.05$, $p = 0.02$), and a lower mean density of aggregated macrophages was found in Ebro Delta compared to the other localities (ANOVA, $F_{(19,2)} = 7.78$, $p = 0.003$) (Table 3). Temporal significant differences in hakes from Barcelona were also found, with higher P of MMCs in individuals from 2007 but a significantly lower P of aggregated macrophages compared to those from 2019 (Chi-squared, $\chi^2 = 16.71$, $p < 0.001$ and $\chi^2 = 9.97$, $p = 0.001$, respectively) (Table 3).

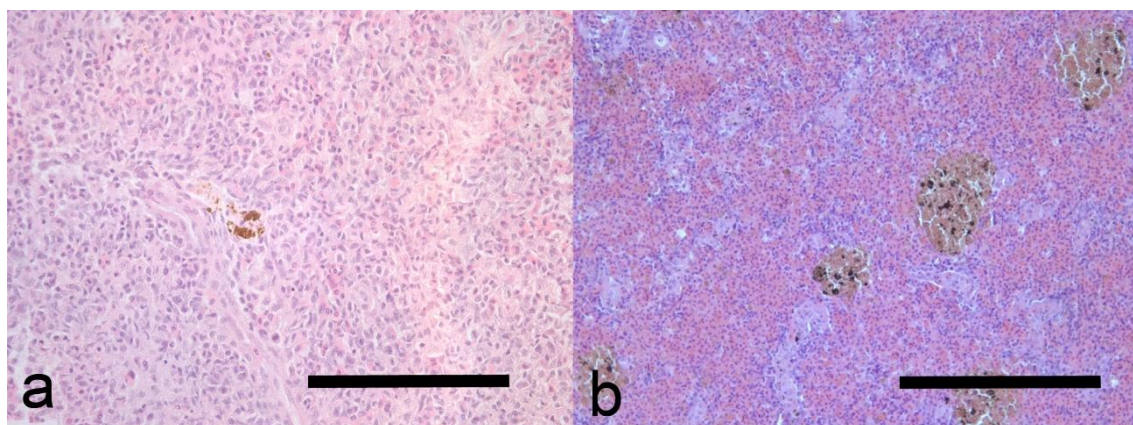


Fig. 6. Histological (H&E stain) sections of alterations identified in the splenic tissue of *Merluccius merluccius*. **a)** Macrophages aggregated containing ceroid pigment. Scale bar = 100 μm . **b)** Melanomacrophagic centres (MMCs) within the splenic parenchyma. Scale bar = 200 μm .

3.1.4.4. Kidney

The histological assessment of the renal tissue revealed the presence of pre-sporogonic stages of coelozoic myxosporean parasites in the lumen of the renal tubules from 63.69% of the specimens. No direct tubular degeneration, damage or host reaction was detected in the tubules with parasites (Fig. 7a). In certain cases, mild lymphocytic infiltration throughout the renal tubules was

detected as well as mineralized or dark material accumulated within some degenerated tubules and, in these cases, granulomas and MMCs were also present nearby (**Fig. 7b**).

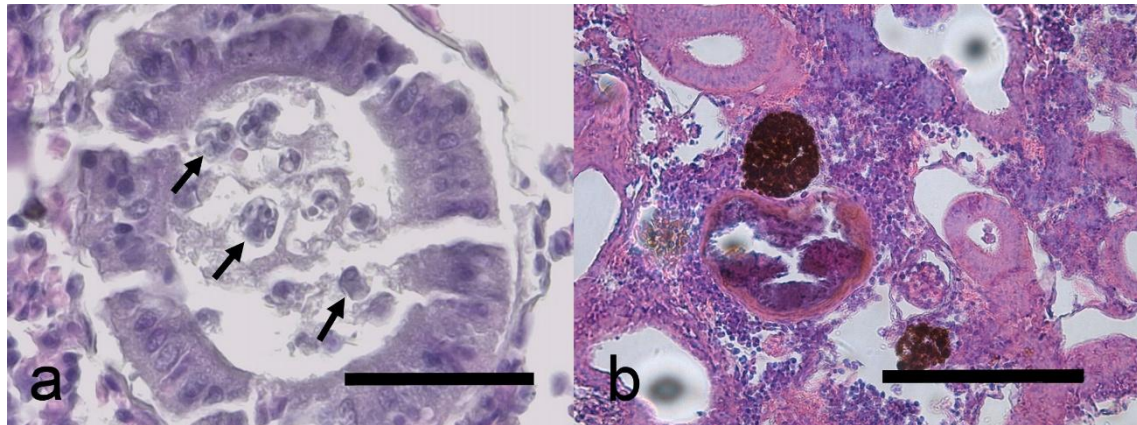


Fig. 7. Histological (H&E stain) sections of alterations identified in the renal tissue of *Merluccius merluccius*. **a)** A renal tubule with coelozoic myxosporean parasites (arrows) Scale bar = 30µm. **b)** A granuloma and melanomacrophage centres (MMCs) within the renal tissue.

3.1.4.5. Digestive tract

Slight alterations were revealed from hake's histological sections of the stomach and intestine such as hyperhemia/vascular congestion identified as an increase of blood cells within the intestinal mucosa in 9.67% of the individuals. Large numbers of rodlet cells were present in the stomach mucosa at almost all hakes from 2019 (**Fig. 8a**). Some scattered granulomas with the same structure as described above in the liver were present in the submucosa-lamina propria, mostly associated with the presence of apparently degraded nematode or digenean larvae and with a scarce inflammatory reaction surrounding the granuloma (**Fig. 8b-c**). Likewise, non-encapsulated digenean metacercariae were detected in 4.83% of the specimens from 2019 located in different layers from the stomach without apparent host reaction (**Fig. 8d**). Viable digeneans, nematodes and cestodes were present within the intestinal lumen but without apparent histological alterations associated to their presence. In addition, a single pathological finding was found associated to the attachment of the scolex of the cestode *C. crassiceps* to the intestinal mucosa (see Chapter 4.1).

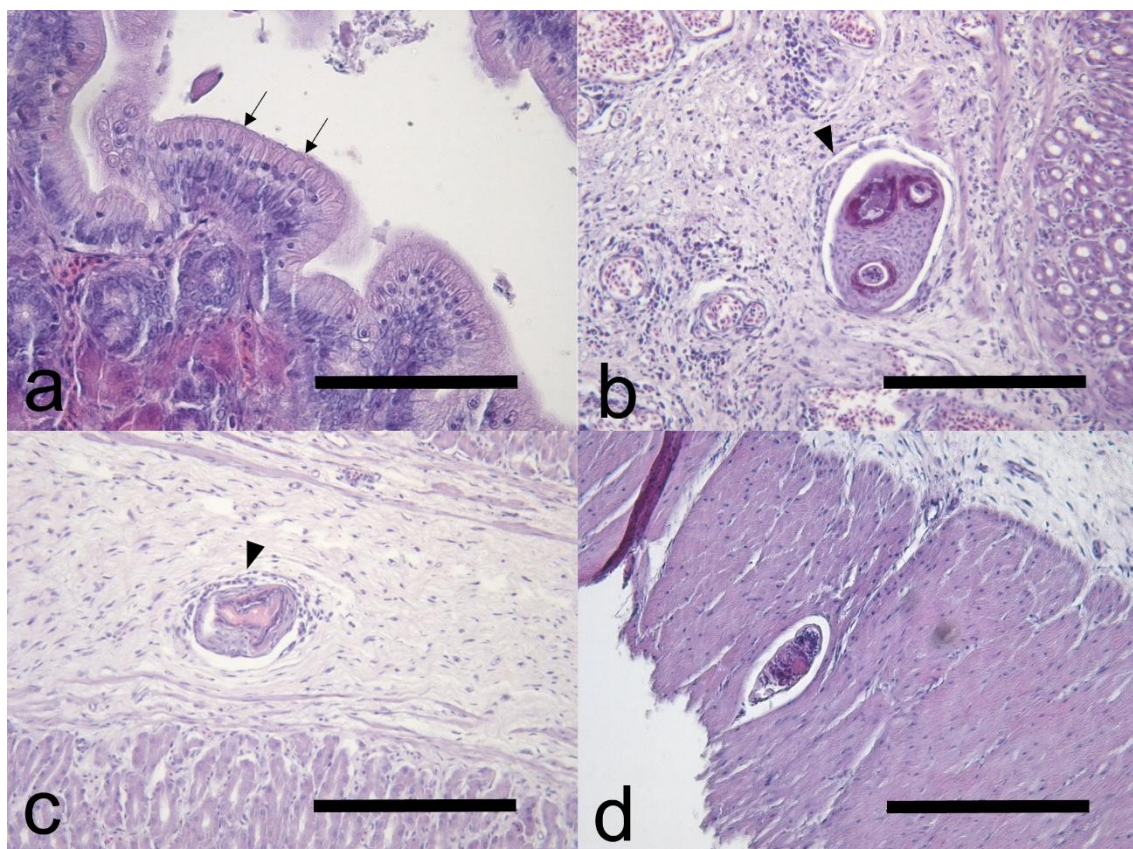


Fig. 8. Histological (H&E stain) sections of alterations detected in the stomach of *Merluccius merluccius*. **a)** Stomach mucosa with rodlet cells (arrows). Scale bar = 100µm. **b)** A non-viable larva within the stomach submucosa-lamina propria causing a mild inflammatory reaction (arrowhead) Scale bar = 200µm. **c)** A granuloma within the stomach submucosa-lamina propria evoking a slight inflammatory reaction (arrowhead). Scale bar = 200µm. **d)** A non-encapsulated migrating digenean metacercaria in the stomach muscular layer without causing any host reaction. Scale bar = 200µm.

3.1.4.6. Gonads

In hake's histological sections from 2019, gonads displayed early gametogenic stages, but in most individuals, gender could not be differentiated by microscopical characteristics since they were immature. Instead, those specimens with early maturing gonads, ovaries showed compact ovigerous lamellae with germline cysts containing oogonia and oocytes in primary stage with basophilic ooplasm surrounded by connective tissue and a thin ovarian membrane (**Fig. 9a**). Likewise, testicles were in an early spermatogenic stage, characterized by the presence of spermatogonia and primary spermatocytes (**Fig. 9b**). No histopathological alterations were detected in the gonadal tissue.

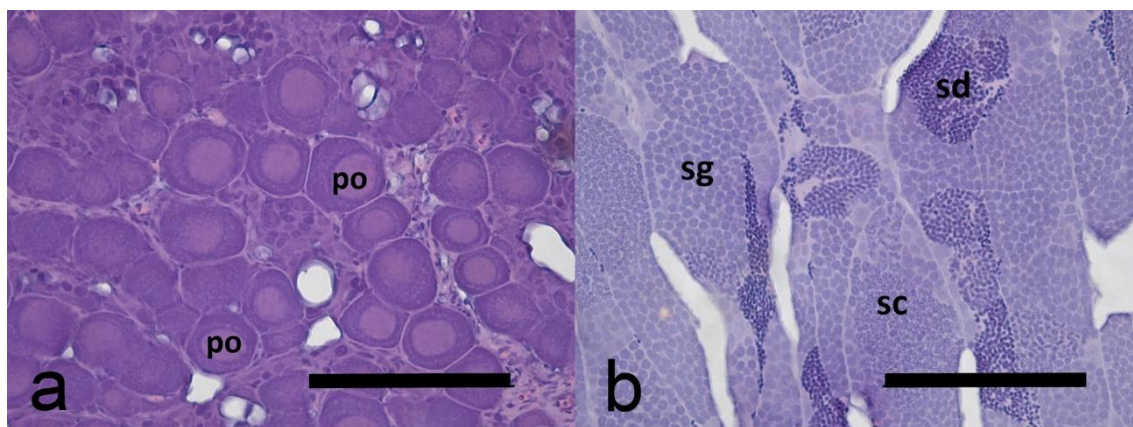


Fig. 9. Histological (H&E stain) sections of the gonads in *Merluccius merluccius*. Scale bar = 100µm. **a)** Early gametogenic ovaries containing primary growth oocytes (po). **b)** Early spermatogenic testes showing predominately spermatocytes (sc) and spermatogonia (sg) and in some cases spermatids (sd).

3.1.5. Associations among fish health indicators and potential explanatory variables

A few significant associations were detected among fish health indicators (i.e. condition indices, parasite species and histopathological alterations). Hakes displaying higher P of extensive hyperplasia in gills presented lower values of Kn (GZM, $z = -2.06$, $p = 0.04$), while those showing higher P of granulomas in the hepatic tissue showed lower values of HSI (GZM, $z = -2.08$, $p = 0.03$). The RDA performed for *M. merluccius* revealed associations between condition indices (Kn, HSI and GSI) and the set of parasites species abundance, SL and environmental variables (see Table 2, Chapter 3.1 M&M) (permutation test, $p = 0.001$), explaining 31.8% of the total variance by the first two axis (**Fig. 10a**). The stepwise selection indicated that fish SL, temperature, turbidity and the parasite *Anthocotyle merluccii* Van Beneden & Hesse, 1863, were the most explanatory variables for the condition indices observed for hakes among localities ($p = 0.001 - 0.018$). Visually, GSI was positively associated with SL and negatively associated with temperature, whereas HSI was negatively related to turbidity.

On the other hand, the RDA relating histopathological alterations with SL, parasites and environmental conditions only explained 18.5% of the total variation by the first two axes (**Fig. 10b**). The parasites *A. spinosicanalis* and *A. merluccii* and oxygen concentration, salinity and turbidity were the most significant explanatory variables of the model ($p = 0.012 - 0.022$). The plot showed that abundance of *A. spinosicanalis* and turbidity were positively associated with MI values of its eggs in gills and negatively related to oxygen concentration. Abundance of *A. merluccii* was positively associated with MD values of splenic aggregated macrophages. Other response variables were not statistically explained by any of the explanatory variables.

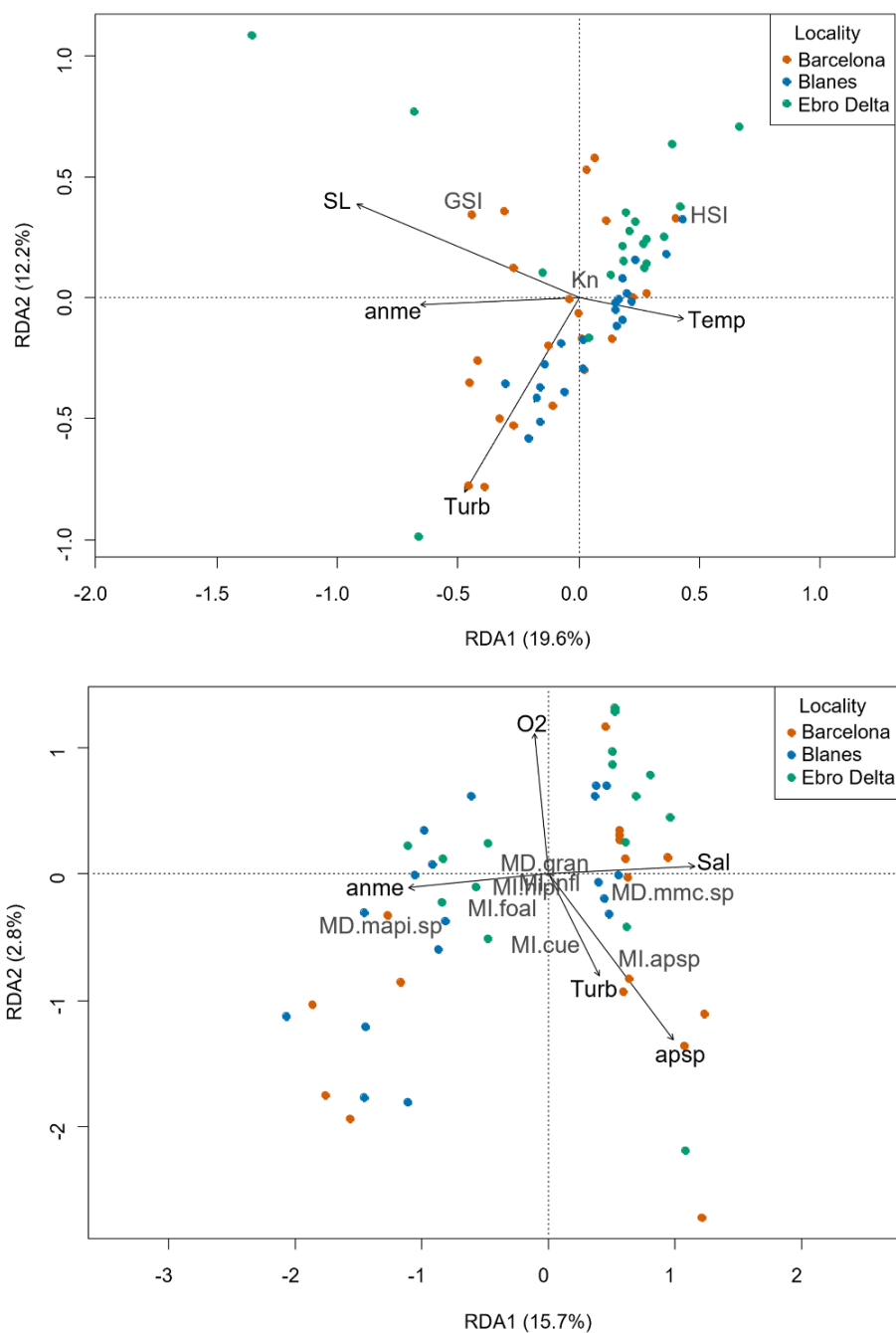


Fig. 10. Redundancy analysis (RDA) on **a)** condition indices and **b)** histopathological alterations of *Merluccius merluccius* constrained by fish size (SL), parasite species and environmental variables most significant for the ordination. Abbreviations: Kn, Le Cren relative condition index; HSI, hepatosomatic index; GSI, gonadosomatic index; MD.MMC.sp, mean density of melanomacrophage centres in spleen; MD.mapi.sp, mean density of aggregated macrophages in spleen; MD.gran.li, mean density granulomas in liver; MD.gran.S, mean density granulomas in stomach; MI.apsp, mean intensity *Aporocotyle spinosicanalis* eggs; MI.CUE, mean intensity cyst of unknown etiology; MI.foal, mean intensity of foci of alteration; MI.infl, mean intensity of inflammation; MI.hipl, mean intensity of hyperplasia; anme, *Anthocotyle merluccii*; apsp, *Aporocotyle spinosicanalis*; Sal, salinity; Temp, temperature; Turb, turbidity; O2, oxygen concentration.

3.2. *Sardinella aurita*

3.2.1. Biometric data and condition indices

For *S. aurita*, fish size ranged from 12.66 to 17.22 cm of SL, with fish captured in autumn being significantly smaller and immature than those from other seasons (K-W, $\chi^2 = 53.76$, $p < 0.001$) (Table 4). Sex ratio was balanced in summer-22, winter-23 and spring-23, but biased to males in summer-23. Regarding Kn, individuals from spring-23 and winter-23 presented significantly higher values compared to the other seasons (ANOVA, $F_{(94,4)} = 6.47$, $p < 0.001$). The HSI was significantly lower in specimens from winter-23 compared to those from the other seasons (ANOVA, $F_{(94,4)} = 15.6$, $p < 0.001$). Significantly higher values of GSI were found in summer individuals from both years (K-W, $\chi^2 = 83.37$, $p < 0.001$). As regards FULL, individuals from spring-23 presented significantly higher values compared to the other seasons (ANOVA, $F_{(95,4)} = 45.47$, $p < 0.001$) (Table 4).

Table 4. Collection data, biological parameters and condition indices are displayed for *Sardinella aurita* specimens from the Catalan coast (NW Mediterranean). The number (n) of fish for each fish species, sampling locality, season and year, mean followed by standard deviation (SD) of standard length (SL), Le Cren relative condition index (Kn), hepatosomatic index (HSI), gonadosomatic index (GSI) and stomach fullness (FULL), percentage of mature individuals and the sex ratio (M:F) are shown. Different superscript letters indicate differences among seasons.

Locality	Season	Year	n	SL (cm)	Kn	HSI	GSI	FULL	Mature (%)	Sex ratio (M:F)
Barcelona	Summer	2022	20	15.59 (0.69) ^a	0.97 (0.07) ^c	0.89 (0.28) ^b	2.89 (1.19) ^a	0.43 (0.16) ^c	100%	1:1
Barcelona	Autumn	2022	20	12.66 (0.48) ^b	1.00 (0.04) ^{bc}	0.90 (0.19) ^b	0.23 (0.12) ^c	0.71 (0.14) ^b	0%	–
Barcelona	Winter	2023	20	15.42 (0.59) ^a	1.03 (0.05) ^{ab}	0.67 (0.15) ^c	0.38 (0.22) ^c	0.82 (0.13) ^b	0%	1:1
Barcelona	Spring	2023	20	16.13 (1.33) ^a	1.05 (0.05) ^a	1.16 (0.23) ^a	0.87 (0.24) ^b	1.19 (0.42) ^a	70%	9:11
Barcelona	Summer	2023	20	17.22 (2.10) ^a	0.96 (0.09) ^c	1.05 (0.19) ^{ab}	2.84 (1.29) ^a	0.48 (0.15) ^c	100%	4:1

3.2.2. Parasitological assessment

All examined *S. aurita* specimens across all seasons were infected by at least one parasite. A total of 11468 parasites belonging to three different metazoan taxa were identified (Table 5). Most of them were located in the digestive tract, while copepods were detected in gills. Among the recovered taxa, digeneans were the most prevalent group, reaching 100% of prevalence, and the main contributors to MI values in each season. Among them, *Aphanurus* sp. Looss, 1907 was the

most prevalent and presented the highest MI values followed by *Bacciger bacciger* (Rudolphi, 1819) Nicoll, 1914, *Parahemiurus merus* (Linton, 1910) Manter, 1940 and *Allopoistholecithum gibbosum* (Rudolphi, 1802) Vainutis & Voronova, 2025.

Overall, TMI was 95.89 parasites/fish, with sardinellas from both summers showing the highest intensities (GZM, $\chi^2 = 116.5$, $p < 0.001$). MSR was 3.5 with no significant differences among seasons, neither for H index values (GZM, $p > 0.05$). Fish from summer-22 and spring-23 presented higher parasite dominance compared to autumn-22 and winter-23 (GZM, $z = 3.15 - 4.04$, $p = 0.01 - 0.007$) (Table 5).

Larger fish presented higher MI values of total digeneans (GLM, $F_{(98,1)} = 28.70$, $p < 0.001$), specifically *Aphanurus* sp. (GZM, $z = 5.09$, $p < 0.001$), *P. merus* (GLM, $F_{(98,1)} = 42.91$, $p < 0.001$) and *A. gibbosum* (GZM, $z = 2.15$, $p = 0.03$). In terms of P, only *A. gibbosum* was found to present significantly higher values in larger fish (GZM, $z = 2.53$, $p = 0.011$). The cestode larvae of the order Tetraphyllidea were also more prevalent (GZM, $z = 2.57$, $p = 0.01$) and displayed higher MI values (GZM, $z = 2.71$, $p = 0.006$) in larger fish (Table 5).

Regarding seasonality, significant higher MI values were detected in summer for all digeneans, except for *B. bacciger*, which also had higher values in autumn (*P. merus*: GZM, $z = 2.02$, $p = 0.04$; *Aphanurus* sp.: GLM, $F_{(95,4)} = 41.42$, $p < 0.001$; *B. bacciger*: GLM, $F_{(95,4)} = 41.42$, $p < 0.001$; *A. gibbosum*: GZM, $z = 2.81$, $p = 0.004$). The same trend was also observed in Tetraphyllidean cestode larvae (GZM, $z = 3.67$, $p < 0.001$). Fish size interacted with seasonal differences only in the case of *P. merus* (GZM, SL*summer-22: $z = 2.02$, $p = 0.04$). Moreover, females captured in spring presented higher infection intensities of *A. gibbosum* compared to males from this season (GZM, $z = 2.05$, $p = 0.04$). In terms of P, only *A. gibbosum* showed significantly higher values in both summer-22 and summer-23 (GZM, $z = 2.26$, $p = 0.02$), and higher P of Tetraphyllidean cestode larvae was detected in spring-23 (GZM, $z = 3.35$, $p < 0.001$), but these differences were associated with fish size.

In addition, microsporidian xenomas were detected in low prevalence in the intestine and pyloric caeca only in four specimens from autumn-22 and summer-23 (Table 5).

Table 5. Prevalence (%) and mean intensity (MI) values followed by the range (in brackets) of the parasites found in specimens of *Sardinella aurita* sampled in each season on the central Catalan coast (NW Mediterranean) are shown. Parasitological descriptors with 95% confidence interval (in brackets) are also displayed. Keys for locations within host: G, gills; K, kidney; I, intestine; S, stomach; PC, pyloric caeca. Different superscript capital and lowercase letters show significant differences ($p < 0.05$) among seasons in the P% and MI of parasites, respectively. Dashes indicate absence of the parasite. X: non-available data. ^aData from histological analyses.

PARASITE TAXA	Location	SUMMER 22			AUTUMN 22			WINTER 23			SPRING 23			SUMMER 23		
		P%	MI (min-max)		P%	MI (min-max)		P%	MI (min-max)		P%	MI (min-max)		P%	MI (min-max)	
Microsporidia	PC, I	–	–		15.00	2.33 (1–4)		–	–		–	–		5.00	3.00 (3–3)	
Myxozoa^a	K	–	–		–	–		–	–		–	–		15.00	X	
Digenea																
<i>Parahemius merus</i>	S	95.00	19.95 ^a (6–53)		90.00	7.44 ^b (1–22)		75.00	2.87 ^c (1–13)		85.00	4.76 ^{bc} (1–9)		100.00	31.25 ^a (4–70)	
<i>Aphanurus</i> sp.	S, I	100.00	143.15 ^a (37–325)		100.00	22.00 ^c (5–53)		100.00	16.60 ^c (3–29)		100.00	70.55 ^b (31–170)		100.00	135.95 ^{ab} (9–420)	
<i>Allopoisthocleithrum gibbosum</i>	PC, I	85.00 ^A	11.00 ^a (1–36)		25.00 ^B (1–8)	3.00 ^b (1–8)		60.00 ^{AB} (1–7)	2.58 ^b (1–7)		30.00 ^B (1–9)	3.33 ^b (1–9)		75.00 ^A (1–31)	12.53 ^a (1–31)	
<i>Bacciger bacciger</i>	PC	95.00	12.74 ^a (1–46)		85.00	20.88 ^a (1–97)		80.00	7.63 ^b (3–15)		100.00	7.40 ^b (3–19)		95.00	37.68 ^a (1–361)	
Cestoda																
Tetraphyllidea larvae sp.	PC, I	70.00 ^B	11.57 ^a (1–42)		35.00 ^C (1–4)	2.14 ^b (1–4)		70.00 ^B (1–8)	3.36 ^b (1–8)		90.00 ^A	4.61 ^b (1–22)		50.00 ^B (1–44)	10.80 ^a (1–44)	
Copepoda																
<i>Clavellisa</i> sp.	G	10.00	2.00 (2–2)		–	–		–	–		–	–		–	–	
Total Prevalence		100.00			100.00			100.00			100.00			100.00		
Total Mean Intensity		154.95 (104.86 – 205.04) ^a			38.2 (26.78 – 49.61) ^c			22.25 (15.42 – 29.07) ^d			73.55 (53.69 – 93.40) ^b			190.5 (123 – 258) ^a		
Total Richness		6			6			5			5			6		
Mean Species Richness		3.65 (3.37 – 3.92)			3.25 (2.91 – 3.58)			3.25 (2.91 – 3.58)			3.75 (3.54 – 3.95)			3.60 (3.24 – 3.95)		
Brillouin Diversity Index		0.65 (0.57 – 0.72)			0.72 (0.58 – 0.85)			0.74 (0.62 – 0.84)			0.58 (0.48 – 0.66)			0.73 (0.63 – 0.83)		
Berger-Parker Dominance Index		0.76 (0.71 – 0.81) ^a			0.64 (0.55 – 0.72) ^b			0.61 (0.53 – 0.68) ^b			0.80 (0.75 – 0.84) ^a			0.70 (0.64 – 0.76) ^{ab}		

The nMDS ordination plot based on a Bray-Curtis dissimilarity matrix (stress = 0.2) showed a partial separation of *S. aurita* parasite assemblages among seasons (Fig. 11). PERMANOVA analysis revealed significant differences on the structure and composition of sardinella's parasite community with respect to SL (structure: $F_{(94,1)} = 19.25$, $p < 0.001$; composition: $F_{(94,1)} = 6.06$, $p < 0.002$) and season (structure: $F_{(94,4)} = 10.35$, $p < 0.001$; composition: $F_{(94,4)} = 3.97$, $p < 0.002$). However, the PERMDISP test indicated significant differences in dispersion within seasons. Post-hoc pairwise comparisons indicated significant differences among all seasons except for summer-22 vs summer-23. The Bray-Curtis similarity index was higher within seasons (ranging between 52 – 70%) than among seasons (ranging between 31 – 58%). The most homogeneous parasite assemblage was observed in spring (70%) whereas specimens from autumn showed the highest intraspecific differentiation (52%). Assemblages from both summer-22 and summer-23 were the most comparable (58%). The Indicator Value (IndVal) analysis identified *Aphanurus* sp. and *A. gibbosum* moderately associated with sardinellas from both summer-22 and summer-23 (IndVal = 0.42 – 0.49, $p = 0.001 – 0.05$) (Fig. 11).

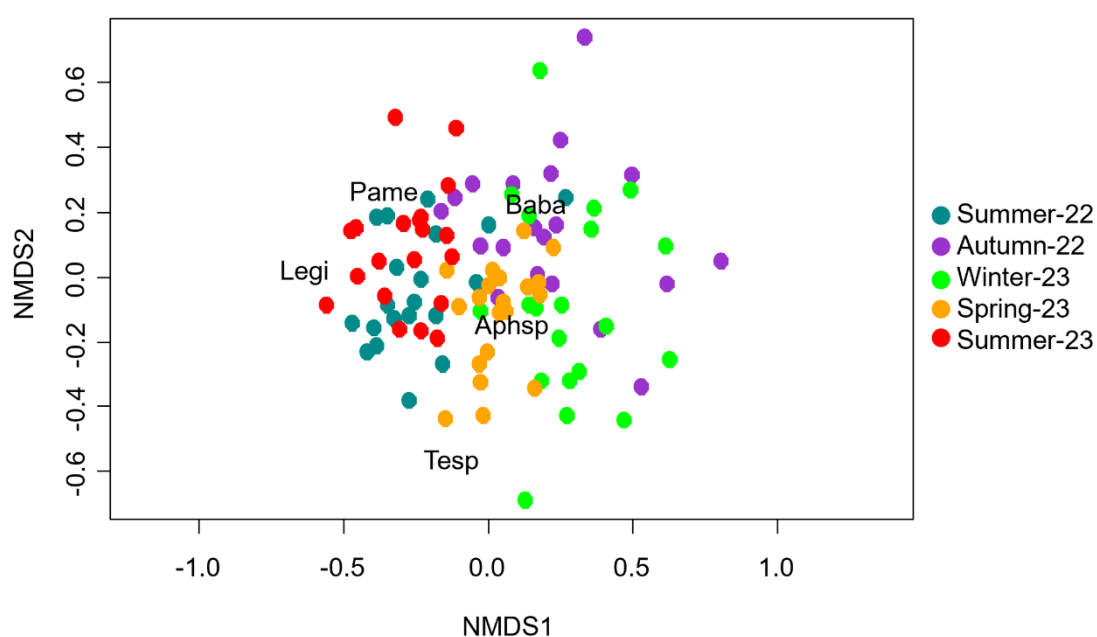


Fig. 11. Non-metric multidimensional scaling (nMDS) ordination plot based on a Bray-Curtis dissimilarity matrix calculated from log-transformed parasite abundance data of *Sardinella aurita* across seasons. Each point represents an individual host coloured according to the region. Labels indicate parasite species significantly associated with specific regions according to the Indicator Value analysis (IndVal): Aphsp, *Aphanurus* sp.; Baba, *Bacciger bacciger*; Legi, *Allopoisthocithum gibbosum*; Pame, *Parahemiurus merus*; Tesp, *Tetraphyllidea* larvae sp.

3.2.3. Histological and histopathological findings

3.2.3.1. Gills

Less than 5% on average of the total filaments examined presented some minor histological alterations affecting the gills in a low intensity (**Table 6**). Similar as in hakes, inflammatory responses mainly composed of granular cells and lymphocytes were observed in round sardinellas and were identified either as discrete foci (i.e. alteration foci) (45.55%), affecting the interlamellar spaces of two to 15 lamellae and to a lesser extent within the filament epithelium, or extensive foci (8.89%), affecting the central core of the whole filament (**Table 6**). These inflammatory responses were, in many cases, associated with the presence of ovoid structures with aspect of embolized blood fluke's eggs (54.44%) (**Fig. 12a-c**). However, these structures were different to those observed in hake, with less defined content and no clear embryos or miracidia present, suggesting degenerative material. These structures were significantly more prevalent in winter-23 and spring-23 (Chi-squared, $\chi^2 = 22.97$, $p < 0.001$). Inflammatory foci followed a similar pattern, displaying higher prevalences from autumn-22 to spring-23 compared to other seasons (Chi-squared, $\chi^2 = 14.69$, $p < 0.001$) (**Table 6**). Epitheliocystis (EPC), present in 31.11% of the examined fish, were significantly prevalent in autumn-22 and spring-23 compared to other seasons (Chi-squared, $\chi^2 = 19.14$, $p < 0.001$) (**Table 6**). Two different typologies of EPC were identified according to their size, staining, position in the lamellae, density of the content and presence of host response (**Fig. 12d-e**): type 1 was a medium sized (30-100 μm) basophilic and finely granular cyst, located intracellularly in the subepithelial space of the lamella, with no evident host reaction (**Fig. 12d**); type 2 was a medium (30-100 μm) basophilic cyst with smooth content, generally found in distal parts of lamellae but sometimes also affecting more than two lamellae, with no host reaction in exception of large cysts that were associated to mild epithelial hyperplasia (**Fig. 12e**). Finally, other EPC-like structures were commonly found and consisted of a small sized (<30 μm) basophilic and granular cyst, located extracellularly between lamellae and with no apparent host response (**Fig. 12f**).

Table 6. Prevalence (%) and mean intensity (MI) or mean density (MD) in terms of number/mm² followed by the standard deviation (SD) of the histopathological alterations found in *Sardinella aurita* are shown. Keys for histopathological alterations: MMCs, melanomacrophage centers. Keys for location within host: G, gills; L, liver; K, kidney; Sp, spleen. Different superscript capital and lowercase letters indicate significant differences in P% and MI or MD, respectively, among seasons.

Histopathology	Location	Summer 2022			Autumn 2022			Winter 2023			Spring 2023			Summer 2023		
		P%	MI (SD)		P%	MI (SD)		P%	MI (SD)		P%	MI (SD)		P%	MI (SD)	
Alteration foci	G	30 ^B	1.00 (0.00)		70 ^A	1.00 (0.00)		65 ^A	1.11 (0.32)		70 ^A	1.00 (0.00)		25 ^B	1.00 (0.00)	
Extensive hyperplasia	G	–	–		5	X		–	–		–	–		–	–	
Extensive inflammation	G	–	–		20	1.25 (0.50)		–	–		15	1.33 (0.58)		5	X	
Epiteliocystis	G	10 ^C	X		70 ^A	4.75 (6.02)		20 ^{BC}	1.25 (0.50)		40 ^{AB}	1.75 (0.89)		15 ^C	1.33 (0.58)	
Ovoid egg-like structures	G	25 ^B	1.38 (0.51)		35 ^B	1.22 (0.44)		80 ^A	1.36 (0.56)		75 ^A	1.22 (0.42)		20 ^B	1.75 (0.89)	
Granuloma	L	–	–		–	–		5	X		5	X		–	–	
MMCs	K	50 ^B	12.63 (9.45) ^c		50 ^B	9.95 (5.86) ^c		95 ^A	19.40 (6.79) ^{ab}		85 ^A	18.71 (10.06) ^{abc}		100 ^A	23.88 (8.97) ^a	
	L	5	X		–	–		–	–		–	–		5	X	
	Sp	50 ^B	17.08 (7.58) ^{ab}		70 ^{AB}	8.67 (6.84) ^b		90 ^A	25.55 (6.85) ^a		90 ^A	13.53 (3.61) ^b		85 ^{AB}	11.66 (8.76) ^b	
Aggregated macrophages	L	35	8.61 (3.17) ^{ab}		40	2.25 (1.03) ^b		55	6.52 (6.07) ^{ab}		40	8.67 (5.92) ^{ab}		60	15.27 (9.37) ^a	
	K	–	–		40	12.14 (4.17)		–	–		10	X		–	–	
Inflammatory focus	L	5	X		20	1.38 (1.13)		5	X		–	–		–	–	
	K	15	6.94 (5.01)		5	X		–	–		–	–		–	–	
Vacuole-like structures	L	50 ^B	X		90 ^A	X		90 ^A	X		80 ^A	X		45 ^B	X	

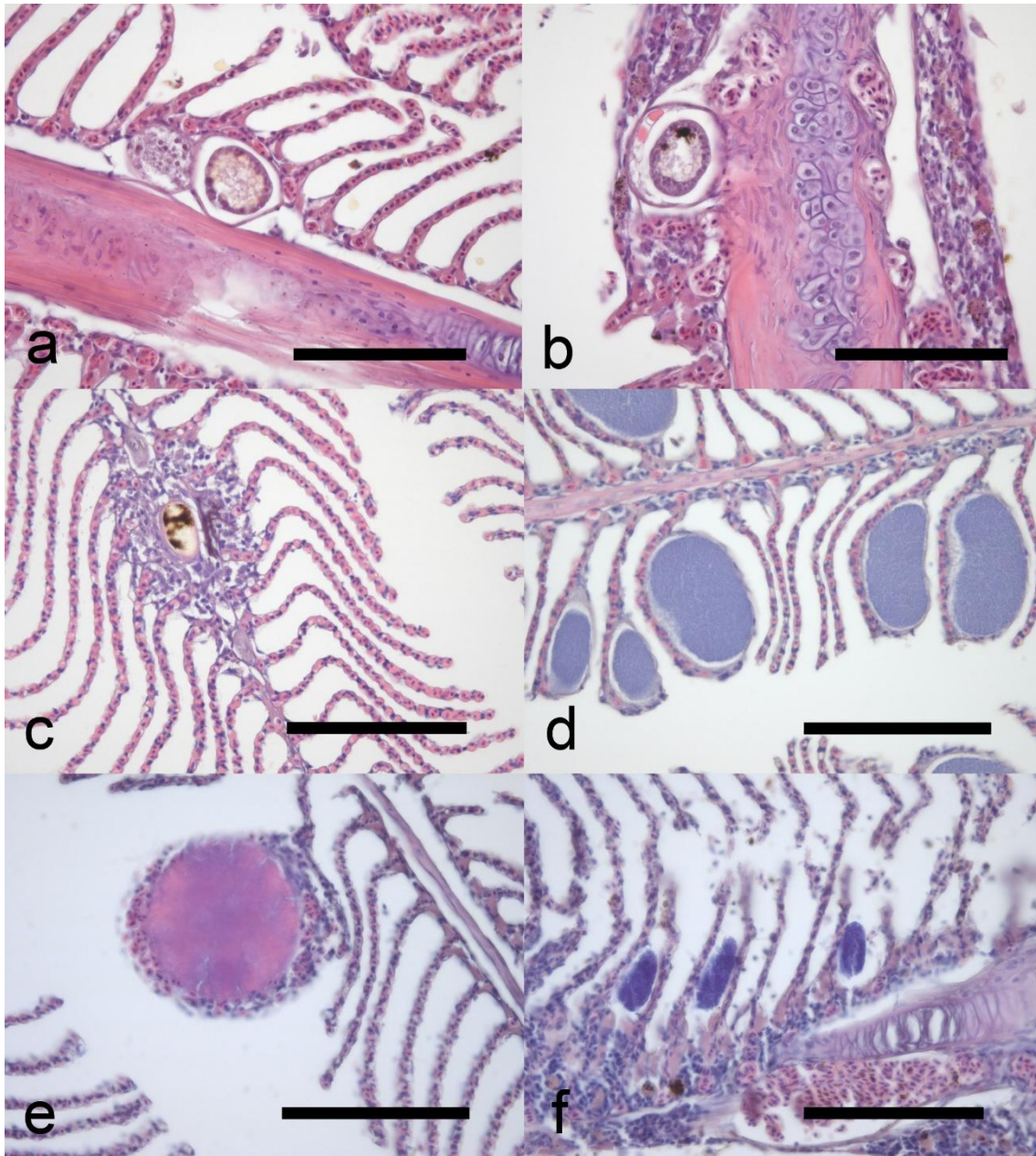


Fig. 12. Histological (H&E stain) gill sections in *Sardinella aurita*. Scale bars= 100 µm. **a)** Two structures resembling blood fluke eggs causing dilatation of the afferent filament artery, **b)** evoking lamellar fusion and **c)** inflammation. **d)** Epitheliocystis (EPC) with medium cysts located intracellularly without causing host reaction (type 1). **e)** EPC characterized by medium intracellular cysts causing inflammation (type 2). **f)** EPC-like structures characterized by small cysts located extracellularly.

3.2.3.2. Liver

In the case the hepatic tissue, similar PAS negative lipid droplets-like structures as those found in hakes (see 3.1.3.2 section) were also found in the cytoplasm of hepatocytes in 76.67% of specimens, being significantly more prevalent in autumn-22, winter-23 and spring-23 compared to both summers (Chi-squared, $\chi^2 = 22.62$, $p < 0.001$) (**Table 6**). Aggregated macrophages were detected in 56.67% of fish (**Fig. 13a**) whereas MMC were nearly absent in this organ. No significant differences were found in the P of aggregated macrophages among seasons (Chi-squared, $p > 0.05$), but significantly higher mean densities were found in fish from summer-23 compared to those of autumn-22 (K-W, $\chi^2 = 24.89$, $p < 0.001$) (**Table 6**). Also, inflammatory foci and small granulomas, as those described in hake, were present in the hepatic tissue affecting only 6% and 2% of the examined fish, respectively, and not in all seasons (**Fig. 13b**).

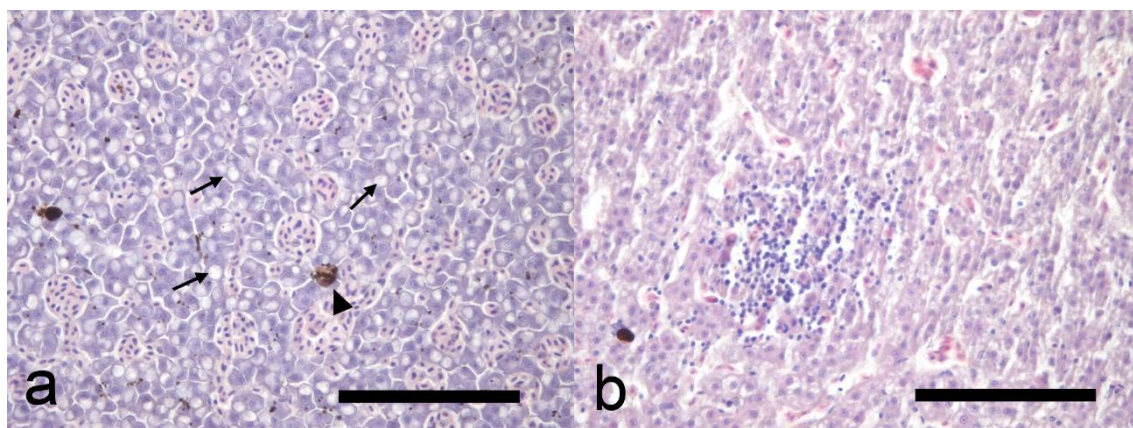


Fig. 13. Histological (H&E stain) sections of alterations identified in the hepatic tissue of *Sardinella aurita*. Scale bars = 100µm. **a)** Hepatocytes lipid droplets-like structures (arrows) and macrophages aggregates (arrowhead). **b)** Inflammatory foci within the hepatic parenchyma.

3.2.3.3. Spleen

The most common alteration in the splenic tissue were MMCs, which were found in 77% of the examined fish (**Fig. 14a-b**), with a similar structure as that described for hakes, although larger in size ($>100 \mu\text{m}$ in diameter) and containing brownish pigments (e.g., lipofuscin). *Sardinellas* from summer-22 presented significantly lower P of MMC compared to those of winter-23 and spring-23 (Chi-squared, $\chi^2 = 13.32$, $p = 0.009$), and higher mean densities were found in winter-23 compared to the other seasons (K-W, $\chi^2 = 24.89$, $p < 0.001$) (**Table 6**).

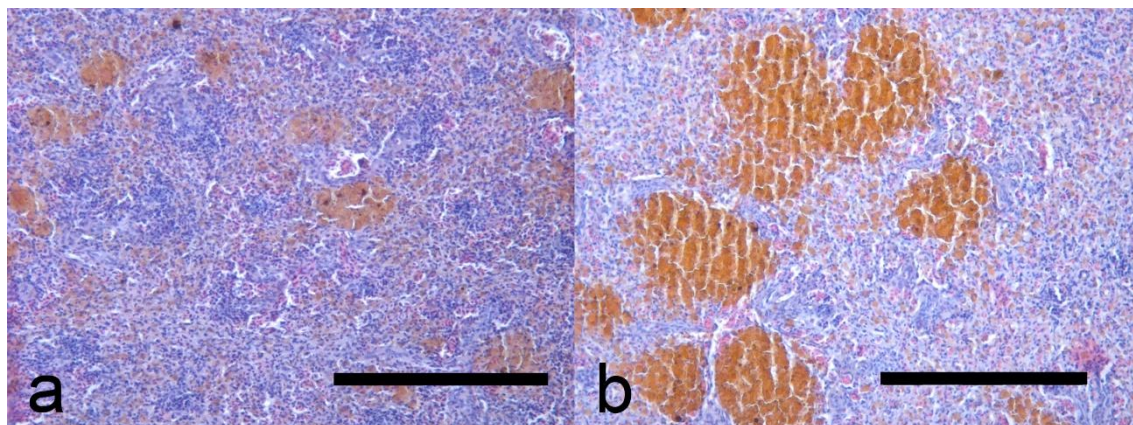


Fig. 14. Histological (H&E stain) sections of a) melanomacrophage centres (MMCs) showing **a)** smaller size and **b)** larger size, identified within the splenic tissue of *Sardinella aurita*. Scale bar = 200µm.

3.2.3.4. Kidney

In the renal tissue, MMCs were also predominantly found in this organ (76% of the examined fish), containing brownish pigments but smaller in size (about 50 µm in diameter) compared to those present in the spleen. *Sardinellas* from summer-22 and autumn-22 presented significantly lower P and MD of MMC compared to other seasons (Chi-squared, $\chi^2 = 25.98$, $p = 0.0005$ and K-W, $\chi^2 = 23.45$, $p = 0.0001$) (**Table 6**). Lymphocytic infiltration was also observed in the renal tissue only in four cases. Similar as in hakes, pre-sporogonic stages of coelozoic myxosporean parasites were found in the renal interstitial tissue only in 15% of sardinellas from summer-23, with no apparent host reaction (**Fig. 15a-b**).

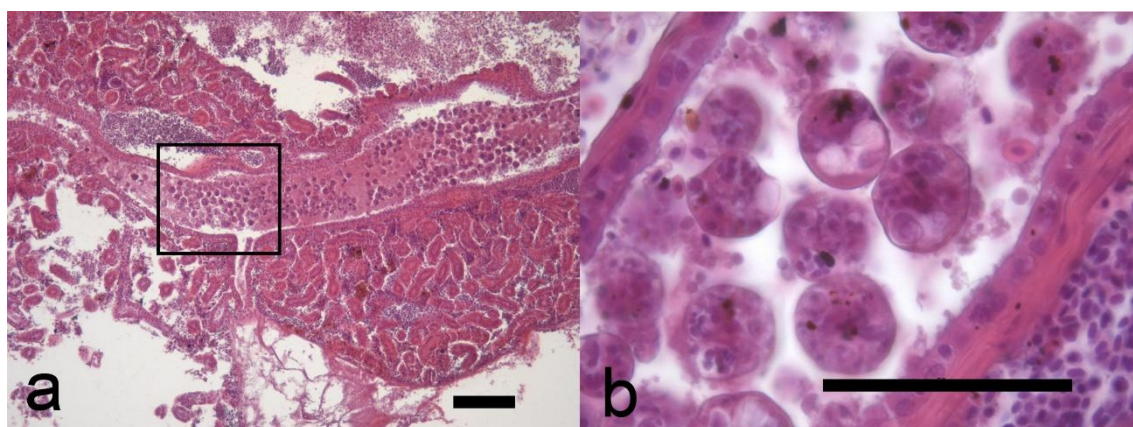


Fig. 15. Histological (H&E stain) sections of alterations identified in the renal tissue of *Sardinella aurita*. **a)** Renal tissue showing collecting ducts with coelozoic myxosporean parasites and some melanomacrophage centres close to renal tubules. Scale bar = 100µm. **b)** Higher magnification of the collecting ducts with coelozoic myxosporean parasites. Scale bar = 30µm.

3.2.3.5. Digestive tract

In the case of the digestive tract, no histopathological alterations were detected in the stomach, intestine or pyloric caeca, nor even associated with the high abundance of digeneans and cestode larvae recovered from the lumen of the digestive tract.

3.2.3.6. Gonads

Three gonadal phases were identified throughout the year following Mustač & Sinovčić (2021) terminology: immature, developing and spawning. The immature phase occurred in individuals collected in autumn-22 and winter-23 and was characterized by ovaries containing oogonia and small primary-growth oocytes (**Fig. 16a**), and testes dominated by primary spermatogonia and spermatocytes (**Fig. 16b**). The developing phase, observed in spring-23, showed enlarged ovaries with primary growth oocytes together with some cortical alveoli oocytes (secondary growth) (**Fig. 16c**), while testes contained spermatocytes, spermatids and in some cases spermatozoa (**Fig. 16d**). The spawning phase was restricted to the summer period, and ovaries had fewer primary oocytes with cortical alveoli and predominate vitellogenic oocytes containing small yolk granules and other oocytes with their cytoplasm filled with yolk (**Fig. 16e**). In some cases, oocytes undergone late germinal vesicle migration. Mature testes were dominated by spermatozoa, although earlier spermatogenic stages were still present (**Fig. 16f**).

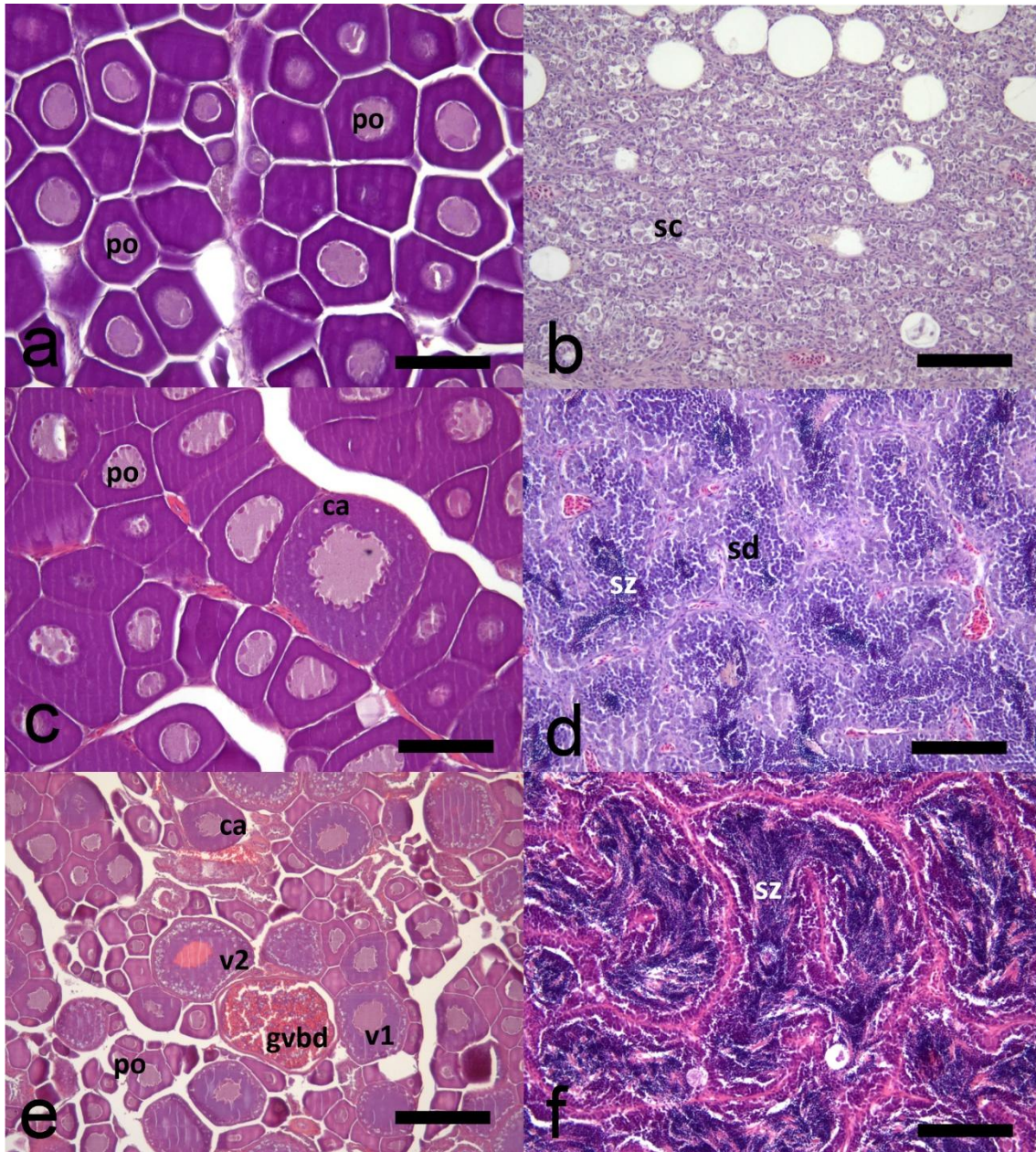


Fig. 16. Histological (H&E stain) sections of the gonads in *Sardinella aurita*. **a)** Immature ovaries and **b)** testes. **c)** Developing ovaries and **d)** testes. **e)** Mature ovaries and **f)** testes. Abbreviations: ca, cortical alveoli oocytes; gvbd, germinal vesicle breakdown; po, primary oocyte; sc, spermatocyst; sd, spermatid; sg, spermatogonia; sz, spermatozoa; v1, early vitellogenic stage; v2, vitellogenic stage. Scale bars: a-d, f, 100 µm; e, 200 µm.

3.2.4. Associations among fish health indicators and potential explanatory variables

Regarding the associations among fish health indicators detected for *S. aurita*, fish displaying higher P of hepatic vacuole-like structures presented lower GSI values (GZM, $z = -3.95$, $p < 0.001$), those with higher MD of splenic MMCs displayed lower values of HSI (GZM, $z = -2.16$, $p = 0.033$) and a positive relationship was detected between MD of macrophages aggregated and GSI (GZM, $z = 3.74$, $p < 0.001$).

Regarding the RDA ordination for *S. aurita* relating condition indices (Kn, HSI and GSI) with fish size and parasites species abundance explained 41% of the total variance by the first two axis (permutation test, $p = 0.001$) (**Fig. 17a**). The stepwise selection indicated that fish SL, stomach fullness and the parasite *Aphanurus* sp., were the most explanatory variables for the condition indices observed for sardinellas among seasons ($p = 0.001 - 0.017$). Visually, GSI was positively associated with SL and *Aphanurus* sp., whereas HSI and Kn were not associated with any explanatory variables.

On the other hand, the RDA relating histopathological alterations with SL and parasites only explained 8.6% of the total variation by the first two axes, showing SL and the parasite *P. merus* as the most explanatory variables (**Fig. 17b**). However, none of the responses could be associated with any of the two explanatory variables.

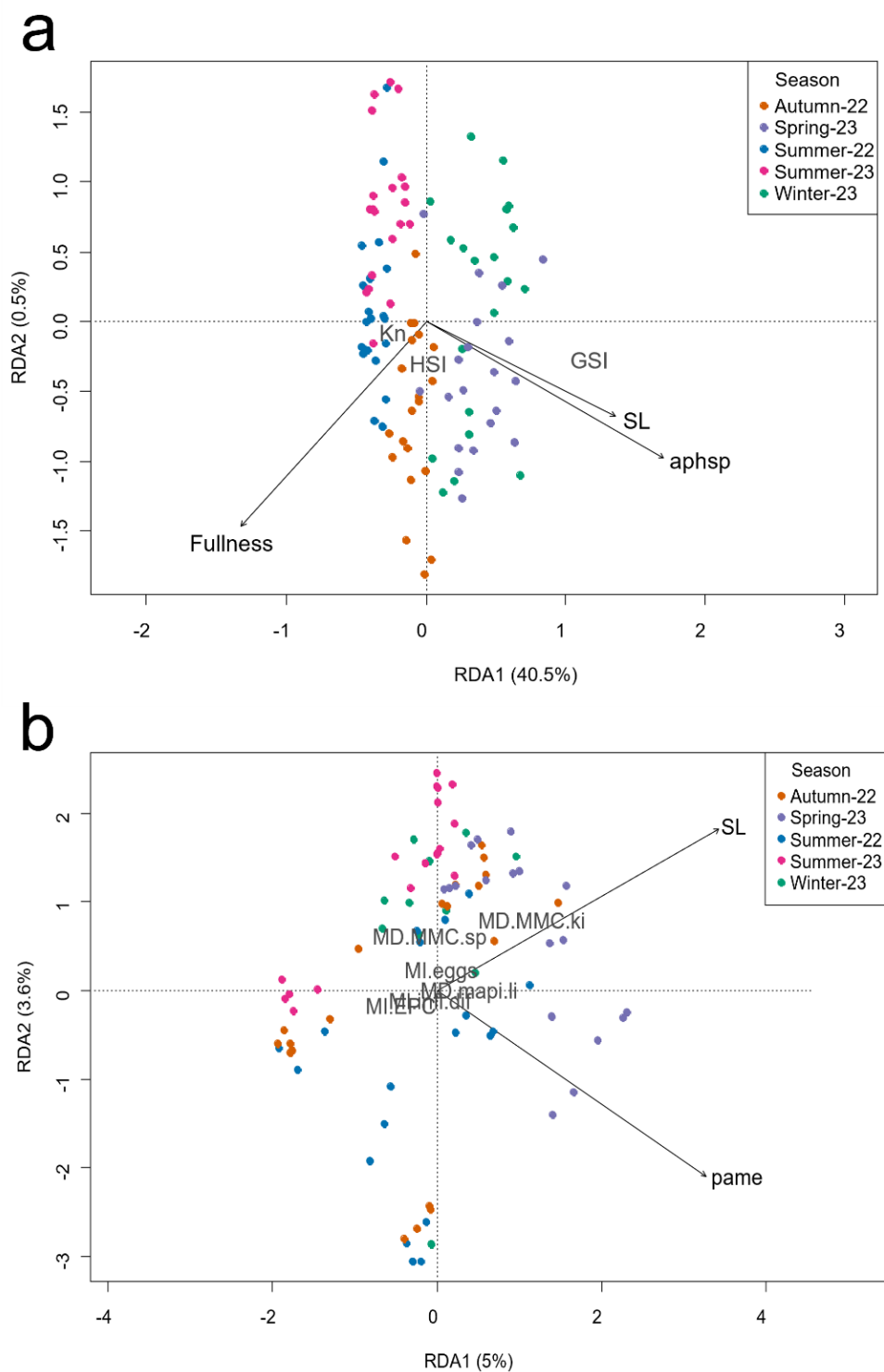


Fig. 17. Redundancy analysis (RDA) on **a**) condition indices and **b**) histopathological alterations of *Sardinella aurita* constrained by fish size (SL) and parasite species most significant for the ordination. Abbreviations: Kn, Le Cren relative condition index; HSI, hepatosomatic index; GSI, gonadosomatic index; MD.MMC.sp and MD.MMC.ki, mean density of melanomacrophage centres in spleen and kidney; MD.mapi.li, mean density of aggregated macrophages in liver; MI.eggs, mean intensity of ovoid structures resembling eggs; MI.EPC, mean intensity of epitheliocystis; aphsp, *Aphanurus* sp.; pame, *Parahemiurus merus*.

4. Discussion

Fish health assessment is a key component of environmental evaluation and the development of effective conservation strategies for natural resources. Understanding the effects of pathogens and diseases on commercially and ecologically important fish species is essential for monitoring ecosystem health and supporting fisheries management. In this context, the present study provides new insights into the condition and main pathologies of *M. merluccius* and *S. aurita* populations along the Catalan coast (NW Mediterranean Sea), evaluating their response to environmental and biological stressors, including parasitic infections. These findings represent a first step towards a broader understanding of the health status of these species and their associated marine ecosystems.

4.1. European hake health assessment

4.1.1. Fish condition and its parasite community

Larger individuals of *M. merluccius* presented higher GSI values, as expected, as this index is an indicator of gonad weight and reproductive investment (Lloret et al., 2014). The RDA identified environmental variables, particularly temperature and turbidity, rather than parasites as main factors associated with the observed variation in condition indices. Consistent with previous studies, spatial variability in food availability and environmental conditions (i.e. temperature and oxygen) are regarded as the most important factors impacting fish health (Lloret et al., 2014). In this sense, the significantly higher HSI and Kn values observed in individuals from off Ebro Delta, also partially reflected in the RDA ordination, could be related to a greater quantity and/or availability of food resources in this area. This hypothesis is supported by the higher fullness values observed in this locality (although not significant), and consistent with the findings of Hidalgo et al. (2008) in hakes from the Balearic Islands. In addition, as indicated by the RDA, the low turbidity levels recorded in this area could indirectly enhance hake condition by improving feeding efficiency (Korman et al., 2020). Moreover, according to Lloret et al. (2002), individuals inhabiting shallower habitats, as in the case of Ebro Delta, were in better condition (i.e. higher available energy reserves) since coastal areas tend to be more productive and with more food resources available compared to deeper waters. On the other hand, individuals from off Barcelona area presented the lowest condition factor (Kn) compared to individuals from the other two localities. Although individuals in this area were slightly more parasitized, no significant correlations were found between condition indices and parasite indicators. It could be hypothesized that certain environmental conditions or factors related to anthropogenic pollution affecting this area could cause a detrimental impact on fish condition (see Chapter 5).

The benthopelagic hake, which occupies a higher trophic level compared to *S. aurita*, hosted a richer parasite community, composed of 22 species and dominated by trophically transmitted helminths (i.e. digeneans, cestodes and nematodes), likely due to its interaction with a broad range of benthopelagic prey (mainly crustaceans), which serve as intermediate hosts for these parasites (Gibson et al., 2002). These findings are consistent with previous studies reporting a higher parasite diversity in fish preying near the seafloor bottom compared to their pelagic counterparts (Dallarés et al., 2014; Constenla et al., 2015). Despite component community was high in richness, infracommunity richness was low, comprising only two or three different species per individual. This could be explained by the heterogeneous prey consumption among individuals and the spatial variation on the distribution of invertebrate intermediate hosts, which limit helminth co-occurrence within single hosts (Poulin, 1997). This relatively low co-occurrence of parasite species within individuals may also contribute to the limited pathological impact observed (see section 4.1.2 for further discussion).

Hake's parasite composition reported in this study is in line with previous literature records (Tirard et al., 1996; Oguz & Bray 2006, 2008; Ferrer-Maza et al., 2014; Carrassón et al., 2019). Particularly, the reported parasite community of adults from *M. merluccius* (specimens > 40 cm in length) in the Gulf of Lion (NW Mediterranean) harboured 19 distinct species (Ferrer-Maza et al., 2014) of which eight have been identified in the present study. In this case, the ontogenetic shift on hake's feeding behaviour towards a more piscivorous diet that takes place in specimens between 20-25 cm length, could explain the lack of some parasite taxa in the examined juvenile specimens (D'Iglio et al., 2022). Indeed, larger fish generally accumulate more helminths (Poulin & Leung, 2011; Cipriani et al., 2018), as reflected by the positive size-intensity relationship observed herein for *C. crassiceps*. Adult's broader diet preying upon both crustaceans and fish could increase the likelihood of ingesting multiple larval infective stages co-occurring within the same intermediate or paratenic hosts, being transmitted simultaneously through the food chain (Marcogliese, 2002). However, this increase in parasite load with host size was not reflected in a decline in their condition, suggesting that hosts may tolerate these infections without major impairment under natural conditions (Hasegawa & Poulin, 2025). Nevertheless, these results should be interpreted with caution, as the size range analysed in the present study was limited and mainly composed of juvenile individuals, reflecting the size class commonly found on the studied continental shelf.

Although the nMDS ordination showed a strong overlap of hake's parasite assemblages among localities, the multivariate analysis revealed significant spatial and size differences in the structure and composition of their parasite communities, as also indicated by the higher Bray-Curtis intraspecific similarity indices compared to the variability observed among localities. Indeed, local

variations in digenean richness were observed along the continental shelf off the Catalan coast, an area characterized by contrasting morpho-bathymetric features, ranging from the deep Blanes Canyon to the smoother, sediment-rich area off the Ebro Delta. This environmental heterogeneity results in a wide array of substrates that support diverse invertebrate and fish communities (Demestre et al., 2000; DeLaHoz et al., 2018), potentially enhancing the availability of different intermediate hosts for a broad range of digenean species (Pérez-del-Olmo et al., 2009). Hakes from Ebro Delta presented a more homogeneous parasite assemblage probably due to the high prevalence of *H. aduncum*, present in almost all specimens but also to local environmental conditions. *H. aduncum* is a widespread generalist nematode that involve harpacticoid copepods as first hosts, amphipods, mysids, euphausiids and chaetognaths as intermediate hosts and fish as paratenic or definitive hosts (Anderson, 2000). It has been suggested that euphausiids and mysids from shallow waters are more parasitized by *H. aduncum* larvae than those of deeper waters, whereas *H. fabri* predominates in demersal environments and infects fish feeding mainly on benthic or epibenthic prey (Rello et al., 2008; Costa et al., 2018b). In this sense, variations in prey availability and/or the layer in which hake feeds along the water column may explain the higher prevalence (although not significant) of *H. aduncum* in Ebro Delta and of *H. fabri* in Blanes. Thus, it could be hypothesized that hakes from Ebro Delta, which is a shallower area, may feed on pelagic crustaceans, consistent with their nocturnal vertical migrations towards superficial waters (Bozzano et al., 2005), whereas hakes from Blanes, which is a deeper area near the canyon of Blanes, could mostly prey on benthopelagic and epibenthic crustaceans. Regarding the temporal comparison in hakes from Barcelona, those sampled in 2007 displayed a greater variability in their parasite infracommunity (as indicated by Bray-Curtis similarity index), likely reflecting temporal changes in environmental conditions and in the availability or distribution of prey. In addition, host size was more heterogeneous in 2007 (18 – 26 cm in length) than 2019 (14.90 – 20 cm in length). Since present results indicated that host size could influence parasite community structure and composition, these temporal differences may be also linked to ontogenetic shifts in hake's feeding behaviour (Ferrer-Maza et al., 2014; D'Iglio et al., 2022).

4.1.2. Pathological and histopathological interpretation

Macroscopic alterations have been reported in other gadoid species, including dermal ulcers and multisystemic necrocentric granulomas related to bacterial infections (e.g. *Francisella noatunensis*) or pollutants (e.g., heavy metals), as described in *Gadus morhua* (see Borucinska & Morka, 2016). In our study though, no skin lesions, the absence of macroscopic alterations (i.e. neoplasms) and histopathological neoplastic changes are consistent with previous studies carried out on *M. merluccius* (Marigómez et al., 2006; Cuevas et al., 2015). The lack of this kind of

alterations could be explained by several factors. First, the present study focused on juvenile individuals, and age is a determinant of disease susceptibility, with neoplastic lesions typically increasing in prevalence in older fish, mostly upon the age of 4 years (Stentiford et al., 2010). In fact, hepatic neoplasia has been widely reported in *Limanda limanda* and in other flatfish species (Feist et al., 2004). These lesions were grossly visible, together with other pathologies such as epidermal hyperplasia and ulcerations of the skin (Stentiford et al., 2010). Second, the absence of lesions may also reflect limited exposure to contaminants (Feist et al., 2004), or a relatively low susceptibility of this species to develop neoplasia. However, it should be noted that the lack of observed alterations does not necessarily imply the absence of environmental impact. For instance, hepatocellular nuclear pleomorphism was described in hakes after the Prestige oil spill in the Bay of Biscay (Marigómez et al., 2006), although its prevalence decreased over time, indicating a recovery from the chemical spill (Cuevas et al., 2015). Finally, the absence of large lesions could also be related to the fitness of the individuals, given that in wild conditions those individuals with reduced fitness due to diseases are likely to not survive, and therefore, fewer chances exist to be caught off commercial fisheries (McVicar, 1997).

Histopathology has been proved as an effective and accurate diagnostic technique to detect changes in the normal structure of any organ. However, its interpretation should be approached with caution, as artifacts can be easily produced during sampling collection, preservation and processing. As Wolf et al. (2015) exhaustively reported, the histological preparation of the gills, along with the kidney and liver, can be technically challenging. Moreover, vascular changes should be interpreted cautiously, as their manifestation can be imitated by artifacts caused during the process of collecting samples. For instance, telangiectasia was detected in almost every examined gill section from this study, particularly in hakes, which typically is resolved via lamellar thrombosis or accumulation of erythrocytes in some gill areas. Thrombotic evidence indicates the prior existence of telangiectasia in gills before necropsy, while a lack of thrombotic changes implies acuteness in lesion duration and questionable pathologic significance (Wolf et al., 2015). Hence, telangiectasia was considered an artifact induced by trawling due to the absence of thrombosis evidence. Likewise, hyperhemia was recurrently detected in intestine sections of hake specimens but was neither accounted as a histopathological finding. For these reasons, the histopathological evaluation of the slides should always be done by trained personnel and always under the supervision of experienced pathologists, specialists in fish diseases and histopathology.

The gills appeared to be the most affected organ. This is not an unusual fact, as gills are in constant close contact with water, pathogens and stressors and many fish pathogens have also a particular tropism for gills (Bernet et al., 1999). Depending on the type and severity of the stressor and

length of exposure, gills can respond in a wide variety of ways. Most of the lesions identified in gill filaments and lamellae (foci or extensive inflammation and hyperplasia) of the present study, were probably mostly related to the mechanical effects caused by the presence of monogeneans, copepods and blood fluke eggs.

Monogeneans can cause focal damage mainly due to their attachment to the gills with their posterior adhesive organs, although in some cases they can also harm the gill structure by their feeding activity. The polyopisthocotylean *Anthocotyle merluccii* has been identified in the herein study and is also well-reported in *M. merluccius* from the NW Mediterranean Sea (Ferrer-Maza et al., 2014; Carrassón et al., 2019) and other species from the genus *Merluccius* (MacKenzie & Longshaw, 1995). This monogenean has four pairs of clamps and uses its anterior-most enormously developed pair of clamps to grasp the inner border of the filament, and the three small remaining pairs of clamps attach to secondary lamellae (Llewellyn, 1956). The putative feeding action of the polyopisthocotylean parasite on the gill tissue should also not be discarded as a source of mechanical damage. Indeed, several histopathological alterations have been reported in other polyopisthocotylean species such as *Sparicotyle chrysophrii* (Van Beneden & Hesse, 1863) Mamaev, 1984 in cultured *Sparus aurata* including lamellar shortening, clubbing and synechiae (Sitjà-Bobadilla & Alvarez-Pellitero, 2009), and even causing anaemia in its host (Riera-Ferrer et al., 2022). In the herein study the pathogenic significance of *A. merluccii* in hake was not important given the low prevalence of the parasite and the low intensity of inflammation detected affecting the lamellae. Similarly, although copepods may cause severe damage to the gill arch and filaments due to their attachment and feeding activity (Colorni & Padrós, 2011), they did not appear to represent a major threat to fish condition in the present study, probably because of their low number and infection intensity. However, histopathological alterations associated with the copepod *Lernaeocera lusci* were still observed and its impact on *M. merluccius* have been reported in other studies (Guillaume et al., 1985), particularly on the fatty acid composition of its host (Telahigue et al., 2019; Hajji et al., 2021). Extensive tissue necrosis and an associated inflammatory response were detected at the attachment site on the gill arch, together with extensive and massive epithelial hyperplasia identified in the surrounding gill filaments. These alterations may be likely caused by the mechanical effect triggered by the presence of the external part of the body of the parasite. In fact, *Lernaeocera* uses its thoracic holdfast to penetrate into the gill arch and reach the main arteries and even cardiac structures (Kearn, 2005). These observations are comparable to those related to other similar parasitic copepod species, such as *Lernaeocera branchialis* (Linnaeus, 1767) to the gills of *G. morhua* (Smith et al., 2007). Likewise, the presence of the blood fluke *A. spinosicanalis* should be considered, as blood flukes have been

reported to be a problem in many cultured fish species as can be accumulated in large numbers inside the gills and blood vessels, obstructing blood circulation, evoking inflammatory responses and reducing the respiratory surface (Padrós et al., 2001; Holzer et al., 2008). In the current study, eggs were only present within the filament arteries indicating that they could not pass through the lamellae capillaries probably due to their size. Eggs presence was significantly associated with focal lamellar fusion and inflammation, which was a common host response associated with eggs. Due to the mild host reaction and low intensity of infection, no relevant detrimental effects on the cardiorespiratory system could be expected. It is also noteworthy to mention that the specimen showing the highest infestation levels of *A. spinosicanalis* eggs in the gills and adult parasites in the heart exhibited liver atrophy and dark pigmentation, together with dilated sinusoids and a marked accumulation of macrophage aggregates and granulomas. These observations suggest that chronic and severe egg accumulation may induce the liver pathologies detected, progressively weakening the host and potentially leading to fish mortality (Bullard & Overstreet, 2002). However, further investigation is needed, particularly regarding the potential histopathological impact of adults inhabiting the heart (see Chapter 4.2).

CUEs appeared to be a significant alteration as they were commonly found among all localities and in both years. Although a significantly higher prevalence was detected in 2007, wide local variations in their prevalence may occur as reported by MacKenzie & Longshaw (1995), in which different prevalences of CUEs were displayed among adjacent samples in *Merluccius hubbsi* Marini, 1933 and *Merluccius australis* Hutton, 1872. Although these structures were first described approximately 50 years ago (MacKenzie, 1979), their etiology remains unknown. Since then, similar lesions were described in different fish species (e.g. Munday & Brand, 1992; Heidel et al., 2002; Diggles 2003; Carreras-Aubets et al., 2012; Dallarés et al., 2014; Constenla et al., 2015, 2022) but, to date, no pathogen has been isolated or identified in association with these alterations. Some studies suggest that they correspond to a form of cartilaginous metaplasia, likely related to an inflammatory response (Heidel et al., 2002), or have been also related to environmental pollution (Carreras-Aubets et al., 2012; Constenla et al., 2015). However, this hypothesis remains controversial due to contradictory observations reported in other studies (Heidel et al., 2002; Constenla et al., 2022).

The morphology of the hepatic parenchyma and the amount of reserve substances such as lipids and glycogen in hepatocytes may vary considerably depending on species, age, sex, nutritional status, season and developmental stage (Wolf et al., 2015). Hepatocytes store excess of energy as glycogen or lipid in the cytoplasm when energy intake exceeds metabolic demands (Wolf et al., 2015). Although hepatocyte vacuolation has been associated with exposure to toxic substances

(Zodrow et al., 2004), intracytoplasmic lipid droplets are often misinterpreted as pathological alterations. This is particularly relevant in hake and other gadiformes, which naturally accumulate large lipid reserves in the liver (Morrison, 1987). In this study, although most of the livers were homogeneous, some of them presented different levels of lipid vacuolization among individuals from the same season. The propensity to store glycogen or lipid seems to vary among fish species and may be also influenced by dietary factors (Wolf et al., 2015).

Moreover, the liver was found to be the most infected organ by nematode larvae, mostly by *H. fabri*, which was mainly recovered in this organ. Reported lesions in gadoid's livers related to the presence of *Hysterothylacium* Ward & Magath, 1917 and *Contracaecum osculatum* (Rudolphi, 1802) Baylis, 1920, resulted in the development of granulomatous changes, inflammation, haemorrhages, atrophy, reduced fat content and destruction of the hepatocytes (Behrens et al., 2023), as a consequence of the direct effects of the nematode larval stages on the hepatic tissue. Moreover, nematode infestation (i.e. *Anisakis* sp.) may also result in changes of muscle's fatty acids profile and a reduction of mineral composition in parasitized hakes (Jouini et al., 2023). Most of the granulomas observed in present study seem to be associated to the migrating nematode larvae, since they were apparent viable or in most cases, degenerating, for their presence also in the stomach. Although a higher prevalence and mean intensity of *H. aduncum* (although not significant) was found in hakes off Ebro Delta, it was not associated with a higher prevalence or density of granulomas in this locality. This granulomatous response had similar ultrastructural features of encapsulation as described in other gadoid fish species (Dallarés et al., 2014, 2016; Behrens et al., 2023). Moreover, although coccidian infections have been described in many fish species, including *Merluccius* spp. (Morrison & Marryatt, 1990), no coccidians were found in this study. This was an unexpected finding, as apicomplexa and particularly coccidians are frequently reported in gadoids, and protozoan infections may be more pathogenic to their host (Hemmingsen & MacKenzie, 2001).

The presence of pigmented macrophages either isolated, aggregated or forming centres in the liver and spleen was noticeable in hake. MMCs primary role is to phagocytize, detoxicate, recycle and act as a long-term storage of endogenous (e.g., cellular debris, erythrophagocytosis, iron recycling) and exogenous materials (e.g. toxic materials, metal deposits) (Agius & Roberts, 2003; Steinel & Bolnick, 2017). Several studies reported MMCs as biomarkers of environmental stress (Carrassón et al., 2008; Basilone et al., 2018). However, hakes from the apparent most polluted locality (i.e. Barcelona) in this study presented a considerably low prevalence of MMCs compared to other localities and to hakes from the same locality in 2007, indicating that other factors such as environmental conditions, stress, age, size and sex of the fish, but also the natural variability of

the different fish species, should be deemed more importantly (Steinel & Bolnick, 2017; Carreras-Colom et al., 2022). In the current study, variability in the intensity of MMCs was noticed among the specimens examined but was not either related to fish size or sex.

The kidney has been highlighted as a target organ of infection for myxosporean parasites (Holzer et al., 2010), mainly related to the action of histozoic myxosporeans such as PKD caused by *Tetracapsuloides bryosalmonae*. However, in this study, only coelozoic myxosporean parasites within the renal tubules, with a lower pathogenic impact, were detected. Sankurathri (1977) described the myxosporean *Myxoproteus meridionalis* Evdokimova, 1977 (formerly *Conispora renalis*) from the renal tubules of *Merluccius productus* Ayres, 1855 from the Strait of Georgia, British Columbia, and mixed infections of *Gadimyxa atlantica* Køie, Karlsbakk & Nylund, 2007 and *Zschokkella hildae* Auerbach, 1909 were found in cultured *G. morhua* (Holzer et al., 2010). However, no major pathological effects were found in the current outcomes, despite of the high prevalence detected. In fact, it has been reported that many coelozoic myxosporidian parasites coexist with their asymptomatic host without causing severe damage (Colorni & Padrós, 2011).

Finally, the histopathological changes detected in the digestive tract tended to be minimal and localized to the region of parasite infection (i.e. digeneans, cestodes and nematodes). Although a high diversity of helminth parasites was found in the lumen of both stomach and intestine, only scattered, slight or negligible host responses were detected. This was not an unexpected observation, as metazoan endoparasites are normally found freely in the lumen of the digestive tract without causing any significant pathogenic effect on the host (Hemmingsen & MacKenzie, 2001). In other hake species, these responses were mainly small focal inflammatory areas and were reported as potentially associated with the site of attachment of the parasite, as for *M. productus* (Meyers et al., 1999). Intestinal cestodes are non or minimally pathogenic, and in most cases, host tissue damage is elicited by the attachment of the scolex to the host (Eiras et al., 2008). Indeed, despite a single finding of the attachment of the bothria of *C. crassiceps* to the intestinal mucosa that caused mild alterations (see Chapter 4.1), no other similar changes were detected in any specimen that indicated the previous attachment of this parasite. Granulomas in the stomach were also present in a lower prevalence than in the liver, but were also the result of larval nematode encapsulation, as described in *Merluccius bilinearis* Mitchill, 1814 (Murchelano et al., 1986). When parasites (nematodes, cestodes or digeneans) were found in the mucosa or submucosa of the stomach, no host reaction was observed. On the other hand, rodlet cells are considered endogenous cells that have been detected in the gastric mucosa in almost all the examined specimens. Although their origin and function are still controverted (Manera & Dezfali, 2004), they have been often described as resident immune cells whose abundance may increase

in response to tissue damage, pathogens or parasites (Sayyaf Dezfuli et al., 2022 and references therein). In gadoids, rodlet cells have been suggested to be associated with the presence of encapsulated larval helminths, as they were reported in the intestinal tract in relation to such infections (Reite, 2005). In the present study, though, they were not involved in any inflammatory responses associated with granulomas of non-viable parasites. Notwithstanding, their occurrence in host responses against other parasites such as protozoans have been observed in several reports (Sitjà-Bobadilla et al., 2016; Sayyaf Dezfuli et al., 2020), providing evidence of its importance in the immune system (Sayyaf Dezfuli et al., 2022). Alternatively, they have also been proposed as potential biomarkers of exposure to contaminants and chemical agents, including microplastics (Pedà et al., 2016) and heavy metals (Sayyaf Dezfuli et al., 2022) (see Chapter 5).

4.2. Round sardinella health assessment

4.2.1. Fish condition and its parasite community

Seasonal variations on the condition indices of round sardinella were evaluated in this study, but no clear associations could be drawn from the RDA ordination plot, since only size was positively associated with GSI, as observed in hakes. Given that parasites did not seem to influence seasonal variations in round sardinellas' condition indices, and as environmental data were not included in the analysis, maybe these variations could be associated with feeding intensity, seasonal changes in environmental conditions (e.g. temperature, oxygen concentration), the quantity and/or composition of available food, and the reproductive stage (Lloret et al., 2014; Brosset et al., 2015). Indeed, *S. aurita* is a capital breeder, which accumulates energy reserves prior to reproduction in summer (Albo-Puigserver et al., 2017), which is in accordance with the observed increase in hepatosomatic and gonadosomatic indices from autumn to spring, together with stomach fullness indicating an increased feeding intensity during this period. In contrast, in summer, the GSI reached its maximum and the HSI was reduced, suggesting a mobilization of their energetic reserves for spawning (Lloret et al., 2014).

The parasite community of the pelagic sardinella, which as fish species occupies a lower trophic level compared to hakes and feeds primarily on zooplanktonic organisms (e.g., copepods, euphausiids), was limited to digeneans and Tetraphyllidean cestode larvae that might use planktonic crustaceans as intermediate hosts, similar to the taxa described by Ramdani et al. (2020) in sardinellas from the Algerian coast (S Mediterranean). Of the 13 parasite species that have been recorded in sardinellas from different locations in the Atlantic and Mediterranean (Ramdani, et al., 2020; Feki et al., 2015), eight have been found in the present study, with

Aphanurus sp. showing the highest prevalences and mean intensities. No nematode larvae were found in the herein sardinellas, which is consistent with previous studies reporting low nematode prevalence (<15%) across different Mediterranean localities such as off Sicily, Gulf of Asinara (off Sardinia), S Adriatic Sea and Gulf of Bejaia (off Algeria) (Costa et al., 2018b; Piras et al., 2014; Goffredo et al., 2019). In contrast, other pelagic species from the same studied area (the Catalan coast), have shown higher infection levels by nematode larvae, particularly *Hysterothylacium* sp. (Frigola-Tepe et al., 2022, 2024; Caballero-Huertas et al., 2023; Ferrer-Maza et al., 2016; Rodríguez-Romeu et al., 2022) (see 4.3 Discussion section from Chapter 6).

The seasonal differences observed for digeneans and Tetraphyllidean cestode larvae could be likely linked to seasonal shifts in round sardinella's diet, which may influence parasite transmission through trophic pathways. Many digenean species use planktonic crustaceans, mainly copepods, as second intermediate hosts (Gibson et al., 2002). In round sardinella, copepods are present in their diet throughout the year, although during warmer months they shift towards a higher intake of large zooplankton such as amphipods and decapods (Albo-Puigserver et al., 2019). The increased sea temperature, primary production and organic material input from river discharges during this period may enhance the development and availability of zooplankton, acting as potential intermediate hosts (Carrassón et al., 2019). Indeed, the increase in prevalence and mean intensity of digeneans and Tetraphyllidean cestode larvae observed in summer, particularly in larger specimens (coinciding with their spawning period), may therefore reflect a higher ingestion of intermediate hosts, as amphipods have been also reported in numerous digeneans species (Poulin, 2025). This observation aligns with results reported by Ramdani et al. (2020), as they also noted an increase in the prevalence of digenean species during this period. However, despite the relatively high parasite infection levels observed during the reproductive period, round sardinella condition did not appear to be negatively affected, suggesting a limited impact of metazoan endoparasites, as discussed previously for *M. merluccius*, and commonly reported in wild fish (Hemmingsen & MacKenzie, 2001). Multivariate analyses supported these patterns since round sardinellas captured in summer from both years showed the highest interspecific similarity. This indicates that parasite assemblages were recurrent across years during this season, likely reflecting the strong influence of seasonal shifts in diet and zooplankton availability in shaping their parasite communities.

4.2.2. Pathological and histopathological interpretation

To date, no relevant macroscopic alterations or neoplastic lesions have been reported in *S. aurita* or other small pelagic fish species, which could be influenced by their short lifespan, limiting the

development and detection of neoplastic lesions. In contrast, these alterations were more frequently described in longer-lived demersal species (Feist et al., 2004). As observed in hakes, Marigómez et al. (2006) also reported hepatocellular nuclear pleomorphism in anchovies after the Prestige oil spill, suggesting that this alteration may be a suitable biomarker of contaminant exposure in short-living pelagic fish, where neoplastic lesions are unlikely to develop within their lifespan (Stentiford et al., 2003), although it was not observed in this study.

Nevertheless, other parasite groups may still cause relevant pathological effects. For instance, massive infections by microsporidians of the genus *Glugea* Thélohan, 1891 have recently been associated with a mortality event in round sardinella from the southern coast of Italy (Mediterranean Sea) (López-Verdejo et al., 2024), where large and abundant xenomas caused mechanical compression of visceral organs, leading to physiological stress and impaired swimming preventing them from feeding (López-Verdejo et al., 2024). However, in the present study, microsporidian xenomas were detected at much less prevalence and intensity, were considerably smaller, and did not appear to cause damage to the visceral organs. However, considering that microsporidian infections may exhibit seasonal variation and seem to likely develop and spread with high temperatures, particularly affecting smaller fish (Mansour et al., 2016; Ramdani et al., 2022), further monitoring and research on the potential impact on round sardinella's health should be performed especially during warmer periods.

Focusing on the observed histopathological alterations, the gills also appeared to be the most affected organ as previously reported for hakes. In *S. aurita*, inflammatory responses in the gills were frequently associated with the presence of ovoid structures resembling blood fluke eggs. However, in contrast to those observed in hakes, these structures did not show any defined content or any evidence of embryos or miracidia, suggesting that they might correspond to degenerated material. Although blood fluke parasites have not been reported in round sardinella neither on other species of the order Clupeiformes, their parasitic origin cannot be completely ruled out, as the occurrence of these structures and associated inflammatory responses showed a marked seasonal pattern, with higher prevalences during winter and spring. This variability could reflect seasonal dynamics in parasite exposure or other causative agents, although the lack of well-defined morphological structure prevents further identification.

On the other hand, EPC was an important pathology recurrent throughout the year, especially in autumn. This disease is characterized by the presence of cysts in the epithelial cells of gills or skin containing a wide range of intracellular bacteria from different phyla (mainly chlamydia and rickettsia) that replicate within host cells, causing high mortalities in cultured fish (Blandford et

al., 2018). In *S. aurita*, EPC may be enhanced by its pelagic schooling behaviour, which could facilitate their transmission among individuals comparable to what occurs in high densities of cultured fish (Blandford et al., 2018). Schooling dynamics are influenced by species' intrinsic characteristics and by environmental factors such as predation pressure, spawning period and food availability, which can vary daily and seasonally (Brehmer et al., 2007) and could partly explain the higher EPC prevalence observed in autumn. Moreover, seasonal environmental stressors such as temperature fluctuations and pollution have been pointed out as factors that could increase EPC prevalence (Crespo et al., 1990; Novacovsky et al., 2021). However, it is difficult to determine how these factors could influence in the prevalence and intensity of EPC from present findings. The three different morphological types of cysts observed in *S. aurita* could indicate the involvement of more than one bacterial agent. EPC types 1 and 2 could be a possible mixed infection involving different bacterial species and affecting different epithelial cell targets along the lamellae. However, given the limitations of histology alone, further molecular and ultrastructural analyses are necessary to clarify the aetiology associated with each cyst type.

Among the histopathological alterations identified in other organs, PAS-negative vacuole-like structures observed in the liver of *S. aurita* livers were similar to those previously reported in anchovies from the same study area and may probably correspond to lipid droplets rather than pathological vacuolation (Rodríguez-Romeu et al., 2022). These structures are commonly related to changes in the nutritional status of fish, reflecting energy storage processes. Certainly, the seasonal increase in vacuolization observed from summer to spring suggests the accumulation of lipid reserves in preparation for the spawning period, in accordance with round sardinella's capital breeding strategy (Albo-Puigserver et al., 2017). Moreover, MMCs were also present in *S. aurita* splenic tissue, as previously discussed for *M. merluccius*, although they appeared larger and more abundant in sardinellas, particularly during winter. Indeed, seasonality was reported to be another contributing factor influencing MMCs densities due to the seasonal environmental variability in temperature, turbidity, salinity but also to differences in the physiological condition of fish and nutritional deficiencies (Carreras-Colom et al., 2022). Moreover, the differences detected between both summer samplings suggest that interannual variability and other environmental or physiological factors beyond seasonal changes may also influence MMCs presence and density.

Regarding parasitic infections in other organs, myxozoa parasites appeared to be scarce in round sardinella. To date, the only reported myxozoa species is *Ceratomyxa truncata* Thélohan, 1895, found at low prevalence the gall bladder of *Sardinella maderensis* (Lowe, 1838) and *S. aurita* from the Senegalese coast (S Atlantic Ocean) (Kpatcha et al., 1996), and also recorded in *S. pilchardus* in the Mediterranean Tunisian coast (Mansour et al., 2022). This limited occurrence suggests that

myxozoan infections are not major components on the parasitic fauna, at least in the studied area, and without causing any apparent host reaction, as observed for hakes.

Ultimately, despite the high intensities of digeneans occurring in the digestive tract, particularly in summer, no host reactions were observed, suggesting that these parasites do not cause damage, as expected, since they are located freely in the lumen of the digestive tract without encapsulating (Hemmingsen & MacKenzie, 2001).

5. Conclusions

In summary, the combined use of complementary health indicators allowed the evaluation of fish health at different biological levels, from individual condition and energetic status to tissue alterations and host-parasite interactions. This study revealed that fish condition and parasite communities are primarily shaped by environmental factors, ontogeny and host trophic ecology. Most of the pathologies and associated infections described for both *M. merluccius* and *S. aurita* were generally focal and produced mild damage. Likewise, parasitic infections typically displayed low intensities indicating that host-parasite relationships were balanced. Therefore, no clear detrimental effects on the health condition of individuals were observed and organ functionality of these individuals is expected to remain uncompromised. Despite the low severity of infections, certain pathologies such as *A. spinosicanalis* eggs infestation in hakes' gills, EPC and microsporidian infections in sardinellas should be given special consideration due to their potentially significant impact on the health of individuals and, consequently, on fisheries. These results highlight that fish condition may vary both at local and temporal scales, as they are constantly influenced by environmental conditions and ecosystem dynamics. Therefore, the absence of severe pathological alterations does not necessarily imply the absence of environmental or biological stressors. Instead, changes in the studied health indicators (i.e. pathology, histopathology, parasitology and condition indices) may provide early warning signals of ecosystem changes or stressors before more evident impacts on fish population occur. Therefore, multidisciplinary approaches as the present one are essential to capture the complexity of factors influencing fish health, highlighting their potential role in long-term monitoring programs to gain a more comprehensive understanding of the general health status and the pathologies to which wild fish populations are prone, essential for developing effective fishery management strategies.

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CHAPTER 4.1

Clestobothrium crassiceps

Muns-Pujadas, L., Constenla, M., Dallarés, S. (2025). A fresh look at *Clestobothrium crassiceps* (Rudolphi 1819) Lühe, 1899 (Cestoda: Bothriocephalidea) two centuries later: first complete description and novel observations from its type-host in the NW Mediterranean Sea. *Parasitology Research*, 124, 138.

<https://doi.org/10.1007/s00436-025-08571-4>.

Abstract

Cleistobothrium crassiceps (Rudolphi, 1819) Lühe, 1899 is the type species of the genus *Cleistobothrium* Lühe, 1899, originally described from *Merluccius merluccius* (Linnaeus, 1758) in the Mediterranean Sea. Despite its apparently wide host and geographic ranges, this species remains poorly understood due to the lack of detailed morphological, genetic and epidemiological data. In the present study, newly collected materials of *C. crassiceps* from *M. merluccius* off Barcelona, Spain (NW Mediterranean) were used to provide the first complete description from its type-host and locality. An integrative approach was applied, combining traditional morphological techniques with confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), molecular, histological and epidemiological data. Confocal microscopy revealed key diagnostic features of the female reproductive system (*i.e.* vagina, Mehli's gland, uterus tube), while SEM analysis showed, for the first time, different distribution patterns of microtriches along the scolex and strobila. Histological observations showed the attachment mode of the scolex to intestinal folds, causing mild epithelial alterations such as attenuation of the intestinal epithelium. Prevalence and intensity of infestation with *C. crassiceps* were higher in larger fish, suggesting a role for dietary shifts and potential paratenic hosts in transmission. Phylogenetic analysis based on newly generated 28S and *cox1* sequences confirmed the monophyly of the genus *Cleistobothrium* and highlighted an intraspecific variation comparable to the genetic divergence observed between congeners *C. splendidum* and *C. cristinae*. Based on these results, a morphological reexamination of paratypes of both species was conducted, proposing *C. cristinae* as a junior synonym of *C. splendidum*.

1. Introduction

The cestode genus *Cleistobothrium* Lühe, 1899, (Bothriocephalidea: Bothriocephalidae) is mainly found parasitizing Gadiformes bony fishes. *Cleistobothrium* species can be morphologically distinguished from those belonging to other bothriocephalid genera by the presence of a sphincter surrounding the anterior aperture of the bothridia (Kuchta et al. 2008). Currently, it includes five valid species, *Cleistobothrium crassiceps* (Rudolphi, 1819) Lühe, 1899, the type species, originally described by Rudolphi (1819) (as *Bothriocephalus crassiceps*) from the European hake *Merluccius merluccius* (Linnaeus, 1758) in the Mediterranean Sea off Naples, Italy; *Cleistobothrium neglectum* (Lönnerberg, 1893) Dronen and Blend, 2003, described from *Raniceps raninus* (Linnaeus, 1758) in the Baltic Sea off Sweden (Lönnerberg, 1893); *Cleistobothrium gibsoni* Dronen and Blend, 2005, described from *Bathygadus macrops* Goode and Bean, 1885 in the Atlantic off the Gulf of Mexico (Dronen and Blend 2005); and *Cleistobothrium splendidum* Gil de Pertierra, Incorvaia and Arredondo, 2011 and *Cleistobothrium cristinae* Gil de Pertierra, Incorvaia and Arredondo, 2011, described from *Merluccius australis* (Hutton, 1872) off the Patagonian shelf of Argentina and from *Merluccius hubbsi* Marini, 1933 captured off the same locality and also off San Matías Gulf in Argentina, respectively (Gil de Pertierra et al. 2011).

Cleistobothrium crassiceps remains a scarcely known species despite apparently being widespread, reported from a wide range of fish hosts. Indeed, it has been reported from different *Merluccius* hosts: the type-host *M. merluccius* (Linnaeus, 1758) in the Mediterranean Sea (Rudolphi 1819), *Merluccius bilinearis* (Mitchill, 1814) off the Canadian and USA coasts (Cooper 1918), *Merluccius albidus* (Mitchill, 1818) off the Canadian coast in the NW Atlantic Ocean (McDonald and Margolis 1995), and *Merluccius productus* (Ayres, 1855) off the USA coast in the NE Pacific Ocean (Wardle 1935) as well as in other teleosts, congeneric hosts: *Dissostichus eleginoides* Smitt, 1898 (Gaevskaja et al. 1990, *Urophycis tenuis* Mitchill, 1814 [as *Urophycis musicki* Cohen and Lavenberg, 1884] (McDonald and Margolis 1995), among others) in the Atlantic Ocean and *Aphos porosus* (Valenciennes, 1837) (Cortés and Muñoz 2009) and *Macruronus magellanicus* Lönnerberg, 1907 in the Pacific Ocean (Oliva 2001).

The original description of *C. crassiceps* provided by Rudolphi (1819) was very brief and included no measurements or drawings. Cooper (1918) provided the first complete and detailed morphological description of this parasite up to the present date based on specimens collected from *M. bilinearis* off the NW Atlantic, Canadian and USA coasts. Given the sparsely detailed original description of this parasite provided by Rudolphi (1819) based on specimens collected off Napoli, Italy, Cooper (1918) assumed conspecificity of his materials with *C. crassiceps* from the Mediterranean Sea based on the few morphological available data (Rudolphi 1819; Molin 1861;

Diesing 1863; Ariola 1896 and 1900; Parona 1899; Barbagallo and Drago 1903). Since then and to date, a few additional studies have focused on specific morphological features of *C. crassiceps*. For instance, Rees (1958) studied in detail the structure of the scolex of this tapeworm and attempted to explore its attachment mode to the intestine of its host but was unable to draw conclusions due to the lack of specimens fixed *in situ* attached to the intestinal mucosa. Later, Gil de Pertierra et al. (2011) discussed the presence of an apical disk in the scolex, a feature that had been previously mentioned and illustrated by Cooper (1918) and thereafter confirmed by Miquel et al. (2012) using scanning electron microscopy (SEM) on specimens obtained from the type-host captured off Girona coast (NW Mediterranean). Despite these contributions, a complete morphological, ultrastructural and genetic characterization of *C. crassiceps* from the type-host and type locality (*i.e.* *M. merluccius* from the Mediterranean Sea) is currently lacking.

Given these considerations, the aim of the present study is to provide, the first complete and detailed morphological redescription including ultrastructural details and genetic characterization of *C. crassiceps* from *M. merluccius* based on newly collected material off Barcelona, Spain, in the Mediterranean Sea. Furthermore, the close interaction of this parasite with its host, its mode of attachment are analysed through a histological assessment and quantitative epidemiological data are provided for the analysed population.

2. Materials and methods

2.1. Sample collection

A total of 182 specimens of European hake were examined for the present study between 2007 and 2023. Of these, 96 specimens were captured in 2007 on board of oceanographic research vessels. Additionally, 62 specimens were captured in 2019 and 24 specimens in 2022-2023 by commercial fishing vessels from the continental shelf of the Balearic Sea (off Barcelona, Spain) at 50-250 m depth and immediately sacrificed by spinal severance (Chervy 2024). Specimens from 2007 were freshly frozen at -20 °C for further parasitological examination, those from 2019 were directly fixed in 10% buffered formalin after performing an abdominal incision for the correct fixation of internal organs and the latest (from 2022-2023) were examined fresh few hours after capture.

Once in the laboratory, fish were measured (standard length, SL), weighed (total weight, TW and eviscerated weight, EW), dissected and all organs were examined under the stereomicroscope for the presence of parasites according to a standardized protocol (Chervy 2024). During the examination of the digestive tract of hakes from 2007 and 2019, tapeworms of *Cleistobothrium*

crassiceps (Rudolphi, 1819) Lühe, 1899 were collected, counted and preserved in 70% ethanol. In the case of hakes from 2022-2023, parasites were killed in warm saline solution and a segment of two specimens was cut off and preserved in 100% molecular grade ethanol for genetic analyses.

2.2. Morphological study

A total of seven cestodes recovered from fresh hakes collected in 2022 were stained with iron acetocarmine, dehydrated through an ethanol series, cleared in clove oil and mounted in Canada balsam (Chervy 2024). Before mounting, the tegument of some pieces of strobila were removed with a scalpel blade to allow a better observation of internal structures (Jones 1990). Illustrations of mounted specimens were performed with a drawing tube attached to an Olympus BH light microscope (Olympus Corporation, Tokyo, Japan) (Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona). Measurements were performed with a stage micrometer and are shown as the range values followed, in parentheses, by the mean, the standard deviation, the number of specimens measured and the number of measurements (the latter only when more than one measurement per specimen was taken). Unless otherwise stated, all measurements are in micrometers (μm).

One of the skinned and stained pieces of strobila was soaked in Canada Balsam and examined through confocal laser scanning microscopy (CLSM) using an Olympus FluoView FV1000 confocal microscope (Servei de Microscòpia i Difracció de Raigs X, Universitat Autònoma de Barcelona). For scanning electron microscope (SEM) examination, one whole specimen was mounted on a SEM stub using conductive double-sided carbon tape and coated with a 40 nm thickness alloy of gold (60%) and palladium (40%) using an Emitech K550X sputter coater and then observed in a Zeiss Merlin high-resolution scanning electron microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) (Servei de Microscòpia i Difracció de Raigs X, Universitat Autònoma de Barcelona) and digital images were obtained. Microthrix terminology follows Chervy (2009).

Finally, an individual with the scolex still firmly attached to the intestinal mucosa of one host collected in 2019 was embedded in paraffin, sectioned into 5 μm serial sections and stained with hematoxylin and eosin (H&E) for histological observations (Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona).

Voucher specimens are deposited in the Museum für Naturkunde (Berlin, Germany) (collection “Vermes”, catalogue Entozoa, No. ZMB E.7761), the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (České Budějovice, Czech Republic)

(IPCAS C-498) and the parasitological collection of the Zoology unit of the Universitat Autònoma de Barcelona (Barcelona, Spain) (Nos. C31-C46).

Three syntypes of *C. crassiceps* (collection “Vermes”, catalogue Entozoa, No. 1807-E) from the Museum für Naturkunde were made available for examination, and high-quality images of several voucher specimens (Nos. USNM1321638-1321644, USNM1349881, USNM1369433, USNM1392752) were provided by the Smithsonian Institution (Washington D.C., United States). Additionally, two paratypes of *C. splendidum* (No. C-600) and *C. cristinae* (No. C-601) were borrowed for examination from the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences.

2.3. Molecular analyses and phylogenetic reconstruction

Total genomic DNA was extracted from the last proglottid of two specimens collected in 2022 using Qiagen™ (Valencia, California, USA) DNeasy® Tissue Kit following manufacturer instructions.

The D1–D3 regions of the nuclear large subunit ribosomal DNA (28S rDNA) and partial fragments of the cytochrome c oxidase 1 (*cox1*) mitochondrial gene were amplified (50 µl total volume) by polymerase chain reaction (PCR) using ExcelTaq™ SMOBIO® PCR Master Mix (SMOBIO Technologies, Hsinchu City, Taiwan) containing 5× concentrated master mix, a mixture of recombinant Taq DNA polymerase, reaction buffer, MgCl₂ (2 mM), dNTPs (0.2 mM), and enzyme stabilizer. Amplifications of 28S rDNA were performed using the primers and conditions described in Fyler et al. (2009). In this case, 0.5 µl of each PCR primer, and 2 µl of extracted gDNA were added to each amplification tube. For amplification of *cox1* mtDNA gene, the primers and conditions used were those described in Scholz et al. (2013) adding 1 µl of each PCR primer and 4 µl of extracted gDNA to each amplification tube. PCR amplicons were sequenced directly for both strands using the primers LSU5 and 1200F in the case of 28S rDNA and PBI-cox1F_seq and PBI-cox1R_seq for *cox1* mtDNA (Faculty of Biosciences, Universitat Autònoma de Barcelona).

Obtained sequences were assembled and edited using BioEdit v7 (Hall 1999) and submitted to GenBank under accession numbers PV258606-PV258607 (28S rDNA), and PV258609-PV258610 (*cox1* mtDNA). Sequence alignments were performed using Muscle implemented in MEGA v6. Cestode species selected for comparative sequences of 28S rDNA and *cox1* mtDNA, devoted to phylogenetic analyses, are shown in Table 1. See details for all selected comparative sequences in Brabec et al. (2015). For both alignment sets, pairwise genetic distance matrices, based on uncorrected p-distance and number of base-pair differences, were generated with MEGA v6.

Table 1. List of cestode species included for sequence alignments of 28S rDNA and *cox1* mtDNA (devoted to phylogenetic analysis) with details on host species, locality and GenBank Accession numbers.

Cestode species	Host species	Locality	GenBank numbers	
			28S rDNA	<i>cox1</i> mtDNA
<i>Clestopbothrium crassiceps</i> (Rudolphi, 1819) Lühe, 1899	<i>Merluccius merluccius</i>	Great Britain, North Sea	KR780884	KR780786
<i>Clestopbothrium crassiceps</i> (Rudolphi, 1819) Lühe, 1899	<i>Merluccius merluccius</i>	Off Barcelona, Mediterranean Sea	PV258606	PV258609
<i>Clestopbothrium crassiceps</i> (Rudolphi, 1819) Lühe, 1899	<i>Merluccius merluccius</i>	Off Barcelona, Mediterranean Sea	PV258607	PV258610
<i>Clestopbothrium cristinae</i> Gil de Pertierra, Incorvaia and Arredondo, 2011	<i>Merluccius hubbsi</i>	Off Patagonia, Atlantic Ocean	KR780901	KR780830
<i>Clestopbothrium splendidum</i> Gil de Pertierra, Incorvaia and Arredondo, 2011	<i>Merluccius australis</i>	Off Patagonia, Atlantic Ocean	KR780920	KR780827
<i>Anantrum</i> n. sp. Overstreet, 1968	<i>Synodus myops</i>	Off Ocean Springs, Atlantic Ocean	-	KR780831
<i>Bothriocephalus scorpii</i> (Müller 1776) Cooper, 1917	<i>Myoxocephalus scorpius</i>	Great Britain, North Sea	-	KR780788
<i>Bothriocephalus</i> cf. <i>carangis</i> Yamaguti, 1968	<i>Uraspis uraspis</i>	Indonesia, Indic Ocean	-	KR780790
<i>Marsipometra hastata</i> Linton, 1898	<i>Polyodon spathula</i>	Off Mississippi, Atlantic Ocean	-	KR780777

For the *cox1* mtDNA sequences dataset, Gblocks 0.91b implemented on the Phylogeny.fr site (Dereeper et al. 2008) was used to select blocks of evolutionarily conserved sites. Maximum likelihood (ML) and Bayesian inference (BI) algorithms were used for phylogenetic tree reconstruction after the determination of the best-fit model of nucleotide substitution with jModelTest v2.1.4 (Darriba et al. 2012) using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), respectively. The best-fitting model selected for ML algorithm was the TIM1 + G model (nst = 6, rates = gamma, ngammacat = 4) while for BI was HKY + G (nst = 2, rates = equal, ngammacat = 4). ML analyses were performed in PhyML v3.0 (Guindon et al. 2010) with a non-parametric bootstrap of 100 replicates. BI analyses were carried out on the Phylo.org site on the CIPRES Science Gateway v3.3 (Miller et al. 2010). Log likelihoods were estimated over 10,000,000 generations using Markov Chain Monte Carlo searches on two simultaneous runs of four chains, sampling trees every 1000 generations. The first 25% of the

sampled trees were discarded as ‘burn-in’ and a consensus topology and nodal support estimated as posterior probability values (Huelsenbeck et al. 2001) were calculated from the remaining trees.

2.4. Data analysis

Infestation parameters such as prevalence (P) and mean intensity (MI) (with the 95 % confidence interval (CI)) of the cestode parasite *C. crassiceps* were calculated following Bush et al. (1997) for each fish size established range (<20 cm and >20 cm standard length), related to the ontogenetic shift in the feeding behaviour and diet of the host (D'Iglio et al. 2022). General fish condition was assessed through Le Cren's relative body condition index ($Kn = EW / (\alpha \times SL^\beta)$) (Le Cren 1951), where α and β are the slope and the intercept of the weight-length relationship representing the entire dataset of sampled fish. Fish body size (standard length, SL), Kn and MI were first plotted for visually assessing data distribution and tested for normality and homoscedasticity using the Shapiro-Wilk test and Levene's test, respectively. Fish size effect and Kn were tested for P and MI using Generalized Linear Models (GZM), applying a logistic distribution for P and a negative binomial distribution for MI.

Data analysis was performed on RStudio software, version 4.1.2. Statistical significance was set at 0.05.

3. Results

3.1. Genetic results and phylogenetic relationships

Two partial identical 28S rDNA sequences (1213 and 1185 nt in length) and two partial *cox1* mtDNA sequences (526 and 549 nt in length) differing by 0.076 % (4 nt) were generated from two specimens of *C. crassiceps* from the Spanish coast, NW Mediterranean Sea.

The two newly generated 28S rDNA sequences of *C. crassiceps* were identical to that of *C. crassiceps* from the North Sea and differed by 0.08% (1 nt) from those of congeneric *C. cristinae* and *C. splendidum*, whose sequences were identical.

A representative phylogenetic tree constructed using *cox1* mtDNA sequences is shown in **Fig. 1**. The two newly generated sequences of *C. crassiceps* formed a supported clade with the sequence of the same species from the North Sea, from which they differed by 0.57% (3 nt). High bootstrap and posterior probability values characterized the clade comprising *C. cristinae* and *C. splendidum*,

which differed by 0.76% (4 nt) between them and by 3.99-4.56% (21-24 nt) and 4.37-4.94% (23-26 nt), respectively, from the newly generated sequences. Overall, all *Clestobothrium* sequences formed a strongly supported monophyletic group clearly differentiated from those of the other Bothriocephalid. With respect to other bothriocephalids included in the analysis, sequences of *Clestobothrium* species differed by 18.06-18.44% (95-97 nt) from *Anantrum* n. sp., by 18.25-18.82% (96-99 nt) from *B. scorpii*, and by 17.68-19.01% (93-100 nt) from *B. cf. carangis*.

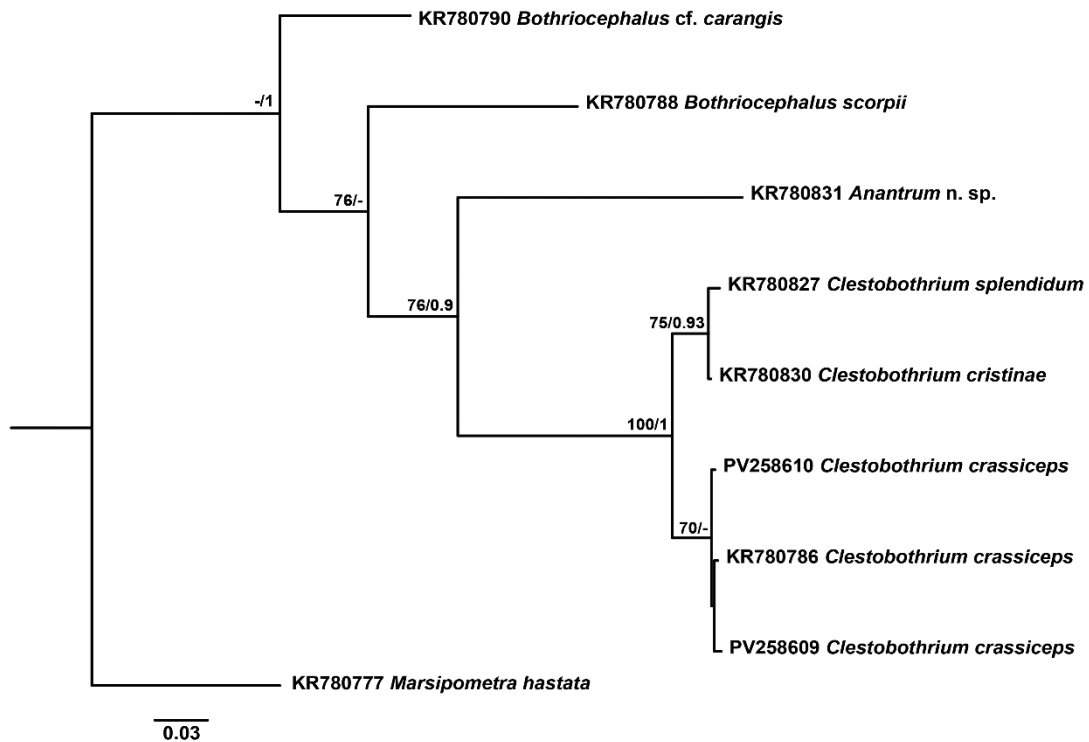


Fig. 1 Maximum likelihood (ML) phylogram reconstructed using newly generated *cox1* mtDNA sequences for *Clestobothrium crassiceps* (PV258609 and PV258610) and retrieved sequences from GenBank for other members. Outgroup: *Marsipometra hastata*. Nodal support from ML and Bayesian inference (BI) analyses indicated as ML/BI. Bootstrap values lower than 70 and posterior probability values lower than 0.9 are omitted. The scale bar indicates the expected number of substitutions per site.

3.2. Species delimitation

As can be readily appreciated in the phylogenetic tree constructed from the highly variable *cox1* gene sequences (**Fig. 1**), *C. splendidum* and *C. cristinae* grouped together forming a highly supported clade, and genetic variation for this gene between these two species is equivalent to that observed between the two newly generated sequences of *C. crassiceps* from the NW Mediterranean. Besides these genetic results, morphological reexamination of paratypes from *C. splendidum* and *C. cristinae* revealed that some features pointed out by Gil de Perterra et al.

(2011) as differential characters to distinguish these two species in their original description should not be considered as such. These authors argued that *C. cristinae* differed from *C. splendidum* in its smaller size and lower number of proglottids, the shape and the length/width ratio of the proglottids and proglottids width/cirrus sac length ratio. These characteristics can vary considerably depending on the worm size either due to allometric growth or intraspecific phenotypic variability. Moreover, Gil de Perterra et al. (2011) also pointed out as differential characters the absence of genital atrium and testes completely surrounded the posterior region of the ovary in *C. cristinae* and the presence of a vaginal sphincter, together with the equatorial location of the ovary in *C. splendidum*. With respect to the first feature, reexamination of type material revealed the presence of a genital atrium in *C. cristinae*, although less conspicuous than in *C. splendidum* probably due to the smaller size of the worms. The location of the testes relative to ovary was observed to be variable among proglottids but in both species testes partially surrounded the posterior region of the ovary. The presence of a vaginal sphincter in *C. splendidum* was not observed in any of the two species. Vaginal microvilli from the terminal part located posterior to the opening of the cirrus-sac, which were clearly visible, might have been misinterpreted as a muscular sphincter. Finally, a post-equatorial location of the ovary was observed both in *C. splendidum* and in *C. cristinae* during reexamination of types.

Therefore, based on the genetic results and the morphological reexamination of the paratypes from *C. splendidum* and *C. cristinae*, synonymization of both species is proposed herein, with *C. cristinae* becoming a junior synonym of *C. splendidum*.

3.3. Morphological redescription of *Cleistobothrium crassiceps* (Rudolphi, 1819) Lühe, 1899

Synonyms: *Bothriocephalus crassiceps* Rudolphi, 1819; *Bothriocephalus pilula* Leuckart, 1819; *Dibothrium crassiceps* (Rudolphi, 1819) Diesing, 1850.

Type-host: European hake *Merluccius merluccius* (Linnaeus, 1758) [as *Merluccius vulgaris* Fleming, 1828 sensu Thompson (1844), Molin (1861), Diesing (1863), Ariola (1896 and 1900), Parona (1899) and Barbagallo and Drago (1903)] (*Gadiformes: Merlucciidae*).

Type locality: Mediterranean Sea, off Napoli, Italy.

Additional hosts and localities: provided as supplemental information in Table S1.

Site of infection: Stomach and intestine.

Redescription

[Based on five whole mounts of gravid worms, one gravid and two mature (non gravid) worms preserved in ethanol, one piece of skinned gravid strobila devoted to confocal electron microscopy and one mature specimen observed with SEM].

Cestodes anapolytic, total length 20–34 (24.2 ± 5.8 ; 5) mm, maximum width 854–1878 (1356 ± 385 ; 5), at level of late mature-early gravid proglottids (at about three fourths of body length in most specimens) (Fig. 2a). Strobila with 34–122 (60.3 ± 41.4 ; 5) immature proglottids, 10–20 (15.6 ± 3.8 ; 5) mature proglottids and 9–37 (25.8 ± 10.5 ; 5) gravid proglottids (**Fig. 2a**). Scolex acraspedote, compact, subglobular, 592–1238 (980 ± 244 ; 6) long, 641–1143 (855 ± 195 ; 7) wide in lateral view, width in dorsoventral view equal to bothridial width; bothridia two in number, tear-shaped in dorsoventral view, with fused posterior margins and very thick and fleshy rims, 534–1202 (894 ± 211 ; 8) long by 529–686 (585 ± 88 ; 3) wide (**Fig. 2b**; **Fig. 3a**), covered with narrow gladiate spinitriches *ca.* 6.5 long by *ca.* 0.75 wide on their basis (**Fig. 3b-c**); bothridial aperture tear-shaped, small, opening into a deep and spacious hollow, surrounded by a thick muscular sphincter; proximal bothridial surface with a more or less pronounced medial longitudinal depression continuous with an apical groove that runs along the apex of the scolex between two fleshy lip-like structures (**Fig. 2b**); scolex apical surface covered with narrow gladiate spinitriches *ca.* 1.7 long by *ca.* 0.45 wide on their basis (**Fig. 3d-e**); Scolex cover of narrow gladiate spinitriches regularly interrupted by oval worn patches each containing one papilla-like structure, consisting in a circular elevation of the tegument *ca.* 1.5 in diameter (**Fig. 3f**). Proglottids craspedote, wider than long, first immature proglottid 108–196 (153 ± 43 ; 5) long by 534–569 (551 ± 24 ; 2) wide; first mature proglottid 137–412 (206 ± 116 ; 5) long by 745–1382 (1059 ± 227 ; 5) wide (**Fig. 2c**); first gravid proglottid 244–441 (318 ± 84 ; 5) long by 863–1634 (1303 ± 301 ; 5) wide (**Fig. 2d**); second-to-last gravid proglottid 458–927 (650 ± 199 ; 5) long by 833–1304 (1144 ± 194 ; 5) wide, last gravid proglottid narrower than preceding gravid proglottids, 510–1439 (794 ± 370 ; 5) long by 637 – 1220 (924 ± 214 ; 5) wide. First immature proglottids covered with narrow gladiate spinitriches *ca.* 3 long by *ca.* 0.5 wide in their basis interspersed with capilliform filitriches with coiled tip, bearing papilla-like structures consisting in small circular protuberances *ca.* 1.5 in diameter (**Fig. 3g-h**). Narrow gladiate spinitriches and bumps gradually disappearing in maturing proglottids until only capilliform filitriches and a smooth surface can be observed (**Fig. 3i**). Velum of all proglottids covered with narrow gladiate spinitriches *ca.* 4 long by *ca.* 0.65 wide on their basis (**Fig. 3j**). Random presence of spurious segmentation in 14.3–58.8 % (33.5 ± 16.9 ; 5) of mature and 24.3–56.7 % (46.8 ± 13.3 ; 5) of gravid proglottids, dorsoventral margins of spurious segments exceptionally reaching medial line of proglottid (**Fig. 2a**; **Fig. 3k**). Boundary between proglottids poorly visible.

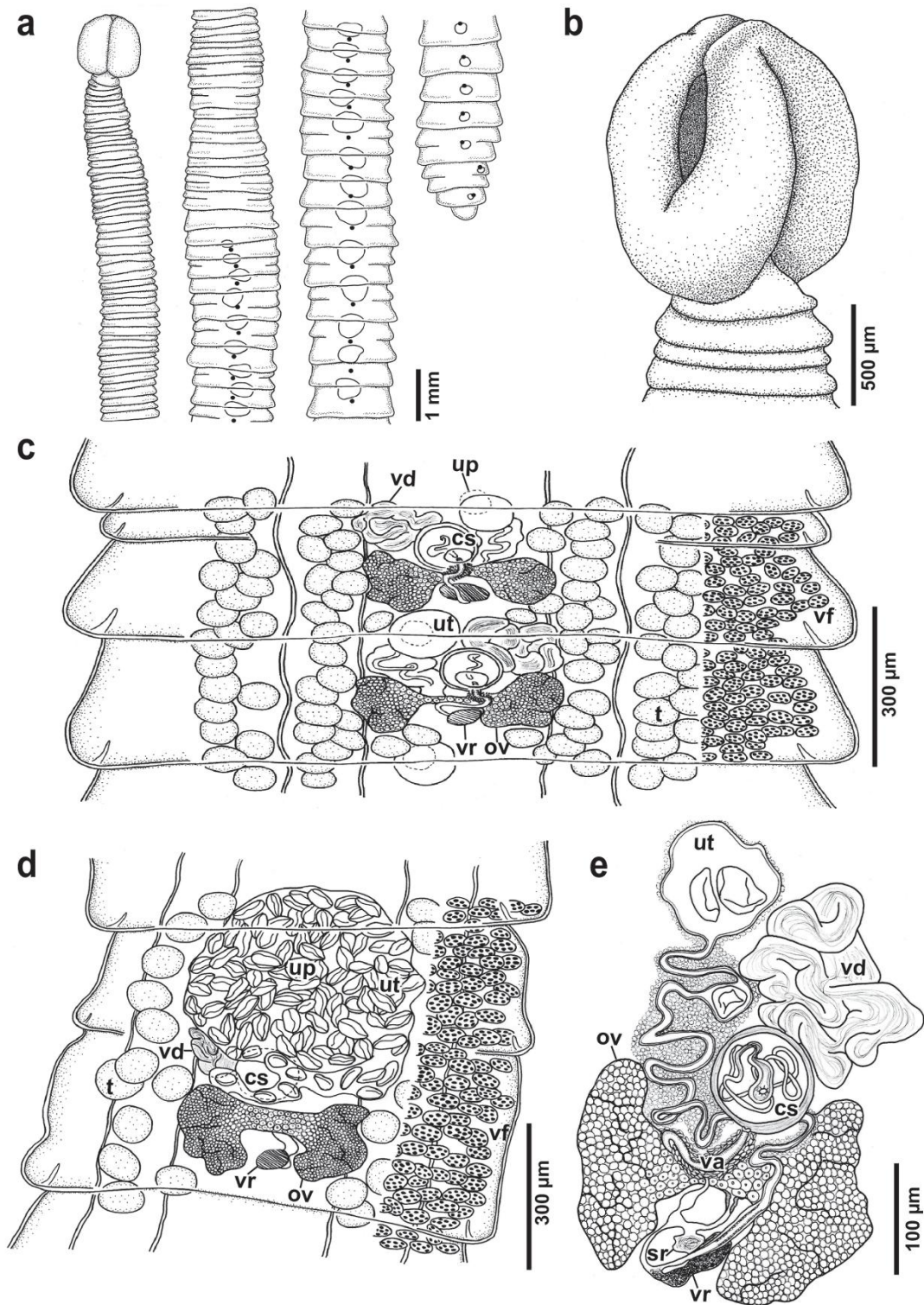


Fig. 2 Line drawings of *Clestobothrium crassiceps* (Rudolphi, 1819) Lühe, 1899 from *Merluccius merluccius* (Linnaeus, 1758) from the Mediterranean Sea off Barcelona, Spain. **a** outline of entire specimen, dorsoventral view; **b** scolex, dorsoventral view; **c** mature proglottids, dorsal view; **d** gravid proglottid, ventral view; **e** genitalia. *Abbreviations:* cs, cirrus-sac; ov, ovary; t, testicles; sr, seminal receptacle; up, uterine pore; ut, uterus; va, vagina; vd, vas deferens; vf, vitelline follicles; vr, vitelline reservoir.

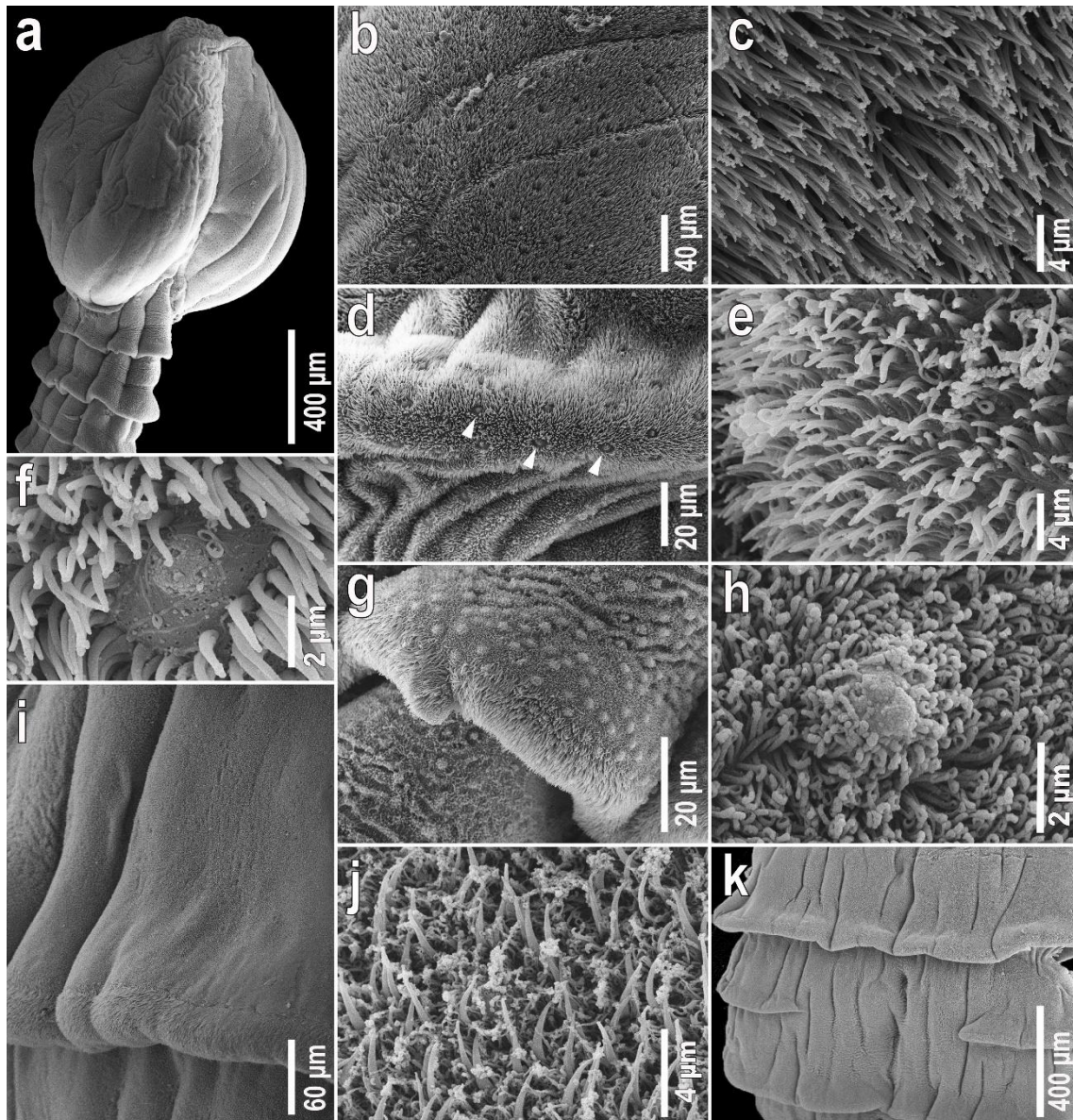


Fig. 3 Scanning electron micrographs of *Clestopothrium crassiceps* (Rudolphi, 1819) Lühe, 1899 from *Merluccius merluccius* (Linnaeus, 1758) from the Mediterranean Sea off Barcelona, Spain. **a)** scolex, dorsoventral view; **b)** gladiate spinitriches covering scolex bothridial surface; **c)** higher magnification of b; **d)** gladiate spinitriches covering the scolex apical surface interrupted by oval worn patches (arrowheads); **e)** higher magnification of d; **f)** detail of oval worn patches with one papilla-like structure; **g)** gladiate spinitriches interspersed with capilliform filitriches covering first immature proglottides; **h)** capilliform filitriches with coiled tip, bearing papilla-like structures; **i)** capilliform filitriches covering the surface of mature proglottides and gladiate spinitriches covering the velum of proglottides; **j)** detail of the gladiate spinitriches covering the velum of proglottides; **k)** mature proglottid with spurious segmentation.

Genital pore dorsal, median, equatorial, immediately posterior to spurious articulations, when present; pore conspicuous, oval, opening into a short hermaphroditic duct or genital atrium (**Fig. 2c**). Cirrus-sac globular to slightly oval (**Fig. 4**), vaguely displaced anteriorly with respect to genital pore, irregularly alternating sinistrally or dextrally with respect to genital pore, 81–98 (93 ± 6.2 ; 6) long by 83–100 (94 ± 5.6 ; 6) wide, thick-walled (**Fig. 2c, e**; **Fig. 4**); cirrus unarmed, coiled inside the cirrus-sac (**Fig. 2e**). Internal or external seminal vesicle not observed. Vas deferens heavily coiled, compact, located antero-lateral to cirrus-sac (**Fig. 2e**), almost reaching anterior margin of proglottid (**Fig. 2c**). Testes slightly oval, 57–88 (67 ± 8.9 ; 5, 15) long by 43–74 (52 ± 8.5 ; 5, 15) wide, arranged in a single layer, distributed across the full length of the proglottid and in two lateral fields each divided into an external narrow field and an internal wider field, external and internal lateral fields separated by excretory ducts (**Fig. 2c-d**); total number of testes per proglottid 30–38 (34 ± 3 ; 5).

Vaginal opening immediately posterior to opening of cirrus-sac within genital atrium (**Fig. 2c**). Vagina posterior to cirrus-sac, sinuous, narrowing as it reaches the ovarian isthmus and runs posteriorly between ovarian lobes (**Fig. 2e**; **Fig. 4**); slightly widening before joining the oviduct near the posterior margin of ovary (**Fig. 2e**). Seminal receptacle present, opening into the vagina just at its union with the oviduct (**Fig. 2e**). Ovary bilobed, H-shaped in dorsoventral view, in posterior region of proglottid, 124–155 (136 ± 12 ; 5) long by 281–438 (338 ± 63 ; 5) wide (**Fig. 2c-d**). Ovarian isthmus ventral, just slightly posterior to cirrus-sac, containing big ova of *ca.* 13 in diameter in fully mature proglottids (**Fig. 2c-e**). Oocapt small, muscular, located just posterior to ovarian isthmus, opening into a narrow oviduct that widens when joining the vagina and then narrows again as it runs towards the posterior margin of the ovary (**Fig. 2e**). At this point, it turns anteriorly and receives the vitelline duct from the vitelline reservoir. Vitelline reservoir irregularly oval in shape, ventral to ovary, 26–33 (29 ± 3.6 ; 3) long by 48–76 (57 ± 17 ; 3) wide (**Fig. 2d-e**). Vitelline follicles circummedullar, interrupted dorsally at level of genital pore (**Fig. 2c**) and ventrally at level of dilated uterus-sac in gravid proglottids (**Fig. 2d**), oval in shape, 24–48 (37 ± 6.2 ; 5, 15) long by 19–38 (25 ± 5.3 ; 5, 15) wide in late mature proglottids, increasing in size in gravid proglottids. Oviduct widening as it runs anteriorly, surrounded by an inconspicuous and diffuse Mehli's gland located at the level of the vitelline reservoir, and then becoming a uterine tube (**Fig. 2e**). Uterine tube long, coiled, extending towards the anterior region of the proglottid laterally to cirrus-sac (on the opposite side to vas deferens) (**Fig. 2e**; **Fig. 4**). Uterus preformed, tubular, irregularly alternating sinistrally or dextrally with respect to genital pore (**Fig. 2c**; **Fig. 4**), enlarged in gravid proglottids forming a circular dilatation full of eggs in anterior region of proglottid (**Fig. 2d**). Uterine pore ventral, median, near the anterior margin of proglottid (sometimes partially covered by velum of precedent proglottid), conspicuous, round (**Fig. 2d**).

Eggs oval, 52–67 (59 ± 4.2 ; 5, 12) long by 36–45 (40 ± 3.3 ; 5, 12) wide, thin-walled, pale-yellow in colour, operculated (**Fig. 2d**).

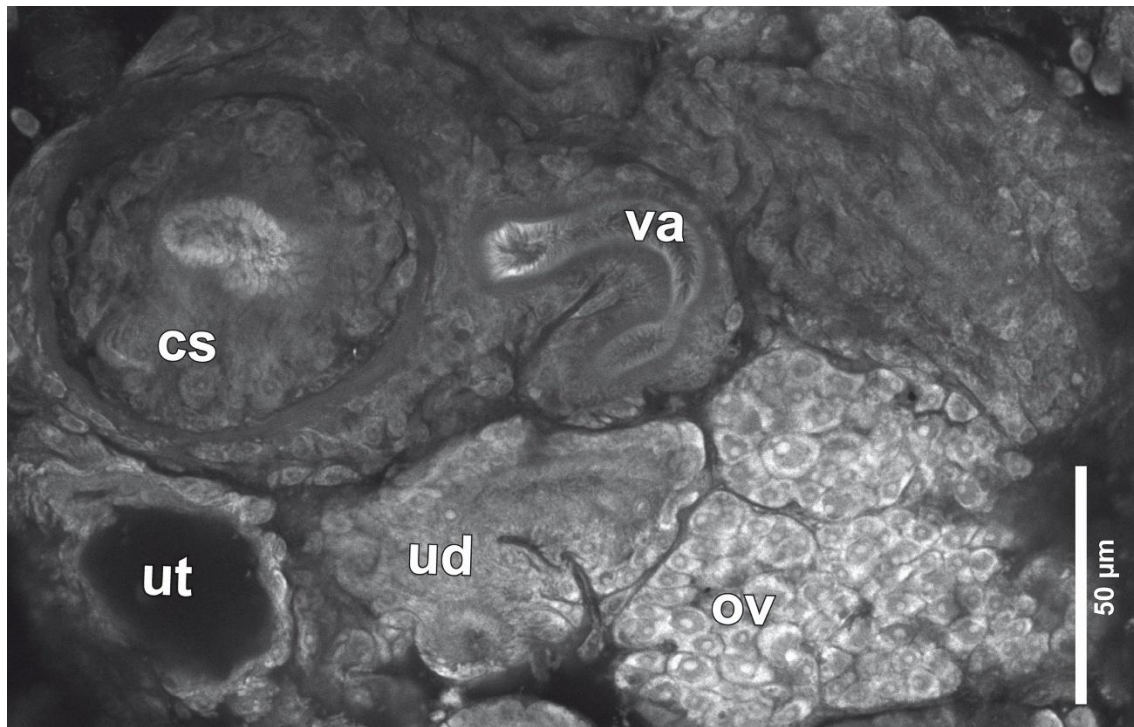


Fig. 4 Confocal laser microscopy micrograph showing genitalia of *Clestobothrium crassiceps* (Rudolphi, 1819) Lühe, 1899 from *Merluccius merluccius* (Linnaeus, 1758) from the Mediterranean Sea off Barcelona, Spain. *Abbreviations:* cs, cirrus-sac; ov, ovary; ud, uterine duct; ut, uterus; va, vagina

Remarks

The current redescription based on new materials of *Clestobothrium crassiceps* from *Merluccius merluccius* off the Mediterranean Sea aligns with Rudolphi (1819) original description. However, a precise comparison was not possible due to the absence of measurements and detailed information in the latter. When comparing our materials with those provided by Cooper (1918) redescription of *C. crassiceps* from *M. bilinearis* off the North Atlantic Ocean, some differences mostly related to size variation become apparent. For instance, Cooper measurements of the scolex were on average smaller than those reported herein (592–1238 μm long by 641–1143 μm wide vs. 640–1080 μm long by 520–900 μm wide), despite falling within the range observed in present samples. Testes were on average smaller in the new material (67 μm long by 52 μm wide vs. 125 μm long by 40 μm wide), and less abundant (30–38 vs. 40–50 per proglottid) compared to those provided by Cooper (1918). The cirrus-sac was more globular to slightly oval in the new specimens, whereas it was more elliptical to slightly oval in the Atlantic ones. Its width was similar in both cases measuring 81–98 μm long by 83–100 μm wide in the Mediterranean samples, and

128-162 μm long by 87-116 μm wide in the Atlantic ones (Cooper 1918). Vitelline follicles were also smaller in present samples than in those described by Cooper (1981) (24-48 μm long by 19-38 μm wide vs. 60 μm long by 30 μm wide), as well as eggs (52-67 μm long by 36-45 μm wide vs. 75 μm long by 40 μm wide), and the ovary was shorter and wider (124-155 μm long by 281-438 μm wide vs. 270 μm long by 130 μm wide). In the present redescription the presence of two types of microtriches which Cooper (1918) already noticed and referred to as “cirri” all over the scolex, and “stouter spinilets” on the posterior borders of the proglottids, was confirmed. Therefore, present results confirm the existence of intraspecific size variation between *C. crassiceps* specimens from the Mediterranean Sea and the Atlantic Ocean, as previously suggested by Gil de Pertierra et al. (2011) based on Rudolphi (1819) type material and Rees (1958) voucher specimens from the Atlantic Ocean. In relation to differences observed between *C. crassiceps* and other congeneric species, most of the differences reported by Dronen and Blend (2003) when they redescribed *C. neglectum* and compared it with the description of *C. crassiceps* provided by Cooper (1918) are in agreement with the present redescription of *C. crassiceps* from the NW Mediterranean Sea. However, Dronen and Blend (2003) highlighted that the eggs of *C. neglectum* were smaller and without operculum and that the ovary was larger compared to the observations made by Cooper (1918) of *C. crassiceps*. In the present case though, we observed that the eggs of *C. crassiceps* were actually slightly smaller than those of *C. neglectum* (52–67 [59] long by 36–45 [40] wide vs. 65–70 [68] long by 32–40 [35] wide), and that the ovary was larger in *C. crassiceps* compared to *C. neglectum* (338 vs. 250 wide). This lack of agreement with Dronen and Blend (2003) comparisons are in all likelihood due to the differences observed between our samples and those provided by Cooper (1918) and highlighted above. Most of the differences indicated by Dronen and Blend (2005) for distinguishing *C. crassiceps* from *C. gibsoni* are consistent with present observations. However, these authors mentioned that the size of the ovary from *C. gibsoni* was wider than the ovary from *C. crassiceps* described by Cooper (1918), but it appeared to be of a similar width compared to present samples (275–400 [340] vs. 281–438 [338] wide). This mismatch is, again, likely due to the differences observed between our samples and those made by Cooper (1918). Moreover, *C. crassiceps* specimens from the Mediterranean also display wider than long mature and gravid proglottids, as observed in *C. gibsoni*. This contrasts with Atlantic specimens of *C. crassiceps*, which Cooper (1918) described as having longer than wider mature proglottids.

Clestopothrium splendidum (now synonymized with *C. cristinae*, see section 3.2.) differs from *C. crassiceps* in having three pairs of osmoregulatory canals on each side of the proglottids (vs. two pairs), and having a straight uterus (vs. sinuous) as Gil de Pertierra et al. (2011) already noted.

Other differences between both species mentioned by these authors were that *C. crassiceps* did not have a genital atrium, that the ovary was posterior and markedly folliculate, and that testes were not surrounding the ovary posteriorly compared to *C. splendidum*, in which the ovary was equatorial and slightly folliculate, and some testes were surrounding the ovary posteriorly. However, in present samples a conspicuous genital atrium receiving the vagina and the ejaculatory duct was observed, the ovary was post-equatorial in both species and was not folliculate, and also that a few testes were usually located posterior to the ovary in both mature and gravid proglottids. Although Gil de Pertierra et al. (2011) mentioned that the presence of two types of microtriches (*i.e.* capilliform filitriches and gladiate spinitriches) was also a feature shared with *C. crassiceps*, present SEM images also revealed the presence of papilla-like structures similar to those found in *C. splendidum* and referred to as tumuli (dome-shaped evaginations) in Gil de Pertierra et al. (2011) description. The distribution of microtriches between the two species is similar on the surface of proglottids but varies on the scolex; capilliform filitriches were observed only in proglottids from *C. crassiceps*, whereas in *C. splendidum* they were covering the central surface of the apical disk from the scolex.

It is worth to mention an interesting feature of the genus *Clestobothrium*: spurious segmentation. For *C. crassiceps* this feature has been described in some detail and illustrated only by Cooper (1918), while Wagener (1854), Diesing (1863) and Johnstone (1909) only referred succinctly to it. Cooper (1918) considered it as a distinctive feature for *C. crassiceps* and distinguished secondary segmentation occurring in the anterior region of the strobila, which is involved in the formation of new proglottids, from what he called “spurious articulations” (following Wagener’s expression “articulatio spuria”) and are observed in the posterior part of the strobila. He remarked that the dorsoventral margins of these false segments never reached the medial line of the proglottid. Dronen and Blend (2003, 2005) described the same spurious segmentations for *C. neglectum* and *C. gibsoni* using the term “secondary pseudostrobilisation”, while Gil de Pertierra et al. (2011) used indistinctively the terms “secondary segmentation” and “spurious articulation”. As said, Cooper (1918) considered these as different phenomena. As regards the present redescription, spurious articulations were frequently noticed at different degrees of development (*i.e.* from incipient constrictions at lateral margins to well-developed margins crossing all the proglottid and almost reaching the medial line) along the whole strobila. This phenomenon should not be confused with incomplete segmentation, which occurs in several bothriocephalid genera (see Kuchta et al. 2008) and that refers to a partial separation of individual proglottids.

3.4. Histological observations

In histological sections, the cestode was observed in the intestinal lumen, attached with the two muscular bothridia to intestinal folds (**Fig. 5a**). The entire tegument of the scolex was covered with spiniriches (**Fig. 5b**), which occasionally came into contact with the epithelial surface and developed adhesions or synechiae between them. Some synechiae were also observed between the spiniriches of the strobila and the intestinal epithelium (**Fig. 5c**). No inflammatory reaction of the fish against the cestode was observed, but there was attenuation (**Fig. 5d**), mild erosion and sloughing of the intestinal epithelium in contact with the parasite (**Fig. 5e**). Multifocal, brown-pigmented intracellular debris was also observed in the intestinal epithelium adjacent to the parasite (**Fig. 5f**).

3.5. Epidemiology

Overall, 41.01% of the examined hakes were infected by *C. crassiceps* with a MI of 1.95 (1.49 – 2.40 CI 95%) individuals per fish. Differences in parameters of infestation of *C. crassiceps* according to the host size were found. Larger specimens (SL > 20 cm) were significantly more infected than the smaller ones (SL ≤ 20 cm) both in terms of P (47.83% vs. 33.72%; GLM, $\chi^2 = 9.11$, $p = 0.003$) and MI (2.39 (1.65 – 3.11 CI 95%) vs. 1.28 (1.10 – 1.44 CI 95%) specimens per fish; GLM, $\chi^2 = 12.91$, $p < 0.001$). No significant differences were detected between Kn and P or MI of *C. crassiceps* (GLM, $p > 0.05$).

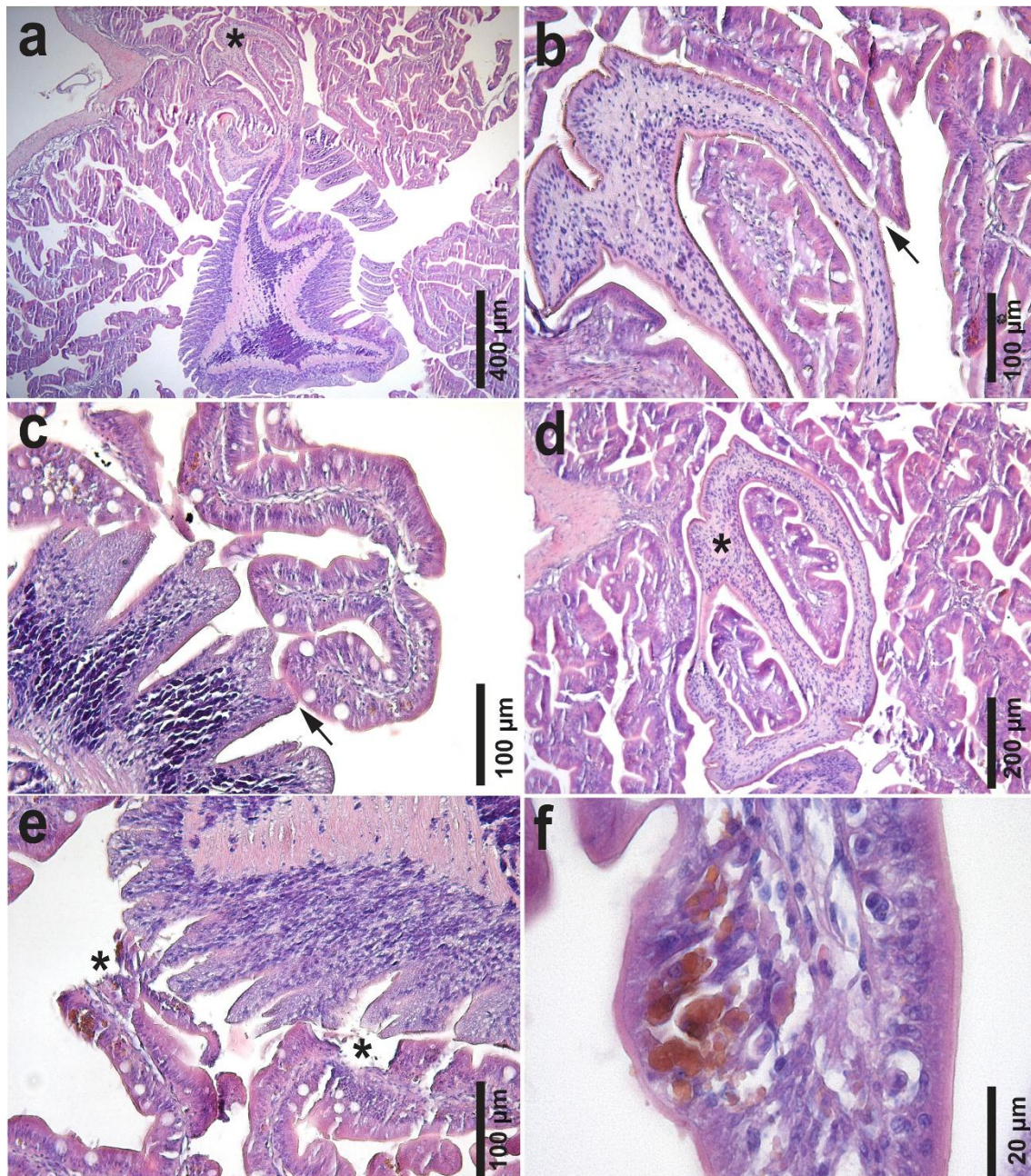


Fig. 5 Histological sections (H&E) of a specimen of *Cleistobothrium crassiceps* (Rudolphi, 1819) Lühe, 1899 attached to the intestinal mucosa of *Merluccius merluccius* (Linnaeus, 1758) from the Mediterranean Sea off Barcelona, Spain. **a)** general view of the scolex (*) attached to the intestinal folds; **b)** detail of a muscular bothridia attached to a portion of the intestinal mucosa. Note the spinitriches in the tegument in close contact with the adjacent intestinal epithelium (arrow); **c)** detail of a synechia (arrow) between the spinitriches of the scolex and the adjacent intestinal epithelium; **d)** attenuation of the intestinal epithelium in close contact with the scolex (*); **e)** sloughing of the intestinal epithelium (*) in contact with the scolex of the parasite. Note brown-pigmented intracellular debris in the intestinal epithelium; **f)** detail of the pigmented intracellular debris.

4. Discussion

The present study provides the first complete morphological redescription of *Cleistobothrium crassiceps* from its type-host (*i.e.* *Merluccius merluccius*) and type locality (*i.e.* the Mediterranean Sea), after the original description provided by Rudolphi (1819). Given the taxonomic challenges associated with species of *Cleistobothrium* and the limited information available on this species, the incorporation of molecular, histological and epidemiological data in this study represents a crucial step for clarifying its diagnostic traits and understanding its potential impact on the fish host.

An integrative approach involving traditional morphological identification and advanced imaging techniques (*i.e.* confocal microscopy, SEM), molecular and ecological data is fundamental for an accurate species characterization (Nadler and De León 2011; Rojas et al. 2025). Morphological features in parasites can be highly variable, not only among developmental stages and host species but also within individuals of the same host and development stage (Kuchta et al. 2024). In this study, confocal microscopy has been proven to be particularly useful in visualizing the distribution of genitalia and female reproductive organs in *C. crassiceps* as some key diagnostic features are challenging to observe with light microscopy. Similarly, it has been also useful for the study of tentacle hook patterns in trypanorhynch cestodes (Elliott 2020; Neitemeier-Duventester et al. 2022).

Phenotypic plasticity can lead to species misidentification, and a good example within the genus *Cleistobothrium* is the misinterpretation of *C. cristinae* as a distinct species from *C. splendidum* (Gil de Pertierra et al. 2011), which actually belong to the same species, as demonstrated here. In the present study, molecular results revealed minimal genetic divergence between these two species, comparable to the intraspecific variation observed within specimens of *C. crassiceps* from the same sampling area and fish host. Moreover, morphological reexamination indicated that previously identified distinguishing traits between these species reported by Gil de Pertierra et al. (2011) were not correctly assessed and that some of them fell within the expected range of phenotypic variation, which further justifies the synonymization of both species. A similar pattern of morphological variability was observed in *C. crassiceps*, potentially linked to host-related or geographical variation. Interestingly, molecular data showed that *C. crassiceps* sequences from the Mediterranean were more closely related to those from the North Sea than to each other, suggesting a genetically homogeneous population of this parasite across the Atlantic and Mediterranean. However, additional genetic data from more specimens and geographic locations

would be necessary to understand the genetic variability and population structure of this species across its whole distribution range.

The morphology of the scolex and their associated structures (*e.g.*, bothridia, acetabula, rostellums) in cestodes determines their attachment mechanisms and the extent of interaction with host tissues. In *C. crassiceps*, the presence of two bothridia suggests an attachment mode adapted to grasping the intestinal folds, although the potential effects on host tissue remain unknown. To date, only Rees (1958) suggested that these bothridia might function similarly to the acetabula in Cyclophyllidea, generating suction and possibly engulfing portions of the intestinal mucosa through the sphincter contraction. However, this hypothesis could not be confirmed to date due to the lack of specimens fixed *in situ* attached to the intestinal mucosa. The present study provides for the first-time a histological section of the scolex of *C. crassiceps* attached to the intestinal mucosa of its type-host, *M. merluccius*, where the engulfing of intestinal folds by the bothridia is confirmed. Although cestodes generally do not cause severe damage to the intestinal wall because they do not penetrate deeply into it (Bosi et al. 2022), the morphology of the scolex and its anchoring structures are responsible for the extent of the damage they can cause to the host (Scholz et al. 2021). The comparison of our findings with those on other Bothriocephalid species highlights both similarities and differences in attachment mechanisms and their associated damage in fish hosts. For example, the Asian fish tapeworm (*Bothriocephalus acheilognathi* Yamaguti, 1934) attaches to the gut wall by its bothria, which engulf the intestinal folds, causing compression of the mucosal epithelium, focal pressure necrosis, haemorrhage, ulceration and focal inflammatory response in cyprinids (Scholz et al. 2012). Although a similar attachment is observed by *C. crassiceps* in the hake's intestine, no significant alterations of the intestinal mucosa in contact with the scolex are observed (neither necrosis, haemorrhages, nor inflammation). The bothridia of *B. scorpii* (Müller, 1776) Cooper, 1917, were reported to function as elongated pincers, gripping the folds of the intestinal mucosa from its hosts *Scophthalmus maximus* (Linnaeus, 1758) and *Rhombus laevis* (Linnaeus, 1758), but this interaction resulted in minor structural changes at the attachment site including reduced epithelial thickness and a slight increase of fibrous tissue (Rees 1958; Davey and Peachey 1968). A more pronounced response caused by *B. scorpii* was observed in *Pseudophycis bacchus* (Forster, 1801). The host responded by depositing collagenous connective tissue around the scolex, leading to villi loss at the attachment area, marked fibrosis, and scolex encapsulation forming a swelling in the fish gut wall (McKinnon and Featherston 1982). Mild histological alterations (synechiae, attenuation of the intestinal epithelium with intracellular waste material and scarce erosion of the intestinal epithelium) have been found associated to the presence of *C. crassiceps* in the intestinal lumen.

Other authors also reported reduction of epithelial thickness associated to the presence of bothriocephalids in the intestine (Rees 1958; Davey and Peachey 1968, Scholz et al. 2012). These changes together would indicate a functional impairment of the intestine, although very mild in the case of *C. crassiceps*. Changes in enzymatic activity and protein synthesis, or even secretion of toxic material is also confirmed in other bothriocephalids (Scholz et al. 2021). Heavy infections by cestodes of this group in other fish species such as *Eubothrium salvelini* (Schränk, 1790) Nybelin, 1922 in coho salmon *Oncorhynchus kisutch* (Walbaum, 1792), have been shown to negatively affect growth, condition and fitness or even leading to fish mortalities (e.g., *Triaenophorus nodulosus* (Pallas, 1781) Rudolphi, 1793 in juvenile salmonids) (see Scholz et al. 2021). However, no significant correlations were detected between hosts' condition factor and parasitological indicators in the present case, indicating that infections by *C. crassiceps* do not compromise hake condition.

Basic aspects of the biology of *C. crassiceps*, such as their life cycle, remain also poorly understood. Though, it can be presumed what has been described for other bothriocephalidean species: planktonic crustaceans (i.e. copepods), where proceroid and plerocercoid larvae develop, may act as first intermediate hosts, and fish species are the definitive hosts, becoming directly infected after consuming the former (Kuchta and Scholz 2007; Scholz et al. 2021). In some cases, a paratenic host (i.e. a fish) is also involved, as it has been reported in *Bothriocephalus gregarius* Renaud Gabrion and Pasteur, 1983 (Morand et al. 1995). In *B. acheilognathi* and *Bothriocephalus pearsei* Scholz, Vargas and Moravec, 1996, it was shown that the transmission of adult parasites can occur from fish to fish through predation by infected host, a phenomenon known as postcyclic transmission (Hansen et al. 2007; Scholz et al. 2012). In the present study, fish size from the definitive host of *C. crassiceps*, *M. merluccius*, appeared to be an important contributing factor influencing *C. crassiceps* abundance, suggesting that host feeding rates and/or dietary shifts are important keys of parasite transmission. Indeed, *M. merluccius* undergoes dietary shifts due to ontogenetic shifts in feeding behaviour according to body size. Juvenile hakes (<15 cm total length) exhibit an opportunistic feeding strategy consuming euphausiids, mysids and decapod crustaceans (Modica et al. 2011; D'Iglio et al. 2022), some of which may act as first intermediate hosts for *C. crassiceps* larvae. As they grow (15-20 cm total length), their diet become more selective dominated by small fishes and suprabenthic crustaceans (Carrassón et al. 2019; D'Iglio et al. 2022), and finally mature hakes (>20 cm total length) adopt a piscivorous diet (D'Iglio et al. 2022). This transition to a more piscivorous diet increases the chances of ingesting infected prey, which could act as paratenic hosts (i.e. fish) for *C. crassiceps*, leading to a higher parasite load in larger individuals, as present findings indicate. This trend was also observed in *Bothriocephalus*

barbatus Renaud Gabrion and Pasteur, 1983 and *B. gregarius*, in which dietary shifts towards a piscivore diet increased the probability of parasitisation while maintaining copepod ingestion as an additional infection route (Robert et al. 1990).

Beyond transmission dynamics, understanding the host range of the cestode *C. crassiceps* is essential to clarify its ecological and evolutionary relationships within the Bothriocephalidea. Bothriocephalideans tapeworms generally exhibit strict host specificity, with about 90% being stenoxenous or oioxenous (infecting one or two host species), while the remaining are euryxenous, infecting a wide range of different host species (Kuchta and Scholz 2007). *Cleistobothrium crassiceps* and several species from the genus *Bothriocephalus* have been found in up to 30 marine fish species, Gadiformes, Scombriformes, Pleuronectiformes and Lophiiformes, amongst others (Cooper 1918; Schmidt 1986). Moreover, *Cleistobothrium* species have been recently reported from latest fish hosts (e.g., *C. neglectum* in *Raniceps ranius*). Thus, some hosts records previously attributed to *C. crassiceps* could correspond to other congeners. The host specificity of many marine taxa remains unclear due to unresolved taxonomy and limited definitive host data (Kuchta and Scholz 2007). In particular, the taxonomic status of several *Merluccius* species which host *C. crassiceps* has remained uncertain for decades, especially from closely related species from the Pacific Ocean (i.e. *Merluccius productus*, *M. angustimanus* Garman, 1899 and *M. hernandezi* Mathews, 1985) that exhibit overlapping morphological traits (Lloris et al. 2005; Silva-Segundo et al. 2011). A recent molecular study suggested that rare *Merluccius* morphotypes from the South Pacific and South Atlantic oceans, previously suspected to be cryptic species, are the result of recurrent hybridization events between *M. gayi* Guichenot, 1848 and *M. australis* or *M. hubbsi* (Pérez et al. 2021). Such taxonomic misidentifications could influence host records for *C. crassiceps* and affect our understanding of its host specificity. Altogether, these taxonomic uncertainties and host range ambiguities highlight the need for integrative taxonomic approaches to accurately assess the host specificity of *C. crassiceps* and its congeners.

5. Conclusions

The present study provides the first complete redescription of *C. crassiceps* from its type-host and type-locality. We highlight the importance of integrative taxonomy combining traditional morphology with advanced imaging techniques and molecular data to avoid species misidentification due to phenotypic plasticity commonly observed in *Cleistobothrium* species. Indeed, despite intraspecific size differences observed between *C. crassiceps* specimens from the Atlantic and Mediterranean, molecular data revealed a genetically homogeneous population of this parasite across both regions. Moreover, genetic results and morphological reexamination supported the synonymization of *C. cristinae* with *C. splendidum*, previously described as distinct species from *M. australis* and *M. hubbsi* off the Patagonian shelf of Argentina. Histology of the *C. crassiceps* specimen attached to the intestinal mucosa of *M. merluccius* provided new insights into the interaction between the attachment structures and host tissue, showing only mild effects. Further studies using an integrative approach as the present study should be more widely applied in parasite taxonomy and ecology across different taxa and regions, to improve our understanding of host-parasite interactions, species boundaries and host specificity in marine ecosystems.

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CHAPTER 4.2

Aporocotyle spinosicanalis

Muns-Pujadas, L., Constenla, M., Dallarés, S., Valmaseda-Angulo, M., Montero, F.E., Padrós, F. Blood intruders: *Aporocotyle spinosicanalis* (Williams, 1958) from its type-host *Merluccius merluccius* and its histopathological impacts in the NW Mediterranean Sea. In preparation.

Abstract

Blood flukes (Trematoda: Aporocotylidae) are common parasites in fish, often causing economic losses in aquaculture due to egg-induced pathologies. In wild conditions, these parasites are often unnoticed and monitoring their pathological impact over time is even more complex. *Aporocotyle spinosicanalis* is a blood fluke species infecting the cardiovascular system of the European hake (*Merluccius merluccius*), a relevant commercial species in the Atlantic Ocean and Mediterranean Sea. Hence, this study aims to provide a genetic characterization of newly collected *A. spinosicanalis* specimens from juvenile *M. merluccius* in the Catalan coast (NW Mediterranean Sea) and describe the histopathological effects of its eggs in the gills as well as the impact of the adults infecting the heart. Phylogenetic analyses based on the large subunit ribosomal DNA (28S rDNA) confirmed the identity, but comparisons with the two available Atlantic sequences showed contrasting levels of divergence. Therefore, although some geographic genetic differentiation may exist, the limited number of sequences and the conservative nature of the 28S marker prevent robust conclusions about population structuring across the hake distribution range. Histopathological examination showed that embolized eggs mainly caused mild inflammatory reactions and lamellar fusion, but in filaments with numerous eggs, especially when these were mature, epithelial lamellar hyperplasia was evident. In contrast, adult flukes elicited more severe host responses including fibrinoid necrosis and lymphocytic myocarditis. In general, hakes were infected at low prevalences (<12% of examined hakes) and displayed mild to moderate infections, with mean intensities of 1.74 (1.43 – 2) eggs/filament. Fish size (>20 cm standard length) contributed to higher infection levels, suggesting a higher exposure due to its host ontogenetic feeding ecology, but no clear detrimental effects on hake condition were detected. Thus, these results highlight that further studies including adult individuals are necessary to explore the host-parasite relationships, assess their lesions severity and its potential associated health impairments.

1. Introduction

Blood flukes (Trematoda: Aporocotylidae Odhner, 1912) are common parasites of many marine and freshwater fish species. They are generally present in the heart, branchial arteries and arterioles but can be also detected in other unusual sites such as the mesentery vessels (Bullard & Overstreet, 2002), pectoral girdles (Montero et al., 2003), brain (Alama-Bermejo et al., 2011) and kidney (Palacios-Abella et al., 2018). Their life cycle involves an invertebrate intermediate host (*i.e.* gastropods, bivalves or polychaetes) in which they asexually multiply through sporocyst stages, and a fish definitive host, where cercariae penetrate, mature and reproduce sexually (Køie, 1982; Power et al., 2020). Pathology associated with aporocotylids is commonly attributed to the release of their eggs, which are distributed via the vascular system and embolize the afferent arteries of fish gills' filaments and capillaries of lamellae depending on their size (Bullard & Overstreet, 2002). Consequently, eggs can induce several host responses in gills including inflammation, vascular obstruction, lamellar fusion, epithelial hyperplasia, haemorrhages and necrosis, especially when miracidia hatch from eggs (see Widdicombe et al., 2024), occasionally leading to significant respiratory impairment or mortality, particularly in aquaculture settings (Ogawa, 2015). Indeed, they are responsible for economic losses in several cultured fish species such as *Sparus aurata* Linnaeus, 1758 (Padrós et al., 2001) and *Seriola dumerili* (Risso, 1810) (Crespo et al., 1992).

Despite their known impact in aquaculture, information about aporocotylid infections in wild fish populations remains scarce. This is due to the limited number of studies focusing diseases in wild fish and the fact that adult flukes are often difficult to detect in their definitive hosts (Cribb et al., 2001), and hosts responses vary depending on species, infection site, intensity, season and environmental conditions (Bullard & Overstreet, 2002), making it even more complex to monitor their pathological impact over time. Among aporocotylids, the genus *Aporocotyle* Odhner, 1900, is of particular importance and includes several species that particularly infect *Merluccius* Rafinesque, 1810, a genus of considerable commercial relevance in global fisheries. To date, five species of *Aporocotyle* have been described from *Merluccius* hosts: *Aporocotyle spinosicanalis* Williams, 1958 described from *Merluccius merluccius* (Linnaeus, 1758) in the North Sea, *Aporocotyle argentinensis* Smith, 1969 described from *Merluccius hubbsi* Marini, 1933 off the Argentinean part of the South Atlantic Ocean, *Aporocotyle australis* Fernández & Durán, 1985 from *Merluccius australis* (Hutton, 1872) off Isla Huafo in Chile (south Pacific Ocean), *Aporocotyle margolisi* Smith, 1967 from *Merluccius productus* (Ayres, 1855) off the Pacific coast of Canada and *Aporocotyle wilhelmi* Villalba & Fernández, 1986 from *Merluccius gayi* (Guichenot, 1848) in Central Chile (south Pacific Ocean). However, no information is currently available on the

pathological impact these parasites may have on *Merluccius* species, as the effects of infections by adult flukes and their eggs remain completely unexplored. Histopathological effects in wild fish species were only reported for *Aporocotyle simplex* Odhner, 1900, the type-species of the genus, in different hosts of flatfish such as *Limanda limanda* (Linnaeus, 1758) and *Hippoglossoides platessoides* (Fabricius, 1780) from the Scandinavian west coast (Thulin et al., 1991). Inflammatory responses, hyperplasia of interlamellar epithelium and hypertrophy of secondary epithelium were reported as common reactions to eggs in gill filaments (Thulin, 1991).

Particularly, *A. spinosicanalis* infects the cardiovascular system of the European hake, an ecologically and economically important benthopelagic fish species widely distributed in the NE Atlantic Ocean and Mediterranean Sea (Oliver & Massutí, 1995; Murua, 2010). This species represents one of the most important target species of fisheries in the NW Mediterranean Sea (FAO, 2025). However, a reduction of their catches, which are mostly based on juveniles, has been observed in the Mediterranean along with a decline of large spawners (>50 cm) (STECF, 2025). In the GSA 6 (Spanish coast), catches have decreased from 3000 tons in 2002 to 1500 tons in 2024 (STECF 2025). Thus, the European hake is considered one of the most vulnerable species included in the Multi-Annual Management Plan (MAP), which is based in several management strategies for the reduction of the fishing effort for the bottom trawling fleet and the closure of specific areas to protect juveniles and spawners (European Commission [EU] (2019), Article 11 EU reg. 2019/1022). Indeed, closing specific coastal areas which are essential recruitment areas for this species would be beneficial for the recovery of its stock biomass (Khoukh & Maynou, 2018). Understanding host-parasite interaction in *M. merluccius* is therefore especially relevant, not only due to its ecological role, but also because health impairments caused by parasitic infections may influence population dynamics and therefore fishery catches. Despite Williams (1958) provided the original description *A. spinosicanalis* based on specimens from *M. merluccius* Atlantic populations, this parasite has received little attention since then, and no genetic data are currently available for Mediterranean populations. This is particularly relevant because genetic studies differentiated Atlantic and Mediterranean European hake populations into two distinct stocks with limited connectivity (Roldán et al., 1998; Cimmaruta et al., 2005). Consequently, genetic differentiation in their parasites may also occur. Moreover, the potential pathological effects of this parasite on its host have never been assessed.

The present study aims to provide a morphological redescription and a genetic characterization of *A. spinosicanalis* from *M. merluccius* based on newly collected specimens from the Balearic Sea (NW Mediterranean Sea). Moreover, the first histopathological description of the alterations

associated with both adults and eggs of *A. spinosicanalis* infection in the gills and heart of *M. merluccius* will be provided, as well as epidemiological values in the study area.

2. Materials & Methods

2.1. Study area and sample collection

A total of 159 specimens of European hake were collected on the continental shelf of the Catalan coast (Balearic Sea, NW Mediterranean) between 2019 and 2025 at 50-112 m depth. Of these, 62 specimens were captured in summer of 2019 (see Table 1 Chapter 3.1 M&M), 21 specimens in winter of 2023 and 31 individuals in spring of 2025 by commercial trawling vessels. After capture, fish from 2019 were immediately fixed *in toto* in 10% buffered formalin for further analysis, after performing an abdominal incision to allow proper fixation of internal organs. On the other hand, fish from 2023 were examined fresh few hours after capture and in the case of hakes from 2025, the heart was immediately fixed in 10% buffered formalin.

At the laboratory, fish were measured (standard length, SL), weighed (total weight, TW and eviscerated weight, EW), and dissected. All organs were examined under a stereomicroscope for the presence of parasites. During the parasitological examination of the heart of hakes from 2019, adult flukes of *A. spinosicanalis* were collected, counted and preserved in 70% ethanol. In the case of fresh *M. merluccius* from 2023, parasites were killed in warm saline solution and preserved in 100% molecular grade ethanol for genetic analyses. The gills of hakes from 2019 and the heart of fish from 2025 were used for histological analysis.

2.2. Molecular and phylogenetic analysis

Genomic DNA extractions from two specimens collected in 2023 were performed using Qiagen™ (Valencia, California, USA) DNeasy® Tissue Kit following manufacturer's protocol. Partial nuclear large subunit ribosomal DNA (28S rDNA) was amplified (50 µl total volume) by polymerase chain reaction (PCR) using ExcelTaq™ SMOBIO® PCR Master Mix (SMOBIO Technologies, Hsinchu City, Taiwan) containing 5× concentrated master mix, a mixture of recombinant Taq DNA polymerase, reaction buffer, MgCl₂ (2 mM), dNTPs (0.2 mM), and enzyme stabilizer. Amplifications of 28S rDNA were performed following Fyler et al. (2009) primers and conditions. In this case, 0.5 µl of each PCR primer, and 2 µl of extracted gDNA were added to each amplification tube. PCR amplicons were sequenced directly for both strands using the primers LSU5F and 1500R.

Obtained sequences were assembled and edited using BioEdit v7 (Hall 1999) and submitted to GenBank under accession numbers PX884878 – PX884879. Sequence alignments were performed using Muscle implemented in MEGA v6, together with available sequences of *Aporocotyle* species described in **Table 1**. In the case of *A. argentinensis*, identical sequences were available from different host species but only one sequence was included in the analysis as representative of the species to avoid redundancy. Pairwise genetic distance matrices (using “uncorrected p-distance” and “number of base-pair differences” models) were calculated in MEGA v6.

Table 1. List of species of aporocotylid digeneans included for sequence alignments of 28S rDNA devoted to phylogenetic analysis with details on host species, locality and GenBank Accession numbers.

Species	Host species	Locality	GenBank Accession numbers	Reference
<i>Aporocotyle spinosicanalis</i> Williams, 1958	<i>Merluccius merluccius</i>	Off Barcelona, Mediterranean Sea	PX884878 – PX884879	Present study
<i>Aporocotyle spinosicanalis</i> Williams, 1958	<i>Merluccius merluccius</i>	Off Orkney Islands, North Sea	AY222177	Olson et al. (2003)
		North Sea	AF167094	Snyder & Loker (2000)
<i>Aporocotyle argentinensis</i> Smith, 1969	<i>Merluccius hubbsi</i>	Off Patagonia, Atlantic Ocean	JX094803	Hernández- Orts et al. (2012)
<i>Aporocotyle mariachristinae</i> Hernández-Orts, Alama- Bermejo, Carrillo, García, Crespo, Raga & Montero, 2012	<i>Genypterus blacodes</i>	Off Patagonia, Atlantic Ocean	JX094802	Hernández- Orts et al. (2012)
<i>Aporocotyle michaudi</i> Santoro, Cipriani, Pankov & Lawton, 2015	<i>Trematomus bernacchii</i>	Ross Sea, Antarctica	KR025807	Santoro et al. (2015)
<i>Aporocotyle margolisi</i> Smith, 1967	<i>Merluccius productus</i>	Off Oregon, Pacific Ocean	MF287915 – MF287916	Hernández- Orts et al. (2017)
<i>Paradeontacylix ibericus</i> Repullés-Albelda, Montero, Holzer, Ogawa, Hutson & Raga, 2008	<i>Seriola dumerili</i>	Off Murcia, Mediterranean Sea	AM489593	Repullés- Albelda et al. (2008)

Gblocks 0.91b implemented on the Phylogeny.fr site (Dereeper et al. 2008) was used to select blocks of evolutionarily conserved sites. Maximum likelihood (ML) and Bayesian inference (BI) algorithms were used for phylogenetic tree reconstruction after the determination of the best-fit model of nucleotide substitution with jModelTest v2.1.4 (Darriba et al. 2012) using the Akaike

Information Criterion (AIC) and the Bayesian Information Criterion (BIC), respectively. The best-fitting model selected for ML and BI algorithm was the HKY+I model (nst = 2, rates = equal, ngammacat = 4). ML analyses were performed in PhyML v3.0 (Guindon et al. 2010) with a non-parametric bootstrap of 100 replicates. BI analyses were carried out on the Phylo.org site on the CIPRES Science Gateway v3.3 (Miller et al. 2010). Log likelihoods were estimated over 10,000,000 generations using Markov Chain Monte Carlo searches on two simultaneous runs of four chains, sampling trees every 1000 generations. The first 25% of the sampled trees were discarded as 'burn-in' and a consensus topology and nodal support estimated as posterior probability values (Huelsenbeck et al. 2001) were calculated from the remaining trees.

2.3. Histopathological analysis

The four gill arches (with their corresponding holobranches) of the left side from hakes sampled in 2019 and the heart of hakes sampled in 2025 were processed by routine paraffin histology, sectioned at 4–5 µm (and into serial sections in the case of hearts) and stained with Haematoxylin and Eosin (H&E). All stained slides were thoroughly screened and examined under the microscope. Images were taken with a Leica camera model CTR5000 connected to a Leica DM500B microscope. A subsample of eggs from 2019 was selected and measures of total length and width were taken through an image-processing software (ProgRes® C3).

Prevalence (P) of eggs infection by *A. spinosicanalis* in gill histological sections was calculated as the percentage of fish with at least one infected holobranch, based on samples obtained in 2019. A semi-quantitative analysis was performed to evaluate the intensity of eggs infection. From each infected holobranch, the total number of eggs within each affected filament was counted and scored as: mild (1) (<5 eggs per filament), moderate (2) (5-15 eggs per filament) and severe (>15 eggs per filament). Mean intensity was calculated as the mean semi-quantitative score per affected individual, excluding those fish without holobranches infected.

2.4. Data analysis

From specimens collected in 2019, prevalence (P) and mean intensity (MI) (with the range interval) of the adult parasite *A. spinosicanalis* were calculated following Bush et al. (1997) for each fish size established range (≤ 20 cm and > 20 cm standard length), related to the ontogenetic shift in the feeding behaviour and diet of the host (D'Iglio et al. 2022). General fish condition was assessed through Le Cren's relative body condition index ($Kn = EW / (\alpha \times TL^\beta)$) (Le Cren, 1951), where α and β are the slope and the intercept of the weight-length relationship representing the entire dataset of sampled fish.

Fish SL, Kn and MI of both adults and eggs and size of eggs were plotted for visually assess data distribution and tested for normality and homoscedasticity using the Shapiro-Wilk test and the Levene's test, respectively. Fish size effect and Kn were tested for P and MI of adults and eggs using Generalized Linear Models (GZM), applying a logistic and a negative binomial distribution, respectively.

Data analysis was performed on RStudio software, version 4.1.2. Statistical significance was set at 0.05.

3. Results

3.1. Genetic results and phylogenetic analysis

Two partial 28S rDNA sequences (1089 and 1086 nt in length) differing by 0.4% (4 nt) were obtained from two specimens of *A. spinosicanalis* from the Catalan coast (NW Mediterranean Sea) (PX884878-PX884879). The resulting alignment was 950 nt in length. Both ML and BI analyses using sequences from **Table 1** resulted in a consensus phylogenetic tree shown in **Fig. 1**. The two newly generated sequences formed a strongly supported clade with two sequences from the same species obtained in the North Sea, from which they differed by 0.1-0.5% (1-5 nt) and by 1.05 – 1.47% (10-14 nt). High bootstrap and posterior probability values also characterized the clade formed by *A. argentinensis* and *A. margolisi*, which differed by 0.6% (6 nt) between them and by 1.79-2.32% (17-22 nt) and by 2.21-2.63% (21-25 nt), respectively, from the newly generated sequences. All mentioned *Aporocotyle* species infecting *Merluccius* spp. formed a strongly supported monophyletic group differentiated from the two species of *Aporocotyle* infecting other groups of fish, *A. mariachristinae* and *A. michaudi* which differed by 5.52-5.79% (50-55 nt) and 6.11-6.53% (58-62 nt), respectively, from the newly generated sequences.

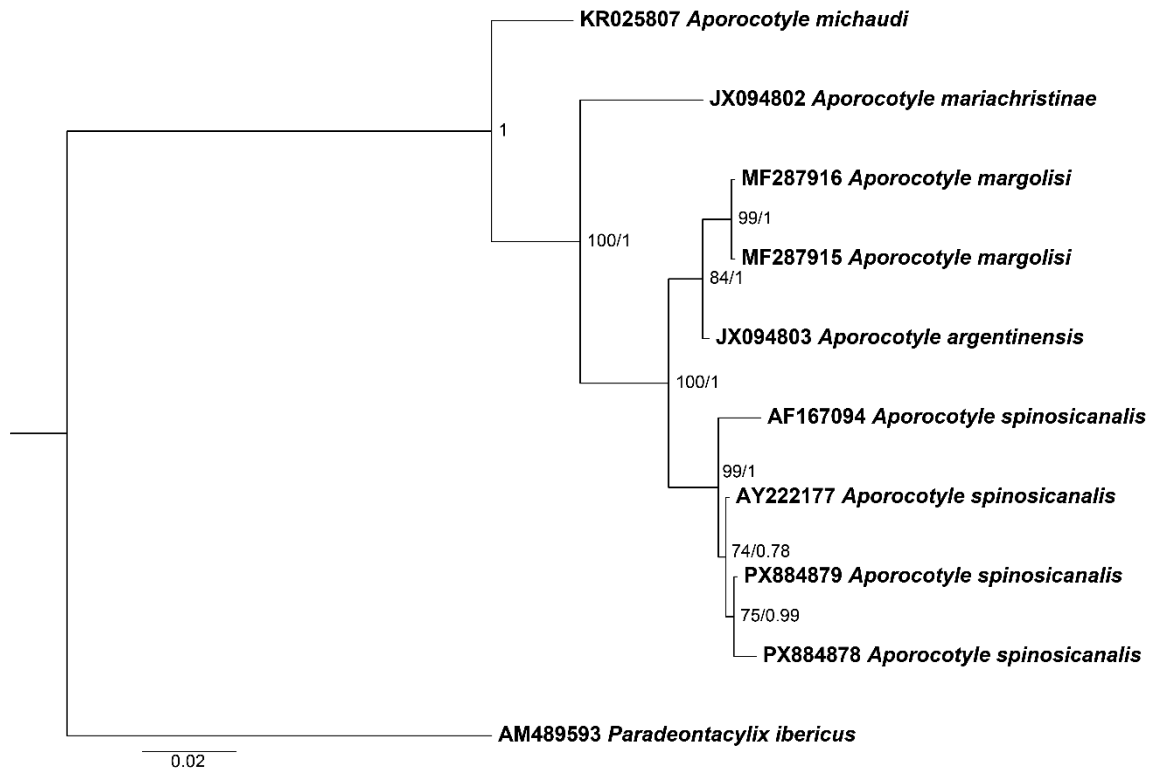


Fig. 1. Maximum likelihood (ML) phylogram reconstructed using newly generated 28S rDNA sequences for *Aporocotyle spinosicanalis* (PX884878-PX884879) and retrieved sequences from GenBank for other species of the genus. Outgroup: *Paradeontacylix ibericus*. Nodal support from ML (bootstrap values > 70%) followed by posterior probabilities from Bayesian inference (BI) analyses (> 0.9) are shown. The scale bar indicates the expected number of substitutions per site.

3.2. Histopathological observations

3.2.1. Gills

Eggs were located inside the afferent arteries of the gill filaments, particularly at the basis of the secondary lamellae (**Fig. 2a**). Eggs were unevenly distributed throughout the gills since filaments devoid of eggs were observed adjacent to filaments filled with eggs. Generally, they were distributed in a single row in medial (48.94%) and distal (31.21%) parts of the filaments, but also in the basal part in a lower proportion (19.15%). In some cases, mostly in distal parts, eggs were clumped together. Adults were observed only in two cases in the afferent arteries of the gill arch.

Embolized eggs consisted of different stages of the parasite, from a heterogeneous aggregation of few cells (immature embryos) to more advanced degree of development (miracidia) and were individually surrounded by a thick shell (**Fig. 2b**). Most of the eggs found were at immature embryo stage (78.72%). Immature embryos measured 32.66–64.58 × 19.62–51.20 (mean 47.01 × 32.91)

μm ($n = 47$) and were equally located in medial and distal parts of the filament. In more developed forms (mature embryo and particularly miracidia), the organisms showed fully developed cilia and was also surrounded by a thick shell (**Fig. 2c**). They were located mainly in the medial and distal parts of the filament and measuring $44.08\text{--}72.93 \times 26.14\text{--}50.01$ (mean 59.02×39.22) μm ($n = 24$). Only 9.93% of the eggs contained apparently degenerating embryos inside their shell but were not encapsulated by any granulomatous inflammatory response (**Fig. 2d**). These eggs measured $46.10\text{--}67.98 \times 29.30\text{--}51.87$ (mean 54.96×37.59) μm ($n = 6$) and were mainly located at the basal part of the gill filaments.

In general, embolized eggs aligned in a single row caused a dilatation of the afferent filament arteries and in some cases, focal inflammatory cell infiltration mainly composed of lymphocytes were observed surrounding the eggs (**Fig. 2a**). This host response was intensified in those filaments with higher number of eggs, clumping together in distal parts (**Fig. 2e**), especially after hatching of miracidia (detected as empty shells), causing extensive inflammation throughout the filament (**Fig. 2f**). Only in two cases, focal lamellar hyperplasia leading to an increase in the thickness of the gill epithelia surrounding miracidia was detected (**Fig. 2g-h**).

3.2.2. Heart

Histological sections of the heart of hakes from 2025 revealed the presence of degenerated and usually encapsulated eggs in the myocardium mainly of the ventricle triggering a granulomatous inflammatory response in 19.35% of the examined specimens. The degenerated eggs were surrounded by concentric layers of fibrous connective tissue and inflammatory cells (fibroblasts, macrophages and lymphocytes) (**Fig. 3a-b**). Adjacent muscle fibres showed mild disorganization and degeneration, but no necrosis was present.

Two adults (measuring 2.48 and 1.74 mm) were found in one single case in the lumen of the heart atrium (**Fig. 3c**) causing extensive fibrinoid necrosis and inflammation in the myocardium (myocarditis), characterized by an accumulation of lymphocytes observed in the spaces among the muscle fibres (**Fig. 3d-f**). Loss of muscle striation and groups of lymphocytes were also observed surrounding the parasite (**Fig. 3e**). A similar inflammatory response was observed in another specimen, but no adult flukes could be found in the surrounding area.

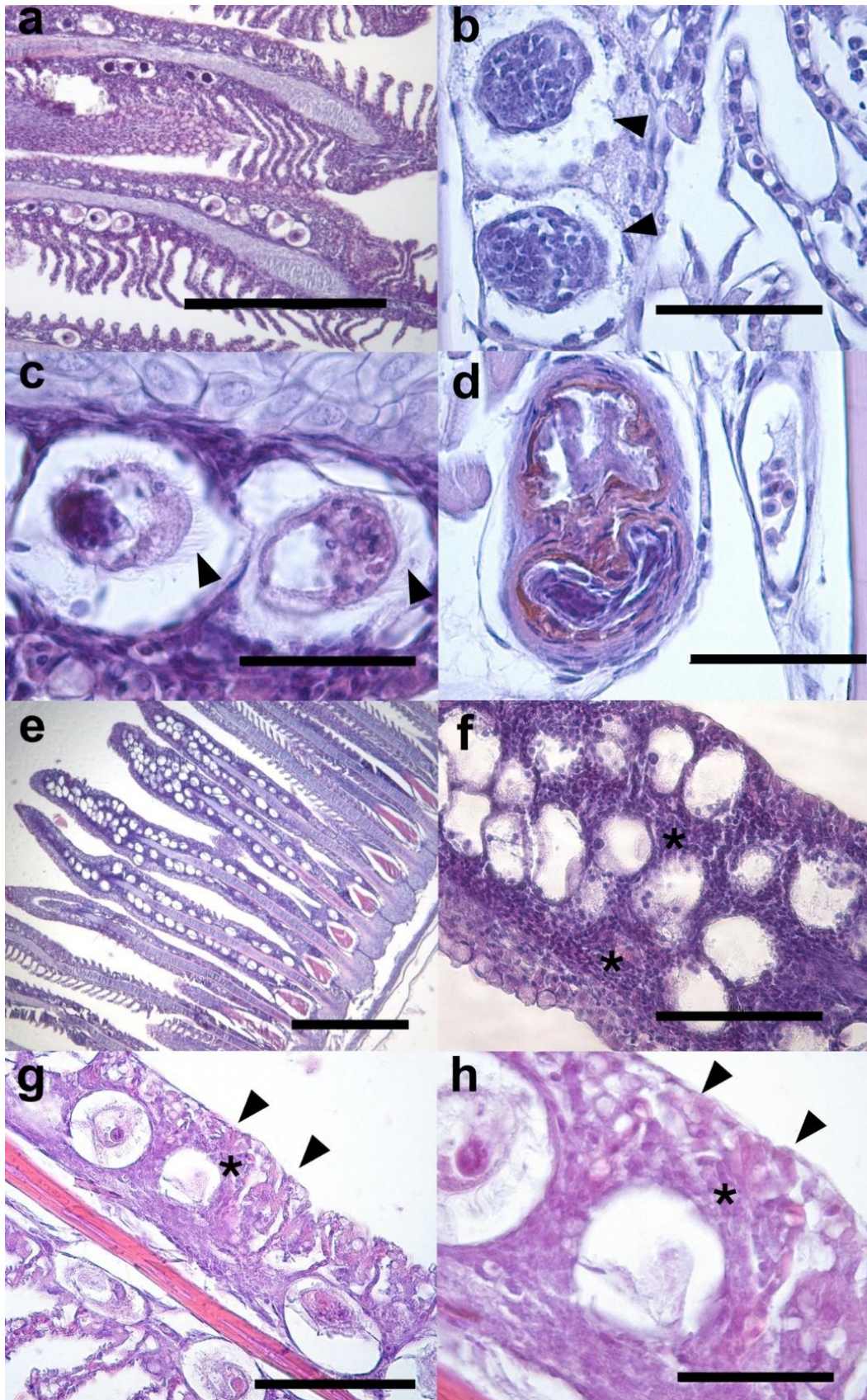


Fig. 2. Histological (H&E stain) gill sections in *Merluccius merluccius* from the Catalan coast (NW Mediterranean) infected with *Aporocotyle spinosicanalis*. **a)** Embolized eggs in medial and distal parts of the afferent filament arteries; **b)** Immature eggs surrounded by a thick shell (arrowheads); **c)** Mature eggs

containing fully developed miracidia with ciliated cells (arrowheads) within the eggshell; **d**) Degenerating egg at the base of the filament; **e**) Filaments with numerous eggs after hatching; **f**) Detail of the holes left after eggs hatching and inflammatory cell infiltration of lymphocytes inside the filament (asterisks); **g**) Mild inflammatory reaction (asterisk) and focal lamellar hyperplasia (arrowheads) surrounding miracidia; **h**) Detail of g. Scale bars: a, e, 400 μ m; b-d, 50 μ m; f-h, 100 μ m.

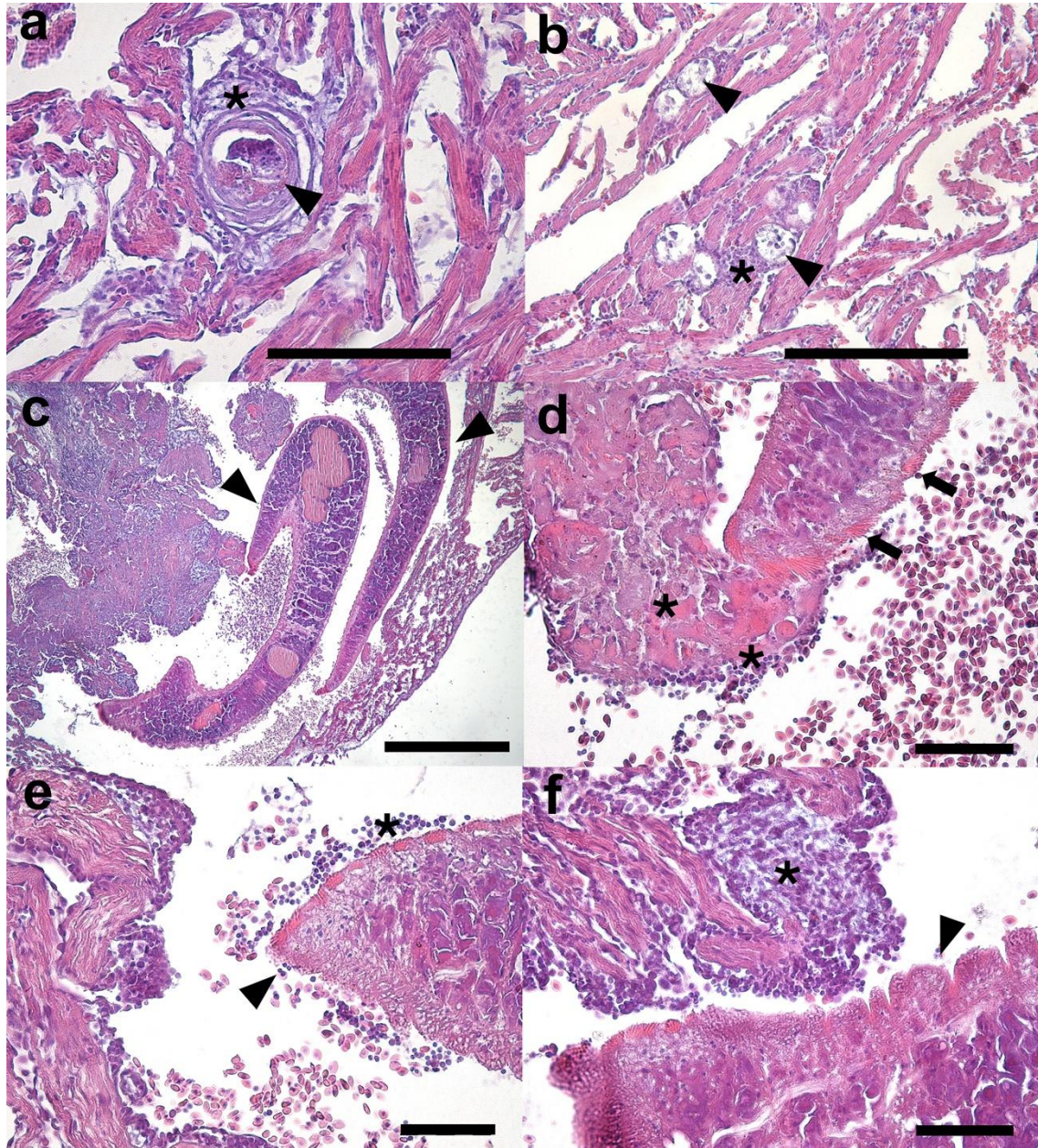


Fig. 3. Histological (H&E stain) sections of the heart of *Merluccius merluccius* from the Catalan coast (NW Mediterranean) infected with *Aporocotyle spinosicanalis*. **a**) Encapsulated egg (arrowhead) surrounded by fibroblasts and inflammatory reaction (asterisk); **b**) Eggs (arrowheads) associated with lymphohistiocytic infiltration (asterisk) in the myocardium; **c**) Two adults of *A. spinosicanalis* (arrowheads) in the heart atrium; **d**) Fibrinoid necrosis (asterisks) and inflammation in the myocardium. Note the presence of clusters of spines from the tegument of the adult (arrows); **e**) Lymphohistiocytic infiltration (asterisk) surrounding the

parasite (arrowhead); **f**) Multifocal areas of lymphohistiocytic infiltration (asterisk) in the myocardium. Scale bars: a, 100 μm ; b, 200 μm ; c, 500 μm ; d-f, 50 μm .

3.3. Infection parameters

During hake's parasitological examination from 2019, *A. spinosicanalis* adults were found in 11.29% of the examined hakes, located in the lumen of the heart ventricle and atrium, with a MI of 3.00 (1–7) flukes per fish. Larger specimens ($\text{SL} > 20 \text{ cm}$) were significantly more infected than smaller ones ($\text{SL} \leq 20 \text{ cm}$), both in terms of P (6.45% vs. 4.84%; GZM, $\chi^2 = 37.24$, $p = 0.01$) and MI of adult flukes (mean of 3.08 (1–7 range) vs mean of 1.00 (1–1 range); GZM, $\chi^2 = 2.37$, $p = 0.004$). A significant strong correlation between SL and MI of adult flukes was also detected (Sperman correlation, $\rho = 0.96$, $p = 0.0004$). A significant association was detected between Kn and MI of *A. spinosicanalis* adults' infection (GZM, $\chi^2 = 6.15$, $p = 0.04$).

Regarding the presence of eggs of the parasite, 9.67% of hakes' gills had eggs in the gills with a MI of 1.74 (1.43 – 2.00) eggs/filament. Following a similar pattern as in blood fluke adults, larger specimens ($\text{SL} > 20 \text{ cm}$) presented significantly higher P of eggs infection (6.45% vs. 3.22%; GZM, $\chi^2 = 31.29$, $p = 0.004$), but no significant differences were found for MI of eggs (mean of 1.69 (1.43–1.85 range) vs. mean of 1.85 (1.70–2.00 range)). Infected fish showed a mean of three infected holobranches (50% of fish with all four holobranches infected), affecting a 10.86% of the total gill surface. No significant associations were found between Kn and P or MI of eggs infection (GZM, $p > 0.05$).

4. Discussion

The present study offers novel insights into the molecular characterization and the histopathological impacts of both adults and eggs of *Aporocotyle spinosicanalis* in the heart and gills from *Merluccius merluccius* off the Catalan coast in the NW Mediterranean Sea.

4.1. Genetics, phylogeny and host specificity

The phylogenetic analyses based on the large subunit ribosomal DNA confirmed the identity of the specimens collected from *M. merluccius* as *A. spinosicanalis*, clustering the two newly sequences with previously reported sequences of this parasite from the North Sea within a supported monophyletic clade. Ribosomal DNA (i.e. 28S and ITS2 rDNA) has been widely used to determine the level of conspecificity among various platyhelminths (Aiken et al., 2007; Pérez-

Ponce de León & Hernández-Mena, 2019), and in the present study, the large subunit ribosomal DNA (28S rDNA) phylogeny revealed clear resolution to discriminate closely related species across broad regions. The genetic divergence observed among *A. spinosicanalis* from the Mediterranean and the North Sea was low in the case of the sequence provided by Olson et al. (2003) and comparable to the level of variation detected between our own sequences. However, the other sequence from the North Sea provided by Snyder & Locke (2000) showed a higher genetic divergence compared to our sequences, which might reflect elevated intraspecific variability, which may be consistent with restricted gene flow among geographically distant populations (Roldán et al., 1998; Cimmaruta et al., 2005), although no information of the location of these parasites was available. This high intraspecific variability also highlights the risk of overestimating parasite diversity based only on morphological traits, as reported for South American species of *Aporocotyle*, where distinct species described from different *Merluccius* hosts (*M. hubbsi*, *M. australis* and *M. gayi*) were later shown to be congeneric (*A. argentinensis*) (Oliva et al., 2022). On the other hand, *A. argentinensis* (ex *M. hubbsi*) and *A. margolisi* (ex *M. productus*), which form a separate clade from *A. spinosicanalis*, reflect the close relationship of their Southwest Atlantic and Northeast Pacific hosts, clearly distinct from the European lineage (*M. merluccius*), as Hernández-Orts et al. (2017) reported. Moreover, the greater divergence observed in *A. mariachristinae* and *A. michaudi*, which infect other fish genera, supports the idea that diversification within *Aporocotyle* may be closely related to host phylogeny (Hernández-Orts et al., 2017), known as the Fahrenholz's rule, which proposes that parasites evolve in parallel with their hosts (Fahrenholz, 1913). However, such congruence does not necessarily imply co-speciation, as parasite diversification can also be shaped by other evolutionary processes such as adaptation in a new host species (termed as host switching) or lineage sorting (i.e. loss or absence of a parasite lineage in a new host species after host divergence) (Page & Charleston, 1998; Lucius & Poulin, 2017).

However, as mentioned above, the large subunit ribosomal DNA is a relatively conserved marker that may underestimate fine-scale genetic diversity (Harrison, 1989; Nolan & Cribb, 2005). Mitochondrial genes such as the mitochondrial cytochrome oxidase 1 (cox1), with a faster evolutionary rate, provide greater sensitivity for detecting more precise species-level divergence, cryptic species and population structure (Harrison, 1989; Pérez-Ponce de León & Nadler, 2010). For instance, Oliva et al. (2022) showed low gene flow through cox1 mtDNA among *A. argentinensis* populations from different *Merluccius* hosts, suggesting a trend towards incipient population speciation, though not statistically supported. Thus, although Hernández-Orts et al. (2017) suggested that diversification within *Aporocotyle* may reflect co-divergence with hake hosts, more molecular studies indicate that apparent host specificity does not always correspond

to distinct parasite lineages (Oliva et al., 2022), highlighting the need for broader genomic data to clarify these evolutionary patterns.

4.2. Host responses to eggs

Aporocotyle spinosicanalis eggs were mostly found in gills filaments, which is also a typical site infection reported for other aporocotylids species. Their distribution could be explained by the adult's localization in the heart and the direction of blood flow through the afferent branchial arteries. Due to their relatively large size, eggs were confined in the afferent filament arteries, without reaching the capillaries of the secondary lamellae, as reported in *Skoulekia bogaraveo* Palacios-Abella, Raga, Mele & Montero, 2018 infecting *Pagellus bogaraveo* (Brünnich, 1768) (Palacios-Abella et al., 2018). Within the filaments, eggs were predominantly located in medial and distal parts, which may represent optimal sites for hatching, possibly due to the vascular architecture and blood flow dynamics that favour their retention in these areas. In contrast, fewer eggs were observed in the basal part of the filament, where most were degenerated, indicating that this site may be not optimal for hatching due to the longer distance with the external environment, as reported in *Paradeontacylix grandispinus* Ogawa & Egusa, 1986 infecting *Seriola dumerili* (Ogawa et al., 1989).

The absence of haemorrhages in the present study could be related not only to the site from which miracidia escape but also to the number of eggs involved and the type of vascular structures affected by hatching (Herbert et al., 1995; Power et al., 2020). In low-intensity infections, single eggs hatching from the primary filament epithelium may cause limited tissue disruption, particularly if the relatively thick epithelium restricts extensive vascular damage (Herbert et al., 1995). In addition, embolized eggs could partially obstruct the affected vessel, limiting the haemorrhage during hatching. Synchronous hatching of multiple eggs, or hatching occurring in the secondary lamellae, may result in greater mechanical damage and higher probability of haemorrhages due to disruption of the thinner epithelial structure (Kirk & Lewis, 1998; Padrós et al., 2001). In the present study, most embolized eggs only caused dilatation of the afferent filament and mild focal inflammation, suggesting a long-term host-parasite coevolutionary relationship (Warren & Bullard, 2023). Nevertheless, this host reaction appeared to be more pronounced when several miracidia accumulated in the same location (most often in distal parts of the filament), and/or associated with egg hatching, consistent with other aporocotylids that have been reported to cause severe lesions such as hyperplasia, clubbing, haemorrhages and granulomatous encapsulation of eggs (Padrós et al., 2001; Holzer et al., 2008; Shirakashi et al., 2012; Ogawa, 2015; Warren et al., 2024). Although dilatation of the afferent filament artery was

evident, no necrosis was detected in distal regions beyond eggs accumulation. This suggests that egg presence did not cause complete obstruction or that occlusion of the artery was insufficient to impair blood supply to distal parts, probably due to the role of the secondary vascular system, a parallel circulatory network that ensures oxygen and nutrient delivery through multiple arterial and capillary connections from the primary arteries rather than from the heart (Panara et al., 2024). In this case, tissue damage may be more associated with the mechanical rupture of eggs during hatching, which can produce microlesions and initiate focal inflammatory responses. These findings are similar to what was observed in *Cruoricola lates* Herbert, Shaharom-Harrison & Overstreet, 1994 (Herbert et al., 1995) and *S. bogaraveo* (Palacios-Abella et al., 2018). When comparing present findings with the only available histopathological description of another species of the genus, *Aporocotyle simplex* Odhner, 1900, their eggs were also reported to be in the afferent filament arteries but induced severe alterations to the gills including interlamellar epithelial hyperplasia and hypertrophy of secondary epithelium (Thulin, 1991). Moreover, granulomatous nodules were common, a marked difference from the herein study, in which eggs were never encapsulated. Taking together, these findings suggest that although *A. spinosicanalis* eggs could potentially obstruct the blood flow when embolized in the afferent filament arteries, it appears to be less pathogenic than other aporocotylids whose eggs reach the secondary lamellae, occlude their vessels and induce hyperplasia, fusion and clubbing of gill lamellae (e.g. *Paradeontacylix balearicus* Repullés-Albelda, Montero, Holzer, Ogawa, Hutson & Raga, 2008 and *Cardicola orientalis* Ogawa, Tanaka, Sugihara & Takami, 2010) (Repullés-Albelda et al., 2008; Shirakashi et al., 2012).

Although most adult flukes were found in the heart, a few individuals were also observed in the branchial artery. This finding may explain the high variability in egg distribution among gill filaments, where filaments completely devoid of eggs were often adjacent to heavily infected ones, as Shirakashi et al. (2012) suggested for *Cardicola orientalis* and *Cardicola opisthorchis* Ogawa, Ishimaru, Shirakashi, Takami & Grabner, 2011, which coinfect the Pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel, 1844). Adults located in the branchial artery could release large amounts of eggs locally, leading to their accumulation in nearby filaments. In contrast, eggs released from adults in the heart would be carried by blood circulation and distributed more evenly across the filaments (Shirakashi et al., 2012). Occasionally, eggs were also found in the ventricular myocardium, where they evoked a granulomatous inflammatory response, a reaction commonly reported in other aporocotylids such as *A. simplex* (Thulin, 1991), *Cardicola laruei* Short, 1953 (McElroy et al., 2020), *Cruoricola lates* (Herbert et al., 1995), *Paradeontacylix* sp. (Ogawa et al., 1989) and *Mugilitrema labowskiae* Warren & Bullard in Warren,

Ksepka, Truong, Curran, Dutton & Bullard, 2024 (Warren et al., 2024). As Ogawa et al. (1989) suggested, the absence of egg encapsulation in gills from present samples but its occurrence in the ventricle indicates a more intense host reaction in the cardiac tissue to isolate and degenerate eggs in a site unsuitable for hatching.

4.3. Host responses to adults

Histopathological effects attributed to adult aporocotylids on the cardiac tissue of fish remain poorly documented compared to the lesions associated with their eggs (Warren et al., 2017, 2024; McElroy et al., 2020; Widdicombe et al., 2024). Most studies report minimal or absent host responses to adults, as in *Cardicola laruei*, where granulomatous myocarditis was exclusively associated with eggs and no cellular reaction was detected with adults (McElroy et al., 2020). Conversely, endocarditis, thickened endocardium, loss of muscle striation in the myocardium, melanin-like pigment and endocardial thrombi, likely blocking normal blood flow and potentially leading to embolism, have been described in association with adults of *Cardallagium anthicum* (Bullard & Overstreet, 2006) Yong, Cutmore, Jones, Gauthier & Cribb, 2017 infecting cultured coho *Rachycentron canadum* (Warren et al., 2017). While in cultured conditions these lesions have been attributed to reduced cardiac output and even acute mortalities, in the wild it is much more challenging to assess their impact in fish hosts' condition and population's structure (McElroy et al., 2020). In this context, the severe but focal lymphocytic myocarditis and fibrinoid necrosis observed in the present study suggests that *A. spinosicanalis* can elicit a marked local inflammatory and immune response probably caused by the direct mechanical damage due to the presence of spines in fluke's tegument which might provoke irritation (Herbert et al., 1995) or by the release of proteins or other substances that may elicit inflammatory response. The detection of similar lesions in the absence of visible flukes may indicate a transient passage of the parasite through the heart, with a resulting immune response persisting even after the parasite had migrated. These findings highlight the potential impacts of *A. spinosicanalis* infections on its host. Myocardial inflammation could compromise the cardiac output, potentially reducing oxygen delivery and overall physiological condition (Poppe & Ferguson, 2006). Such impairment may also increase susceptibility to secondary bacterial or viral infections, further affecting fish health. However, teleost hearts can repair and regenerate (Poppe & Ferguson, 2006), and post-infection recovery seems possible, as suggested by endocardial scarring in young cobias from the Gulf of Mexico with lesions compatible with adult flukes (Warren et al., 2017).

4.4. Life cycle and incidence of infection

The incidence of aporocotylid infections in natural populations is likely shaped by a combination of environmental and host-related factors (McElroy et al., 2020). The life cycle of *A. spinosicanalis* remains unknown, as is the case for most aporocotylids. So far, complete life cycle has been described for only 11 freshwater species and eight marine species (see Warren & Bullard, 2023). Among these, one species of *Aporocotyle* (i.e. *A. simplex*) and six species of *Cardicola* Short, 1953 use terebelliform polychaetes of the families Terebellidae or Polycirridae as intermediate hosts (e.g. Kjøie, 1982; Cribb et al., 2011; Shirakashi et al., 2016; Ogawa et al., 2017). Because these genera are not closely related, it has been suggested that most marine actinopterygian-infecting aporocotylids likely rely on polychaetes as intermediate hosts (Power et al., 2020), indicating long-term evolutionary relationships (Warren & Bullard, 2023). In contrast, freshwater aporocotylids generally use gastropods, while some species infecting chondrichthyans use bivalves, reflecting three well-supported clades of aporocotylids (Cribb et al., 2017; Warren & Bullard, 2019; Power et al., 2020; Cutmore et al., 2023). These observations suggest that other *Aporocotyle* species may also rely on polychaetes to complete their life cycle. In the study area, approximately 38% of the polychaete species recorded from the western Mediterranean have been recorded, with *Ditrupa arietina* (O. F. Müller, 1776) and *Owenia fusiformis* Delle Chiaje, 1844 dominating sandy environments and *Lumbrineris latreilli* Audouin & Milne Edwards, 1833, *Hilbigneris gracilis* (Ehlers, 1868) and *Sternaspis scutate* (Ranzani, 1817) being more abundant in muddy bottoms at depths of 10 – 50 meters (Sardà et al., 2014). Further studies investigating the polychaete assemblages in the seafloors and depth ranges where infected hakes were collected could help identify potential intermediate hosts.

Environmental conditions can strongly influence these dynamics. Abiotic factors such as water temperature, salinity and pH can modulate parasite transmission by influencing miracidia survival, cercaria release and the development of sporocysts and adults, and the availability of intermediate and definitive hosts (Pietrock & Marcogliese, 2003; Koprivnikar et al., 2010; McElroy et al., 2020). Seasonal variations are also important, since the development and release of cercariae from intermediate hosts is often synchronized with specific periods of the year, resulting in marked seasonal fluctuations in infection prevalence (Bullard & Overstreet, 2002; Holzer et al., 2008). Indeed, higher prevalences of aporocotylids are typically observed during summer, as warmer temperatures increase cercarial productivity and release, likely due to an increased metabolic activity of the intermediate host (Poulin, 2006; McElroy et al., 2020; Selbach & Poulin, 2020). For example, in *Thunnus orientalis*, peaks of infection by *Cardicola* spp. occur in summer and early autumn (Sugihara et al., 2017; Shirakashi et al., 2017), similar to what was observed in

Cardicola laruei infecting seatrout (McElroy et al., 2020). In this study, present findings showed that the probability of infection can occur during the whole year, as presence of eggs and adults have been detected in summer of 2019, winter of 2023 and spring of 2025. Although seasonality was not tested in this study, the existence of seasonal patterns cannot be dismissed.

Beyond environmental drivers, host-related traits such as ecology and life history may also modulate exposure to infection. In the present study, infection prevalence of *A. spinosicanalis* was higher in larger hakes, indicating that fish size influences exposure to the parasite. This finding aligns with Smith & Williams (1967), who also reported higher prevalences in larger individuals. This pattern could be explained by hake habitat and its size-dependent distribution. Hake eggs and larvae are pelagic (Olivar et al., 2003), so juvenile fish might be presumably uninfected when first descending to the seafloor. Both juvenile and adult hakes perform diel vertical migrations, ascending to the water column at night to feed and remaining near the seafloor during daylight (D'Iglio et al., 2022), where they may encounter cercariae due to the proximity with the intermediate host. Consequently, adults may have more chances to be infected due to their longer time near the sea bottom, whereas juveniles might have more limited opportunity for contact with the intermediate host due to their recent arrival to the seafloor (Smith & Williams, 1967). Thus, given that the present study focused on juvenile specimens, further research including adult individuals is needed to determine whether parasite infection increases with host size as well as to assess lesions severity and their potential associated health impairments.

5. Conclusions

In conclusion, the present study provides new molecular and histopathological data on *Aporocotyle spinosicanalis* infecting *Merluccius merluccius* from the northwestern Mediterranean Sea. Phylogenetic analyses confirmed the identity of the parasite and revealed contrasting levels of genetic divergence among geographically distant populations, although additional markers are needed to better resolve intraspecific diversity. Histopathological observations showed that eggs mainly caused mild vascular dilatation and focal inflammation in gills, whereas adults and degenerated eggs in the heart induced more severe inflammatory lesions, including myocarditis and fibrinoid necrosis. Infection prevalence increased with host size, suggesting cumulative exposure through the host's life span. Overall, these findings improve our understanding of the biology and pathogenic potential of *A. spinosicanalis* and highlight the need for further studies on its life cycle, transmission dynamics and impacts on wild hake populations.

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CHAPTER 5

Anthropogenic pollutants and health impacts

Muns-Pujadas, L., Dallarés, S., Constenla, M., Padrós, F., Carreras-Colom, E., Grelaud, M., Carrassón, M., Soler-Membrives, A. (2023). Revealing the capability of the European hake to cope with micro-litter environmental exposure and its inferred potential health impact in the NW Mediterranean Sea. *Marine Environmental Research*, 186, 105921. <https://doi.org/10.1016/j.marenvres.2023.105921>.

Muns-Pujadas, L., Constenla, M., Dallarés, S., Carreras-Colom, E., Soler-Membrives, A. Padrós, F. Assessing the seasonal uptake of micro-litter by round sardinella (*Sardinella aurita* Valenciennes, 1847) and its potential health impact in the NW Mediterranean Sea. *Continental Shelf Research*. Under review.

Abstract

This chapter presents two complementary scientific approaches regarding the assessment of the potential impact of contaminants on the same target fish species from the Catalan coast (NW Mediterranean Sea). The first study was conducted on *Merluccius merluccius* for the characterisation of anthropogenic items (AIs) (microplastics and non-synthetic elements) in the water and ingested by fish at two different times (12 years apart) and three different localities. The second one addressed the characterisation of AIs ingested by *Sardinella aurita* during a year (seasonally). Heavy metals concentrations on fish muscle tissue were also characterised in both studies and their potential impacts in the health of both species were assessed. This evaluation was developed through a multidisciplinary approach integrating condition indices, parasites and histological techniques. Their approaches differed according to sampling objectives, offering valuable information on the spatial and seasonal dynamics of these anthropogenic pollutants and their potential biological implications.

For *M. merluccius*, juvenile specimens and water samples were analysed for AIs off Blanes, Barcelona and Ebro Delta in 2019, including a temporal comparison with hakes from 2007 off Barcelona. Heavy metals levels were assessed from hakes' muscle from the same three localities in 2019. Cellulosic fibres were predominant (77.8% of samples) in both fish and water samples, except for Barcelona. A mean of 0.85 fibres/m³ from the surrounding water was observed, while fish ingested a mean of 1.39 (SD = 1.39) items/individual. No differences in ingested AI abundance and composition off Barcelona between years were found. Small AIs from the water matched ingested AI composition. Hakes did not ingest large fibres despite being present in the environment, probably due to their feeding behaviour. Heavy metals concentrations did not show spatial variability, but mercury was positively correlated with size. No adverse health effects or parasites aggregations were detected to be potentially related to AI ingestion or metal exposure. The second study focused on *S. aurita* specimens collected seasonally between 2022 and 2023 off Barcelona to assess temporal variations in AI ingestion. Moreover, additional specimens were caught in one season to evaluate heavy metal accumulation in muscle tissue. Fish ingested a mean of 2.27 (SD = 2.13) items/individual, with the highest abundance recorded in winter and the lowest in summer, being cellulosic fibres the dominant type (73.56% of AIs). Seasonal variations on AI ingestion were consistent with environmental (i.e. rainfall rates, water stratification) and biological (i.e. feeding behaviour, reproduction) factors. No health effects or parasite aggregations could be attributed to AI exposure, even during the season with the highest ingestion rates. Similarly, no clear adverse effects from metals exposure were detected, as histopathological changes encountered were likely associated with other biological and environmental factors.

1. Introduction

Anthropogenic activities, including urbanization, industrialization and agriculture, result in the discharge of diverse pollutants, including materials, particles, chemicals, pharmaceutical compounds and other contaminants, such as heavy metals (e.g., Hg, Cd, Pb, As), into aquatic ecosystems, with potential adverse effects for organisms due to their persistence and bioaccumulative potential (Yancheva et al., 2015). For the last two decades, growing concern about the impact and potential toxicity of certain anthropogenic stressors such as anthropogenic items (AIs), including microplastics (MPs, < 5 mm; Hartmann et al., 2019) and non-synthetic fibres with an anthropogenic origin (e.g. cellulose, cotton) (Cole et al., 2011; Ryan et al., 2019; Suaria et al., 2020), have been on the spotlight due to their ubiquity and persistence in the marine environment and particularly within the digestive tract of marine biota (Fossi et al., 2018). These pollutants are particularly relevant in semi-enclosed areas such as the Mediterranean Sea, where intense coastal development, maritime traffic and riverine inputs favour their accumulation in marine food webs (Mancuso et al., 2023). Within this context, monitoring frameworks have been implemented to achieve a good environmental status across European seas, such as the Marine Strategy Framework Directive (MSFD, 2008/56/EC) and the Integrated Monitoring and Assessment Programme for the Mediterranean Sea (IMAP, 2016). These programmes addressed a broad range of descriptors, including microplastics and heavy metals, and promoted the use of biomarkers (e.g. histopathological changes in target tissues) and bioindicators (e.g. fish species) to monitor spatial and temporal trends regarding pollution for environmental risk assessment (Bellas et al., 2016; Fossi et al., 2018). In this context, fish are valuable indicators as they are continuously exposed to environmental pollutants, which can absorb and accumulate in their tissues (Yancheva et al., 2015). Variations in trophic level, habitat use and feeding strategies among benthic, benthopelagic and pelagic species influence the uptake of AIs and heavy metals, offering complementary insights into pollutants distribution across marine compartments and their potential biological effects (Fossi et al., 2018; Higuieruelo et al., 2025; Oros, 2025 and references therein).

As an example of benthopelagic species, the European hake, *Merluccius merluccius* (Linnaeus, 1758) has been recommended as a possible small-scale bioindicator species of microplastic and heavy metals pollution in FAO-GSAs due to its commercial and ecological importance, its trophic position and its wide distribution throughout the Mediterranean Sea (Oliver & Massutí, 1995; Fossi et al., 2018; Giani et al., 2019; Salvaggio et al., 2020; Inal et al., 2026). Moreover, owing to its benthopelagic feeding behaviour (Cartes et al., 2004) and since many of its preys move vertically following nictemeral rhythms, feeding takes place at different levels in the water column

(Bozzano et al., 1997), so hakes may be exposed to both AIs and heavy metals from the near-bottom environment as well as from the water column. On the other hand, small pelagic fish species such as the round sardinella, *Sardinella aurita* Valenciennes, 1847, are particularly prone to ingest AIs due to their planktivorous feeding behaviour and high exposure to low-density synthetic items, such as polyethylene and polypropylene, which predominantly float in the water column (Sambolino et al., 2023). Particularly, the round sardinella can switch from particulate to filter-feeding behaviour according to seasonal variations in food availability (Bachiller et al., 2021) and might also be particularly exposed to ingesting AIs from the water column, potentially following seasonal patterns influenced by the environmental conditions. However, the specific impacts of environmental and anthropogenic stressors on wild species remain poorly quantified and the underlying mechanisms remain unclear. Factors such as climate change, shifts in food availability (Feuilloley et al., 2022), as well as exposure to pollutants including AIs and heavy metals (Avio et al., 2020; Shabaka et al., 2020; Bachiller et al., 2021; Zeghdani et al., 2023) have been suggested to affect the condition of these species, potentially leading to trophic mismatches and alterations of the energy transfer dynamics within the pelagic ecosystem, as observed in sardines and anchovies (Albo-Puigserver et al., 2019; Saraux et al., 2019).

Previous studies have reported considerable variability in the type and quantity of AIs ingested by hakes (Fossi et al., 2018; Giani et al., 2019; Mancuso et al., 2019; Bianchi et al., 2020; Avio et al., 2020) and by sardinellas in different localities from the Mediterranean Sea, mainly in the central basin (Avio et al., 2020; Shabaka et al., 2020; Zeghdani et al., 2023). This variability could be attributed to the presence of different types and concentrations of AIs in the different sampling areas and to the different assessment methodologies used in these studies. This bias likely induced by the lack of standardized methods and protocols for the assessment of ingested AIs are hindering comparisons among studies (Lusher et al., 2017; Cowger et al., 2020). Moreover, none of these studies have related hake ingestion of AIs to their concentration in the surrounding environment and, furthermore, none of them have assessed their potential effect on fish health condition.

On the other hand, heavy metals concentrations in the muscle of hakes have been reported in several areas from the Mediterranean such as the Ionic and Adriatic Seas, the coast of Algeria (southern Mediterranean), the Gulf of Lion and the Spanish coasts (northwestern basin) (Falcó et al., 2006; Cossa et al., 2012; Perugini et al., 2014; Llull et al., 2017; Ramos-Miras et al., 2019; Salvaggio et al., 2020; Kaddour et al., 2022). Similarly, metal concentrations in round sardinella have also been reported in the Aegean Sea, the Adriatic Sea, the Strait of Messina (Sicily) and the northern African coasts (i.e. Tunisia, Libya, Egypt) (Tepe et al., 2009; Storelli et al., 2003; Lo Turco

et al., 2013; Annabi et al., 2018; Khalifa et al., 2010; Monier et al., 2023). As expected, spatial variability on metals accumulation has also been observed across studies due to the influence of environmental, anthropogenic and biological factors. Environmental parameters such as pH, salinity, temperature, and dissolved organic matter can modulate metal bioavailability and uptake by affecting their chemical speciation and solubility. For instance, elevated temperatures can increase metabolic and respiratory rates and uptake through gills while salinity and pH may alter the proportion of bioavailable ionic forms (Oros et al., 2025 and references therein). In addition, anthropogenic pressures (e.g., urban discharges, industrial activities, agriculture) determine local contamination levels whereas biotic factors such as size, age, diet, and trophic position play an important role in metal bioaccumulation and biomagnification in food webs (especially for methylmercury) through dietary intake (Storelli et al., 2003). Among these studies, only Salvaggio et al. (2020) and Annabi et al. (2018) assessed the toxicological effects of heavy metals in hakes and sardinellas, respectively, through histopathological analyses, while other studies did not evaluate their impact on the health condition of these species.

To investigate the adverse effects of these pollutants on aquatic organisms, different biomarkers at different biological levels have been applied in ecotoxicological studies (van der Oost et al., 2003 and references therein). Subcellular and cellular biomarkers including biochemical enzymes, oxidative stress indicators and physiological responses have been commonly applied to assess exposure to heavy metals and other chemical pollutants in fish populations (van der Oost et al., 2003 and references therein; Shah & Parveen, 2022). Although these biomarkers are sensitive, specific to certain pollutants and respond rapidly to exposure, their interpretation in terms of adverse effects at the organism level is often challenging, particularly under complex and variable field conditions (Dabrowska et al., 2012; Yancheva et al., 2015, 2016). On the other hand, histopathological alterations are considered among the most effective tools for evaluating toxic effects of chemical pollutants (Au, 2004; Dabrowska et al., 2012; Yancheva et al., 2016; Salamat & Zarie, 2016). In both wild and experimental conditions, histopathological analyses have been mostly applied to benthic flatfish species (e.g. *Platichthys flesus*, *Limanda limanda*, *Solea solea*), revealing cause-effect associations between persistent organic pollutants and heavy metals exposure and toxicopathic liver lesions such as non-neoplastic lesions (e.g. inflammation, necrosis, degeneration), pre-neoplastic changes (e.g., foci of cellular alteration) and neoplasms (e.g., tumors) (e.g. Vethaak & Wester, 1996; Bernet et al., 1999; Stentiford et al., 2003; Myers et al., 2003; Au, 2004; Feist et al., 2004; Lang et al., 2006). Although histopathological lesions are non-specific to pollutants (Salamat & Zarie, 2016), several studies have described alterations following exposure to heavy metals in different fish species due to their well-known chronic

sublethal effects. Most of the reported histopathological alterations in gills include hyperplasia, hypertrophy of epithelial cells, epithelial lifting, lamellar fusion, aneurysm and necrosis. In liver, inflammation, vascular alteration, vacuolization, pyknotic nuclei, melanomacrophage centers and hemorrhage whereas in kidney, damaged glomeruli and renal tubule, necrosis, hypertrophied renal tubule, bile duct proliferation and granulomas were commonly observed (Thophon et al., 2003; Shah & Parveen, 2022; Zahran et al., 2025 and references therein).

Regarding AIs ingestion, assessing their potential impacts on wild marine organisms remain particularly challenging, not only due to the complexity of natural systems but also because the underlying mechanisms and their magnitude remain unclear. Under experimental conditions, numerous studies have explored several debated mechanisms through which MPs may affect fish health, including physical damage to the digestive system, the release of plastic-associated additives, their potential role as vectors for persistent organic pollutants and heavy metals, and their possible translocation to other organs (Cole et al., 2011; Rochman et al., 2013; Avio et al., 2015; Lu et al., 2016; Pedà et al., 2016; Jovanović, 2017; Lusher et al., 2017; Kögel et al., 2020). Some studies have reported the translocation of MPs to organs such as liver, gills and muscle (Lu et al., 2016; Zeytin et al., 2020), although these translocation mechanisms are still poorly understood and debated (Burns & Boxall, 2018; Batel et al., 2020). Increasing attention has been given in particle size, since translocation seem to be more plausible for small particles (particularly nanoplastics, $<1\ \mu\text{m}$) rather than microplastics. In this context, some recent critical assessments have suggested that part of the reported presence of MPs in internal organs, especially in muscle, may be influenced by methodological limitations including contamination during sample processing, insufficient quality control procedures, the difficulties in polymer identification and the reported size particles ($>100\ \mu\text{m}$) which some of them may be biologically not possible due to the size of biological barriers (Lin et al., 2023; Di Fiore et al., 2024). Moreover, it was suggested that other items present within fish digestive tracts such as parasites could increase the surface area for adhesion and retention of AIs ingested (Hernandez-Milian et al., 2019; Pennino et al., 2020). Indeed, Hernandez-Milian et al. (2019) reported that parasites aggregations in the intestine could favour the retention and accumulation of MPs, but other mechanisms could not be disregarded.

Taking this altogether, it is important to note that these experimental conditions do not fully reflect the natural marine environment, where concentrations, particle types and exposure durations differ considerably (Hossain & Olden, 2022). Indeed, there is currently no clear evidence that AIs ingestion alone causes negative health effects in wild fish populations, as organism health in natural environments results from the interaction of multiple stressors such as infectious

agents, environmental conditions, nutritional status and reproductive period, making it necessary to assess biological responses through an holistic framework that integrates different indicators (e.g. condition indices, histopathology and parasitology) (Carreras-Colom et al., 2020, 2022a; Rodríguez-Romeu et al., 2020, 2022; Higuieruelo et al., 2024).

The present study aims to characterize the ingestion of anthropogenic items (AIs), the accumulation of heavy metals, and their potential health impact in two fish species with contrasting feeding strategies in the Balearic Sea (NW Mediterranean Sea). For *M. merluccius*, juvenile specimens captured in 2019 from three sampling areas with different anthropogenic pressure levels (Blanes, Barcelona and Ebro Delta) were analysed (i) to characterize ingested AIs, (ii) compare them with those present from their surrounding environment (i.e. near-bottom water), (iii) assess temporal changes in AIs ingestion within a 12 years-gap from the most polluted locality, (iv) quantify heavy metal levels in muscle and (v) examine potential impacts on health through condition indices, histological alterations and parasite indicators. For *S. aurita*, specimens were seasonally captured in 2022-2023 from Barcelona with the following purposes: (i) to investigate seasonal variations in AIs ingestion, (ii) to discuss the influence of seasonal environmental and biological drivers on AIs uptake based on external data, (iii) to quantify metal concentrations in muscle and (iv) to evaluate their potential impact on fish health through condition indices and histological alterations in different target organs.

2. Materials and methods

2.1. Study area and data collection

A total of 111 *M. merluccius* specimens were captured on different localities (Blanes, Barcelona and Ebro Delta) along the continental shelf of the Catalan coast (NW Mediterranean) in summer 2007 and 2019 (see Table 1 and Fig. 1, Chapter 3.1 M&M). Immediately upon capture, 82 hakes were fixed *in toto* in 10% buffered formalin, until further AIs analysis was developed, while 29 hakes were frozen at -20 °C for subsequent metal analyses (see Table 1, Chapter 3.1 M&M). Environmental samples of near-bottom water were collected during fishing trawls in 2019 to ensure that they were taken in exactly the same surrounding environment as hake specimens. The filtered material from water samples was collected using a WP2-net (200µm mesh size), equipped with a mechanical flowmeter that was towed horizontally at a velocity of 2-3 knots and at 5-10 m above the seafloor along each trawl (ca. 30 minutes). Filtered water samples were

immediately fixed in 10% buffered formalin until further AIs extraction (see below). In 2019, environmental variables were also recorded at each locality (see Table 2, Chapter 3.1 M&M).

Moreover, 110 *S. aurita* specimens were seasonally collected between 2022 and 2023 off Barcelona (see Table 1 and Fig. 1, Chapter 3.1 M&M). Immediately upon capture, 100 sardinellas ($n = 20$ specimens per season) were fixed *in toto* in 10% buffered formalin, until further AIs analysis was developed, while 10 sardinellas were frozen at -20°C for subsequent metal analyses (see Table 1, Chapter 3.1 M&M). Due to the lack of recorded environmental conditions, environmental data of the study area for each season (i.e. sea surface temperature, chlorophyll-a concentrations, average volume of the Besòs and Llobregat rivers and rainfall trends) were provided from external databases (see Table 3 and Fig. 2, Chapter 3.1 M&M).

2.2. Anthropogenic items analysis

At the laboratory, after taking biometric data for each specimen from both studied species (Chapter 3.1 M&M), fish were dissected and the stomach and intestine were preserved in filtered 70% ethanol for visual inspection of AIs following the protocol described in Chapter 3.3.1.1 M&M. In 2019, near-bottom water samples were also inspected for AIs as explained in Chapter 3.3.1.2 M&M.

The isolated AIs from each sample (fish contents and near-bottom water samples) were classified according to its shape, measured and classified into five different typologies according to the visual aspects of their morphology (see Chapter 3.3.1.3 M&M). Then, a representation of at least 30% of fibres of each typology were identified by micro-FTIR and FTIR spectroscopy. Likewise, all synthetic AIs (belonging to categories B, C, D and E), which by their morphological features were doubtful, were also analysed. Moreover, a representative subsample of non-synthetic AIs from category A was also analysed to represent at least 10% of all cellulosic-like AIs found at each sampling point. The resulting spectra were processed and identified following the procedure described in Chapter 3.3.1.4 M&M. Several preventive measures were taken during fish dissection, water samples and inspection of AIs to avoid airborne contamination (see Chapter 3.3.1.5 M&M).

2.3. Heavy metals analysis

Concentrations of cadmium (Cd), lead (Pb), zinc (Zn), copper (Cu), nickel (Ni), and arsenic (As) from muscle samples of *M. merluccius* and *S. aurita* were analysed at the Servei d'Anàlisi Química (SAQ, Chemistry Analytical Service) from Universitat Autònoma de Barcelona (UAB) and at the Instituto Español de Oceanografía (IEO-CSIC, Vigo, Spain), respectively. Total mercury (Hg)

concentration from both species was determined at the IEO-CSIC. Laboratory procedures of determination of heavy metals is described in Chapter 3.3.2 M&M. Considering the fact that no seasonal changes were detected in heavy metals concentrations of *S. aurita* in previous studies from the Mediterranean Sea (Lo Turco et al., 2013; Copat et al., 2015; Monier et al., 2023), a single batch of ten frozen fish from winter 2022 was used for these analyses (see Table 1 Chapter 3.1 M&M).

Metals concentrations are reported in mg/kg dry weight to reduce variability from tissue water content and to assess bioaccumulation in muscle tissue and converted to wet weight for comparison with literature.

2.4. Fish health condition analysis

Fish condition indices (Kn, GSI and HSI), feeding intensity (FULL), parasite community composition and histopathological alterations of both fish species were previously analysed in Chapter 4, following Chapter 3.2 M&M procedures. These data were also used in the present chapter to further explore the potential impacts of AIs ingestion and heavy metals concentrations on fish condition.

Regarding parasitology, only prevalence (P) and mean intensity (MI) of the main taxonomic groups (e.g. Nematoda, Digenea, Cestoda, Monogenea, etc.) were calculated in this chapter.

For the histological assessment (following Chapter 3.2.3 M&M procedure), the digestive tract was evaluated due to its direct contact with AIs and the liver was selected as a target organ for the assessment of potential changes associated to the presence of toxic additives within the AIs. The potential changes induced by heavy metals were evaluated in all examined organs from Chapter 4. Each sample was qualitatively examined for the identification of specific disorders which could be potentially related to heavy metals, the ingestion of AIs or their associated toxic substances. Prevalence and mean intensity or mean density of histological alterations found in each fish was performed in Chapter 4. Rodlet cells (RC) intensity only present in *M. merluccius* was calculated from a random subsample of 10 specimens per locality through a semi-quantitative analysis from histological sections of the stomach mucosa. The quantitative assessment of RC was considered in this study as their increase in number is related to exposure to physical and chemical injuries (Manera & Dezfali, 2004), and their presumed relationship to MPs exposure was suggested by Pedà et al. (2016). Three fields of view were randomly selected and counts of the number of rodlet cells were performed on each section at 400x of magnification, and scored according to cluster partitioning around medoids (PAM) as: low (1) (<11 RC per observation field), medium (2) (12-16

RC per observation field) and high (3) (>17 RC per observation field). Mean intensity was calculated from the average semi-quantitative value of each individual.

2.5. Data analysis

For each fish individual from both species, AIs prevalence (P, as the proportion of fishes of the total sample containing AIs), mean abundance of AIs (MA, as the mean number of AIs ingested in all analysed fish), and mean intensity of AIs (MI, as the mean number of AIs ingested excluding those individuals without AIs) were calculated for fibres and fragments. In addition, the total length of fibres occupying the digestive system of fish (TLAI, as the sum of all fibres lengths), mean fibre length (ML, as the mean of all fibres lengths) and mean fibre diameter (MD, as the mean of all fibres diameter) were calculated also for each individual fish. All the aforementioned calculations were performed for the total of AIs, and separately for synthetic and non-synthetic in the case of fibres. All variables were tested for normality and homoscedasticity using the Shapiro-Wilk test and the Levene's test, respectively. Data distribution was also plotted for visual assessment.

To assess differences in P, MA, MI of fragments and P, MA, MI, ML, TLAI, MD of either the total of fibres and separately for synthetic and non-synthetic fibres among localities and years (in the case of *M. merluccius*) and seasons (in the case of *S. aurita*), General (GLM) or Generalized Linear Models (GZM) (logistic model for P and negative binomial model for MA, MI, ML and TLAI) were used, with SL and FULL set as covariates (previously calculated in Chapter 4), to account for potential size and feeding intensity effects on AIs ingestion. Then, using organs (i.e. stomach, intestine and pyloric caeca) of individual fish as replicate samples, differences among organs (considering globally all localities or seasons) were tested for P using GZM with logistic distribution, and for MA, MI, TLAI, ML and MD of either the total of fibres and separately for synthetic and non-synthetic fibres using GZM with negative binomial distribution. Post-hoc pairwise comparisons were conducted using estimated marginal means tests (EMM, emmeans function) with Holm adjustment. Differences in ML of fibres from water samples among localities were tested using Kruskal-Wallis tests and Dunn's multiple comparison tests to explore significant effects.

For *M. merluccius*, fibres lengths from the digestive tract and from water samples were classified into four size clusters by partitioning around medoids (PAM) applying the k-medoids algorithm on a matrix of dissimilarity, using the Manhattan distances to calculate dissimilarities. Likewise, rodlet cells (RC) intensity was also classified into three clusters by PAM. Each cluster was represented by one object which is located at its centre. The k clusters are established by assigning each object

of the dataset to the nearest of the central objects, thus objects that show a high level of similarity are grouped together, while objects which are most dissimilar are assigned to different clusters (Kaufman & Rousseeuw, 1990). To assess differences among sampled localities and between years (off Barcelona) and organs as well as between fish and environmental samples in fibres size clusters proportions and AIs typologies, Chi-Squared or Fisher-exact tests (when sample size was too small) were applied. Also, Fisher-exact tests were used to detect differences in RC categories among localities and pairwise tests were performed to determine which pairs of categories contributed to the overall significance.

Differences in hake's heavy metals concentrations among localities were also tested using GLM or GZM (gamma distribution) with SL as covariate. Post-hoc pairwise comparisons were conducted using estimated marginal means tests (EMM, emmeans function) with Holm adjustment. When significant interactions between localities and fish size were found, size was mean-centred prior to analysis to better interpret these differences, and pairwise comparisons among locations were conducted at representative body sizes corresponding to the mean body size and ± 1 standard deviation.

For both species, possible associations among AIs (MA and TLAI) and histological alterations (i.e. granulomas, inflammation areas), rodlet cells intensity and parasites were tested using GZMs (for prevalence) and Spearman correlation tests (for mean density of histological alterations and mean intensity of parasites). Moreover, a correlation matrix was built to test possible associations among AIs descriptors (MA, TLAI and ML), RC and parasite intensities, condition indices and FULL (previously calculated in Chapter 4) by means of Pearson's and Spearman's correlation tests. These correlations were performed using individuals for which all the above-mentioned variables were measured.

In the case of metal concentrations, since they were measured from a different subset of individuals at each locality, individual-level correlations between metals and condition indices, parasites and histopathological alterations could not be performed. Instead, potential associations were explored using multivariate analyses at the population level, considering each locality or season as an independent population. Therefore, Redundancy analyses (RDA) were carried out to visualize potential associations of fish responses (i.e. condition indices and histopathological alterations) and parasite intensities with anthropogenic pollutants (i.e. anthropogenic items and heavy metals concentrations), as explanatory variables, with SL and stomach fullness as covariables. A backwards stepwise selection procedure based on a permutation test was performed to select the most significant explanatory variables. The

statistical significance of the constrained ordination and explanatory variables was assessed using 999 permutations. Finally, a Multiple Factor Analysis (MFA) was used in the case of *S. aurita* to simultaneously assess the relationship among Als parameters, heavy metal concentrations, intensity of main parasite groups found in the digestive tract, histopathological alterations and fish health condition indices, and to visualize and differentiate groups of samples according to these variables. This multivariate analysis enables analysing and visualizing individuals that are characterized by different sets of variables (both quantitative and qualitative) which are structured into groups. Thus, the differences within groups are minimized whilst differences among groups are maximized.

Data analysis was performed using RStudio software, version 4.1.2, and using the PAMK function implemented in the package “fpc”, the packages “dunn.test”, “Hmisc”, “vegan”, “FactoMineR” and “factoextra”. Statistical significance was set at 0.05.

3. Results

3.1. The case of the benthopelagic *Merluccius merluccius*

3.1.1. Spatial and temporal patterns of Als ingested

A total of 41 of the 62 analysed hakes from 2019 contained Als in their digestive tracts. A significant major P of Als was noticed off Blanes (90.9%), followed by Ebro Delta (63.1%) and Barcelona (42.8%) (GZM, $\chi^2 = 10.289$, $p = 0.006$). A total of 95 Als were found within the digestive tracts of *M. merluccius*. The vast majority were fibres (97.8% of the total). Only one fragment (0.03 mm²) and one film (2 mm²) were detected in the intestine of two different individuals in Barcelona and Blanes, respectively. MA of Als found per individual was 1.39 (SD=1.39) and MI of Als per individual was 2.13 (SD=1.18). The ML of fibres found was 1.56 (SD=1.24) mm, their MD was 0.03 (SD=0.07) mm and the mean TLAI found per individual was 3.43 (SD=2.67) mm.

No differences of MA, MI, ML, TLAI and MD were observed between stomach and intestine ($p > 0.05$). Blanes was the location with the highest P of non-synthetic Als (GZM, $\chi^2 = 15.314$, $p = 0.003$) and MA of both synthetic (GZM, $\chi^2 = 6.388$, $p = 0.03$) and non-synthetic (GZM, $\chi^2 = 8.121$, $p = 0.03$), and global Als (GZM, $\chi^2 = 7.247$, $p = 0.03$) (**Table 1**). No significant differences among localities were found for the values of MI, TLAI, ML and MD of either synthetic or non-synthetic Als (GZM, $p > 0.05$) (**Table 1**).

A total of 7 of the 20 analysed hakes off Barcelona from 2007 contained AIs in their digestive tracts. A total of 13 AIs were found within their digestive tracts (**Table 1**). No significant differences in terms of P, TLAI, MA, MI and ML of ingested AIs were found between 2007 and 2019 in Barcelona.

3.1.2. AIs from environmental water samples

A total of 180 AIs were found in near-bottom water samples. This is equivalent to an overall average concentration of 0.85 AIs/m³ (0.52 AIs/m³ off Blanes, 1.53 AIs/m³ off Barcelona and 0.50 AIs/m³ off Ebro Delta) (**Table 1**). All AIs detected in environmental samples were fibres. The ML of these fibres was 14.65 (SD=14.29) mm and the MD was 0.02 (SD=0.004) mm. Significant higher values of ML of synthetic fibres off Barcelona were detected compared to the other localities (K-W, $\chi^2 = 26.15$, $p < 0.01$) (**Table 1**).

Table 1. Prevalence of AIs ingested by fish and mean (standard deviation, SD) of the abundance, intensity, sum of total lengths (TLAI) and mean length (ML) of the AIs (synthetic and non-synthetic) found in digestive contents of hakes in 2007 and 2019, as well as mean (SD) of the abundance, TLAI and ML in water samples, from each sampled locality in 2019. Different superscript letters indicate significant differences among localities.

		Blanes	Barcelona		Ebro Delta	Total
		2019	2019	2007	2019	2019
Digestive content	n	22	21	20	19	
Prevalence (%)	Synthetic	40.91	14.28	10.00	21.05	25.80
	Non-synthetic	86.36 ^b	38.09 ^a	35.00	57.89 ^a	61.29
Mean abundance (n/ind)	Synthetic	0.60 (0.85) ^a	0.14 (0.36) ^b	0.15 (0.49)	0.26 (0.56) ^b	0.34 (0.65)
	Non-synthetic	1.50 (1.14) ^a	0.52 (0.75) ^b	0.50 (0.76)	1.11 (1.41) ^b	1.05 (1.18)
Mean intensity (n/ind)	Synthetic	1.44 (0.73)	1.00 (0.00)	1.50 (0.71)	1.25 (0.50)	1.31 (0.60)
	Non-synthetic	1.74 (1.05)	1.38 (0.52)	1.43 (0.53)	2.22 (1.20)	1.78 (1.02)
TLAI (mm/ind)	Synthetic	3.78 (2.54)	1.37 (0.66)	2.29	2.99 (2.99)	2.71 (2.52)
	Non-synthetic	2.41 (1.97)	1.52 (1.07)	1.43 (0.95)	2.99 (2.22)	2.36 (1.91)
ML (mm/ind)	Synthetic	2.54 (1.29)	1.37 (0.66)	1.15	2.11 (1.58)	2.27 (1.30)
	Non-synthetic	1.34 (0.84)	1.35 (1.16)	0.87 (0.37)	1.32 (0.69)	1.34 (0.87)
Environmental samples	n	1	1		1	
Mean Abundance (n/m ³)	Synthetic	0.21	1.33		0.06	0.53
	Non-synthetic	0.31	0.19		0.44	0.32
TLAI (mm/m ³)	Synthetic	0.07	0.27		0.11	0.15
	Non-synthetic	0.12	0.03		0.05	0.07
ML (mm/AI)	Synthetic	1.25 (0.49) ^a	22.40 (12.92) ^b		6.58 (5.63) ^a	20.68 (13.56)
	Non-synthetic	2.00 (1.76)	1.34 (0.99)		1.60 (1.13)	1.66 (1.30)

3.1.3. Als size classification

Fibres were classified into four different size classes based on the PAM algorithm: small (≤ 1.15 mm), medium (1.16 – 2.62 mm), large (2.63 – 6.39 mm) and extra-large (≥ 6.40 mm). The most abundant size category within the digestive tracts of hakes was small (46.2%), followed by medium (39.8%) and the less abundant size category was large (14%). No extra-large fibres were found in fish samples. No significant differences in the size of fibres ingested by hakes were found among localities nor between organs and years (Chi-squared, $p > 0.05$). In near-bottom water samples, small was the most abundant fibre size class off Blanes (55.6%) and Ebro Delta (48.5%), contrary to Barcelona, where the most abundant size class was extra-large (79.1%). In this case, significant differences in the size of the fibres from water samples were found among localities (Chi-squared, $\chi^2 = 73.86$, $p < 0.001$). Fibre size distribution from water samples was significantly different to that of fibres ingested by hakes in Barcelona (Chi-squared, $\chi^2 = 11.79$, $p = 0.008$), but not in the case of the other localities (Fig. 1, Table S1).

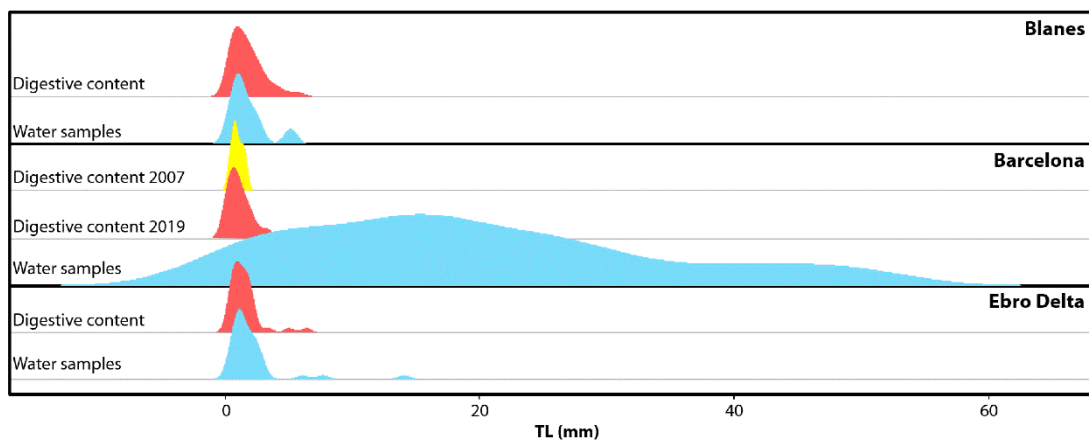


Fig. 1. Density standardized charts showing the distribution of the total mean length (ML, mm) of fibres from the digestive tract of *Merluccius merluccius* in 2019 (red colour), 2007 (yellow colour) and from near-bottom water samples (blue colour) according to the sampled locality.

3.1.4. AIs polymer characterization

The 100% of the resulting spectrums from AIs belonging to category A were identified as cellulose. From category B, 86.3% of the spectrums were identified as polyethylene terephthalate (PET), 9.1% as polyamide (PA) and 4.5% as cellulose. In the case of category C, 100% of the resulting spectra corresponded to acrylic. Regarding category D, 96.2% of the resulting spectra matched to PA and 3.8% to PET. Finally, 75% of the resulting spectra from AIs belonging to typology E were identified as polypropylene (PP) and 25% as PET (**Fig. 2**).

Of the 108 AIs found in the hake's digestive tract in 2007 and 2019, cellulose was the most frequent polymer in all localities (overall prevalence 71.3%). The rest of the AIs were of synthetic composition, mostly PET (overall prevalence ca. 20%), but also acrylic and PP. No significant differences among localities nor years were noticed in terms of polymer composition (Chi-squared, $p > 0.05$).

Regarding the 180 AIs found in near-bottom water samples, cellulose was the most frequent polymer in Blanes and Ebro Delta. However, PA fibres were predominant in Barcelona. In this case, significant differences were found in polymer composition among localities (Chi-squared, $\chi^2 = 134$, $p < 0.001$). Composition of the AIs found in near-bottom water samples was significantly different from that of the AIs ingested by hakes in Barcelona (Chi-squared, $\chi^2 = 97.8$, $p < 0.001$) (**Fig. 3**).

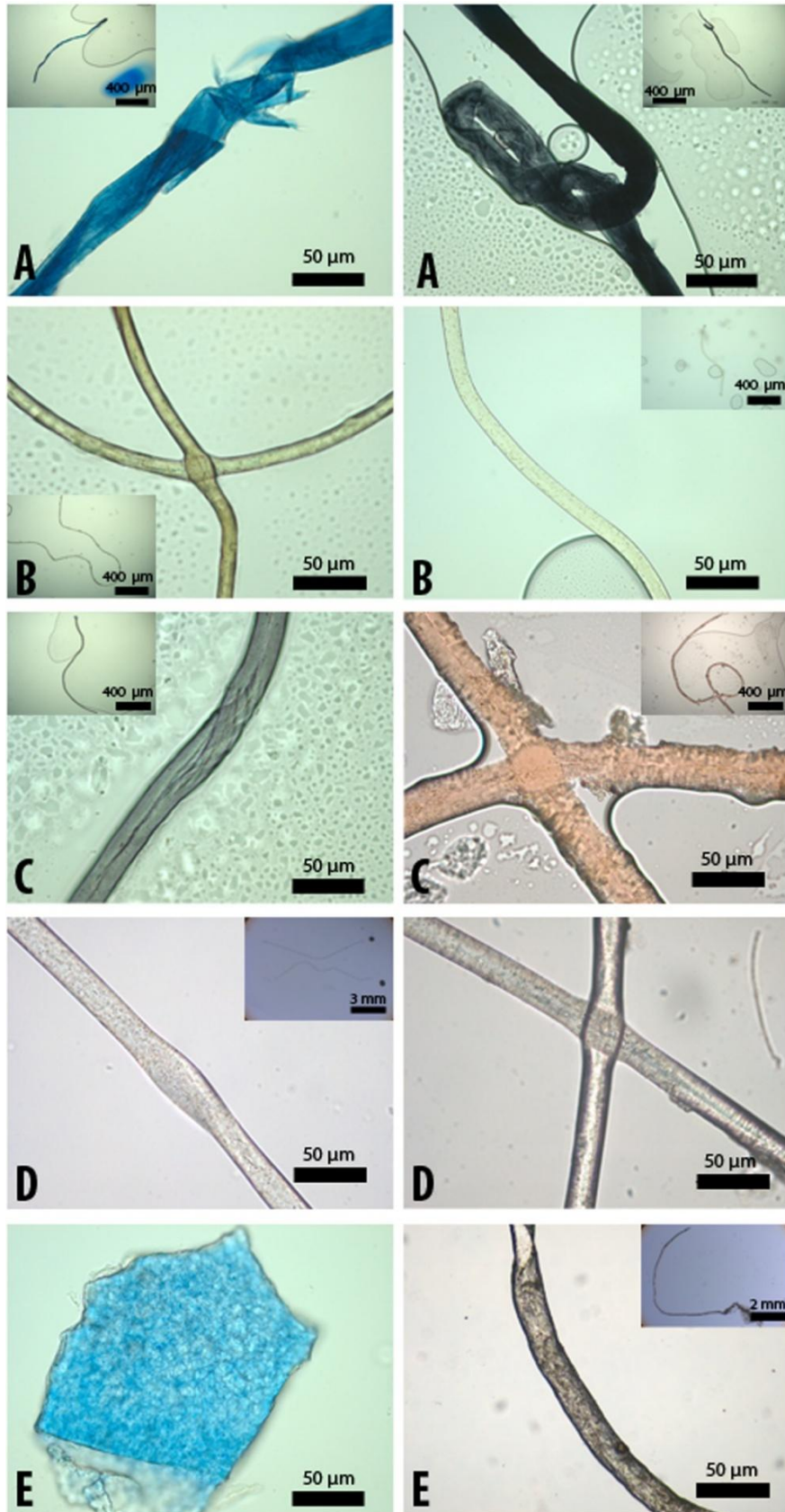


Fig. 2. Images of AIs representative of the five different typologies of polymers found in the digestive tract of *Merluccius merluccius* and in the surrounding near-bottom water samples. Each category (A to E) is described in Table 2 from Chapter 3.3.1.3 M&M.

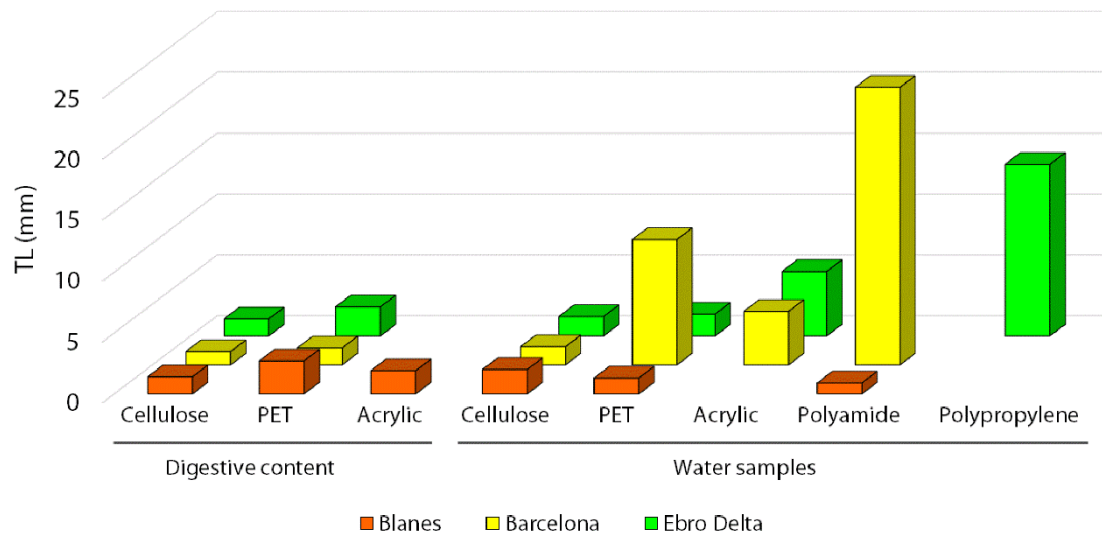


Fig. 3. Mean size (ML) and polymer composition of the AIs found in the digestive content of *Merluccius merluccius* and in water samples from each sampling area: off Blanes, off Barcelona and off Ebro Delta.

3.1.5. Heavy metals concentrations

Heavy metals concentrations in hake's muscle from each locality are shown in **Table 2**. Individuals from Blanes analysed for heavy metals were significantly larger than those from Barcelona and Ebro Delta ($K-W = 24.64$, $p < 0.001$). Significant interactions between fish length and location were detected for Zn (GLM, $F_{(2, 23)} = 4.11$, $p = 0.03$) and Cd (GZM, $z = 2.46$, $p = 0.02$). Pairwise comparisons at specific fish sizes showed that small individuals (<18.86 cm) from Barcelona had significantly higher Zn levels than those from Blanes (emmeans, $t = 2.91$, $p = 0.024$), and higher Cd levels than those from Blanes and Ebro Delta (emmeans, $t = 2.68$, $p = 0.02$ and $t = 4.51$, $p = 0.0005$, respectively). No significant differences among locations were observed at medium (18.86 cm) and larger (>18.86 cm) sizes. Hakes from Blanes displayed significant higher concentrations of Hg compared to those from Barcelona and Ebro Delta, but it was related to size (GZM, $z = 5.79$, $p < 0.001$). Spearman correlations indicated that fish length was positively correlated with concentrations of Hg ($\rho = 0.66$, $p < 0.001$) and negatively correlated with Zn ($\rho = -0.72$, $p < 0.001$). No other metal concentration was correlated with fish size.

Table 2. Number (n), mean and (standard deviation) of standard length (SL) and heavy metals concentrations in muscle tissue of *Merluccius merluccius* specimens captured off three localities from the Catalan coast (NW Mediterranean Sea) are provided. Metallic elements analysed are nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) expressed in mg/kg dry weight (dw) and wet weight (ww). <LOD indicates below the limit of detection (see Chapter 3.3.2 M&M). Number of individuals with values <LOD and >LOD are indicated between brackets. Significant differences among localities are indicated by different superscript letters.

Blanes		Barcelona		Ebro Delta	
n	9	10	10	10	
SL	24.22 (2.95) ^a	18.33 (1.47) ^b	14.58 (0.45) ^c		
dw		dw		dw	
ww		ww		ww	
Ni	<LOD(5)/0.19 (0.14)(4)	<LOD(5)/0.04 (0.03)(4)	<LOD(4)/1.01 (1.86)(6)	<LOD(4)/0.20 (0.36)(6)	<LOD(6)/0.98 (0.33)(4)
Cu	1.19 (0.47)	0.24 (0.09)	1.19 (0.16)	0.24 (0.03)	0.97 (0.33)
Zn	14.33 (0.95)	2.91 (0.22)	15.77 (1.59)	3.19 (0.25)	17.23 (0.96)
As	17.25 (5.18)	3.49 (0.99)	17.9 (3.5)	3.63 (0.73)	20.5 (4.11)
Cd	<LOD(8)/0.003(1)	<LOD(8)/0.001(1)	<LOD(4)/0.004 (0.001)(6)	<LOD(4)/0.001 (0.0003)(6)	<LOD(9)/0.004 (0.0003)(1)
Pb	<LOD(4)/0.39 (0.56)(5)	<LOD(4)/0.08 (0.12)(5)	<LOD(2)/0.12 (0.13)(8)	<LOD(2)/0.03 (0.027)(8)	<LOD(2)/0.11 (0.07)(8)
Hg	0.52 (0.03) ^a	0.11 (0.007)	0.27 (0.08) ^b	0.05 (0.02)	0.29 (0.08) ^b
					0.05 (0.01)

3.1.6. Potential health impacts from AIs and heavy metals

Histopathological analyses did not reveal alterations in the mucosa of the stomach or the intestine that could be eventually associated to AIs abrasion (see Chapter 4). However, high prevalences (>90%) and a high number of rodlet cells (RC) in the stomach mucosa (see Fig. 8 Chapter 4) and some granulomas were detected in all localities. Mean RC intensity differed among localities with hakes from Ebro Delta exhibiting higher mean RC intensities (mean $\pm$ SD: 2.10 ± 0.88) compared to Blanes (1.70 ± 0.95) and Barcelona (1.60 ± 0.84) but were not significantly correlated with AIs ingestion nor either parasite intensity from the digestive tract. Findings and changes observed in the histopathologic evaluation of the liver consisted of lipidic metamorphosis, granulomas, macrophages aggregates and small inflammation areas (see Chapter 4). However, none of them could be related to AIs ingestion since no clear statistical relationships were found between the prevalence and mean densities of these observations with levels of AIs ingested by individuals. Similarly, no associations were found between parasite P or MI and levels of AIs ingested by fish (GZM, $p > 0.05$).

The correlation matrix indicated no clear relationships among AIs parameters and fish condition indices, stomach fullness or parasites (previously described in Chapter 4). General AIs parameters (abundance, sum of lengths and mean lengths) were positively correlated among them ($\rho > 0.6$), with MA and TLAI of non-synthetic fibres showing high correlation values with total AIs MA ($\rho = 0.93$, $p < 0.001$ and $\rho = 0.92$, $p < 0.001$), respectively. ML of synthetic AIs displayed high correlation values with ML of total AIs ($\rho = 0.82$, $p < 0.001$) (**Fig. 4**).

Apart from these histopathological observations, macrophages aggregates and melanomacrophage centres were also found in the splenic and renal tissue, displaying higher mean densities in hakes from Barcelona compared to Ebro Delta (see Chapter 4). However, no correlations between levels of heavy metals and histopathological findings could be performed at the individual level due to methodological limitations.

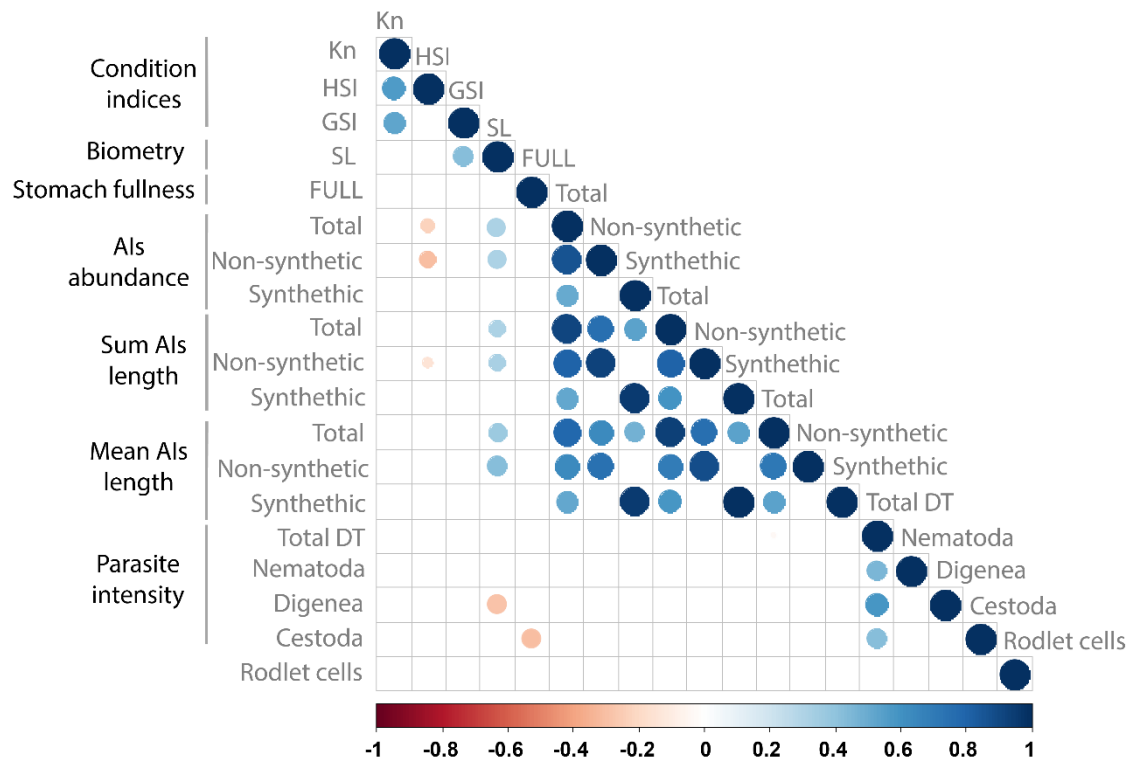


Fig. 4. Correlation matrix indicating positive (blue) and negative (red) significant ($p < 0.05$) correlations among total AIs abundance (Total), non-synthetic fibres (Non-synthetic) and synthetic fibres (Synthetic) abundance, sum of total AIs lengths (Total), non-synthetic fibres (Non-synthetic) and synthetic fibres (Synthetic) sum of lengths, mean of total AIs lengths (Total), non-synthetic fibres (Non-synthetic) and synthetic fibres (Synthetic) mean of lengths, fish standard length (SL), stomach fullness (FULL), Le Cren relative condition index (Kn) and hepatosomatic index (HSI), total intensity of parasites from the digestive tract (Total DT), and of main parasite taxa (i.e. nematodes, digeneans and cestodes), and rodlet cells intensity found within *Merluccius merluccius* digestive tracts. Circle size and colour intensity are proportional to the value of the correlation coefficient.

The RDA relating potential associations of AIs, metals concentrations and environmental variables with condition indices (Kn, HSI and GSI) and SL and stomach fullness as covariates at the population level only explained 20.14% of the total variation by the first two axes (**Fig. 5a**). The global model was significant (permutation test, $F_{(58,3)} = 4.88$, $p = 0.008$) and the most significant explanatory variables of the model was turbidity ($p = 0.004$), followed by non-synthetic AIs and salinity. Most of the constrained variation was captured by the first RDA axis (RDA1, $F = 13.57$, $p = 0.003$). The explanatory variables (turbidity and non-synthetic AIs) were significantly associated with RDA1 axis, but correlations were moderate ($r = 0.42$, $p = 0.0007$ and $r = 0.28$ and $p = 0.02$, respectively), whereas HSI and Kn were strongly negatively correlated with RDA1 axis ($r = -0.99$, $p < 0.001$ and $r = -0.55$, $p < 0.001$, respectively). However, no clear associations were detected between condition indices and pollutants.

On the other hand, the RDA relating AIs, metals concentrations and environmental variables with histopathological observations explained 30.25% of the total variation by the first two axes (**Fig. 5b**). The model was significant (permutation test, $F_{(54, 7)} = 3.46$, $p = 0.004$) and turbidity, Pb and synthetic AIs were the most significant explanatory variable of the model (permutation test, $F = 8.65$, $p = 0.001$, $F = 4.65$, $p = 0.03$ and $F = 3.66$, $p = 0.04$, respectively). The first RDA axis explained most of the constrained variation (RDA1, $F = 22.58$, $p = 0.009$). The variables synthetic AIs and Cd were significantly but moderately associated to RDA1 axis ($r = 0.27$, $p = 0.03$ and $r = -0.35$, $p = 0.004$). For response variables, mean density values of splenic aggregated macrophages and melanomacrophage centres were strongly correlated with RDA1 axis ($r = -0.85$, $p < 0.001$ and $r = 0.58$, $p < 0.001$, respectively). Visually, mean density values of splenic aggregated macrophages were positively associated with Cu levels and negatively related with Pb, Zn and Ni. No other histopathological alterations were related to pollutants.

Finally, RDA analyses relating parasite intensities with AIs and heavy metals concentrations did not reveal any significant association ($p > 0.05$, RDA not included).

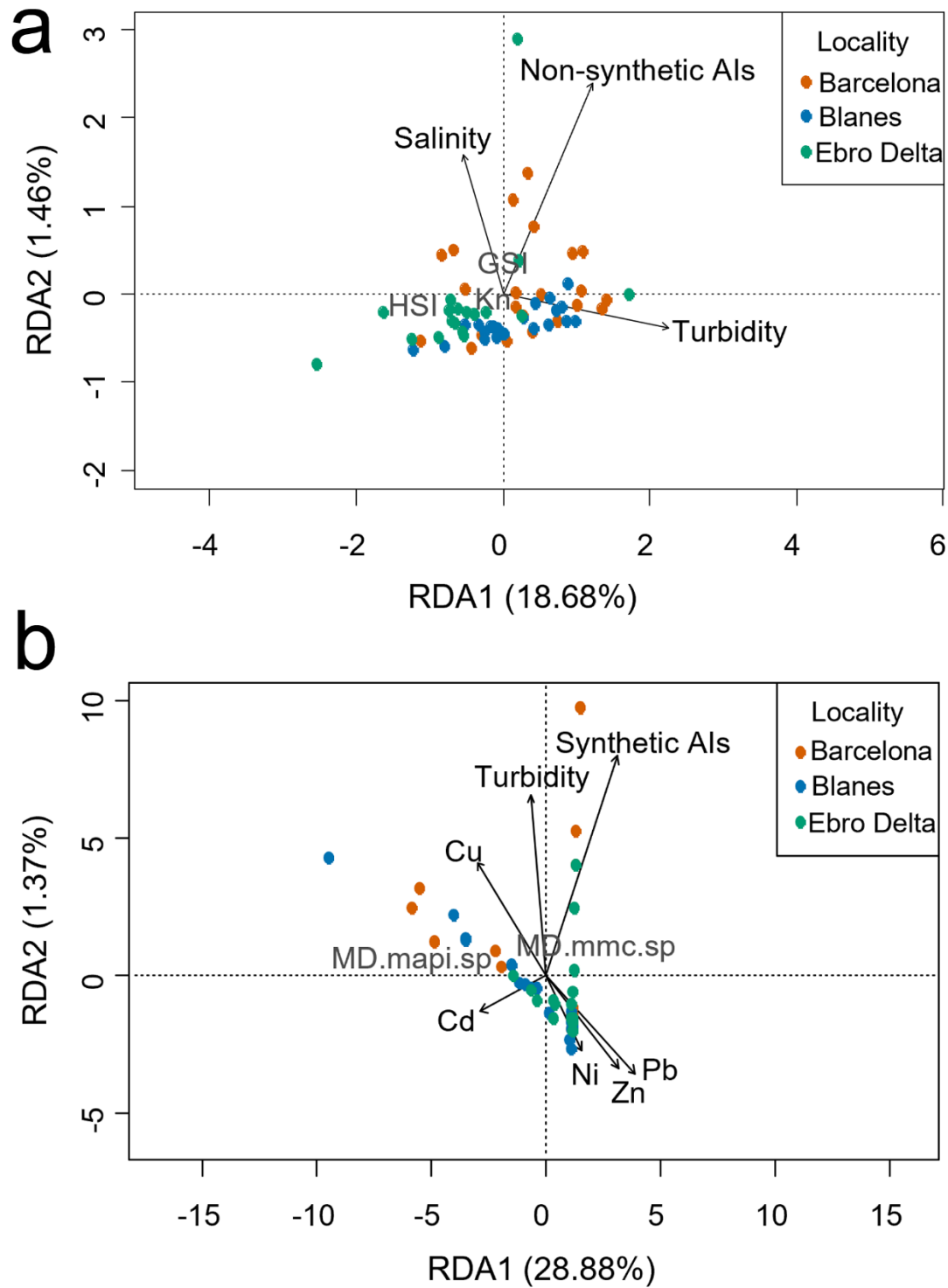


Fig. 5. Redundancy analysis (RDA) on **a)** condition indices and **b)** histopathological alterations of *Merluccius merluccius* constrained by anthropogenic items (Als) ingested and metal concentrations in muscle tissue most significant for the ordination. Abbreviations: Kn, Le Cren relative condition index; HSI, hepatosomatic index; GSI, gonadosomatic index; MD.mmc.sp, mean density of melanomacrophage centres in spleen; MD.mapi.sp, mean density of aggregated macrophages in spleen.

3.2. The case of the pelagic *Sardinella aurita*

3.2.1. Seasonal patterns of AIs ingested

A total of 80 of the 100 individuals of *S. aurita* examined contained at least one AI within the digestive tract, accounting for a total of 227 AIs. The vast majority were fibres (93.39%), from which 78.77% were non-synthetic, 18.86% were synthetic and 1.88% could not be identified. Only 15 fragments were found, and all of them were made of synthetic polymers.

From overall AIs (including fragments and fibres), seasonal differences were observed for P, MA and MI of ingested AIs. Fish from winter-23 and summer-22 showed the highest levels of AIs ingestion compared to those from summer-23 (GZM, P: $z = 2.77$, $p = 0.005$; MA: $z = 4.41$, $p < 0.001$; MI: $z = 2.78$, $p = 0.04$) (Fig. 6).

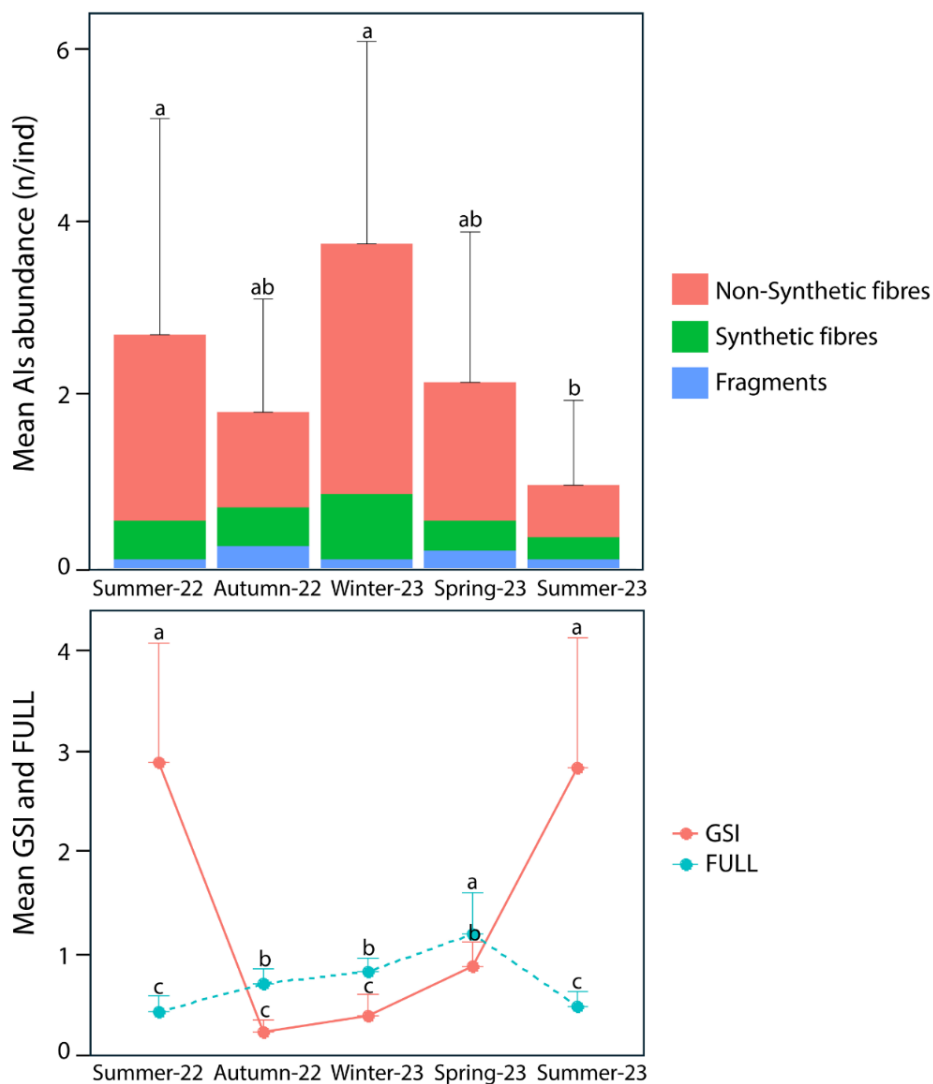


Fig. 6. Seasonal mean abundance values ($\pm$ standard deviation) of total anthropogenic items (AIs) per individual (n/ind), including non-synthetic and synthetic fibres and fragments recovered from the digestive

tract of *Sardinella aurita* specimens collected in the Balearic Sea (NW Mediterranean) are shown. Mean gonadosomatic index (GSI) and stomach fullness index (FULL) ($\pm$ standard deviation) for each season are also displayed. Different superscript letters indicate significant differences among seasons.

Fragments were found in only 7% of fish, ranging from 0.25 to 2.13 mm², with a mean area of 0.85 (SD = 0.69) mm². Fish from spring-23 showed the highest MA of fragments, with a significant interaction between season and FULL (GZM, $z = -1.97$, $p = 0.048$) (**Table 3**). No significant differences were observed among seasons for P and MI of fragments (GZM, $p > 0.05$).

Regarding fibres, they were found in 78% of fish. Most fibres size (96.66%) ranged from 0.05 to 4.85 mm (ML = 1.45 (SD=0.90) mm). Moreover, eight fibres larger than 5 mm, ranging between 5.37 and 8.72 mm (ML = 6.26 (SD=1.26) mm), and two exceptionally long fibres of 14.18 and 21.04 mm were also found. Fish from winter-23 displayed significantly higher values of P of non-synthetic fibres compared to those from summer-23 (GZM, $z = -2.80$, $p = 0.04$) (**Table 3**). A similar significant trend was observed for the MA of total fibres and non-synthetic fibres, with higher values in specimens from winter-23 compared to those from summer-23 (GZM, total: $z = -4.33$, $p < 0.001$ and non-synthetic: $z = -4.13$, $p < 0.001$) and autumn-22 (GZM, total: $z = -3.04$, $p = 0.02$ and non-synthetic: $z = -2.88$, $p = 0.03$, respectively) (**Table 3**). Specimens from winter-23 also presented significantly higher MI values of total fibres compared to summer-23, with a significant interaction detected between season and FULL (GZM, $z = -2.09$, $p = 0.03$). TLAI was significantly higher in fish from spring-23 compared to summer-23 and ML of non-synthetic fibres ingested by fish from spring-23 were also higher compared to other seasons (GZM, total TLAI: $z = -2.11$, $p < 0.001$; non-synthetic TLAI: $z = -3.15$, $p < 0.001$; synthetic TLAI: $z = -3.35$, $p < 0.001$; non-synthetic ML: $z = 2.33$, $p = 0.04$) (**Table 3**).

Overall, 41.51% of the fibres were recovered from the stomach, 37.73% from the intestine, and 20.75% from the pyloric caeca. Significantly lower P and MA of total and non-synthetic fibres were found in the pyloric caeca compared to the stomach and intestine (GZM, total P: $z = -1.90$, $p = 0.05$ and MA: $z = -2.41$, $p = 0.015$; non-synthetic P: $z = -2.42$, $p = 0.015$ and MA: $z = -2.43$, $p = 0.014$). The stomach was the organ which presented significantly higher values of TLAI of both total and non-synthetic fibres (GZM, $z = 2.92$, $p = 0.003$ and $z = 2.18$, $p = 0.028$, respectively). No significant differences were observed for MI, ML and MD of fibres among organs ($p > 0.05$).

Table 3. Prevalence (P%) of fragments and fibres (synthetic, non-synthetic and total) ingested by fish and mean with the standard deviation (SD) of the intensity (MI), abundance (MA), sum of total lengths (TLAI), total lengths (ML), and diameter (MD) of the fibres found in the digestive tracts of *Sardinella aurita* in each season from the Balearic Sea (NW Mediterranean). Significant differences among seasons are indicated by different superscript letters.

		SUMMER22	AUTUMN22	WINTER23	SPRING23	SUMMER23
Fragments						
P (%)	Synthetic	10.00	25.00	10.00	15.00	10.00
MA (n/ind)	Synthetic	0.10 (0.31) ^b	0.25 (0.44) ^{ab}	0.10 (0.31) ^b	0.20 (0.52) ^a	0.10 (0.31) ^b
MI (n/ind)	Synthetic	1.00 (0.00)	1.00 (0.00)	1.00 (0.00)	1.33 (0.58)	1.00 (0.00)
Fibres						
P (%)	Synthetic	35.00	45.00	55.00	30.00	5.00
	Non-synthetic	80.00 ^{ab}	60.00 ^{ab}	95.00 ^a	70.00 ^{ab}	45.00 ^b
	Total	80.00	75.00	100.00	70.00	65.00
MI (n/ind)	Synthetic	1.29 (0.67)	1.00 (0.00)	1.36 (0.67)	1.17 (0.41)	1.25 (0.50)
	Non-synthetic	2.69 (2.33)	1.83 (0.83)	3.05 (2.09)	2.29 (1.33)	1.33 (0.50)
	Total	2.81 (1.87)	2.12 (1.11)	3.75 (2.31)	2.87 (1.96)	1.58 (0.90)
MA (n/ind)	Synthetic	0.45 (0.76)	0.45 (0.51)	0.75 (0.85)	0.35 (0.59)	0.25 (0.55)
	Non-synthetic	2.15 (2.35) ^{ab}	1.10 (1.12) ^{bc}	2.90 (2.15) ^a	1.60 (1.54) ^{abc}	0.60 (0.75) ^c
	Total	2.60 (2.66) ^{ab}	1.55 (1.39) ^{bc}	3.65 (2.25) ^a	1.95 (1.93) ^{abc}	0.90 (0.85) ^c
TLAI (mm/ind)	Synthetic	2.85 (2.71) ^{ab}	3.19 (6.72) ^{ab}	1.83 (1.47) ^{ab}	4.89 (5.73) ^a	1.81 (1.13) ^b
	Non-synthetic	4.18 (3.90) ^{ab}	2.20 (1.12) ^{ab}	4.16 (3.47) ^{ab}	5.33 (3.14) ^a	1.71 (1.06) ^b
	Total	5.43 (4.46) ^b	3.44 (6.03) ^b	4.96 (3.97) ^b	6.93 (4.70) ^a	1.88 (1.07) ^b
ML (mm/fibre/ind)	Synthetic	2.35 (2.09)	3.19 (6.72)	1.39 (0.97)	3.60 (3.17)	1.57 (1.20)
	Non-synthetic	1.51 (0.74) ^{ab}	1.27 (0.50) ^b	1.33 (0.47) ^b	2.59 (1.93) ^a	1.30 (0.75) ^b
	Total	1.66 (0.94)	1.75 (2.59)	1.38 (0.66)	3.06 (2.32)	1.43 (0.89)
MD (mm/fibre/ind)	Synthetic	0.05 (0.094)	0.02 (0.005)	0.02 (0.007)	0.02 (0.006)	0.02 (0.003)
	Non-synthetic	0.02 (0.005)	0.02 (0.005)	0.02 (0.003)	0.02 (0.003)	0.02 (0.008)
	Total	0.03 (0.03)	0.02 (0.005)	0.02 (0.004)	0.02 (0.003)	0.02 (0.007)

3.2.2. Als polymer characterization

From the total of Als that were analysed through micro-FTIR or FTIR, 92.18% of the obtained spectra were considered valid. Regarding fibres, four polymers confirmed by micro-FTIR matched the visual typologies defined in the present study (**Fig. 7**). Fibres from category A were non-synthetic since 93.61% of the resulting spectrums corresponded to cellulose. The rest of fibres from categories (B-D) matched with three synthetic polymers: 71.42% of the spectrums from category B were identified as PET, 66.66% of the spectra from category C corresponded to acrylic and 75.00% of the spectra from category D were identified as PA (**Fig. 7**). In the case of fragments, four polymers were identified by FTIR corresponding to PP, PA, PET and PE (**Fig. 7**).

As a result, the global composition (including both fragments and fibres) consisted, in decreasing order of importance in terms of abundance, of cellulose (73.56%), polyethylene terephthalate (PET) (14.10%), polyamide (PA) (3.96%), polypropylene (PP) (3.08%), acrylic (1.32%) and polyethylene (PE) (0.88%). In the case of fibres, cellulose was the most abundant polymer across all seasons, whereas for fragments, PP showed a higher proportion in spring-23 (**Fig. 8**). No significant differences in polymeric composition were found among seasons nor organs (Chi-squared, $p > 0.05$).

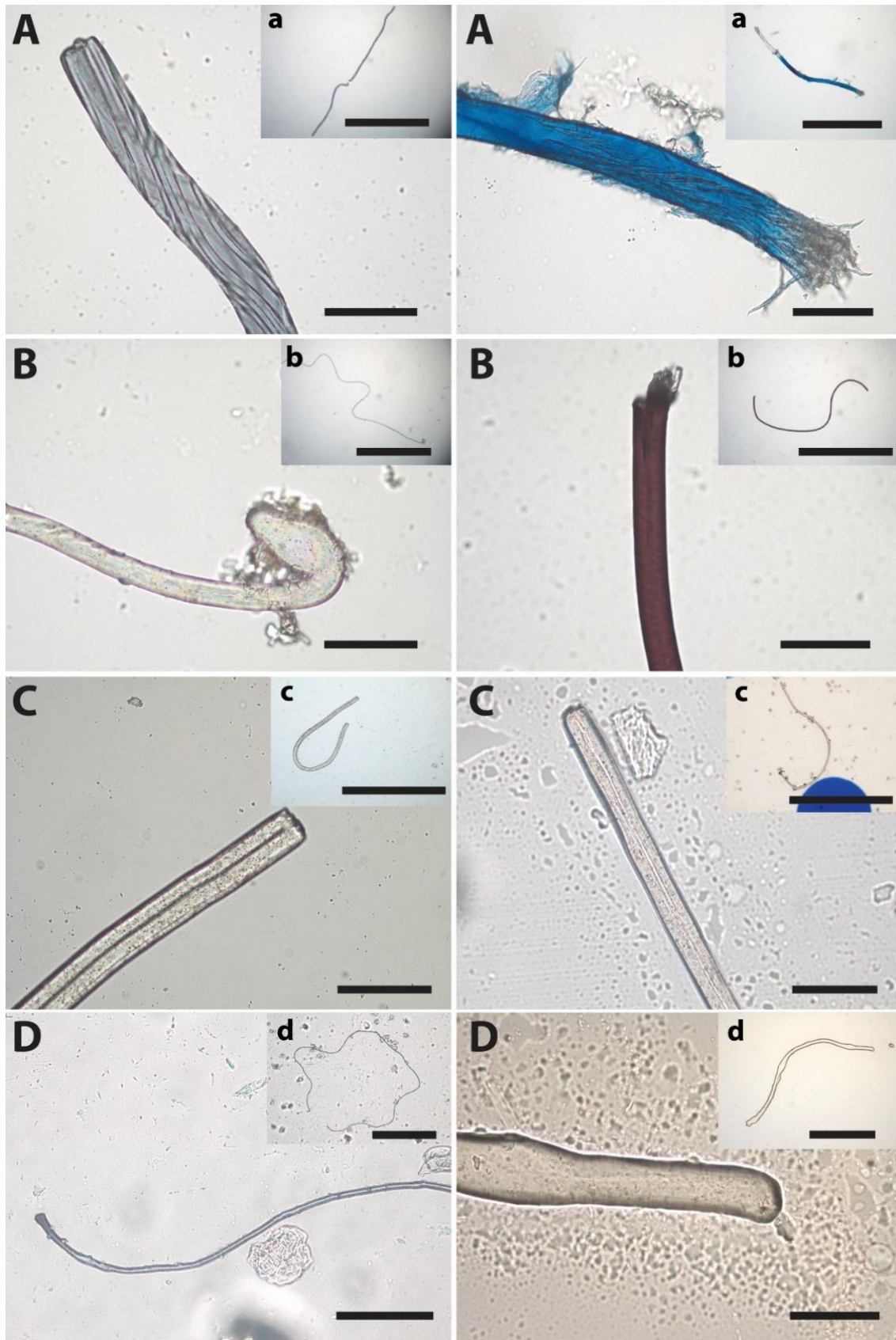


Fig. 7. Images of representative fibres of the four different typologies of polymers found in the digestive tract of *Sardinella aurita* specimens collected in the Balearic Sea (NW Mediterranean). Each category (A to D) is described in Table 1. Scale bars = A–D, 100 μm ; a–b, 1000 μm ; c, 500 μm ; d, 400 μm .

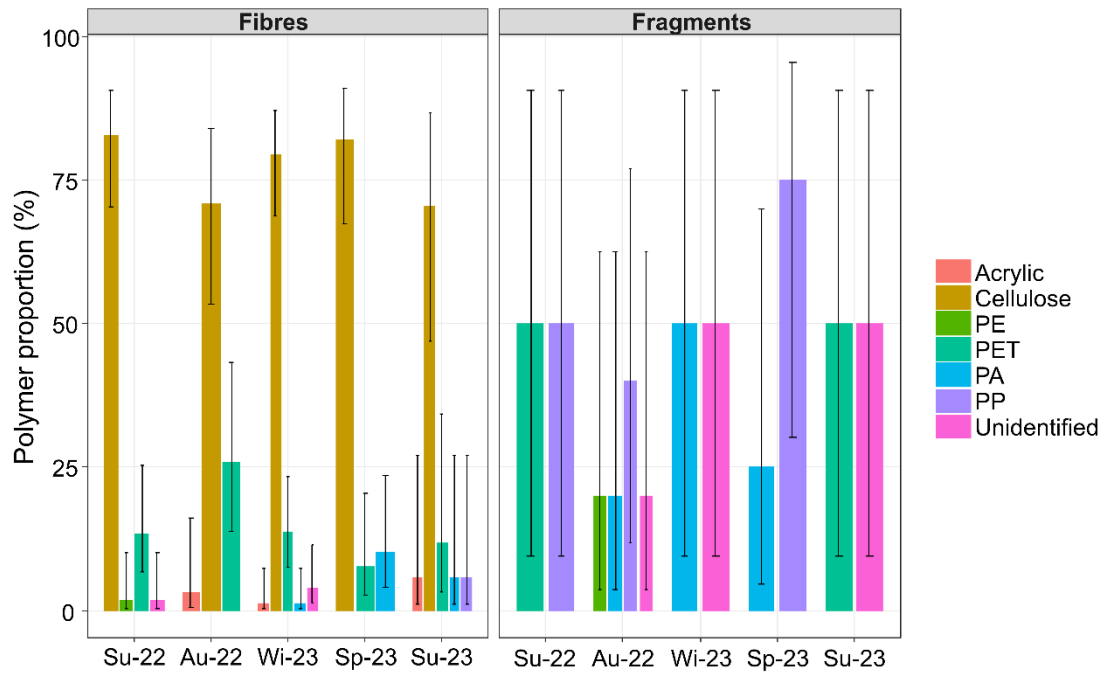


Fig. 8. Seasonal polymer composition of anthropogenic items (AIs) ingested by *Sardinella aurita* in the Balearic Sea (NW Mediterranean). Bars represent the proportion (%) of each polymer per season, separately for fibres and fragments. Error bars indicate 95% confidence intervals. Seasons abbreviations: Su22, summer 2022; Au22, autumn 2022; Wi23, winter 2023; Sp23, spring 2023; Su23, summer 2023.

3.2.3. Heavy metals concentrations

Heavy metals concentrations in round sardinella's muscle off Barcelona are shown in **Table 4**. Individuals analysed for heavy metals measured a mean of 15.00 ± 0.84 cm in standard length. Pearson correlations indicated that fish length was negatively correlated with concentrations of Pb ($\rho = -0.87$, $p = 0.002$), whereas no other metal concentration was correlated with fish size.

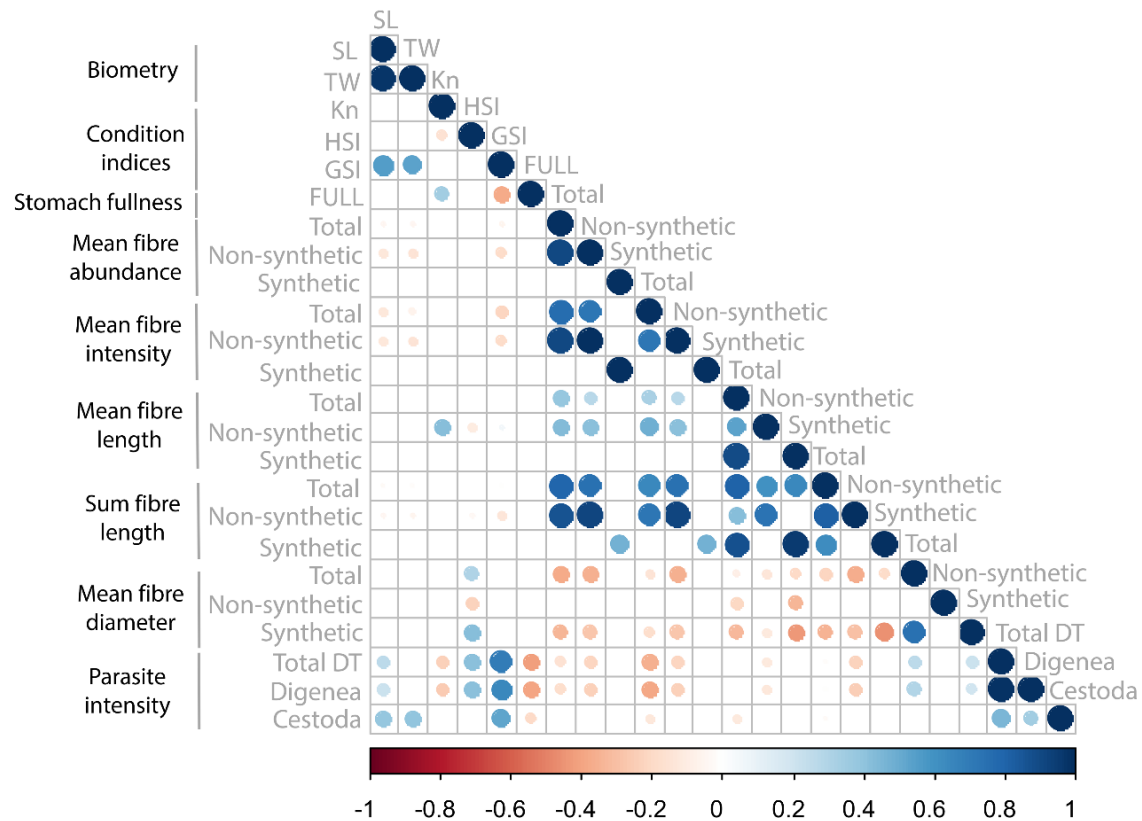
Table 4. Mean and standard deviation (SD) values of heavy metals from *Sardinella aurita* musculature captured off Barcelona (NW Mediterranean Sea) are given. Metallic elements analysed are nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) expressed in mg/kg dry weight (dw) and wet weight (ww).

Heavy metal concentrations	mg/kg dw	mg/kg ww
Ni	0.037 (0.016)	0.009 (0.004)
Cu	4.86 (0.73)	1.17 (0.22)
Zn	21.94 (2.04)	5.26 (0.56)
As	25.50 (8.61)	6.14 (2.21)
Cd	0.003 (0.0005)	0.001 (0.0001)
Pb	0.18 (0.12)	0.04 (0.03)
Hg	0.36 (0.09)	0.08 (0.02)

3.2.4. Potential health impacts from AIs and heavy metals

No relevant histopathological alterations or damage (e.g. haemorrhages or inflammation) were observed in the mucosa of the stomach, intestine and pyloric caeca, which are the organs in direct contact with AIs. Other histopathological alterations observed included PAS negative vacuole-like structures, inflammatory foci and granulomas in the hepatic tissue, aggregated macrophages and melanomacrophage centres in liver, kidney and spleen (see Chapter 4). None of these histopathological alterations could be linked to AIs ingestion as no significant statistical relationship was found between their prevalence or mean density values and the levels of AIs ingested by fish. Likewise, no clear associations between AIs ingestion and parasite prevalence or intensity were found (GZM and Spearman correlations, $p > 0.05$). No correlations between levels of heavy metals and histopathological findings could be performed at the individual level due to methodological limitations.

General parameters of fibre load (MA, MI, TLAI, ML and MD) were positively correlated among them ($\rho > 0.6$). However, no strong correlations were found among fibre descriptors and biological parameters or parasites, although some significant associations were observed: Kn and non-synthetic ML ($\rho = 0.43$, $p = 0.035$), HSI and total MD ($\rho = 0.48$, $p = 0.02$), HSI and synthetic MD ($\rho = 0.52$, $p = 0.01$), GSI and total MI ($\rho = 0.25$, $p = 0.002$), GSI and non-synthetic MI ($\rho = 0.25$, $p = 0.01$), MI of digeneans and total fibre MA ($\rho = -0.28$, $p = 0.004$), non-synthetic MA ($\rho = -0.22$, $p = 0.02$) and synthetic MA ($\rho = -0.23$, $p = 0.02$) (**Fig. 9**).



were related to higher condition factor and fullness whereas those from autumn were mainly characterized by higher mean intensities of epitheliocystis. Overall, parasites and condition indices were the group of variables that contributed the most to explain the model (Fig. 10a).

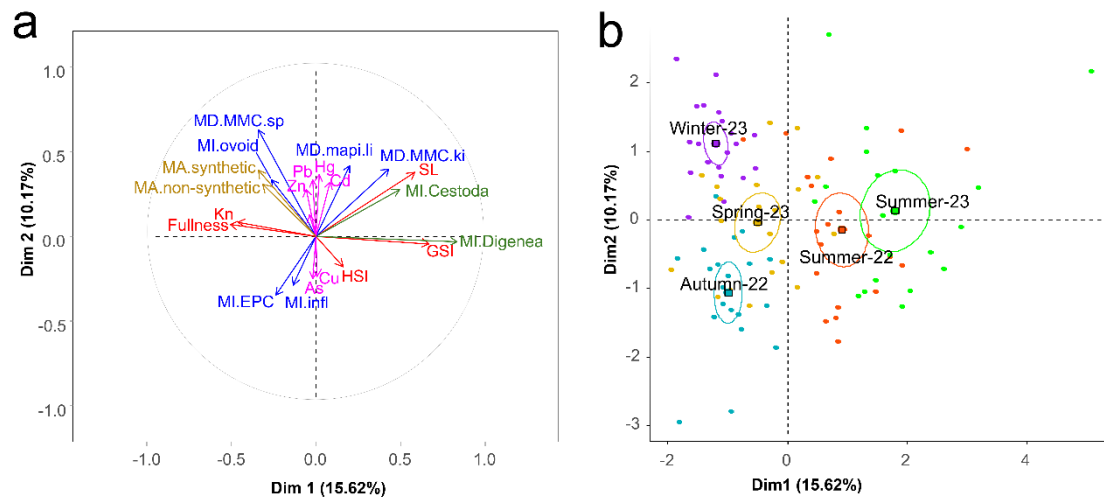


Fig. 10. a) Multiple Factor Analysis (MFA) displaying relationships among AIs mean abundance (synthetic and non-synthetic) in *Sardinella aurita* digestive tract, concentrations of heavy metals in muscle, fish health condition indices (Kn, HSI, GSI), stomach fullness and standard length (SL), mean intensity of main parasite taxa (Digenea and Cestoda), histopathological alterations (MD.MMC.sp and MD.MMC.ki, mean densities of melanomacrophages centres in the spleen and kidney, respectively; MD.mapi.li, mean densities of macrophages pigmented in liver; MI.ovid, mean intensity of ovoid structures in gills; MI.EPC, mean intensity of epitheliocystis and MI.infl, mean intensity of inflammation in gills). **b)** Factor map of the MFA results for *S. aurita* individuals from each season. Each individual is represented by a dot.

4. Discussion

4.1. The case of the benthopelagic *Merluccius merluccius*

This is the first study that simultaneously quantifies Als ingestion by the European hake off the Catalan coast (NW Mediterranean) in relation to Als present in the near-bottom water layer, and heavy metals concentrations in their muscle tissue. This multidisciplinary approach allows the assessment of the simultaneous behaviour of factors of different nature, which is usually complex and difficult to assess.

4.1.1. Methodological considerations of Als ingestion studies

Microplastics studies have demonstrated that a wide range of Als shapes can be found in the marine environment (Lusher et al., 2017; Hartmann et al., 2019; Suaria et al., 2020). This study highlight that fibres were the most abundant Als (i.e. 97.8%) in both fish digestive tracts and in water samples, in agreement with previous studies from the Mediterranean Sea (Bellas et al., 2016; Compa et al. 2018; Suaria et al., 2020; Rodríguez-Romeu et al., 2020; Carreras-Colom et al., 2020, 2022a), confirming their dominance in micro-litter composition in this area.

However, most of the studies regarding MPs continue to rely on digestion methods for their isolation, which can disintegrate cellulosic items (Treilles et al., 2020). Thus, these approaches are at great risk of underestimating the total abundance of Als. As a result, comparisons of present results regarding the total amount of Als with published data can be inaccurate, so outcomes of these studies are only compared to our synthetic data. Furthermore, although some studies have excluded non-synthetic fibres from their results to avoid overestimation of Als due to airborne contamination (Dehaut et al., 2016; Pfeiffer & Fischer, 2020; Santini et al., 2022), the present study demonstrates that working on isolation devices can effectively prevent background contamination of the samples and thus provide reliable results. As a matter of fact, several studies focusing on micro-litter and avoiding digestion methods have highlighted a much greater presence of cellulosic fibres both in biotic and abiotic compartments (Sanchez-Vidal et al., 2018; Suaria et al., 2020; Rodríguez-Romeu et al., 2020). For all this, it is of major importance to avoid destructive digestion techniques that can potentially degrade cellulose-based fibres (Dehaut et al., 2016; Treilles et al., 2020) and to establish a standardized methodology to characterize micro-debris (Cowger et al., 2020; Concato et al., 2023).

4.1.2. Characterization of ingested and environmental Als

Most Als ingested by hakes were cellulosic fibres smaller than 5 mm and similar fibre sizes (<2.6 mm) were detected both in hakes and water samples off Blanes and Ebro Delta, in accordance

with previous findings from the Gulf of Cadiz (fibres size <3.1 mm) (Bellas et al., 2016). The predominance of cellulosic fibres has also been widely reported in the deep-sea (Sanchez-Vidal et al., 2018) and inside the digestive tract of other fish and crustaceans (Carreras-Colom et al., 2018, 2020; Rodríguez-Romeu et al., 2020, 2022). Its origin is mainly associated with textile industries and households, through wastewater treatment plants and domestic washing machines discharges (Browne et al., 2011). Their long-term accumulation in the marine environment may be explained by the high release of fibres from natural fabrics during laundering and by the slow degradation of chemically treated or dyed fibres (Suaria et al., 2020).

While several studies have focused on MP in surface waters (e.g., de Haan et al., 2019; Suaria et al., 2020) and seafloor sediments (e.g., Sanchez-Vidal et al., 2018), information on near-bottom water layers remains scarce, making comparisons difficult. However, few studies have demonstrated that, due to their properties (e.g., shape, density and chemical composition), most MPs are likely to spread into deeper layers of the water column, where they can interact with marine biota (Reisser et al., 2015; Avio et al., 2020), resulting in a highly heterogeneous distribution (Suaria & Aliani, 2014). This spatial distribution could be influenced by several factors such as urbanization, industry, river inputs, different strategies of wastewater treatment plants, fishing activities, local pollution sources, density properties of micro-debris and resuspension processes likely caused by bottom-trawl fishing vessels (Paradis et al., 2018). The observed spatial differences could also be explained by the different morphology of the associated continental shelves. Indeed, the depositional continental shelf off Barcelona, characterized by a smooth bathymetry, is influenced by the Llobregat and Besós rivers that drain densely urbanized areas, and thus discharge huge amounts of AIs that can easily be accumulated there (Durán et al., 2014). In Blanes, the nearby submarine canyon could have a role in the AIs that arrive from inland sources and probably accumulate them in the deep sea (Tubau et al., 2015). In contrast, the lower abundance of AIs/m³ detected in water samples off Ebro Delta suggests that this sampling area might not receive the direct influence of Ebro River discharge and consequently a low land-based AIs input, due to its distance from the coast compared to the other areas. Besides, in this microtidal and low-energy continental shelf, resuspension processes are rare (Palanques et al., 2002), so AIs could sediment more rapidly and thus would not be detected at the near-bottom water. In fact, this is in accordance with the turbidity levels recovered from the present study, which were lower compared to the other two sampling areas (see Table 2, Chapter 3.1 M&M). Considering only synthetic AIs, concentrations observed off Blanes were comparable to those reported by Baini et al. (2018) in the north Tyrrhenian Sea (0.22 (SD= 0.57) items/m³), and by

Lefebvre et al. (2019) in the Gulf of Lions (0.23 (SD= 0.20) items/m³), whose samplings were performed at different depths of the water column.

Hakes from the Catalan coast appeared to be regularly exposed to AIs, although ingestion levels were generally lower than those reported for hakes from the Tyrrhenian (82.5%), Ionian (48%, 1.75 (SD= 2) AIs/ind) and Adriatic Seas (30.6%, 1.09 (SD= 0.30) AIs/ind) (Giani et al., 2019; Bianchi et al., 2020). Differences among studies may reflect both varying anthropogenic pressures and methodological approaches. Compared with other species from nearby areas, hake ingestion levels were lower than those reported in benthic fish but similar or lower than those observed in pelagic species, suggesting that these differences could be explained by fish feeding modes or by the heterogeneous distribution of AIs in the water column (Bour et al., 2018).

Finally, similar AI ingestion levels in hakes from Barcelona in 2007 and 2019 indicate that this area has been exposed to AIs over time. Previous studies from the same area also detected similar ingestion levels after a ten year-gap, but reported changes in polymer composition, reflecting shifts in textile production and usage trends (Carreras-Colom et al., 2020; Rodríguez-Romeu et al., 2020, 2022). However, considering that local effects on AIs dynamics may exist in this area, no clear evidence of temporal trends for the levels of AIs could be drawn from the present results.

4.1.3. Comparison between environmental and ingested AIs

For a contextualized interpretation of AIs ingestion in hakes, it is important to consider the abundance and distribution of AIs in the surrounding environment, since higher environmental concentrations may increase the probability of exposure to them, with the consequent active or passive ingestion of these particles while feeding (Fossi et al., 2018), foraging or drinking (Roch et al., 2020). Although some studies have detected a clear relationship between the levels of AIs ingested by marine species and their proximity to highly anthropized areas (Alomar et al., 2016; Giani et al., 2019; Rodríguez-Romeu et al., 2020), in the present study, lower levels of ingested AIs were found off Barcelona, a densely populated coastal area with high concentrations of AIs in the environment. This highlights the difficulty of establishing direct relationships between environmental availability and ingestion, as water samples are specific to a certain depth and AIs distribution in the water column is heterogeneous and poorly characterized (Rios-Fuster et al., 2022). This limitation is particularly relevant for species that perform diel vertical migrations to feed, as the European hake, which may actively or passively ingest AIs from different water layers. Therefore, comparisons between environmental and ingested AIs should be interpreted with caution.

These inconsistent results could be explained by the size distribution of fibres from the environment and by the particular feeding behaviour of hakes. Ontogenetic shifts in the feeding behaviour and diet in juvenile hakes allow the transition from an opportunistic feeding behaviour based on euphausiids, mysids and decapods crustaceans (Modica et al., 2011) to a more selective foraging behaviour (D'Iglio et al., 2022) dominated by small and fast-moving fishes and suprabenthic crustaceans of the suborder Gammaridea, the orders Mysidacea, Euphausiacea and natantian decapods (Carrassón et al., 2019). This specialization may reduce the accidental ingestion of large fibres. In fact, the size and polymer composition of ingested fibres were similar to those detected in the environment off Blanes and the Ebro Delta. However, water samples off Barcelona contained a wider size range dominated by extra-large polyamide fibres that were absent in hake digestive tracts. When considering only fibres within the size range ingested by hakes, AIs concentration in Barcelona decreased from 1.53 to 0.26 items/m³, a lower value than the concentration of AIs from the other two localities, but consistent with the lower levels of AIs ingested by hakes from this area. These findings suggest that hakes may selectively avoid or be unable to ingest fibres above a certain size threshold.

To sum up, while the studied surrounding waters contained a mean of 0.85 items/m³, hakes ingested a mean of 1.39 (SD=1.39) items/individual. Despite local variability was observed in environmental fibre size distributions, micro-debris detected in the environment and in fish were generally consistent for fibres smaller than 6 mm. This supports the potential use of European hake as a bioindicator species for monitoring small-sized micro-debris in near-bottom waters.

4.1.4. Accumulation of heavy metals in hake muscle

For most analysed metals, concentrations in hake muscle were within the ranges previously reported for Mediterranean and Atlantic waters (Falcó et al., 2006; Ramos-Miras et al., 2019; Salvaggio et al., 2020; Kontas et al., 2022; Molera Arribas et al., 2025). However, some metals showed clear size and locality patterns, highlighting the importance of considering both biological and environmental factors when assessing metal accumulation. Coastal urban and industrial discharges, particularly through rivers, represent important sources of heavy metals to the marine environment, favouring their accumulation in shallow sediments, as reported off Barcelona (Palanques et al., 2017). Metal concentrations in fish are also influenced by habitat, diet, trophic level, physiology, metabolism, environmental availability and fish size (Liang et al., 1999; Madgett et al., 2021; Higuieruelo et al., 2025).

The negative relationship observed between Zn concentration and fish size may reflect ontogenetic changes in metabolism, diet and detoxification capacity. Smaller individuals generally

show higher feeding and metabolic rates, which may increase trace metal uptake and accumulation (Canli & Atli, 2003; Merciai et al., 2014). Consequently, net metal accumulation (the difference between uptake and depuration), is often higher in juvenile stages (Canli & Atli, 2003). As fish grow, dietary shifts, increased mobility and more efficient detoxification mechanisms may reduce Zn concentrations. Higher Zn and Cd levels in smaller hakes from Barcelona likely reflect local anthropogenic inputs associated with urban coastal environments, potentially enhanced by physicochemical conditions (e.g., temperature, salinity, pH) affecting metal bioavailability (Oros, 2025). In the case of Cd, Barcelona was the locality where this metal was mostly detected above the limit of detection, supporting the influence of local sources on small individuals compared to other localities.

In contrast, Hg concentrations increased with fish size, consistent with bioaccumulation processes. Mercury, particularly methylmercury (MeHg), accumulates over time and biomagnifies through trophic webs (Cresson et al., 2014; Higuero et al., 2025). This likely explains the higher Hg concentrations observed in hakes from Blanes, which were significantly larger than those from other localities. Hg levels detected in this study (0.05–0.11 mg/kg ww) were similar to those previously reported from the Catalan (0.09 mg/kg ww) and Valencia coasts (0.13 – 0.16 mg/kg ww) (Perelló et al., 2014; Yusà et al., 2008), but higher from those reported from the Ionian Sea (0.009 – 0.01 mg/kg ww) (Salvaggio et al., 2020). In contrast, higher Hg levels were reported in hakes from nearby regions such as the Balearic Islands and the Gulf of Lions (0.3–0.6 mg/kg ww) (Llull et al., 2017; Cossa et al., 2012), as well as from the Adriatic Sea (0.59 mg/kg ww) (Perugini et al., 2014) and the Aegean Sea (1.9–2.4 mg/kg dw) (Kontas et al., 2022). These differences could be likely related to the larger fish sizes analysed in those studies compared to the present one (29.0–56.7 cm vs. 14.58–24.22 cm). In this context, hake ontogenetic dietary shift towards a more piscivorous diet are likely to further enhance Hg accumulation, explaining the opposite size trend observed with Zn (Cossa et al., 2012).

Interestingly, Hg levels in Mediterranean hakes remain higher than those reported for Atlantic individuals of similar size (0.06 mg/kg ww) (Cossa et al., 2012), supporting the so-called “Mediterranean mercury anomaly”. This phenomenon has been linked to the higher methylation potential of Hg in the Mediterranean Sea, where the relatively shallow oxygen minimum zone favours methylmercury incorporation into shallow trophic webs (Cossa & Coquery, 2005; Cresson et al., 2014) with direct food safety implications (see Chapter 6).

4.1.5. Association of anthropogenic pollutants to hake health status

Despite the growing knowledge from experimental studies on the effects of MPs and their additives on fish (Kögel et al., 2020), their impacts under wild conditions remain poorly understood (Kwak et al., 2022; Concato et al., 2023). Experimental results vary greatly depending on exposure levels and the type, size and shape of the particles used, and most studies focus on PE and PS, while common polymers found in wild fish, such as PP, PA and cellulose, remain understudied (Hossain & Olden, 2022).

In *M. merluccius*, AIs appeared to pass through the digestive tract in the same way as digested prey contents, since no differences were observed between stomach and intestinal AI abundance. This is consistent with experimental studies indicating that MPs typically pass through the digestive system within hours to a few days, supporting the idea that the exposure to MPs is generally short-term (Rodríguez-Romeu et al., 2024; Dawson et al., 2025). Besides, no digestive tract alterations potentially associated with AIs were detected, consistent with previous studies (Jovanović, 2017; Batel et al., 2020; Rodríguez-Romeu et al., 2020, 2022). Tissue alterations due to the direct impact of AIs exposure or their toxic additives have been described through histopathological findings in the intestine (*e.g.* edema, necrosis, epithelial desquamation and lymphocyte infiltration) and liver (*e.g.* hepatocyte vacuolation, hypertrophy and blood sinusoid congestion) (Pedà et al., 2016; Lu et al., 2016). However, some of these studies should be taken with caution due to potential inaccuracies in the interpretation of the histopathological findings, as some fish pathologists have warned (Baumann et al., 2016; De Sales-Rebeiro et al., 2020; Hossain & Olden, 2022). Moreover, rodlet cells, previously associated with parasitic infections and contaminant exposure, including MPs (Pedà et al., 2016; Dezfuli et al., 2022; Manera, 2024 and references therein), were observed in almost all hakes independently of AI ingestion levels. Experimental studies reported increased rodlet cells abundance after exposure to MPs, metals and herbicides (Pedà et al., 2016; Dezfuli et al., 2006; Giari et al., 2008), but AI concentrations used experimentally were generally much higher than those found in wild fish. Moreover, the origin and specificity of rodlet cells as biomarkers remain controversial under natural conditions (Dezfuli et al., 2022).

Although the AIs detected herein were likely too large to cross the intestinal barrier (Burns & Boxall, 2018), additives or adsorbed contaminants may still be released and absorbed, potentially affecting organs such as the liver. Experimental studies have described hepatic alterations associated with MPs, including glycogen depletion, inflammation, oxidative stress and necrosis (Rochman et al., 2013; Lu et al., 2016). Nevertheless, no associations between AI levels and liver

alterations were observed in this study. Instead, these changes were more likely related to natural metabolic variability or nematode infections. This is not surprising considering that studies finding tissue alterations associated with MPs have been conducted using very high levels of MPs, much higher than the levels detected in this study.

At the individual level, Als ingestion did not appear to negatively affect fish condition, nutritional state or feeding activity, as no associations with condition indices or stomach fullness were detected. Similar outcomes were reported in sardines, anchovies (Compa et al., 2018; Rodríguez-Romeu et al., 2022) and red mullets (Rodríguez-Romeu et al., 2020). Likewise, parasite intensity was not associated with Als exposure, suggesting that parasites seem not to interfere in the accumulation of Als within the digestive tract, as reported by Rodríguez-Romeu et al. (2020). This contrasts with what Hernandez-Milian et al. (2019) suggested in grey seals. Moreover, Pennino et al. (2020) reported a positive relationship between MPs and parasites in anchovies, suggesting that individuals inhabiting more polluted areas ingested higher amounts of MPs and therefore more likely to be infected by parasites.

On the other hand, exposure to toxic contaminants, such as heavy metals, may weaken fish immune system, potentially increasing susceptibility to parasitic infections and pathologies (Wester et al., 2002; Marigómez et al., 2006). Although several histopathological lesions were described in hakes after the Prestige oil spill in the Bay of Biscay (Atlantic Ocean), such as inflammation, atrophy, necrosis, apoptosis, neoplasms, foci of cellular alteration and melanomacrophage centres in liver, their prevalence decreased several years after, indicating recovery from chemical exposure (Marigómez et al., 2006; Cuevas et al., 2015). In contrast, the present study provides no clear evidence of histopathological alterations directly attributable to metal exposure. Although individual correlations could not be performed due to methodological limitation, this is supported by the low proportion of variance explained by the RDA, the absence of neoplastic changes, and the fact that observed inflammatory and progressive changes (e.g., gill hyperplasia) and hepatic granulomas were more likely associated with parasitic infections, as discussed in Chapter 4. Besides, the RDA plot indicated an association between higher densities of splenic aggregated macrophages and Cu concentrations, this pattern should be interpreted with caution given the low explanatory power of the model. While Cu exposure has been shown to induce lesions such as aneurysm, edema and lamellae fusion in gills and necrosis, vascular haemorrhage, dilated sinusoids and vacuole degeneration in the hepatic tissue in experimental studies (Hoseini et al., 2016), such responses were not found in the present study. Similarly, melanomacrophage centres in liver and kidney observed in other species following Cu exposure have been linked to hepatocyte death and tissue recycling processes (Hoseini et al., 2016), but

these structures are also known to vary naturally with species, age, size and sex, and are not exclusively indicative of contaminant exposure (Carreras-Colom et al., 2022b), as discussed in Chapter 4. Overall, these results indicate that histopathological alterations and fish condition indices in the studied hakes are largely driven by other biological and environmental factors rather than metal contamination. Nevertheless, further studies focusing on adult hakes would be necessary to evaluate whether ontogenetic shifts in diet and habitat could lead to higher accumulation of metals and potential health effects not detected in juveniles.

4.2. The case of the pelagic *Sardinella aurita*

This study also provides novel insights into seasonal patterns of Als ingestion in round sardinella and heavy metals accumulation on their muscle off the Catalan coast (NW Mediterranean Sea), with a special focus on their health impacts. As a first approach to evaluate health effects through histopathological analysis, this study provides a basis for assessing seasonal variability in Als exposure, although no clear effects on fish health were detected.

4.2.1. Geographic variability in Als ingestion among pelagic fish

Micro-litter ingestion has been widely reported in pelagic fish across the Mediterranean (Table S2). In the present study, round sardinella showed lower Als ingestion levels than those reported for the same species in the Adriatic and southern Mediterranean, particularly along the Algerian and Egyptian coasts (Avio et al., 2020; Zeghdani et al., 2023; Shabaka et al., 2020), where extremely high abundances were reported, even though these studies only considered synthetic items. Similar lower ingestion levels have also been observed in anchovy, hake and red mullet from the Catalan coast compared to other Mediterranean regions, especially the Adriatic and Tyrrhenian Seas (Rodríguez-Romeu et al., 2020, 2022; present study). However, small-scale variability has been also reported within the Balearic Sea (Rodríguez-Romeu et al., 2022; Compa et al., 2018), suggesting that both methodological differences and feeding behaviour may influence Als ingestion patterns (Filgueiras et al., 2020; Giani et al., 2023; Rodríguez-Romeu et al., 2024).

Most studies reported that pelagic fish primarily ingest low-density synthetic polymers such as PE, PP and PS (Table S2), commonly found in surface waters (Suaria et al., 2016). Given the higher density of cellulose, it would not be expected to remain at the surface and be available to pelagic species. Surprisingly, present findings revealed a predominance of non-synthetic fibres (*i.e.* cellulose) ingested by round sardinella, in agreement with observations for round sardinella,

Atlantic mackerel and horse mackerel from the Adriatic Sea (Avio et al., 2020). In contrast, anchovies from the same area as the present research ingested more synthetic particles (*i.e.* PE) (Rodríguez-Romeu et al., 2022), suggesting differences in feeding behaviour and with anchovies likely feeding in surface waters (Suaria et al., 2016; Lopes et al., 2020), whereas round sardinellas might feed throughout the water column. Indeed, fibres are mainly introduced in coastal environments through atmospheric deposition and wastewater discharge via submarine outfalls, whose outflow is often below the surface, representing a direct pathway in the water column (Koester, 2018). Local factors such as rainfall, river discharges and proximity to coastline can also influence fibres availability to the marine environment (see section 4.2.2). Moreover, despite their relatively high density, cellulosic fibres can remain suspended in the water column for long periods due to their filamentous shape and low sinking velocity (Bagaev et al., 2017; Lenaker et al., 2019). Their persistence may also be enhanced by slow degradation processes associated with dyes and chemical products used during their manufacture (Suaria et al., 2020), explaining their dominance in round sardinella's digestive tracts.

4.2.2. Seasonal environmental drivers of Als availability in the water column

Als ingestion varied seasonally, suggesting that Als availability in the water column may fluctuate with environmental and/or local factors such as rainfall rates, river runoffs and seasonal oceanographic dynamics (Avio et al., 2020; Mancuso et al., 2023 and references therein). The Barcelona coast represents a hotspot for Als pollution due to intense anthropogenic pressures. The Besòs and Llobregat rivers, which flow through highly urbanized and industrialized areas, may play a key role in transporting large volumes of nutrients as well as land-based Als into the sea, especially during periods of high rainfall (Sanchez-Vidal et al., 2013). Consistent with this, the significantly lower levels of Als ingestion observed in summer of 2023 coincided with relatively dry conditions (see Fig. 2, Chapter 3.1 M&M), supported by reduced river inputs (see Table 3, Chapter 3.1 M&M), likely limiting Als availability in the water column. Conversely, higher Als ingestion in summer of 2022 followed a spring with higher rainfall levels (see Fig. 2, Chapter 3.1 M&M), suggesting that increased river runoff (see Table 3, Chapter 3.1 M&M) may have contributed to greater land-based discharges into the sea. These observations highlight the role of precipitation regimes on the discharge of Als into the marine environment.

In addition, the development of a thermocline during summer at 20-30 meters (Sabatés et al., 2007) limits the vertical mixing of the water column, as supported by the recorded high SST and low chlorophyll-a concentrations (see Table 3, Chapter 3.1 M&M). Under these stratified conditions, *S. aurita* may be constrained to surface and coastal waters due to warmer

temperatures (Sabatés et al., 2008; Maynou et al., 2020) were inputs from wastewater and urban runoff, particularly cellulosic items, may be also higher (Santonicola et al., 2023). In autumn, decreasing SST indicated the onset of water column mixing, typically associated with increased productivity (Sabatés et al., 2007). During this period, a higher abundance of fragments (although not significant among seasons) was recovered from round sardinellas, suggesting that fragments may oscillate below the surface and potentially sink to deeper depths (Kooi et al., 2017). Indeed, AIs distribution may also be influenced by biofouling, marine snow formation, biological ingestion and egestion, weathering, apart from the mixing mechanisms of winds and currents (see Mancuso et al., 2023). Winter mixing of the water column (Sabatés et al., 2007) might lead round sardinellas move more actively throughout the water column, increasing their exposure to AIs and potentially explaining the significantly higher AIs ingestion observed during this period. Additionally, seasonal human activities may contribute to fibre inputs, as people wear more clothing during colder seasons (Erlandson et al., 2005) leading to a 700% increase in washing machine use (Takuma et al., 2006; Browne et al., 2011). Therefore, higher amounts of fibres may enter the marine environment through wastewater treatment plants effluents and river discharges (Ben-David et al., 2021). Finally, the significantly long fibres ingested by round sardinellas from spring may indicate a recent arrival of these pollutants from local sources, as Carreras-Colom et al. (2020) also suggested in *Aristeus antennatus* during the same season. Environmental processes such as mechanical abrasion, UV exposure, biofouling and oxidation tend to progressively fragment and shorten fibres during transport, indicating longer resident times in the marine environment (Sørensen et al., 2021). However, considering that rainfall was relatively scarce in spring 2023 (see Fig. 2, Chapter 3.1 M&M), which aligns with the moderate levels of AIs abundance observed, the predominance of longer fibres might be rather explained by fish feeding dynamics and prey selection patterns and not so much by water discharge or stratification (see section 4.2.3 for further discussion).

4.2.3. Seasonal biological drivers of AIs ingestion by round sardinella

Beyond environmental factors, seasonal variations in AIs ingestion might also be influenced by shifts in feeding intensity and behaviour (filter-feeding vs. particulate feeding) in response to seasonal variations in food availability, as observed in sardines (Rodríguez-Romeu et al., 2024). Round sardinella displays a generalist diet, combining filter-feeding on calanoid copepods but with an important opportunistic active predation upon larger prey (*i.e.* decapods and euphausiids) (Bachiller et al., 2021). Apart from the reduced rainfall recorded in spring-summer 2023, which could partially explain the observed low AIs ingestion during summer of 2023, (as mentioned in section 4.2.2), it also coincides with the lowest feeding intensity, as fish redirect almost all their

energy to meet the high demands of reproduction (Lloret et al., 2014), leaving less energy available for feeding. Indeed, their spawning period occurs in early summer (from May to July) (Tsikliras, 2008), as reflected by the increasing GSI values, reaching its peak in summer. Moreover, the predominance of larger prey (0.5–0.9 mm; e.g., euphausiids, amphipods and decapods) during summer and autumn (Albo-Puigserver et al., 2019; Tsikliras et al., 2005) may promote a shift towards particulate-feeding (Bachiller et al., 2021). This feeding behaviour may reduce the probability of passive fibre ingestion (Rodríguez-Romeu et al., 2024) while increasing the likelihood of fragment ingestion, as these may be mistaken for prey (Capone et al., 2020), in line with the higher abundance of fragments observed in autumn. In contrast, higher feeding intensity during winter and spring, consistent with their capital breeding strategy of accumulating the maximum energy reserves prior to reproduction (Albo-Puigserver et al., 2017), may increase Als exposure. During winter, when large zooplankton is scarce, round sardinella relies mainly on filter-feeding on microplankton (<0.1 mm in size) (Albo-Puigserver et al., 2019). Therefore, the probability of accidentally ingesting fibres rather than fragments increases, as supported by present findings. Finally, an increased consumption of gelatinous zooplankton (*i.e.* salps) has been reported in spring (Albo-Puigserver et al., 2019). Salps are efficient particle feeders capable of capturing suspended micro-debris (ranging from 2 to 1000 µm in size) from the water column, acting as potential vectors for Als (Wieczorek et al., 2019). Consequently, round sardinellas could have either confused Als, and particularly fragments (supported by present findings), by gelatinous preys due to their similar appearance as it was reported in *Trachurus mediterraneus* (Rios-Fuster et al., 2019) or indirectly uptake Als through salp predation (Cole et al., 2011).

4.2.4. Heavy metals accumulation

The Barcelona coast is known to accumulate metals in shallow coastal sediments due to the proximity of urban cities, port activities and river mouths (e.g. the Besòs River), that increase the transport and accumulation of metals on the coastal sediments (Pinedo et al., 2014). However, pelagic fish are generally less exposed to heavy metals due to their low trophic level and planktivorous feeding behaviour compared to demersal species (Higueruelo et al., 2025; Oros et al., 2025). Levels of metals previously recorded in round sardinella from other areas were considerably lower than the detected herein. For example, Hg levels were four times lower than those from the Adriatic Sea (0.3 mg/kg w.w.) (Storelli et al., 2003), Pb and Cd ranged between five to 17 times and ten to 300 times lower, respectively, compared to those from the Strait of Messina (Lo Turco et al., 2013) and from the Eastern Mediterranean (Tepe, 2009). Regarding As and Ni, our results appeared to be twice and 18 times lower, than those reported in the Strait of Messina (Lo Turco et al., 2013). Higher levels of Cd and Zn were observed in *S. aurita* from the Canary Islands

in the Atlantic Ocean (Lozano-Bilbao et al., 2024). In contrast, heavy metal levels detected in this study were similar to other pelagic species from the same area, such as *Engraulis encrasicolus* (for Cu, As, Hg) and *Trachurus mediterraneus* (for Cd, Ni, Zn), and much lower than other benthic (i.e. *Nephrops norvegicus*, *Scylliorhinus canicula*) and benthopelagic species (i.e. *Micromesistius poutassou*, *Phycis blennoides*, *M. merluccius*) from the same region (Carreras-Colom et al., 2022; Higuieruelo et al., 2025; present study). This could be explained by the fact that hakes were juveniles, likely had a similar exposure time to metals and had not yet shifted to a more piscivorous diet that would increase their trophic level and hence bioaccumulate metals. As previously explained in the discussion section of *M. merluccius*, negative correlations between fish length and some metal concentrations have been well documented (Canli & Atli, 2003; Merciai et al., 2014). In this case, Pb levels were higher in smaller sardinellas, a relationship that has been reported in other studies (Merciai et al., 2014). They suggest that it could be determined by higher feeding and metabolic rates from small individuals compared to larger fish, as previously discussed in *M. merluccius* Discussion section. Variations in enzymatic processes involved in their accumulation, growth-related changes in body proportions and shifts in feeding diet and behaviour may also explain influence on these accumulation patterns (Liang et al., 1999; Merciai et al., 2014).

4.2.5. Effects of anthropogenic pollutants on fish health

Als were found throughout the digestive tract, though in a lower abundance in the pyloric caeca, suggesting limited accumulation in specific organs and an easy egestion, as previously observed for *M. merluccius*. Furthermore, the absence of histopathological alterations in the digestive tract suggests that Als did not cause any significant mechanical effects or damage that could compromise its function or structural integrity. This may be expected considering the small size range of ingested Als relative to the digestive system (Jovanović et al., 2018; Rodríguez-Romeu et al., 2020, 2022). Similarly, as previously reported for the herein *M. merluccius*, in this study we did not observe any alteration in the liver or other tissues at the histological level that could potentially be associated to Als, even in the season in which fish ingested more Als (i.e. winter).

Under wild conditions, establishing causal relationships between Als ingestion and histopathological effects, growth, condition or reproductive impairment is far more challenging, due to the influence of multiple intrinsic and extrinsic factors, including environmental variability, age, diet and reproductive status. Moreover, the relatively low levels of Als detected may have limited the identification of potential associations with adverse health effects. However, statistically significant relationships should be also interpreted with caution, as they do not

necessarily imply direct causality and may be influenced by confounding factors or natural variability in fish populations. For example, Compa et al. (2018) suggested that lower body condition in wild sardines could be linked to high levels of AIs ingested, but this relationship was not observed neither in round sardinellas nor in anchovies, sardines, European hakes or red mullets from nearby areas (Lefebvre et al., 2019; Rodríguez-Romeu et al., 2020, 2022; present study). Similarly, a recent experimental study using wild sardines exposed to realistic concentrations of fibres found in natural conditions highlighted that the observed reduction in body condition and weight was not due to MPs ingestion but was likely influenced by their feeding strategy (filter-feeding vs. particulate-feeding) (Rodríguez-Romeu et al., 2024). Consistently, no clear associations between AIs ingestion, parasite aggregations and condition indices were detected in *S. aurita*.

The most prevalent alterations observed such as aggregated pigmented macrophages, MMCs, and vacuole-like structures, were likely linked to the natural variability of fish populations rather than being a consequence of AIs exposure (see Chapter 4 for further discussion) and have been widely reported in other fish species from the same study area (Rodríguez-Romeu et al., 2020, 2022; Higuieruelo et al., 2024). Regarding metal exposure, this study attempted to explore potential toxicological effects of heavy metals in *S. aurita* specimens. Although metals were detected in muscle tissue, no histopathological alterations typically associated with metal toxicity were observed (see *M. merluccius* discussion section from this chapter). Among the lesions commonly linked to metal exposure, MMCs and the presence of vacuole-like structures in the hepatic tissue were the only findings recorded. Annabi et al. (2018) reported nuclear pyknosis, fibroblast proliferation and accumulation of lipid droplets in the hepatic tissue as responses to metal exposure in the Gulf of Gabes (Tunisia coast, southern Mediterranean), displaying higher levels of Pb than present findings. Although hepatic vacuolation has been described as a pathological response to toxic chemicals (Zodrow et al., 2004), the observed vacuole-like structures likely reflect variations in the nutritional status of fish, as discussed in Chapter 4. Moreover, MMCs are non-specific and are known to vary with species, age, physiological condition and environmental factors (Carreras-Colom et al., 2022b). Therefore, present results do not provide evidence of adverse impact related to the recorded levels of metal exposure in this species, also supported by the lack of significance in the multivariate analyses.

It should also be noted that the assessment of fish health in this study, based on histopathological screening and condition indices may not detect subtle, sub-lethal or long-term physiological effects associated with AIs exposure. This is particularly relevant considering the potential effects of plastic-associated additives from MPs and the potential role of MPs as vectors for persistent

organic pollutants and heavy metals. Some experimental studies have reported hepatic stress responses including glycogen depletion, fatty vacuolation and cell necrosis in *Oryzias latipes* after a 60-day exposure to MPs associated with PAHs and PCBs (Rochman et al., 2013), and Lu et al. (2018) reported enhanced cadmium toxicity due to MPs, resulting in oxidative damage and inflammatory responses in zebrafish tissues. In contrast, others have found no evidence for a relevant transfer of contaminants via MPs and suggest that, under natural conditions, their contribution to chemical exposure is negligible compared with the direct transfer routes through biota, making the underlying mechanisms still debated and their magnitude remain unclear (Burns & Boxall, 2018; Batel et al., 2020). These effects may be more pronounced for nanoplastics ($< 1\mu\text{m}$) due to their higher surface-area-to-volume ratio promoting additive leaching (Ivleva et al., 2017; Batel et al., 2020), and their ability to cross the gastrointestinal barrier, potentially affecting other organs.

5. Conclusions

This study provides a comprehensive assessment of anthropogenic items (AIs), including microplastics, and trace metals in two ecologically distinct fish species (*Merluccius merluccius* and *Sardinella aurita*) from the NW Mediterranean Sea, highlighting both spatial and seasonal variability in exposure and uptake. AIs were widely present in the environment and ingested by both species, with cellulosic fibres consistently dominating over synthetic ones, regardless of differences in environmental availability. In hake, ingestion patterns appeared to be strongly influenced by fibre size distribution and ontogenetic feeding behaviour, supporting its suitability as a bioindicator of near-bottom micro-debris. In contrast, round sardinella showed marked seasonal variability in AI ingestion, driven by environmental processes such as vertical mixing and rainfall, as well as species-specific feeding strategies, with higher uptake during winter associated with filter-feeding activity. Trace metal concentrations in both species were generally within previously reported ranges, with variations linked to size, habitat, and biological factors. In hake, mercury exhibited a clear bioaccumulation trend with size, while in sardinella, metal concentrations were comparatively low and not associated with observed tissue alterations. Importantly, no clear evidence of adverse effects of AIs or metal exposure was detected in either species, as condition indices, histopathological findings, and parasitological descriptors showed no consistent relationship with contaminant levels. Overall, these findings suggest that, under current environmental conditions, the impact of microplastics and trace metals on fish health appears limited. However, the observed species-specific, spatial, and seasonal patterns underline the importance of adopting integrated and multidisciplinary approaches to monitoring. Potential limitations such as sample size, the lack of assessment of physiological or biochemical responses, the possibility of subtle or long-term impacts not captured in the present study, the limited sampling period and area and the use of specimens obtained from commercial fisheries (underrepresenting other age groups) should also be considered. Continued research combining ecological, temporal, and physiological indicators is essential to improve early detection of potential impacts and to better understand the long-term consequences of pollutant exposure in marine ecosystems.

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Supplementary material

Table S1. Percentage of fibres of each size category found in *Merluccius merluccius* digestive tracts (DIG) and in near-bottom water samples (NBW) for each sampled locality.

	Blanes		Barcelona		Ebro Delta	
	DIG (%)	NBW (%)	DIG (%)	NBW (%)	DIG (%)	NBW (%)
Small	42.2	55.6	61.5	8.5	45.7	48.5
Medium	37.8	27.8	30.8	1.6	45.7	36.4
Large	20	16.7	7.7	10.9	8.6	9.1
Extra-large	0	0	0	79.1	0	6.1

Table S2. Comparative table on micro-litter ingestion data by pelagic fish species in different areas from the Mediterranean Sea based on available publications. Target species, sampled locality, number of analysed fish (N), isolation method used for AIs recovery, AIs prevalence (P%), mean abundance and mean size followed by standard deviation when available, or size range (min– max) when mean size was not available, the dominant polymers found and the reference are provided for each study. Results considering both synthetic (syn) and non-synthetic (non-syn) items are indicated separately when available to facilitate the comparison with studies based only on synthetic items and with present results. Dashes indicate absent data.

Species	Locality	N	Isolation method	P (%)	Mean abundance (n/ind)	Size (mm)	Polymers	Reference
<i>Engraulis encrasicolus</i>	Gulf of Lions	105	Visual inspection	15	0.18 (0.20)	–	Cellulose, wool	Compa et al. (2018)
	Gulf of Lions	84	Visual inspection	11	0.11 (0.31)	1.81 (1.52)	PET	Lefebvre et al. (2019)
	Adriatic Sea	80	Digestion + filtration	91	1.25	0.04–2.22	PVC	Renzi et al. (2019)
	Adriatic Sea	30	Digestion + density-separation	26.6	0.47 (0.86)	0.054–0.76	PE, PP	Mistri et al. (2022)
	Balearic Sea (off Barcelona)	150	Visual inspection	syn= 60 non-syn= 40	syn= 1.23 (1.68) non-syn= 0.67 (1.09)	1.99 (1.21)	PE, cellulose, PET	Rodríguez-Romeu et al. (2022)
	Gulf of Lions (GSA07)	103	Visual inspection	60	1.5	<5	–	Pennino et al. (2020)
	Ligurian Sea	100	Visual inspection	35	0.34 (0.29)	0.56–7.70	PE, PP	Capone et al. (2020)
	Tyrrhenian Sea	30	Digestion + filtration	26.7	0.3 (0.10)	0.1–0.3	PAN, PET, PE	Valente et al. (2023)
	Tyrrhenian Sea	77	Digestion + filtration	21	0.3 (0.7)	0.1–5	PE, PP, PET	Giani et al. (2023)
	Balearic islands	24	Visual inspection	0	0.00	–	–	Rios-Fuster et al. (2019)
<i>Trachurus trachurus</i>	Spanish peninsular coast	15	Visual inspection	6.67	0.07 (0.26)	111.27 (7.32)	–	Rios-Fuster et al. (2019)
	Alboran Sea	51	Digestion + filtration	0	0.00	–	–	López-Martínez et al. (2022)
	Tyrrhenian Sea	30	Digestion + filtration	43.3	0.63 (0.17)	0.1–1	PAN, PET, PP, PE, PS	Valente et al. (2023)
	Tyrrhenian Sea	43	Digestion + filtration	14	0.18 (0.4)	0.1–25	PP, PS, silicone, PA	Giani et al. (2023)
	N Adriatic Sea	10	Density-separation + filtration	10	2.00 (0.00)	–	cotton	Avio et al. (2020)
	C Adriatic Sea	10	Density-separation + filtration	syn= 10 non-syn=60	syn=1.00 (0.00) non-syn= 1.33 (0.52)	0.1–0.5	PE, cotton	Avio et al. (2020)
	S Adriatic Sea	8	Density-separation + filtration	syn= 12.5 non-syn= 87.5	syn= 1.00 (0.00) non-syn=8.00 (8.20)	1–5	cotton	Avio et al. (2020)

(Continued)

Species	Locality	N	Isolation method	P (%)	Mean abundance (n/ind)	Size (mm)	Polymers	Reference
<i>Scomber scombrus</i>	Tyrrhenian Sea	30	Digestion + filtration	33.3	0.47 (0.14)	0.1–5	PET, PAN, PA, PS	Valente et al. (2023)
	Tyrrhenian Sea	42	Digestion + filtration	29	0.35 (0.60)	0.3–25	PET, viscose, PP	Giani et al. (2023)
	Balearic Sea (Balearic Islands)	2	Digestion + filtration	100	2.5 (0.7)	2.57 (0.89)	PE, PS	Compa et al. (2022)
	N Adriatic Sea	10	Density-separation + filtration	syn=30 non-syn=90	syn=1.3 (0.58) non-syn=4.22 (1.71)	0.1–1	PS, PP, cotton	Avio et al. (2020)
	S Adriatic Sea	10	Density-separation + filtration	70	1.3 (0.5)	0.01–1	PE, PA	Avio et al. (2020)
	Alboran Sea	40	Digestion + filtration	35	0.37 (1.03)	4.56 (2.68)	PE, cellulose	López-Martínez et al. (2022)
	Aegean Sea	12	Digestion + filtration	50	1.08 (0.1)	0.72 (0.86)	PET	Eryasar et al. (2024)
<i>Sardinella aurita</i>	S Adriatic Sea	9	Density-separation + filtration	syn= 33.3 non-syn=55.5	syn= 1.3 (0.6) non-syn= 2.2 (0.84)	0.1–5	PE, PP, cotton	Avio et al. (2020)
	Balearic Sea (off Barcelona)	150	Visual inspection	syn=45 non-syn=70	syn=0.60 (0.82) non-syn=1.67 (1.84)	syn=1.96 (2.40) non-syn= 1.57 (1.15)	Cellulose, PET, PA, PP	Present study
	Gulf of Bejaia (Algeria coast)	120	Digestion	72.5	7.83	–	–	Zeghdani et al. (2023)
	Egypt coast	33	Digestion	9	1450 (3207)	0.025–1	PE, PP	Shabaka et al. (2020)

CHAPTER 6

Fish consumption: nutritional benefits and human health risks

Muns-Pujadas, L., Constenla, M., Dallarés, S., Soler-Membrives, A., Padrós, F., Sala, R. (2025). The round sardinella (*Sardinella aurita* Valenciennes, 1847) from the NW Mediterranean: a healthy and safe choice for human consumption.

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Muns-Pujadas, L., Constenla, M., Dallarés, S., Padrós, F., Fioravanti, M.L., Caffara, M., Gustinelli, A. Distribution of zoonotic anisakid nematodes in two commercial Mediterranean fish species. In preparation.

Abstract

Fish are recommended by the European Food Safety Authority (EFSA) due to their high content of omega-3 polyunsaturated fatty acids (PUFAs), providing benefits for human health. Small pelagic fish are especially relevant because of the high levels of these fatty acids. However, the decline of traditional commercial species such as sardines and anchovies has increased interest in thermophilic species as potential alternative fishery resources. At the same time, fish consumption may involve health risks associated with heavy metals and zoonotic parasites. This chapter evaluates the commercial viability of the round sardinella (*Sardinella aurita*) as an alternative pelagic resource through the assessment of its nutritional composition (proximate and fatty acid profile), heavy metal concentrations and zoonotic parasites in specimens seasonally collected off Barcelona (NW Mediterranean). Potential risks associated with the consumption of European hake (*Merluccius merluccius*) from the same area were also evaluated. In addition, infection levels of zoonotic nematodes in both species were compared across several Mediterranean regions. Results showed that round sardinella is a lean fish due to its low lipid content, which varied seasonally, being highest in spring (1.69%) and lowest in summer (0.30–0.62%). Despite this, high proportions of omega-3 PUFAs (46.7–53.7% of total fatty acids) were observed during summer and autumn. These seasonal differences were likely related to feeding ecology, reproduction and breeding strategy. A 100 g portion of round sardinella consumed during summer and autumn provided 206–290 mg of EPA+DHA, meeting recommended daily intake values. Heavy metal concentrations in both fish species were below European Commission regulatory limits, indicating that their consumption is generally safe. However, consumption of both species by infants should be limited to three times per week due to potential methylmercury exposure. No zoonotic nematodes were detected in round sardinella from any sampled region, neither in hakes from the Balearic Sea, although infections with *Hysterothylacium aduncum* (52.62%) and *Hysterothylacium fabri* (19.58%) were common. These species are generally considered non-pathogenic, although occasional allergic reactions have been reported. In contrast, hakes from the Adriatic Sea showed a potential anisakiasis risk, as 64.04% were infected with *Anisakis pegreffii*, occurring in visceral organs, abdominal cavity and musculature, with infection levels increasing with fish size. Geographical differences in nematode distribution were likely related to trophic ecology and environmental conditions. Overall, round sardinella represents a healthy and safe alternative pelagic resource. Promoting its consumption, particularly in autumn after spawning, could contribute to more sustainable fisheries by redistributing fishing pressure among pelagic species. In hake, anisakiasis risk in some regions could be minimized through rapid evisceration, freezing and proper cooking before consumption.

1. Introduction

Fish represents an important component of the human diet, providing high-quality protein, minerals including iron, zinc, calcium, and nutrients such as vitamins and essential amino acids, which are essential for proper physiological function and metabolic processes for human health (see Noreen et al., 2025). Moreover, fish stands out for its favourable lipid profile, rich in long-chain polyunsaturated fatty acids (LCPUFA), particularly omega-3 fatty acids (n-3) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found in high proportions in the tissue of marine fish and certain shellfish (Strobel *et al.*, 2012; Amoussou *et al.*, 2022). These n-3 LCPUFA are essential for human beings since they play a key role in human growth and development as they are not synthesised sufficiently by the human body (Wiktorowska-Owczarek *et al.*, 2015). Due to their many cardiovascular, neurological, immunological, and other physiological benefits (Calder, 2015; Zárate *et al.*, 2017; Tacon *et al.*, 2020), the European Food Safety Authority (EFSA) established a daily dietary recommendation for EPA and DHA consumption (EFSA, 2012a). Moreover, apart from n-3 LCPUFA, it is also important to consider the proportion of other FA to evaluate the nutritional value of these dietary lipids due to their role in human metabolism and their association with the prevention of coronary heart disease. Thus, two nutritional quality indices were proposed, an atherogenic index (AI) and a thrombogenic index (TI) (Ulbricht & Southgate, 1991; Simopoulos, 2002). However, the value of fish as a food source cannot be assessed solely in terms of its nutritional contribution, but it must also be evaluated from a safety perspective, considering the potential presence of pathogenic biological agents such as parasites and chemical contaminants such as heavy metals (see Chapter 5) which can accumulate in tissues and represent health risks (Sidhu, 2003).

In Mediterranean countries, fish and seafood are integral components of the well-known “Mediterranean diet”, recognised as a healthy dietary pattern with local, traditional and seasonal products (de Juan et al., 2024). The Mediterranean diet remains a key nutritional and cultural reference in Spain. However, its actual adherence has declined in recent years, with studies showing that young groups, particularly adolescents and young adults, are progressively shifting their dietary patterns towards more Westernized eating habits, with a higher intake of ultra-processed foods (Alfaro-González et al., 2025). Spain is the second largest consumer country of fishery products from the European Union accounting for approximately 42 kg per capita in 2024, with Atlantic salmon (*Salmo salar* Linnaeus, 1758) (12%), European sardine (*Sardina pilchardus*, Walbaum, 1792) (10%) and European hake (*Merluccius merluccius*, Linnaeus, 1758) (9%) representing the most consumed species and a substantial proportion of total fish intake (European Commission, 2026).

Proximate composition (i.e. moisture, protein and lipid content), and fatty acid (FA) composition have been widely studied on important Mediterranean commercial fish species including the European hake, European anchovy (*Engraulis encrasicolus*, Linnaeus, 1758) and European sardine, revealing temporal, seasonal and spatial variations (Roncarati et al., 2012; Pethybridge et al., 2014; Biton-Porsmoguer et al., 2020; Jouini et al., 2023; Traina et al., 2024). Based on these studies, the European hake is considered a valuable fish resource due to its protein, mineral and fatty acid content. Although its lipid content is lower compared to other fatty fish, ranging between 0.9% and 1.9%, its fatty acid profile is characterized by a relatively high proportion of polyunsaturated fatty acids (30.85 – 50.7%), particularly DHA (Roncarati et al., 2012; Traina et al., 2024). On the other hand, fatty fish such as sardines and anchovies are characterized by higher lipid content and higher proportions of n-3 LCPUFA, therefore their regular intake is recommended to meet dietary requirements for EPA and DHA consumption (Kris-Etherton et al., 2002). However, in recent years a decline in lipid content has been reported in sardines and anchovies from the NW Mediterranean, which may reflect a deterioration in their nutritional condition, likely linked to a reduced food availability associated with the ongoing effects of sea warming and also to fishing pressure (Saraux et al., 2019; Biton-Porsmoguer et al., 2020; Frigola-Tepe et al., 2025). Within this context, exploring alternative fish resources to diversify fishing pressure is becoming relevant, particularly in regions where the consumption of fish is rooted but stocks show signs of malnourishment (Biton-Porsmoguer et al., 2020; Vila-Belmonte et al., 2024; Ollé-Vilanova et al., 2026). Thermophilic pelagic species such as the round sardinella (*Sardinella aurita* Valenciennes, 1847) have expanded their distribution across colder areas from the Mediterranean Sea (Sabatés et al., 2006; Tsikliras, 2008) and may represent an emerging resource (Vila-Belmonte et al., 2024; Ollé-Vilanova et al., 2026). Despite an increase in landings of this fish species has been observed in the last ten years (FAO, 2025), it has mostly been used as bait or for fishmeal production in the Mediterranean (Tsikliras & Antonopoulou, 2006), although in African countries it's an important fish resource (Thiaw et al., 2020). Consequently, its nutritional status for human consumption has received far less attention due to its low economic value. Only a few studies focusing on this topic have been carried out to date, most of them focused on a specific period. Among these, Ben Rebah et al. (2010) documented annual changes in total lipid content in specimens from off the Tunisian coast and FA composition in individuals collected in February, while Abouel-Yazeed (2013) provided similar data for specimens collected in March off the coast of Egypt. On the Catalan coast (NW Mediterranean Sea), Vila-Belmonte et al. (2024) recently reported differences in the FA composition according to the specimen's maturity stage (from May to September) and Ollé-Vilanova et al. (2026) provided both muscle and mesenteric fat content

from a specific period (March to November). However, no studies regarding the seasonal variation of the FA composition of this fish species have been undertaken to date.

Besides nutritional benefits, wild fish consumption may involve health risks associated with parasites and anthropogenic contaminants such as heavy metals (reported in Chapter 5) (Storelli et al., 2003; Cipriani et al., 2018a,b). Certain metals, including Pb, Cd, Hg, As and Ni, can accumulate in fish tissues and pose risks to human health (Castro-González & Méndez-Armenta, 2008), leading to the establishment of international limits regulations (European Commission Regulation (EC) No 2023/915) and consumption recommendations, especially for vulnerable groups such as children and pregnant women (EFSA, 2014; EFSA, 2020). Mercury is particularly harmful due to its neurotoxic, digestive and immune system effects (WHO, 2024), while Pb and Cd may affect neurological, renal, skeletal and respiratory systems (WHO, 2019, 2023a). Even essential trace elements such as Cu and Zn (EFSA, 2017) can become toxic at high concentrations such as liver damage and gastrointestinal symptoms (Bosch et al., 2016), so regulatory limits have also been set in these cases (Nauen, 1983; BOE, 1991). Due to its higher trophic level and benthopelagic habitat, European hake is expected to accumulate more heavy metals than round sardinella, and Hg levels exceeding regulatory thresholds have been reported in large hake specimens from some Mediterranean areas (Perugini et al., 2014; Cossa et al., 2012). In contrast, despite its lower trophic position, elevated Pb and Cd concentrations have also been detected in round sardinella from several coastal areas of the Aegean Sea (eastern Mediterranean) (Tepe, 2009). Since the Mediterranean Sea is considered a hotspot for heavy metals, continuous monitoring of commercial fish species remains essential to ensure seafood safety (Storelli & Barone, 2013; Cresson et al., 2014; Higuieruelo et al., 2025).

Zoonotic parasites also represent an important food safety concern, particularly when raw or undercooked fish is consumed. Anisakiasis, caused mainly by *Anisakis pegreffii* Campana-Rouget & Biocca, 1955 and *Anisakis simplex* (Rudolphi, 1809) Dujardin, 1845 larvae, is one of the best-known fish-borne zoonoses (Mattiucci et al., 2022). These parasites have complex life cycles involving crustaceans, fish and cephalopods, and cetaceans as definitive hosts (Klimpel & Palm, 2011; Mattiucci et al., 2018). Larvae, after being ingested by fish, penetrate the intestinal wall and encyst on the surface of abdominal organs or migrate to the musculature. Humans become accidental hosts when consuming raw, smoked, marinated salted or undercooked infected fish, and the ingestion of viable larvae can cause gastrointestinal (e.g., nausea, vomiting and epigastric pain) or allergic reactions (Mattiucci et al., 2013, 2022). *A. pegreffii* is commonly identified as the most prevalent larvae species infecting various fish species across Mediterranean regions, although other nematode species such as *Skrjabinisakis physeteris* (Baylis, 1923) Safonova,

Voronova & Vainutis, 2021 (formerly *Anisakis physeteris*) and *Anisakis typica* (Diesing, 1860) Baylis, 1920 have been also reported and their abundance vary across areas (Mattiucci et al., 2004; Cipriani et al., 2018a,b; Mattiucci et al., 2018). Infection levels and parasite distribution vary greatly among fish species and Mediterranean regions, depending on environmental conditions, food-web structure and host availability (Mattiucci et al., 2018 and references therein). The European hake has been extensively studied due to its commercial relevance, showing important geographic differences in prevalence, intensity and muscle migration of anisakid larvae, with direct implications for food safety (Mattiucci et al., 2004; Valero et al., 2006; Ferrer-Maza et al., 2014; Piras et al., 2014; Costa et al., 2018; Cipriani et al., 2018 Goffredo et al., 2019). Generally, these studies focused on adult hakes, with little information on juvenile specimens, which are especially relevant in fishing grounds from the continental shelf (ICATMAR, 2025). In contrast, zoonotic parasites in round sardinella remain poorly studied, mostly restricted to North African waters (Feki et al., 2015; Ramdani et al., 2020), where it has a higher consumption as fresh fish in the local markets (Thiaw et al., 2020). Available data generally indicate low prevalence or absence of zoonotic species, with occasional records of *A. pegreffii* or *Hysterothylacium* spp. (Piras et al., 2014; Costa et al., 2018; Goffredo et al., 2019), another nematode genus that, although it has less or negligible zoonotic potential, can occasionally cause allergic reactions (Roca-Geronès *et al.*, 2020). To date, only *A. pegreffii* and *A. simplex* (s.s) have been reported in fish musculature, whereas the zoonotic potential of other anisakid species remains unclear (Mattiucci et al., 2018), highlighting the importance of continuous monitoring studies, particularly in regions where fish consumption is a fundamental component of population's diet

In this context, fish consumption should be understood as a balance between its nutritional benefits and the need to guarantee its safety. The implementation of good practices throughout the entire production chain, from capture or aquaculture to processing and distribution is essential to maximize its benefits while minimizing their associated risks. Hence, this study aims to assess *S. aurita* seasonal nutritional status (proximate composition including protein and lipid content, and fatty acid composition) from the coast of Barcelona (NW Mediterranean Sea) and its potential risks for human consumption through the analyses of the presence of heavy metals and zoonotic parasites. Moreover, a risk assessment of heavy metals and zoonotic parasite exposure associated with the consumption of the well-established fish species *M. merluccius* was also conducted. In addition, a comparison of the distribution and infection levels of zoonotic parasites in both fish species from different regions of the western (Balearic Sea) and central Mediterranean Sea (Adriatic and Tyrrhenian seas) is also provided.

2. Materials & Methods

2.1. Study area and sample collection

For the nutritional analysis, a total of 100 *S. aurita* specimens were seasonally captured aboard commercial purse seiner fishing vessels on the continental shelf of the Catalan coast off Barcelona (NW Mediterranean Sea) between 2022 and 2023 (20 individuals per season including two summer periods) (see Table 1 and Fig.1 Chapter 3.1 M&M). Fish were measured, weighted, eviscerated and stored in ice before further preservation in laboratory conditions. For heavy metal analyses, 10 *M. merluccius* and 10 *S. aurita* specimens were also collected on the coast of Barcelona and preserved frozen at -20 °C for subsequent metal analyses (see Table 1, Chapter 3.1 M&M).

Additionally, for the zoonotic parasites assessment, a total of 260 *M. merluccius* and 238 *S. aurita* specimens were sampled between 2019 and 2025 from different areas from the Western (Balearic Sea) and Central Mediterranean Sea (Tyrrhenian and Adriatic seas) (see Table 1 and Fig.1 Chapter 3.1 M&M). After capture, fish were immediately maintained fresh or frozen except for fish captured on the continental shelf of the Catalan coast (Balearic Sea), which were fixed in toto in 10% buffered formalin on board. Posteriorly, fish were transported to the laboratory for further parasitological analysis.

2.2. Analysis of fillet proximate composition and fatty acids

Once in the laboratory, fillet weight (from both the right and left sides) was measured for each specimen, and then were frozen and stored at -20°C until lyophilisation (for 72 hours). After lyophilisation, dried fillets were weighed again for moisture determination. For each season, skinless fillets were grouped into five groups of four individuals each and were homogenised with a grinder. Analytical determinations of chemical composition (i.e. crude protein, total lipid and fatty acid composition) were performed according to AOAC (Association of Official Agricultural Chemists) International methods as described in Chapter 3.4 M&M.

To estimate the nutritional quality of the lipids, atherogenic (AI) and thrombogenic (TI) indices were calculated according to Ulbricht & Southgate (1991) using the following formulas. AI and TI values lower than 1 were considered beneficial to human health.

$$AI = \frac{[C12:0 + (4 * C14:0) + C16:0]}{(\sum MUFA + \sum n - 6 + \sum n - 3)}$$

$$TI = \frac{[C14:0 + C16:0 + C18:0]}{\left[(0.5 * \sum MUFA) + \left(0.5 * \sum n - 6 + (3 * \sum n - 3) + \left(\frac{\sum n - 3}{\sum n - 6} \right) \right) \right]}$$

Moreover, the nutritional intake contribution of EPA+DHA from round sardinella consumption was estimated according to Costa *et al.* (2013) using the following formula:

$$\text{Nutritional contribution (\%)} = [(C \times M)/(DRI)] \times 100$$

where C is the mean concentration of EPA+DHA (mg kg⁻¹), M is the meal portion consumed (kg) and DRI is the dietary reference intake value (mg) established by the European Food Safety Authority (EFSA, 2012a). The DRI of EPA and DHA for preventing cardiovascular diseases ranges between 250 and 500 mg/day. Hence, in the present study the minimum DRI of 250 mg was used for the calculations. Moreover, taking into account the fact that the annual consumption of fish and seafood in Spain was around 42 kg per capita in 2024, representing 115g daily per capita (European Commission, 2026), and that the mean fillet weight of one round sardinella specimen is approximately 25 g, a meal portion of four individuals representing 100 g/day of fillet was used for the calculation.

2.3. Analysis of heavy metals

Concentrations of mercury (Hg), cadmium (Cd), lead (Pb), zinc (Zn), copper (Cu), nickel (Ni), and arsenic (As) from muscle samples of *M. merluccius* and *S. aurita* were analysed as described in Chapter 3.3.2 M&M. To calculate the metal intake contribution of a meal portion of 60 g/day, 85 g/day and 100 g/day of the studied fish fillet in an infant (12 kg of assumed weight), children (35 kg of assumed weight) and adult person (70 kg of assumed weight), respectively (AESAN, 2008), the following formula by Costa *et al.* (2013) was applied:

$$\text{Contribution (\%)} = [(C \times M)/(BW \times TDI \text{ or } TWI)] \times 100$$

where C is the mean concentration of heavy metal (mg kg⁻¹) in the meal, M is the meal portion consumed (kg), BW is the body weight (kg), TDI or TWI is the tolerable daily or weekly intake (mg kg⁻¹), respectively, established by the Expert Committee on Food Additives (JECFA) of the FAO/WHO (2010a,b) and by the European Food Safety Authority (EFSA, 2009; 2012b; 2020; 2023). Following the conservative approach by EFSA, total Hg concentration was converted to MeHg using a conversion factor of 1 (EFSA, 2012b). For As, no TWI is currently established for fish and seafood consumption.

2.4. Parasitological examination

At the laboratory, after taking fish biometric data (Chapter 3.1 M&M), the maturity stage and sex were determined and the gonadosomatic index (GSI) was calculated as specified in Chapter 3.2.1 M&M, from specimens collected in the Adriatic and Tyrrhenian Seas (central Mediterranean) and

off Valencia (western Mediterranean). Data from fish caught off the Catalan coast (western Mediterranean) analysed in Chapter 4 was also used in the present chapter.

From 20 specimens of each locality, the digestive tract and the abdominal cavity including mesenteries, liver, gonads, kidney, spleen and swimbladder were visually examined for the presence of nematodes under a stereomicroscope. For the rest of fish individuals, the viscera (including all abdominal organs) were also analysed using the UV-press method as indicated in Chapter 3.2.2 M&M. The whole fillet muscle of all examined fish individuals was also examined using the UV-press method. Nematodes recovered from fixed fish were cleared and examined as semi-permanent mounts in glycerine, whereas those from fresh or frozen fish were preserved in 95% ethanol for molecular analysis (see Chapter 3.2.2 M&M).

2.5. Data analysis

All variables used for analyses were tested for normality using the Shapiro-Wilk test and for homoscedasticity using the Levene's test. Data distribution was also plotted for visual assessment.

Fish size effect was first tested for fillet moisture, crude protein and total lipid content and fatty acid composition of *S. aurita*, using General Linear Models (GLM) for parametric data or Generalised Linear Models (GZM) for non-parametric data with Poisson distribution models. Since no interactions with fish size were detected, differences among seasons for fish biometric data (SL and EW) and for proximate composition and fatty acid composition were tested using ANOVA and Tukey's HSD post hoc tests for parametric data or Kruskal-Wallis and Dunn's multiple comparison tests for non-parametric data.

For the analysis of nematodes, fish size effect and sex were tested for prevalence (P) and mean intensity (MI) of each nematode species using GZM (with logistic model for P and negative binomial models for MI). Then, GZM were also carried out to test significant differences among localities for P and MI of nematode species. Also, differences among localities for P and MI of nematode species according to their site of infection (musculature, digestive tract and abdominal cavity including mesenteries, liver and gonads) were also tested with GZM. Post-hoc pairwise comparisons were performed using estimated marginal means tests (EMM, emmeans function) with Holm adjustment. Differences on the relative abundance (%) of each nematode species according to the total nematode abundance for each locality were tested using Chi-squared tests.

The length at which 50% of fish were infected by anisakid nematodes was inferred using a binomial logistic regression based on a GLM. The probability of infection was calculated as: $P = 1/(1 + \exp(-\beta_0 + \beta_1 \cdot SL))$, where P is the probability of infection at a given length, and β_0 and β_1

are the intercept and slope estimated parameters. The length at which 50 or 95% of individuals were derived from the model (Roa et al., 1999). In addition, the relationship between fish size and anisakid abundance was evaluated using the Spearman correlation coefficient.

Data analysis was performed using RStudio software, version 4.1.2. Statistical significance was set at 0.05.

3. Results

3.1. Proximate fillet composition

The size (SL) and weight (EW) of round sardinella used in this study as well as the proximal and fatty acid composition of its fillets are present in **Table 1**. Fish ranged from 13.39 to 15.59 cm of SL and from 26.16 to 42.44 g of EW, being those from autumn significantly smaller and lighter than those from the other seasons (ANOVA, $F_{(19,4)} = 8.32$, $p < 0.001$ and $F_{(20,4)} = 5.23$, $p = 0.004$, respectively) (**Table 1**). In contrast, fish fillet yield (the proportion of the edible fillet weight with respect to the EW) was highest (60.97%) in autumn (**Table 1**). No fish size interaction was observed in terms of crude protein and total lipid content (% wet weight) in *S. aurita* fillets. Significant differences were observed regarding proximal composition between seasons but were not linked in any case to fish size. Crude protein ranged from 16.70 to 20.32% of wet weight, with winter-23 being the season in which individuals displayed significantly lower values compared to other seasons (K-W, $\chi^2 = 17.34$, $p = 0.001$). Moisture (ranging from 73.35 to 76.84%) was found to be inversely proportionate to the total lipid content, with spring-23 being the season with significantly lower values of moisture (K-W, $\chi^2 = 13.37$, $p = 0.009$) and higher values of lipid content compared to the other seasons (ANOVA, $F_{(20,4)} = 46.66$, $p < 0.001$). In general, total lipid content in all seasons was below 2% (between 0.30 and 1.69%). Differences were also found between years in the same season, such as the fact that significantly lower levels of lipid were found in summer-22 compared to summer-23 (Wilcoxon, $W = 13$, $p < 0.001$) (**Table 1**).

Table 1. Number (n) and mean with the standard deviation (SD) of the standard length (SL), eviscerated weight (EW), edible fillet weight (FW), fillet yield (FY in %) in relation to EW, moisture crude protein, and total lipid content (in % wet weight), fatty acid profiles (% of total fatty acids) of the muscle of specimens of *Sardinella aurita* sampled in each season in the central Catalan coast (NW Mediterranean) are given. Mean with the standard deviation for the ratios calculated between omega-6 fatty acids (n-6) and omega-3 fatty acids (n-3), between C16:1 (PO) and C16:0 (P) as well as for the atherogenic index (AI), thrombogenic index (TI) for each season are provided. The nutritional intake contribution (%) of EPA+DHA (NC) for the consumption of *S. aurita* for each season was also calculated. Different superscript letters show significant differences ($p < 0.05$) among seasons. Dashes indicate absence of the fatty acid.

	SUMMER 22	AUTUMN 22	WINTER 23	SPRING 23	SUMMER 23
n	20	20	20	20	20
SL (cm)	15.59 (0.69) ^a	13.39 (0.65) ^b	15.07 (0.51) ^a	15.35 (2.63) ^a	15.51 (0.69) ^a
EW (g)	42.44 (5.37) ^a	26.16 (4.53) ^b	38.59 (4.60) ^a	39.52 (11.67) ^a	39.36 (5.04) ^a
FW (g)	20.15 (3.01) ^a	15.94 (3.02) ^b	22.60 (2.85) ^a	22.45 (7.11) ^a	21.15 (3.32) ^a
FY (%)	47.47	60.93	58.56	56.80	53.73
Moisture (%)	76.84 (0.61) ^a	76.56 (0.65) ^a	76.56 (0.65) ^a	73.35 (0.98) ^b	76.59 (0.87) ^a
Crude protein (%)	19.69 (0.75) ^a	20.30 (0.82) ^a	16.70 (0.45) ^b	18.78 (0.91) ^a	20.32 (0.40) ^a
Total lipid (%)	0.30 (0.06) ^d	0.67 (0.22) ^{bc}	0.92 (0.04) ^b	1.69 (0.36) ^a	0.62 (0.02) ^c
C13:0	—	—	—	0.29 (0.08)	—
C14:0	2.16 (0.59) ^b	2.20 (0.68) ^b	9.32 (0.53) ^a	8.99 (2.04) ^a	1.81 (0.36) ^b
C15:0	0.86 (0.11)	0.89 (0.13)	1.77 (0.18)	1.88 (0.56)	0.64 (0.04)
C16:0 (P)	26.81 (1.71) ^c	22.64 (0.80) ^c	45.82 (0.34) ^a	35.69 (7.17) ^b	22.21 (1.00) ^c
C16:1 (PO)	1.25 (0.34) ^c	1.31 (0.39) ^c	4.93 (0.40) ^b	6.56 (0.60) ^a	1.12 (0.21) ^c
C17:0	1.60 (0.14) ^{bc}	1.43 (0.09) ^c	2.24 (0.23) ^a	1.89 (0.48) ^{ab}	1.23 (0.05) ^c
C18:0	10.03 (0.35) ^b	7.35 (0.18) ^c	12.04 (0.81) ^a	8.79 (1.91) ^{bc}	8.66 (0.36) ^{bc}
C18:1n9c	4.65 (0.40) ^b	4.36 (0.39) ^b	7.03 (0.22) ^a	7.24 (1.23) ^a	4.05 (0.30) ^b
C18:1n7	2.42 (0.26) ^b	2.09 (0.20) ^b	3.87 (0.19) ^a	4.43 (0.37) ^a	2.43 (0.17) ^b
C18:2n6c	1.58 (0.12) ^{ab}	1.93 (0.35) ^a	0.48 (0.08) ^c	1.23 (0.62) ^b	1.75 (0.14) ^{ab}
C18:3n3	—	0.72 (0.10)	—	0.85 (0.28)	0.54 (0.08)
C20:0	—	0.37 (0.01) ^b	0.76 (0.15) ^a	0.47 (0.14) ^b	—
C20:1n9	—	0.39 (0.05) ^c	4.65 (0.71) ^a	2.07 (0.45) ^b	0.52 (0.02) ^c
C20:2	—	0.39 (0.02)	—	0.39 (0.03)	—
C20:4n6	1.86 (0.19) ^a	1.93 (0.08) ^a	—	0.93 (0.26) ^b	2.14 (0.13) ^a
C20:5n3 (EPA)	3.89 (0.28) ^a	6.12 (0.32) ^a	0.99 (0.13) ^b	4.28 (3.35) ^a	4.89 (0.41) ^a
C22:6n3 (DHA)	42.88 (3.17) ^a	46.41 (3.18) ^a	4.69 (0.69) ^c	14.09 (9.14) ^b	48.26 (2.27) ^a
C24:1n9	—	—	1.42 (0.10) ^a	0.98 (0.24) ^b	—
Total SFA	40.61 (2.79) ^c	34.00 (1.75) ^c	70.18 (2.06) ^a	55.84 (11.74) ^b	33.92 (1.78) ^c
Total MUFA	5.90 (0.74) ^b	6.06 (0.84) ^b	18.03 (1.43) ^a	16.85 (2.53) ^a	5.69 (0.53) ^b
Total n-3 PUFA	46.77 (3.46) ^a	53.25 (3.59) ^a	5.67 (0.82) ^c	19.23 (12.78) ^b	53.69 (2.76) ^a
Total n-6 PUFA	3.44 (0.32) ^a	3.86 (0.43) ^a	0.48 (0.08) ^c	2.16 (0.87) ^b	3.89 (0.28) ^a
Total PUFA	50.21 (3.77) ^a	57.11 (4.02) ^a	6.15 (0.90) ^c	21.39 (13.65) ^b	57.58 (3.04) ^a
EPA/DHA	0.09 (0.004) ^b	0.13 (0.015) ^b	0.21 (0.05) ^a	0.30 (0.09) ^a	0.10 (0.01) ^b
n-6/n-3	0.07 (0.002) ^b	0.07 (0.007) ^b	0.08 (0.009) ^{ab}	0.11 (0.04) ^a	0.07 (0.004) ^b
PO/P	0.05 (0.01) ^c	0.06 (0.017) ^c	0.11 (0.01) ^b	0.18 (0.04) ^a	0.05 (0.008) ^c
AI	0.63 ^c	0.50 ^c	3.44 ^a	1.87 ^b	0.47 ^c
TI	0.70 ^b	0.57 ^b	1.00 ^a	1.34 ^a	0.58 ^b
NC (%)	28.26	116.08	14.20	199.11	82.21

3.2. Fatty acid fillet composition

In relation to fatty acid composition in round sardinella fillets, significant differences were observed in different seasons, but no fish size effect was observed (**Table 1**). Winter-23 and spring-23 were the seasons with the highest proportions of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). On the other hand, summer-22, autumn-22 and summer-23 were the seasons with the highest concentration of polyunsaturated fatty acids (PUFA) (**Fig. 2**).

The percentage of SFA ranged between 33.92% and 70.18% of total fatty acids. The highest content (70.18%) was observed in winter-23 (K-W, $\chi^2 = 21.60$, $p < 0.001$), followed by spring (55.84%). In both seasons, C16:0 (palmitic acid) was the most abundant (K-W, $\chi^2 = 21.45$, $p < 0.001$), representing 63.91% and 66.58% of the total SFA, respectively (**Table 1, Fig. 2**). Higher proportions of both C18:0 (stearic acid) and C14:0 (miristic acid) were also obtained in winter-23 (ANOVA, $F_{(20,4)} = 15.65$, $p < 0.001$; K-W, $\chi^2 = 17.71$, $p = 0.001$, respectively), as well as in spring-23 for C14:0 (K-W, $\chi^2 = 17.71$, $p = 0.001$).

As for SFA, significant differences were obtained in relation to MUFA content, which ranged between 5.69% and 18.03% of the total fatty acids. Winter-23 and spring-23 were the seasons with a significantly higher percentage of MUFA (K-W, $\chi^2 = 21.54$, $p < 0.001$) (**Table 1, Fig. 2**) and C18:1n9c (oleic acid) (K-W, $\chi^2 = 19.13$, $p < 0.001$), the most abundant MUFA in each season (between 38.99 and 78.81% of total MUFA). Spring-23 presented a higher proportion of C16:1 (palmitoleic acid) (38.93% of total MUFA) (K-W, $\chi^2 = 18.58$, $p < 0.001$) but a lower content of C20:1n9 (eicosanoic acid) than in winter-23 (ANOVA, $F_{(11,3)} = 49.55$, $p < 0.001$). The PO/P ratio (C16:1/C16:0) displayed low values in all seasons but was significantly higher in spring-23 (K-W, $\chi^2 = 19.28$, $p < 0.001$) (**Table 1**).

In the case of PUFA content, significant variations were also found between seasons. The percentage of PUFA ranged between 6.15% to 57.58% of total fatty acids. The most abundant PUFA were n-3 PUFA, specifically C22:6n3 (DHA) representing between 65.87 and 85.4% of the total PUFA (**Table 1**), which is reflected in the n-6/n-3 ratio. Total PUFA and DHA were significantly higher in summer-22, summer-23 and autumn-22 compared to winter-23 (K-W, $\chi^2 = 14.91$, $p = 0.001$ and $\chi^2 = 13.53$, $p = 0.003$, respectively) and spring-23 (K-W, $\chi^2 = 14.91$, $p = 0.002$ and $\chi^2 = 13.53$, $p = 0.003$, respectively). The EPA content was lower but followed a similar pattern to DHA, being significantly higher during summer-22, summer-23 and autumn-22 and also in spring (K-W, $\chi^2 = 13.57$, $p = 0.008$) (**Table 1, Fig. 2**). Regarding the EPA/DHA ratio, higher values were obtained for winter-23 and spring-23 (K-W, $\chi^2 = 21.12$, $p < 0.001$), due to a noticeable decrease of DHA in

this period. Other PUFA such as C18:2n6, C18:3n3 and C20:4n6 were also present in almost all seasons but in a low proportion, representing less than 10% of the total PUFA.

The lowest AI and TI values were obtained for summer-22, summer-23 and autumn-22 (K-W, $\chi^2 = 24.00$, $p < 0.001$ and $\chi^2 = 24.00$, $p < 0.001$, respectively), ranging from 0.47 to 0.70; whereas in winter-23 and spring-23 these ranged from 1.00 to 3.44. The estimated nutritional intake contribution of EPA+DHA, assuming a consumption of 100 g of round sardinella per day, ranged from 14.20 to 199.11%, being higher in spring 23 and autumn 22 (**Table 1**).

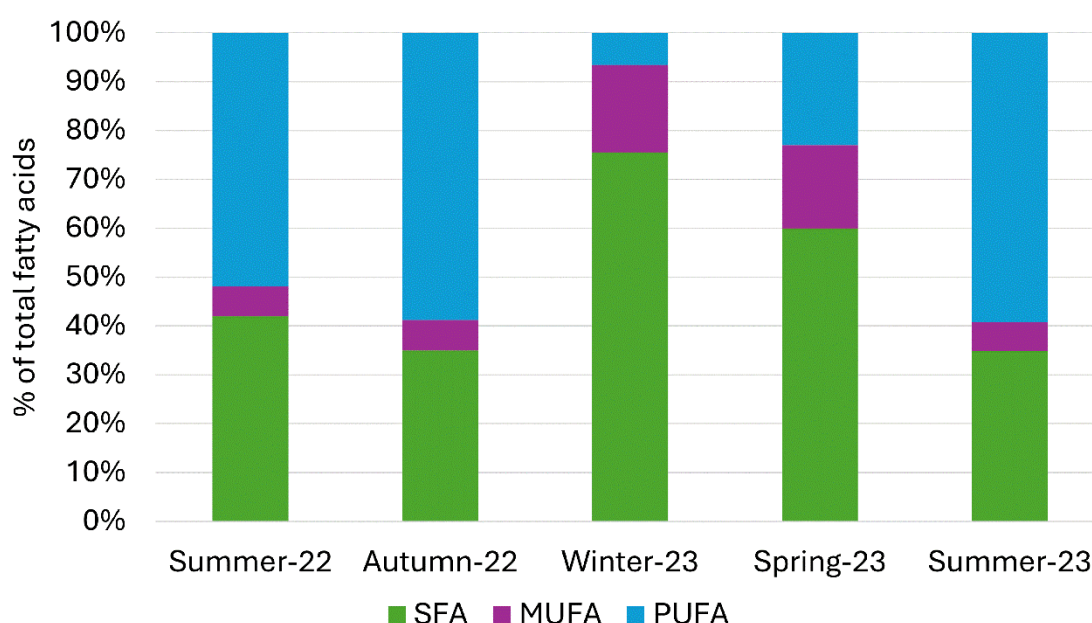


Fig. 2. Seasonal variation of the percentage of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids with respect to the total fatty acid composition in the flesh of *Sardinella aurita* captured in the central Catalan coast (NW Mediterranean).

3.3. Heavy metals

While heavy metal concentrations in the musculature of *M. merluccius* and *S. aurita* were described in Chapter 5, the corresponding maximum permissible concentration and the tolerable intake levels (TDI or TWI) for each heavy metal are summarized in **Table 2**. Overall, values detected in muscle samples of analysed fish were below the established limit for consumption for each metal. Zn and As displayed the highest concentrations compared to other metals in both species. Both species presented similar metal concentrations although hakes showed slightly higher concentrations of Ni and Pb whereas round sardinellas displayed higher concentrations of Cu, Zn, As and Hg.

The estimated metal intake contribution of consuming a portion of 60 g, 85 g or 100 g of the examined fish by infants, children and adults, respectively, under each frequency scenario (once, three and five times per week) is also presented in **Table 2**. Overall, the analysed metals did not exceed the established tolerable limits, except for MeHg, whose intake contribution exceed the tolerable weekly limits in infants consuming both hake and round sardinella five times per week (105.04% and 164.33% of the established TWI, respectively) but falling within the established thresholds when consuming these fish species three times per week. Considering all consumption frequencies, the contribution of MeHg intake from hake and round sardinella to the established TWI for an adult of 70 kg ranged between 6.00 – 30.01% and 9.39 – 46.95% respectively. (**Table 2**). For Cd, consuming hake and sardinella represented 0.06 – 0.28% and 0.04 – 0.18% of the TWI for adults, respectively, whereas for Pb, its contribution ranged from 0.15 – 0.75% for hake and 0.24 – 1.21% for sardinella (**Table 2**). For a child of 30 kg, MeHg exposure due to hake and round sardinella consumption represented between 11.90 – 59.52% and 18.62 – 55.87%, respectively. For the rest of cases, metal intake contributions were lower than those detected for MeHg in all scenarios (**Table 2**).

Table 2. Mean and standard deviation (SD) values of heavy metals (Ni, Cu, Zn, As, Cd, Pb, Hg) from *Merluccius merluccius* and *Sardinella aurita* musculature collected off Barcelona (NW Mediterranean) are given in mg/kg wet weight. For each metal, maximum permissible concentration (MPC) is also provided according to the European Commission, Regulation 2023/915 for Cd, Pb and Hg, BOE (1991) for Cu and FAO/WHO (1989) for Zn. Heavy metals intake contribution (%) by consuming raw flesh of both *M. merluccius* and *S. aurita* depending on the frequency of consumption (once a week, three times per week and daily) and the consumer (infant of 12 kg weight, child of 30 kg weight and adult of 70 kg), considering a meal portion of 60 g (infant), 85 g (child) and 100 g (adult) of fish fillet per day. TDI: tolerable daily intake (mg/kg bw); TWI: tolerable weekly intake (mg/kg bw). Dashes indicate absence of data.

Heavy metal	Mean (SD)	MPC	TDI	TWI	Contribution (%)			3 portions/week			5 portions/week		
					Infant	Child	Adult	Infant	Child	Adult	Infant	Child	Adult
Merluccius merluccius													
Ni	0.202 (0.36)	–	0.013	–	1.11	0.63	0.32	3.33	1.89	0.95	5.55	3.15	1.59
Cu	0.242 (0.03)	20.0	0.5	–	0.03	0.02	0.01	0.10	0.06	0.03	0.17	0.10	0.05
Zn	3.193 (0.25)	40.0	0.65	–	0.35	0.20	0.10	1.05	0.60	0.30	1.75	0.99	0.50
As	3.634 (0.73)	–	–	–	–	–	–	–	–	–	–	–	–
Cd	0.001 (0.0003)	0.05	–	0.0025	0.19	0.11	0.06	0.58	0.33	0.17	0.97	0.55	0.28
Pb	0.026 (0.03)	0.3	–	0.025	0.52	0.30	0.15	1.57	0.89	0.45	2.61	1.48	0.75
Hg	0.05 (0.02)	0.5	–	0.0013	21.01	11.90	6.00	63.02	35.71	18.01	105.04	59.52	30.01
Sardinella aurita													
Ni	0.009 (0.004)	–	0.013	–	0.05	0.03	0.01	0.14	0.08	0.04	0.24	0.08	0.07
Cu	1.17 (0.22)	20.0	0.5	–	0.17	0.09	0.05	0.50	0.28	0.14	0.84	0.28	0.24
Zn	5.26 (0.56)	40.0	0.65	–	0.58	0.33	0.17	1.73	0.98	0.50	2.89	0.98	0.83
As	6.14 (2.21)	–	–	–	–	–	–	–	–	–	–	–	–
Cd	0.001 (0.0001)	0.05	–	0.0025	0.12	0.07	0.04	0.37	0.21	0.11	0.62	0.21	0.18
Pb	0.04 (0.03)	0.3	–	0.025	0.85	0.48	0.24	2.54	1.44	0.73	4.23	1.44	1.21
Hg	0.08 (0.02)	0.3	–	0.0013	32.87	18.62	9.39	98.60	55.87	28.17	164.33	55.87	46.95

3.4. Zoonotic nematodes

3.4.1. Fish biological data

Specimens of *M. merluccius* (SL: 14.5 – 34.0 cm) and *S. aurita* (SL: 12.0 – 22.4 cm) showed significant differences on SL and GSI among localities, with hakes from the northern and southern Tyrrhenian Sea and sardinellas from the northern Balearic Sea being larger and displaying higher GSI values than those from other localities (SL: K-W, $\chi^2 = 181.87$ and 53.23 , $p < 0.001$, respectively and GSI: K-W, $\chi^2 = 124.6$ and 40.48 , $p < 0.001$, respectively). Overall, specimens from both species were mostly immature and the sex ratio varied between species and localities, showing a predominance of females in hakes (Table 3).

Table 3. Biometric data of the examined fish host species *Merluccius merluccius* and *Sardinella aurita* sampled in different localities from the Mediterranean Sea, including number of fish (n) and mean values followed by standard deviation of standard length (SL) and gonadosomatic index (GSI). Percentage of immature individuals and sex ratio (M:F) are also displayed for each locality. For each fish host, different superscript letters indicate statistically significant differences among localities.

Species	Zone	Locality	n	SL (cm)	GSI	Immature (%)	Sex ratio (M:F)
<i>Merluccius merluccius</i>	N Balearic Sea	Blanes	22	19.40 (3.16) ^{cd}	0.16 (0.18) ^c	72.72%	2:11
		Barcelona	21	17.31 (1.31) ^d	0.04 (0.04) ^c	80.95%	1:4
		Ebro Delta	19	17.82 (2.55) ^d	0.28 (0.57) ^{bc}	89.47%	4:5
	S Balearic Sea	Valencia	15	24.47 (2.57) ^b	0.32 (0.15) ^{bc}	100%	1:4
	N Adriatic Sea	Rimini	89	23.69 (1.73) ^b	0.53 (0.27) ^b	85.39%	1:80
	N Tyrrhenian Sea	Rosignano	20	25.70 (3.70) ^a	0.78 (0.32) ^a	90%	3:7
	C Tyrrhenian Sea	Roma	20	20.25 (1.98) ^c	0.40 (0.22) ^b	80%	1:1
	S Tyrrhenian Sea	Naples	40	28.18 (2.32) ^a	0.44 (0.21) ^b	92.50%	3:17
		Salerno	14	27.50 (1.50) ^a	0.78 (0.32) ^a	78.57%	5:9
	N Balearic Sea	Barcelona	100	15.40 (1.92) ^a	1.44 (1.42) ^a	55%	13:12
<i>Sardinella aurita</i>	N Adriatic Sea	Rimini	39	15.15 (1.35) ^{ab}	0.41 (0.24) ^b	100%	1:2
		Scardovari	22	14.72 (1.14) ^{ab}	0.36 (0.22) ^b	100%	12:7
	S Adriatic Sea	Bari	77	14.41 (1.39) ^c	0.42 (0.28) ^b	96.11%	7:8

3.4.2. Nematode parasites

A total of 440 nematode larvae were recovered from 59.61% of the examined *M. merluccius* specimens across the different sampled areas. Visual inspection and morphological analysis revealed a total of 204 *Anisakis* spp. larvae in 35.38% of fish, 228 *Hysterothylacium* spp. in 30.38% fish and eight *Capillaria* sp. in 0.77% of fish. In contrast, no nematodes were detected in any of the examined *S. aurita* individuals across the sampled localities.

For the nematodes recovered from *M. merluccius*, PCR-RFLP analysis were performed in all *Anisakis* larvae and on 63.43% of *Hysterothylacium* spp. of the total individuals from this genus recovered, as the remaining specimens from the N Balearic Sea were morphologically identified in Chapter 4. This resulted in distinct RFLP patterns that allowed the identification of two confirmed zoonotic species: 86.76% were *Anisakis pegreffii* and 9.31% *Anisakis pegreffii* x *Anisakis simplex* hybrid. In addition, 3.92% were identified as *Skrjabinisakis physeteris*, a species with uncertain zoonotic potential. Moreover, two species of the genus *Hysterothylacium* with limited zoonotic relevance were detected: 66.08% of them were identified as *H. fabri* and 33.92% were *H. aduncum* (Fig. 3). Co-infections by anisakid and *Hysterothylacium* species occurred only in 6.54% of hakes.

Morphological identification revealed that *A. pegreffii* larvae were characterized by the presence of a long muscular ventricle, a boring tooth at the anterior end and a mucron at the posterior part (Fig. 4 a-c), whereas *S. physeteris* also presented an anterior boring tooth but a shorter ventricle and lacked a posterior mucron (Fig. 4 d-e). *Anisakis pegreffii* x *Anisakis simplex* hybrids were morphologically identical as *A. pegreffii*. In contrast, both *Hysterothylacium* species presented an anterior end with three lips and papillae and a cactus tail in their posterior end (Fig. 4 f, h). In some cases, *Hysterothylacium* male specimens were distinguished by the presence of two spicules (Fig. 4i). *H. aduncum* differed from *H. fabri* by the presence of a longer intestinal caecum (Fig. 4 g, j). *Capillaria* sp. specimens were females characterized by the presence of a stichosome, which is an oesophagus surrounded by glandular cells (stichocytes), and an ovary and eggs could also be distinguished (Fig. 4 k-l).

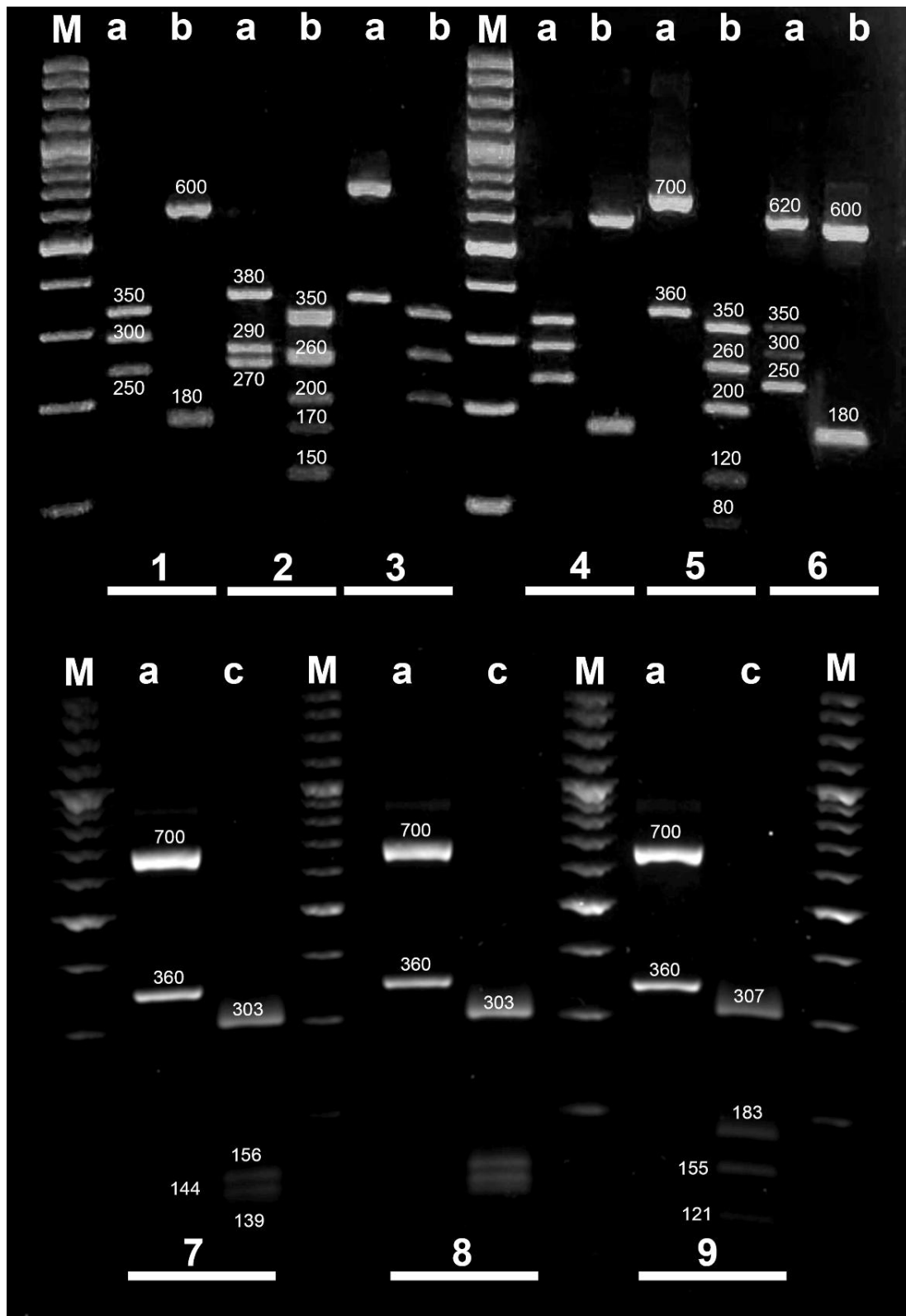


Fig. 3. Restriction fragment length polymorphism patterns (molecular weight marker 100 base pairs, M) obtained with the restriction enzymes *HinfI* (lanes a), *HaeIII* (lanes b) and *AluI* (lanes c). Lanes 1, 4, *Anisakis pegreffii*; lane 2, *Skrjabinisakis physeteris*; lanes 3, 5, *Hysterothylacium* sp.; lane 6, *Anisakis pegreffii* x *Anisakis simplex* hybrid; lanes 7 – 8, *Hysterothylacium fabri*; lane 9, *Hysterothylacium aduncum*. Base pairs of each fragment for each species are also shown.

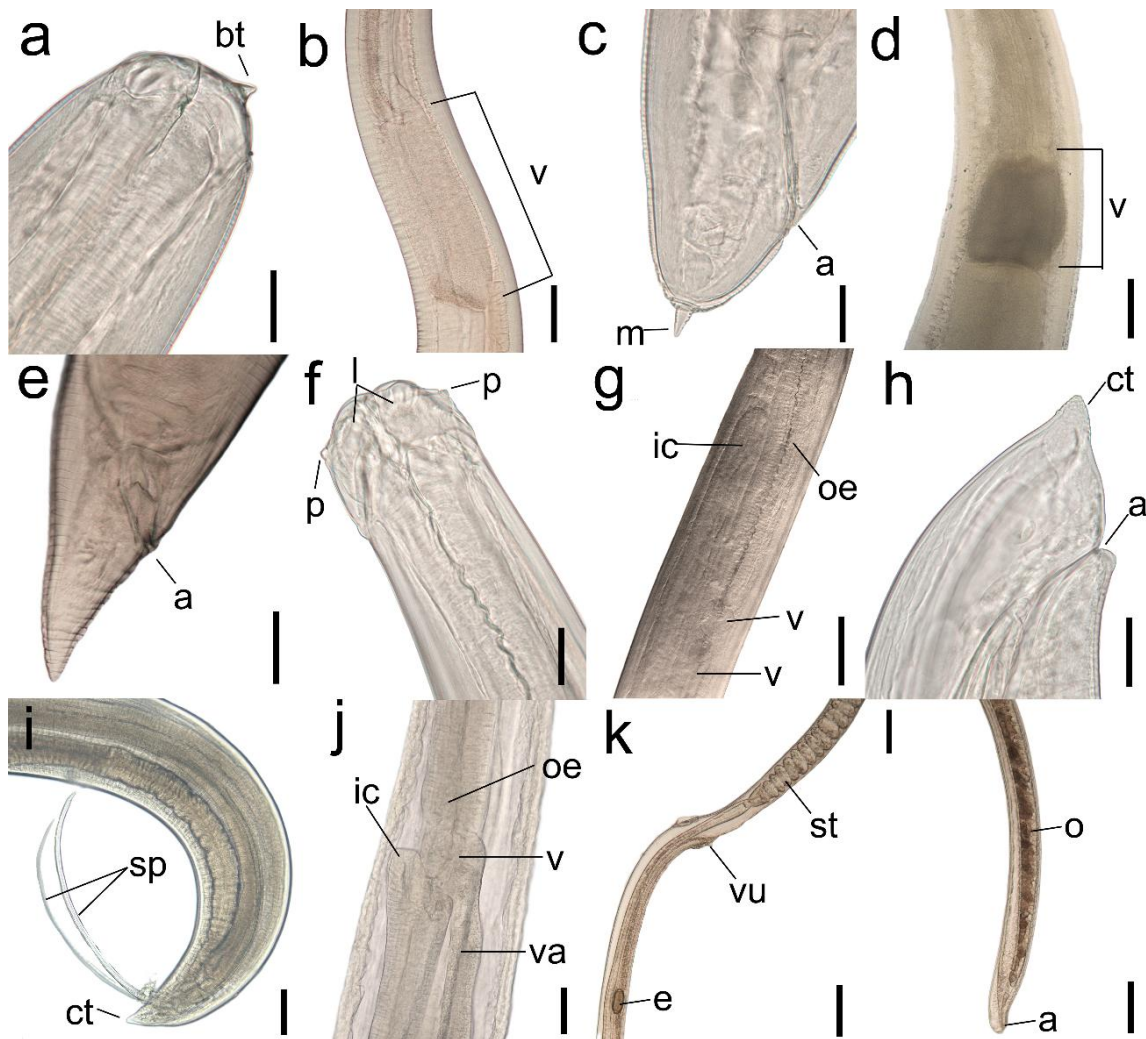


Fig. 4. Microphotographs showing main morphological characteristics of nematodes from the digestive tract of *Merluccius merluccius* collected in the Mediterranean Sea. **a)** Anterior end of *Anisakis pegreffii* showing the boring tooth (bt). Scale bar = 50 μ m. **b)** middle part of *A. pegreffii* with a muscular ventriculus (v). Scale bar = 200 μ m. **c)** Posterior end of *A. pegreffii* showing the mucron (m) and the anus (a). Scale bar = 50 μ m. **d)** Ventriculus (v) of *Skrjabinisakis physeteris*. Scale bar = 200 μ m. **e)** Tail without mucron and the anus (a) of *S. physeteris*. Scale bar = 50 μ m. **f)** Anterior end of *Hysterothylacium aduncum* showing two lips (l) and papillae (p). Scale bar = 50 μ m. **g)** Detail of oesophagus (oe), intestinal caeca (ic), ventriculus (v) and ventricle appendix (va) of *H. aduncum*. Scale bar = 200 μ m. **h)** Posterior end of *H. aduncum* showing the cactus tail (ct) and the anus (a). Scale bar = 50 μ m. **i)** Posterior end of *H. aduncum* showing the cactus tail (ct) and two spicules (sp). Scale bar = 100 μ m. **j)** Detail of oesophagus (oe), intestinal caeca (ic), ventriculus (v) and ventricle appendix (va) of *Hysterothylacium fabri*. Scale bar = 200 μ m. **k)** Medium region of *Capillaria* sp. showing the stichosome (st), vulva (vu) and one egg (e). Scale bar = 200 μ m. **l)** Posterior end of *Capillaria* sp. with the ovary (o) and anus (a). Scale bar = 200 μ m.

For the total nematodes, 27.66% were found freely in the lumen of the digestive tract, particularly in the intestine, whereas 52.60% of larvae were found encysted in the abdominal cavity, mostly in the liver and mesenteries and in a few cases in the gonads. The remaining 14.09% of larvae were found encysted in the muscle. Specifically, 29.94% of *A. pegreffii* and 42.11% of *A. pegreffii* x *A. simplex* hybrids were found in the muscle. Moreover, all individuals of *S. physeteris* and a high proportion of *A. pegreffii*, *A. pegreffii* x *A. simplex* hybrids and *H. fabri* were present in the abdominal cavity, found equally encysted in mesenteries and in the liver. The rest of species were mostly found within the digestive tract: *H. aduncum* was equally found in both stomach and intestine and *Capillaria* sp. and *Raphidascaris* sp. were only found in the intestine (Fig. 5).

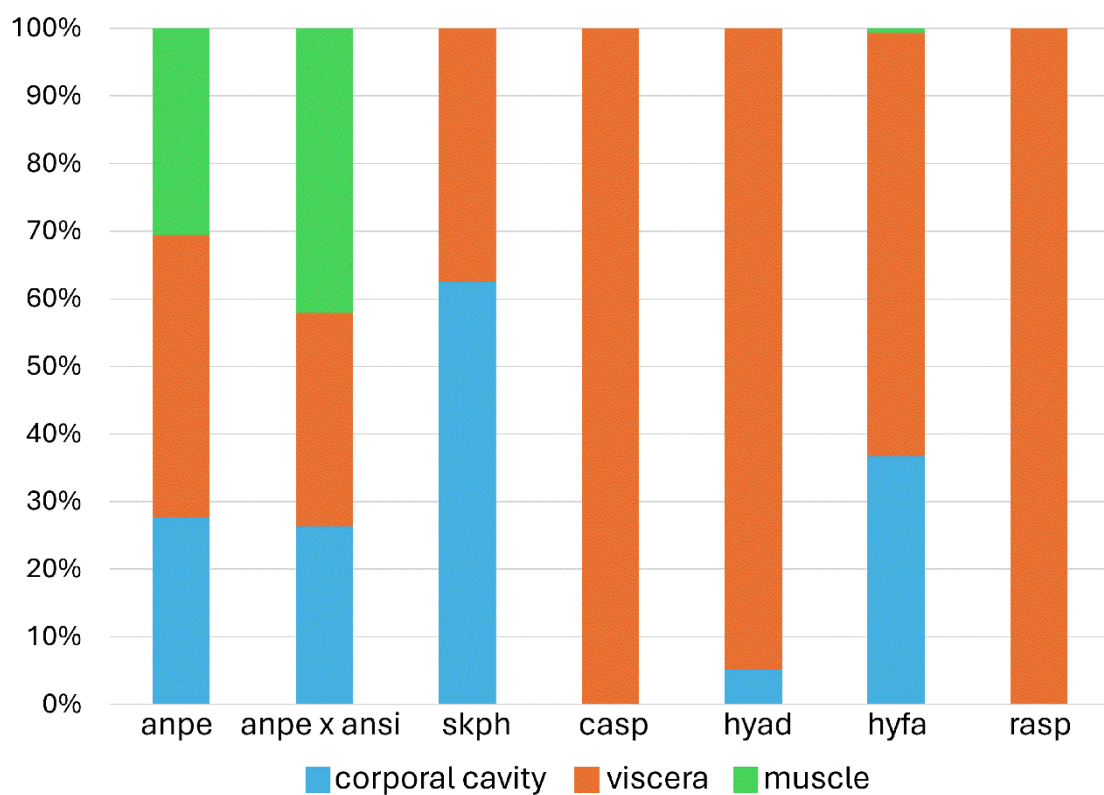


Fig. 5. Proportion of nematode species identified in different locations within *Merluccius merluccius* specimens from the Mediterranean Sea. Abbreviations: anpe, *Anisakis pegreffii*; anpexansi, *Anisakis pegreffii* x *Anisakis simplex* hybrid; skph, *Skrjabinisakis physeteris*; casp, *Capillaria* sp.; hyad, *Hysterothylacium aduncum*; hyfa, *Hysterothylacium fabri*; rasp, *Raphidascaris* sp.

Significantly higher mean intensities of nematodes were found in larger (GZM, $z = 3.95$, $p < 0.001$) and mature fish (GZM emmeans, $z = 2.58$, $p = 0.009$). Particularly, P of *A. pegreffii* (GZM, $z = 4.32$, $p < 0.001$) and *A. pegreffii* x *A. simplex* hybrid (GZM, $z = 2.49$, $p = 0.01$) as well as MI of *H. fabri* (GZM, $z = 2.03$, $p = 0.04$), increased with fish size. Females were significantly more parasitized by *A. pegreffii* compared to males (GZM emmeans, $P: z = 4.04$, $p < 0.001$), but less infected by *H. fabri* (GZM emmeans, $P: z = 2.74$, $p < 0.001$) compared to males and immature fish. Conversely, smaller and immature fish showed significantly higher P of *H. aduncum* (GZM, $z = -5.04$ and $z = 5.06$, $p < 0.001$, respectively).

The minimum size at which hakes were found infected by zoonotic nematodes (i.e. *A. pegreffii* and *A. pegreffii* x *A. simplex* hybrids) measured 19.50 cm SL. The estimated size at which 50% of individuals were infected (L50) was 26.49 cm SL, increasing to 36.29 cm SL for a 95% of infection probability (L95) (**Fig. 6a**). When considering larval location, the L50 increased to 27.86 cm SL for hakes harbouring larvae in the abdominal cavity or musculature, with estimated values of 30.19 cm SL for larval occurrence only in the abdominal cavity and 37.72 cm SL only in the muscle. A significant positive and moderate correlation was observed between hake SL and anisakid mean abundance (Spearman rho = 0.50, $p < 0.001$), showing higher infection levels in larger individuals (**Fig. 6b**).

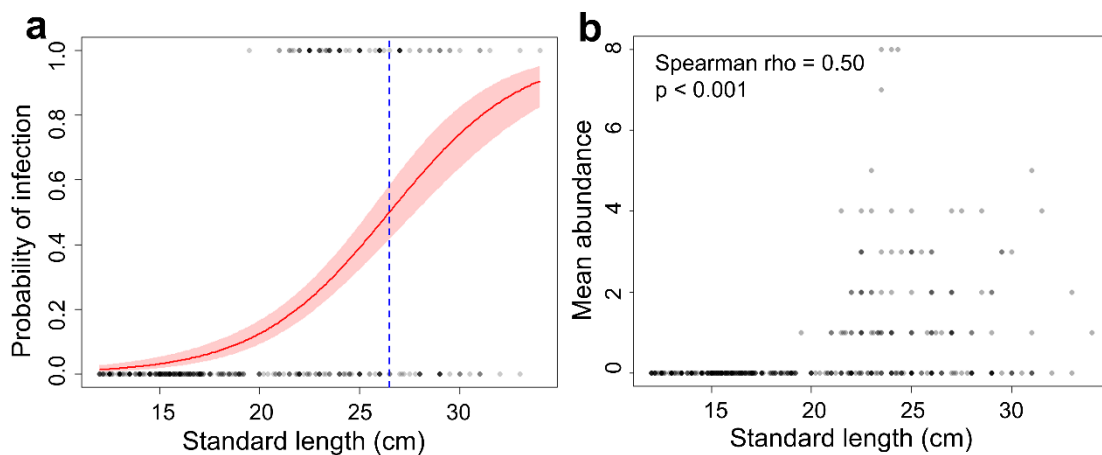


Fig. 6. a) Probability of infection by anisakid species in *Merluccius merluccius* with the estimated size (standard length) at which 50% of individuals are infected (L50). The blue line indicates the length at which 50% of hakes were infected by anisakids (26.49 cm SL). **b)** Spearman correlation analysis between *M. merluccius* standard length (cm) and mean abundance of anisakids per fish.

Infection data on the prevalence (P) and mean intensity (MI) of each nematode species infecting *M. merluccius* across the sampling localities are displayed in **Table 4**. Overall, the most prevalent and abundant nematode found across all regions was *A. pegreffii* (40.13% with a total mean abundance of 0.68 (1 – 6) individuals/fish) followed by *H. fabri* (34.01% and a total mean abundance of 0.56 (1 – 21) individuals/fish). Particularly, *A. pegreffii* was significantly predominant in hakes off Rimini (Adriatic Sea) and off Naples (southern Tyrrhenian Sea), accounting for 84.90% and 70%, respectively (Chi-squared, $\chi^2 = 98.48$, $p < 0.001$ and $\chi^2 = 59.84$, $p < 0.001$) (**Fig. 7**), with hakes off Rimini displaying higher P compared to hakes off Naples and Toscana (GZM emmeans, $z = 3.12 - 3.51$, $p = 0.008 - 0.003$, respectively). In the Tyrrhenian Sea, *H. fabri* dominated in almost all localities (74.44 – 100%) except off Naples (**Fig. 7**), showing the highest levels of infection (P and MI) in hakes off Salerno and Toscana compared to fish from the Balearic Sea, Napoli and Roma (GZM emmeans, P: $z = 3.3 - 3.17$, $p = 0.02 - 0.04$; MI: GZM emmeans, $z = 3.3 - 3.8$, $p = 0.002$) (**Table 4**). In the Balearic Sea, *H. aduncum* dominated the northern part (46.15 – 90.32%) (Chi-squared, $\chi^2 = 73.87$, $p < 0.001$), and larger fish from Blanes were more infected in terms of P (GZM, Blanes*SL: $z = 2.26$, $p = 0.02$) but showed similar intensity values than the rest of localities (**Table 4**). *S. physeteris* represented a notable proportion in the southern Balearic Sea (off Valencia) (Chi-squared, $\chi^2 = 55.17$, $p < 0.001$), co-occurring with *H. fabri* (**Fig. 7**), but no significant differences were detected in terms of P or MI among localities.

Moreover, significant differences in P and MI of nematode species according to their site of infection (musculature, abdominal cavity and digestive tract) were found across localities (**Table 4**). Particularly, hakes off Rimini were more infected by *A. pegreffii* in the muscle compared to those off Napoli (GZM emmeans, P: $z = 2.65$, $p = 0.02$). *H. fabri* encysted in the abdominal cavity was found at higher P and MI in fish off Salerno compared to those from Napoli and Blanes (GZM emmeans, P: $z = 3.22 - 3.35$, $p = 0.008 - 0.04$; MI: $z = 3.02 - 4.01$, $p = 0.001 - 0.04$). Moreover, a significantly higher percentage of fish from Toscana also had *H. fabri* in the abdominal cavity than those from Napoli (GZM emmeans, P: $z = 3.08$, $p = 0.02$). Larger hakes off Blanes and Rimini were significantly more infected by *H. aduncum* in their digestive tract (GZM, P: Blanes*SL: $z = 2.09$, $p = 0.03$) (**Table 4**).

Table 4. Number (n), prevalence (P%), mean abundance (MA) and mean intensity (MI) followed by (range values) of the nematodes recovered from *Merluccius merluccius* in different locations from the Balearic, Adriatic and Tyrrhenian Seas (Mediterranean Sea) are provided. Abbreviations: anpe, *Anisakis pegreffii*; anpe x ansi, *Anisakis pegreffii* x *Anisakis simplex* hybrid; skph, *Skjabinisakis physeteris*; casp, *Capillaria* sp.; hyad, *Hysterothylacium aduncum*; hyfa, *Hysterothylacium fabri*; rasp, *Raphidascaris* sp. Different superscript capital and lowercase letters show significant differences in the P% and MI, respectively, of nematodes among localities.

Zone	Locality	Species	n	Musculature		Abdominal cavity		Digestive tract		Total	
				P%	MI	P%	MI	P%	MI	P%	MI
Balearic	Blanes	hyad	12	–	–	4.76	1.00 (1–1)	27.27	1.83 (1–3)	31.82	1.71 (1–3)
		hyfa	13	–	–	31.82	1.00 (1–1) ^b	13.64	1.43 (1–3)	45.45	1.30 (1–3) ^b
		rasp	1	–	–	–	–	4.55	1.00 (1–1)	4.55	1.00 (1–1)
	Barcelona	hyad	17	–	–	4.76	1.00 (1–1)	52.38	1.45 (1–2)	52.38	1.55 (1–2)
		hyfa	8	–	–	9.52	2.00 (1–3)	19.05	1.00 (1–1)	14.29	2.33 (1–4) ^b
N Adriatic	Ebro Delta	hyad	28	–	–	5.26	1.00 (1–1)	68.42	2.08 (1–5)	73.68	2.00 (1–5)
		hyfa	2	–	–	5.26	2.00 (2–2)	–	–	5.26	2.00 (2–2) ^b
	Valencia	hyfa	2	–	–	–	–	13.33	1.00 (1–1)	13.33	1.00 (1–1) ^b
		skph	5	–	–	33.33	1.00 (1–1)	–	–	33.33	1.00 (1–1)
		anpe	126	33.71 ^A	1.47 (1–3)	37.08	2.12 (1–8)	6.74	1.00 (1–1)	64.04 ^A	2.39 (1–8)
N Tyrrhenian	Rimini	anpe x ansi	6	2.25	1.00 (1–1)	4.49	1.00 (1–1)	–	–	6.74	1.00 (1–1)
		hyad	18	–	–	2.25	1.00 (1–1)	11.24	1.30 (1–3)	14.61	1.38 (1–3)
		anpe	2	5.00	1.00 (1–1)	–	–	–	–	10.00 ^B	1.00 (1–1)
	Rosignano	anpe x ansi	6	10.00	1.50 (1–2)	–	–	–	–	20.00	1.50 (1–2)
		hyad	1	–	–	5.00	1.00 (1–1)	–	–	5.00	1.00 (1–1)
C Tyrrhenian	Roma	hyfa	49	–	–	45.00 ^A	2.63 (1–6)	35.00	3.00 (1–9)	50.00 ^A	5.10 (2–13) ^a
		hyfa	2	–	–	10.00	1.00 (1–1)	–	–	10.00 ^B	1.00 (1–1) ^b
		anpe	25	10.00 ^B	1.00 (1–1)	22.50	2.11 (1–4)	7.50	1.00 (1–1)	32.50 ^B	2.08 (1–4)
	Naples	anpe x ansi	5	5.00	1.00 (1–1)	7.50	1.00 (1–1)	–	–	10.00	1.25 (1–2)
		hyfa	3	–	–	7.50 ^B	1.00 (1–1) ^b	–	–	7.50 ^B	1.00 (1–1) ^b
S Tyrrhenian	Salerno	skph	3	–	–	7.50	1.00 (1–1)	–	–	7.50	1.00 (1–1)
		anpe	12	28.57	1.00 (1–1)	28.57	1.75 (1–3)	7.14	1.00 (1–1)	50.00	1.63 (1–4)
		anpe x ansi	2	7.14	1.00 (1–1)	–	–	–	–	14.29	1.00 (1–1)
	casp	hyad	8	–	–	–	–	14.29	4.00 (1–7)	14.29	4.00 (1–7)
		hyfa	1	–	–	7.14	1.00 (1–1)	–	–	7.14	1.00 (1–1)
		hyfa	67	7.14	1.00 (1–1)	57.14 ^A	7.38 (1–21) ^a	21.43	2.33 (1–4)	57.14 ^A	6.09 (1–21) ^a

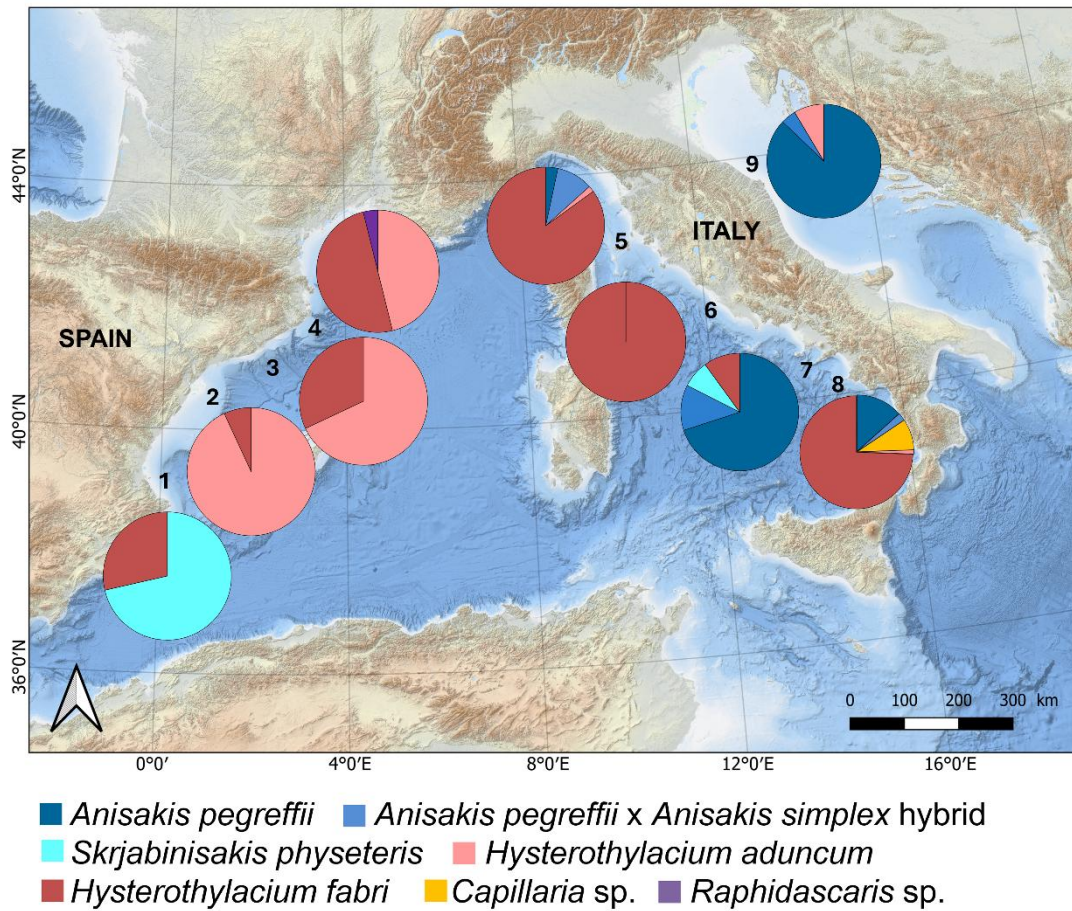


Fig. 7. Circular plots showing the relative abundance (%) of nematodes species from the total nematodes recovered in *Merluccius merluccius* specimens from each sampling point across the Western (1 – 4) and Central Mediterranean Sea (5 – 9). Sampling localities in the Balearic Sea include Valencia (1), Ebro Delta (2), Barcelona (3) and Blanes (4); localities in the Tyrrhenian Sea include Rosignano (5), Roma (6), Napoli (7) and Salerno (8); and the Adriatic Sea locality is Rimini (9).

4. Discussion

4.1. The nutritional value of *Sardinella aurita*

This study provides new insights on the seasonal variation in proximate and fatty acid composition of round sardinella from the central Catalan coast (NW Mediterranean), highlighting its potential as a commercial fish resource in this region.

Depending on the lipid content of their flesh, fish can be classified into three categories: lean fish (<5 % fat), mid-fat fish (5–10 % fat), and fatty fish (10–25 % fat) (Huss, 1995; Taşbozan & Gökçe, 2017). Despite being a small pelagic fish species, *S. aurita* could not be considered a fatty fish, as lipid content from the fillet remained below 2% throughout the year. Instead, it should be classified as a lean fish, such as the Atlantic cod (*Gadus morhua* Linnaeus, 1758) from the Atlantic Ocean (0.8 % fat content; Zeng *et al.*, 2010) and European hake from the Central Mediterranean Sea (0.90–1.38 % fat; Roncarati *et al.*, 2012). Our outcomes showed lower fat contents for the whole fillet (with white and red muscle) than those previously reported for *S. aurita* in the Gulf of Gables (Southern Central Mediterranean) more than a decade ago, where white muscle contained 1.32–5.55 % fat and red muscle contained 6.99–18.50% fat content (Ben Rebah *et al.*, 2010). This difference could reflect geographic and temporal variations in the abundance and composition of plankton communities, as was also noted in sprat *Sprattus sprattus* (Linnaeus, 1758) from the Baltic Sea (Usydus *et al.*, 2012). In fact, inter-annual variations in primary production may influence lipid reserves that fish accumulate until the beginning of their spawning period, as was noticed in the Pacific sardine (*Sardinops sagax* Jenyns, 1842) (Litz *et al.*, 2010). Thus, the lower lipid content observed in summer 2022 compared to summer 2023 (spawning period) may reflect interannual differences in food supply and primary production during the preceding months (autumn 2021 to spring 2022), potentially influencing fish condition. Indeed, a decrease in primary production has been related to a decline in the lipid content in sardines (1.78–5.86% fat) and anchovies (0.89–2.46% fat) from the Catalan coast in recent years (Nunes *et al.*, 2018), resulting in a reduction in the stock size and body condition of these populations (Biton-Porsmoguer *et al.*, 2020), affecting the sustainability of fisheries (ICATMAR, 2020). This decreasing trend and/or a shift in the abundance and composition of phytoplankton communities in the Mediterranean have also been pointed out in other studies, probably as a response to global warming (Fullgrabe *et al.*, 2020; Feuilloley *et al.*, 2022). Accordingly, the sardine and the European anchovy from the northwestern Mediterranean should also be considered a mid-fat and lean fish, respectively, rather than fatty fish. Moreover, reproductive stage and breeding strategy are important factors linked to the seasonal variations round sardinella lipid content, as reported in

other fish species (Pethybridge *et al.*, 2014; Biton-Porsmoguer *et al.*, 2020). The highest lipid values were observed in spring, indicating the maximum energy accumulation during the autumn-winter period before reproduction, thus supporting a capital breeding strategy (Albo-Puigserver *et al.*, 2017). In contrast, the lowest values were noted in summer, coinciding with the spawning stage, when fat reserves were previously mobilised for gonadal development (Lloret *et al.*, 2014). This is in line with studies of round sardinella from the Catalan coast (Vila-Belmonte *et al.*, 2024), from the Gulf of Gabes (Ben Rebah *et al.*, 2010) and from the Adriatic Sea (Mustać & Sinovčić, 2012), but also from the Indian oil sardine (*Sardinella longiceps* Valenciennes, 1847) and the fringescale sardinella (*Sardinella fimbriata* Valenciennes, 1847) from the Cochin coast (Indian Ocean) (Som & Radhakrishnan, 2013), and other pelagic fish species such as the sardine in the NW Mediterranean (Pethybridge *et al.*, 2014; Biton-Porsmoguer *et al.*, 2020).

Fatty acid composition also varied considerably depending on the species, diet, feeding behaviour, reproductive status, sexual maturity, seasonality and environmental conditions such as temperature (Taşbozan & Gökçe, 2017). Dietary variations largely contribute to seasonal differences in fish fatty acid composition (Pethybridge *et al.*, 2014; Biton-Porsmoguer *et al.*, 2020; Vila-Belmonte *et al.*, 2024). Focusing on this, round sardinella displays a generalist and opportunistic feeding behaviour mainly based on zooplankton, although dietary seasonal variations are also reported, suggesting a strong adaptability to food availability (Albo-Puigserver *et al.*, 2019). However, this dietary input might not always reflect flesh FA composition when diet changes have occurred recently, as muscle tissue require time to incorporate new FA levels (Xu *et al.*, 2020). To link the diet of round sardinella with the observed seasonal differences in FA composition, data from Albo-Puigserver *et al.* (2019) has been used, as this study described the diet of this fish species from the same area (Catalan coast) ten years ago. In winter, microplankton including tintinnids (rich in n-3 LCPUFA; Desvillettes *et al.*, 1997) and diatoms (rich in C16:1 and EPA) and to a lesser extent, copepods (i.e. *Acartia* spp. and *Euterpina acutifrons*, rich in C16:1 and n-3 LCPUFA) (Dalsgaard *et al.*, 2003; Merquiol, 2022), were reported to be prevalent in the diet of round sardinella (Albo-Puigserver *et al.*, 2019). The high proportion of SFA, particularly C16:0, together with the low PO/P (C16:1/C16:0) and EPA/DHA (<0.5) ratios, suggests a predominantly phytoplankton diet rather than zooplanktonic, but likely dominated by other microplankton groups. This finding aligns with observations made by Biton-Porsmoguer *et al.* (2020) in sardines from the Catalan Sea. In fact, these authors also revealed a shift and/or reduction in the proportion of diatoms in sardine's diet compared to that of ten years ago in the Gulf of Lion (Pethybridge *et al.*, 2014). Thus, correlating the FA results herein with existing literature on fish diet might not always be accurate as changes in plankton composition and abundance within a

ten-year period have occurred in this region (Nunes *et al.*, 2018). In spring, the predominance of planktonic tunicates of the family Salpidae in round sardinella diet (Albo-Puigserver *et al.*, 2019), which are rich in SFA (C16:0 and C14:0), MUFA (C16:1 and C18:1n9) and to a lesser extent, DHA (Merquiol, 2022), is in accordance with the high levels of SFA and MUFA detected in fish fillets. In contrast, both summers and autumn were characterized by a lower SFA and MUFA proportions alongside an increase in PUFA levels, particularly DHA. This pattern may reflect an increased consumption of large zooplankton (0.5–0.9 mm) such as decapod larvae, amphipoda and copepoda (Albo-Puigserver *et al.*, 2019), particularly rich in n-3 LCPUFA (Merquiol, 2022). Simultaneously, a reduction in total lipid content was observed during this period, indicating the mobilisation of these stored lipids to meet reproductive demands. Although PUFA are also crucial for gonadal and egg development, they can be incorporated from the diet to maintain the fluidity of cell membranes and their structure (Garrido *et al.*, 2008). Similar patterns have also been reported in spawning European anchovies from the same area during the same period (spring/summer) despite having different reproductive strategies (capital vs income breeding strategies) (Biton-Porsmoguer *et al.*, 2020). Therefore, round sardinella may rely on both stored reserves and the energy obtained from its summer dietary input to meet the high demands of reproduction (Albo-Puigserver *et al.*, 2019).

Regarding implications for human health, global recommendations issued by WHO (WHO, 2023b) and previous studies have demonstrated that the dietary replacement of SFA with unsaturated fat, including MUFA and PUFA, decreased the risk of suffering from cardiovascular diseases (Siri-Tarino *et al.*, 2015; Kris-Etherton & Krauss, 2020). Also, an excessive intake of n-6 PUFA and a very high n-6/n-3 ratio promotes the development of many chronic diseases that are highly prevalent in Western societies such as cardiovascular disease, cancer, diabetes, arthritis and inflammatory and autoimmune diseases (Simopoulos, 2002, 2008; EFSA, 2012a). The n-6/n-3 ratio in Western diets is considered 15/1 to 16.7/1, far above the 5/1 optimal ratio that is beneficial for human health (Simopoulos, 2008; Molverismyr *et al.*, 2022). Even lower ratios (<1) have been proven to significantly reduce blood triglyceride concentration and increase high-density lipoprotein cholesterol concentration (Li *et al.*, 2021). In this study, round sardinellas showed favourable n-6/n-3 ratios throughout the year, comparable to those reported for commercial important fish species such as wild and farmed seabass (*Dicentrarchus labrax* Linnaeus, 1758), and seabream (*Sparus aurata* Linnaeus, 1758) (Amoussou *et al.*, 2022) and wild Atlantic salmon (*Salmo salar* Linnaeus, 1758) (Molverismyr *et al.*, 2022). Hence, the consumption of round sardinella would contribute to reducing the n-6/n-3 ratio in the human diet. Likewise, AI and TI values below 1 in both summers and autumn indicate potential cardio-protective benefits. According to the DRI of

EPA+DHA recommended by EFSA to prevent chronic disease (EFSA, 2012a), consuming 100g of round sardinella per day (~4 individuals) from summer 2023 and autumn 2022 appeared to be the best (82.21-116.08% of DRI), providing an intake of 206 and 290 mg of EPA+DHA per day, respectively. Hence, round sardinella would be a good candidate for consumption during autumn, when n-3 LCPUFA levels are highest, and AI and TI values are optimal. Additionally, it would allow this fish species to spawn undisturbed by fishing pressure during summer, which is crucial to ensure their viability. Moreover, although fish catches in the studied area mostly focus on sardines and the European anchovy (with spring being the peak catch period), a decline in the abundance of both species has been observed during autumn (ICATMAR, 2020). For this reason, focusing most catches on round sardinella during this period for human consumption would support sustainable fishing by distributing fishing pressure across different species and seasons and simultaneously enhance the seasonal diversification of these fish species in the market.

4.2. Heavy metals exposure from fish consumption

As mentioned above, another aspect to take into consideration related to fish as a food product is the potential presence of contaminants of anthropogenic origin. Concentrations of Cd, Pb and Hg content from both *M. merluccius* and *S. aurita* from the coast of Barcelona fall within the limits set by European legislation (Commission Regulation No 2023/915). These results are consistent with previous studies of fish sold in Catalan markets (Falcó et al., 2006; Martí-Cid et al., 2008; Perelló et al., 2014; González et al., 2019). However, González et al. (2019) advised special caution for hake consumption, as this species is among the most frequently consumed by infants and was identified as the main contributor to MeHg intake. Accordingly, they recommended limiting the consumption of hake to two or three times per week. The present study showed similar results for infants, since consuming hake and round sardinella five times a week (representing 300 g/week for an infant of 12 kg weight) exceeded the established thresholds for TWI of MeHg. Instead, consuming both species to a maximum of three times per week would be safe for infants whereas for adults it does not suppose any risks even consuming both species five times per week. It is also important to consider that hakes analysed in this study were juvenile specimens with an average standard length of 17.31 cm, whereas no information on hake's length was provided by González et al. (2019), which analysed specimens from different markets. Given that heavy metals bioaccumulate with size, fish length is an important factor to consider in risk assessment studies. Indeed, higher metal concentrations were detected in adult hakes (33.5 – 43.7 cm) from the Algerian coast (southern Mediterranean) (Djellouli et al., 2023), but were also higher in juvenile hakes of similar sizes as in the present study from the Adriatic Sea (Perugini et al., 2014). Similarly, round sardinellas from eastern and southern parts of the Mediterranean also detected higher

concentrations in specimens of similar sizes as herein (Storelli et al., 2003; Lo Turco et al., 2013; Tepe et al., 2009). Therefore, these findings indicate that not only fish size but also the region of origin are fundamental factors that should be reported to improve monitoring studies of risk assessment and provide more accurate recommendations for fish consumption.

Regarding other metals, inorganic As is considered toxic for human health and therefore limits have been established for the main contributors such as rice, grain-based products and drinking-water (EFSA, 2014; 2024). Its presence in fish is generally low because the non-toxic organic compound (i.e. arsenobetaine) predominates (EFSA, 2024). Therefore, fish consumption is not considered a major health risk in terms of As exposure (Copat, 2015), despite slightly higher contributions from fish and seafood consumption were reported in Italy and Spain compared to other European countries (EFSA, 2014). Moreover, elevated total As concentrations have been reported in sediments from the coast of Barcelona, exceeding the toxic effect threshold. These authors argued that natural sources of As from the Mediterranean Sea might differ from those of North American waters, where these ecotoxicological limits have been established, highlighting the need for a re-evaluation according to current levels of As (Carreras-Colom et al., 2022). Similarly, the European Food Safety Authority established a tolerable daily intake for Ni, but its absorption from food is considered lower than that from drinking water, making fish an unlikely significant route of Ni intake (EFSA, 2020). In addition, the low concentrations of Cu and Zn detected in this study, all below maximum permissible limits, and their insignificant contribution to metal intake indicate that consuming hake and round sardinella does not pose a risk for human health. Hence, consumption of both species would be recommended following the recommendations from the Spanish Agency of Food Safety and Nutrition of consuming fish between three and four times per week for all age groups of consumers (AESAN, 2022).

4.3. Zoonotic nematodes distribution across Mediterranean regions

In this study, both zoonotic and non-zoonotic nematodes were detected in *M. merluccius*, with clear geographic differences in infection levels across the Mediterranean. In contrast, no nematode infections were observed in *S. aurita*, indicating no apparent zoonotic risk for their consumption.

4.3.1. Zoonotic relevance to consumers

Among the anisakid species identified, *A. pegreffii* was the only confirmed causative agent of anisakiasis, a zoonotic disease acquired through the ingestion of L3 larvae in fish flesh and associated with gastrointestinal symptoms such as nausea, vomiting, abdominal pain, fever and diarrhoea (Mattiucci et al., 2013, 2022). In this study, *A. pegreffii* larvae were first detected in

hakes from 19.5 cm SL onwards, and infection probability increased with fish size, reaching 50% of hakes infected at 26.49 cm SL and almost 100% in individuals larger than 36 cm. These findings indicate that anisakiasis risk increases with fish size, becoming particularly relevant in larger hakes.

A. pegreffii was mainly detected in abdominal organs, especially the liver, but also in the musculature, demonstrating its capacity to migrate into edible tissues. Although L3 larvae in their paratenic hosts neither develop nor feed, previous studies on *A. simplex* suggest that larval distribution within paratenic hosts might be influenced by lipid-rich tissues, explaining their frequent presence in fish liver, as observed in *G. morhua* and *Trachurus trachurus* (Linnaeus, 1758) (Strømnes, 2014; Cresson et al., 2023). Its migratory ability to the musculature has also been documented in anchovies, both in alive fish and through *post-mortem* migration (Cipriani et al., 2016), representing a potential risk for consumers. Moreover, *post-mortem* migration increases when fish are stored at 5 – 7°C after their capture, whereas storage below 2°C considerably reduces larval migration. Therefore, rapid gutting and refrigeration below 2°C immediately after capture are recommended, while freezing at -20°C for at least 24 hours remains the most effective preventive measure (Cipriani et al., 2016; European Regulation (EU) No 1276/2011). Nevertheless, because hakes are generally consumed cooked, the risk of anisakiasis for consumers is expected to be low.

The zoonotic potential of *S. physeteris* remains uncertain, as no clinical anisakiasis cases have been attributed to this species. Moreover, it has not been observed in the examined fish muscle, in agreement with Cipriani et al. (2018a), suggesting limited capacity to migrate into musculature. Similarly, *Hysterothylacium* species are considered to have low or negligible zoonotic relevance because fish act as definitive hosts and larvae are usually restricted to visceral organs and abdominal cavity (Cipriani et al., 2016). However, a low proportion of *H. fabri* was detected in the musculature of examined hakes off Salerno, and allergic reactions associated with *H. aduncum* have occasionally been reported (Roca-Geronès et al., 2020). Although their zoonotic importance appears limited, these parasites may still affect consumer perception and fish marketability due to their visibility in edible tissues.

4.3.2. Drivers of *Anisakis* (Family: Anisakidae) distribution

Special attention should be given to geographic origin, as hakes from the central Mediterranean (i.e. Adriatic Sea) showed higher prevalence of *A. pegreffii*, whereas hakes from the western basin (i.e. Balearic Sea), presented higher prevalence of *H. fabri* and *H. aduncum*. The predominance of *A. pegreffii* in the Adriatic Sea agrees with previous studies describing it as the dominant anisakid species in the Mediterranean Sea occurring in several fish species (Mattiucci et al., 2018).

Differences among regions suggest that local environmental conditions, food-web structure, as well as the presence of intermediate and definitive hosts may shape the distribution of these species, as observed in anchovies, hakes and Atlantic mackerels (Cipriani et al., 2018a,b; Levsen et al., 2018, Mattiucci et al., 2018). The Adriatic Sea is a narrow basin characterized by a water cyclonic circulation allowing constant nutrient upwelling due to river inputs (e.g. Po River), sustaining high primary production and abundant intermediate and definitive hosts (mainly bottlenose dolphins) (Cipriani et al., 2018a,b; Mattiucci et al., 2018), explaining the observed high larval infection levels compared to other localities. However, higher prevalence (87.5% vs. 64.04) and abundance (9.29 vs. 1.62 ind/fish) of *A. pegreffii* in hakes of similar sizes compared to the present study (23 – 28 cm vs. 19.5 – 29.5 cm) were reported in the Adriatic Sea (Cipriani et al., 2018a). The same authors also observed even much higher abundances (98.9 ind/fish) in larger hakes (23 – 64 cm) (Cipriani et al., 2018a), supporting host size role in anisakid infection (Mattiucci et al., 2018 and references therein). Similarly, in the present study, although the size range of analysed fish was narrower, larger and mature hakes showed higher infection levels than smaller ones. Females were also more parasitized, although this likely reflects the female-biased sex ratio of the sampled population. This higher anisakid infection in larger fish is consistent with the reported accumulation of third-stage larval (L3) during host lifespan before infecting its definitive host, enhanced by their ontogenetic dietary shifts towards a more piscivorous diet, increasing exposure to infected paratenic hosts and reinfections (Mattiucci et al., 2018; Cipriani et al., 2018a; D'Iglio et al., 2022).

Although the Adriatic Sea is considered a hotspot for *A. pegreffii* infection in both demersal and pelagic fish (Cipriani et al., 2018a,b), no nematodes were found in the pelagic *S. aurita*, suggesting that host trophic ecology might limit exposure to anisakis larvae rather than a lack of parasite availability in the area. Previous studies also reported low anisakid prevalence in sardinellas from the same or nearby Mediterranean regions (Piras et al., 2014; Goffredo et al., 2019; Ramdani et al., 2020) and from the Atlantic Ocean (Martin-Carrillo et al., 2022). These observations could be related to the feeding habits and prey composition of *S. aurita*. The examined sardinellas were caught during late winter/early spring, when their diet is dominated by microplankton and planktivorous Salpidae (Albo-Puigserver et al., 2019), which are not intermediate hosts of *Anisakis*. *S. aurita* could still acquire L3 larvae through copepod consumption over time, particularly during summer, when intermediate hosts such as copepods and decapod larvae are more frequently consumed (Klimpel & Palm, 2011; Hure & Mustač, 2020). However, experimental studies suggest that copepods alone are insufficient for transmission because L3 larvae are not yet fully developed within copepod hemocoel (Køie, 2001). Instead, larvae need larger

crustaceans such as euphausiids to fully develop and become infective to fish and cephalopod paratenic hosts (Kjøie, 2001), which are particularly important as intermediate hosts for *Anisakis* compared to other crustaceans (Busch et al., 2012). These observations could explain that higher infections have been associated with dietary shifts towards euphausiid consumption in *S. pilchardus* and *T. trachurus* (Gutiérrez-Galindo et al., 2010; Cresson et al., 2023). Therefore, the absence of *Anisakis* larvae observed in *S. aurita* from the Adriatic Sea may be related to their small size, the lack of ingestion of euphausiids and/or the ingestion of copepods that were either not parasitized by *Anisakis* or harboured larvae not yet developed to infect round sardinellas.

Another region of potential zoonotic relevance is the southern Tyrrhenian Sea, particularly the Gulf of Naples, where *A. pegreffii* predominated in hakes and showed higher intensity values than other co-infecting species, such as *A. pegreffii* and *A. simplex* (s.s.) hybrids and *S. physeteris*. This predominance may be associated with the complex bathymetry of the area, characterized by submarine canyons and steep shelf-slopes (Aiello & Sacchi, 2022). Similar geomorphological conditions have been described near Sicily, where abrupt slopes and upwelling processes promote the vertical migration of mesopelagic fish such as myctophids and sternoptychids towards shallow waters, representing important prey for juvenile hakes (Modica et al., 2015; D'Iglio et al., 2022). Since several myctophid species have been identified as hosts of *A. pegreffii* in the Mediterranean, they may act as paratenic hosts (Mateu et al., 2015; Gaglio et al., 2018), thereby explaining their possible transmission in examined hakes from the Gulf of Naples.

Moreover, putative hybrids between *A. pegreffii* and *A. simplex* (s.s.) were detected at low proportions in Adriatic and Tyrrhenian regions, as previously reported in other species (Macchioni et al., 2021; Caffara et al., 2025). Although in sympatric areas such as the Spanish and Portuguese Atlantic coast and the Alboran Sea (western Mediterranean), hybridization between these two cryptic species may occur (Mattiucci et al., 2004, 2016; Cipriani et al., 2018a), their detection in allopatric areas should not be interpreted as current hybridization. Instead, it could reflect historical gene flow or shared ancestral polymorphisms that persisted in current populations (Mladineo et al., 2017; Mattiucci et al., 2018). Moreover, the genetic marker used in this study (i.e. PCR-RFLP of the ITS region of the rDNA) has limited resolution for detecting true hybridization events and may lead to misidentification (Mattiucci et al., 2016). Instead, multi-marker nuclear loci analysis would therefore be more recommended to accurately identify hybrids. Consequently, the detected putative hybrids should be conservatively considered *A. pegreffii*, the parental species occurring in the Mediterranean Sea (Mattiucci et al., 2016). Despite this limitation, PCR-RFLP remains as a useful, low-cost and simple genetic tool for analysing large numbers of samples compared with sequencing, as it provides restriction patterns for the identification of closely

related ascaridoid species based on length polymorphism through restriction enzymes (D'Amelio et al., 2000; Espiñeira et al., 2010).

Other anisakid species such as *S. physeteris* appeared more restricted to regions where their definitive hosts, sperm whales (mainly *Physeter macrocephalus*, but also *Kogia sima*) are common such as the Alboran Sea, the gulf of Valencia, the Adriatic and Tyrrhenian Seas (Mattiucci et al., 2004; Mateu et al., 2015; Cipriani et al., 2022). Particularly, the highest prevalence detected in this study was in hakes from the Gulf of Valencia, although mean intensities remained low, consistent with previous reports for hakes in the Alboran Sea (Cipriani et al., 2018a) and in other fish species (Mattiucci et al., 2018). This low infection levels suggests that *S. physeteris* probably relies mainly on deep-dwelling squid species rather than fish as their intermediate or paratenic hosts (Mattiucci et al., 2018; Cipriani et al., 2022), as reported for *Todarodes sagittatus*, *Illex argentinus* and *Ommastrephes bartramii* (Picó-Duran et al., 2016) which are the main prey for sperm whales (Palomba et al., 2021).

Another relevant finding is the absence of zoonotic nematodes in hakes and sardinellas from the western Mediterranean. Previous studies have also reported lower prevalences of *Anisakis* larvae in *M. merluccius* from the Balearic Sea and southern Spanish coast compared to other Mediterranean and Atlantic regions (Mattiucci et al., 2004; Barcala et al., 2018; Cipriani et al., 2018a) and in other fish such as *E. encrasicolus*, *S. pilchardus*, *M. barbatus*, *Micromesistius poutassou* (Risso, 1827), *T. trachurus* and *Scomber scombrus* Linnaeus, 1758 (Valero et al., 2000, 2006; Rello et al., 2008, 2009; Gutiérrez-Galindo et al., 2010; Ferrer-Maza et al., 2016; Cipriani et al., 2018b; Levsen et al., 2018; Rodríguez-Romeu et al., 2020, 2022). However, in the Gulf of Lions, higher prevalences of *Anisakis* species were recorded in specimens of *M. merluccius*, also probably because these were larger than those studied herein (Ferrer-Maza et al., 2014). Although the western Mediterranean hosts abundant cetacean populations (e.g., bottlenose, striped and the Risso's dolphins and fin whales) within the Mediterranean Cetacean Migration Corridor (ACCOBAMS, 2021), the low prevalence of *A. pegreffii* suggests that transmission may not depend only on cetacean presence. Stable trophic interactions and favourable local environmental conditions, such as those occurring in the semi-enclosed Adriatic Sea, may also be important.

4.3.3. Drivers of *Hysterothylacium* (Family: Raphidascarididae) distribution

Hysterothylacium species, particularly *H. fabri*, showed a broad distribution across the Mediterranean, with different infection levels among regions. In the southern Tyrrhenian Sea, hakes examined off Salerno were infected not only by *A. pegreffii*, but also co-occurred with *H. fabri*, which infected almost 60% of hakes and showed considerably higher mean intensity values

(6.09 *H. fabri*/fish vs. 2.39 *A. pegreffii*/fish). Similar mean intensity infection values were observed in the northern Tyrrhenian Sea (5.10 *H. fabri*/fish), whereas lower infections occurred in central Tyrrhenian and Balearic Seas. These findings differ from those reported by Cipriani et al. (2018a), who did not detect *Hysterothylacium* sp. in the Tyrrhenian Sea or in the western Mediterranean. In contrast, Angelucci et al. (2011) found that 42% of hakes from the coast of Sardinia (Tyrrhenian Sea) were infected by *Hysterothylacium* larvae showing high mean intensities (5.8 individuals/fish), whereas 30% were parasitized by *Anisakis* sp. larvae in lower mean intensities (1.7 individuals/fish).

Species of *Hysterothylacium* are widespread generalist nematodes with complex life cycle involving copepods as first intermediate hosts, crustaceans, chaetognaths and echinoderms as intermediate or paratenic hosts, and numerous fish species acting as paratenic or definitive hosts, as described for *H. aduncum* (Køie, 1993; Anderson, 2000; Pérez-del-Olmo et al., 2008; Busch et al., 2012). While *H. aduncum* is commonly found infecting euphausiids and planktivorous fish (e.g., *E. encrasicolus* and *T. trachurus*), the life cycle of *H. fabri* is still not completely known. In this study, the presence of third and fourth stage larvae together with adults of *H. aduncum* and *H. fabri* indicates that *M. merluccius* is a suitable definitive host for both nematodes, as reported for other gadoid species (Køie, 1993; Klimpel & Rückert, 2005; Dallarés et al., 2014, 2016).

The widespread occurrence of *H. fabri* across sampled regions, except the Adriatic Sea, suggests a broad ecological adaptability. High infection levels of *H. fabri* in hakes off Salerno and *H. aduncum* in Barcelona and Ebro Delta may be related to local seasonal or annual variations in environmental conditions and prey availability. Several prey species commonly consumed by juvenile hakes (20 – 30 cm length) in the southern Tyrrhenian Sea, including *E. encrasicolus*, *S. pilchardus*, *Boops boops* (Linnaeus, 1758) and *T. trachurus* (D'Iglio et al., 2022) are intermediate or paratenic hosts of both nematodes (Rello et al., 2008, 2009; Roca-Geronès et al., 2020; Macchioni et al., 2021; Rodríguez-Romeu et al., 2022). Since the abundance of these prey species can vary annually (D'Iglio et al., 2022), fluctuations in their availability may influence parasite transmission to hakes. Moreover, the opportunistic feeding behaviour of hakes (Bozzano et al., 2005) may increase the consumption of infected prey, potentially explaining the high infection intensities observed in some areas. Similar regional differences in infection levels linked to intermediate or definitive host occurrence have been reported, for example in *M. barbatus* from the south-eastern Spanish coast (Morales-Yuste et al., 2024).

Finally, as reported in Chapter 4, round sardinellas from the NW Mediterranean neither those captured off Italian coasts were infected by *Hysterothylacium* species. This contrast with what was

reported in other pelagic species. For instance, sardines from the Catalan coast exhibited both spatial and seasonal variability showing higher prevalences (56.2%) in Ebro Delta (south) compared to Costa Brava (north) and in spring, coinciding with early planktonic blooms which may increase the availability of intermediate hosts such as copepods (Frigola-Tepe et al., 2022, 2024; Caballero-Huertas et al., 2023). Similarly, anchovies were also reported to host *H. aduncum* and *H. fabri* across different localities from the Catalan coast (Ferrer-Maza et al., 2016; Rodríguez-Romeu et al., 2022). Although round sardinella occupies a higher trophic level compared to sardines and anchovies due to its generalist and higher trophic plasticity (Albo-Puigserver et al., 2019), it suggests that this host species may feed upon different preys that may be less parasitized by nematode larvae and therefore has a low susceptibility to be infected (Rello et al., 2008).

4. Conclusions

This study highlights the potential of *S. aurita* as an alternative fish resource in the NW Mediterranean, providing valuable insights into both its nutritional quality and associated health risks. Despite being a lean species with low lipid content, round sardinella exhibits a high proportion of omega-3 long-chain polyunsaturated fatty acids, particularly DHA, especially during summer and autumn, meeting recommended EPA+DHA intake levels. Both *S. aurita* and *M. merluccius* from the Balearic Sea showed low concentrations of heavy metals within European regulatory limits, indicating a low risk for consumers under normal dietary conditions. However, mercury exposure may pose a concern for infants under high consumption scenarios. Importantly, the absence of zoonotic nematodes in both species from this region further supports their safety, reducing the risk of anisakiasis. Regional differences were evident, particularly when compared to other Mediterranean areas such as the Adriatic Sea, where *M. merluccius* showed a higher prevalence of *Anisakis pegreffii*, emphasizing the role of geographic origin and fish size in parasite distribution. In contrast, *S. aurita* appears less exposed to zoonotic nematodes, likely due to ecological factors. The presence of nematodes with less or negligible zoonotic potential such as *Hysterothylacium* spp. in other regions reinforces their limited relevance to human health. Overall, both species from the Balearic Sea can be considered safe for consumption, while round sardinella, particularly when caught in autumn, represents a nutritionally beneficial option. Promoting its consumption could contribute to more sustainable fisheries by diversifying fishing pressure across species and seasons. Furthermore, its favourable nutritional profile supports its potential for broader applications, including its use in processed and value-added seafood products.

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CHAPTER 7

General discussion

1. Integrative fish health assessments under global change

The multidisciplinary approach applied in the present thesis, aligned with a One Health perspective provided an integrated assessment of fish health, potential stressors and food safety aspects relevant to fisheries and consumers, focusing in a consolidated and an emerging commercial fish species from the Catalan coast (NW Mediterranean). By combining diverse disciplines, this work contributed to a broader understanding of how fish populations respond to biological, environmental and anthropogenic factors, essential to support sustainable fisheries management under the current context of global change (Lloret et al., 2012).

An important outcome of this thesis is that, although both studied species did not showed evidence of disease impact or health impairment, fish health varied across spatial and temporal scales. These variations were more likely associated with environmental conditions and food availability rather than direct effects of parasitism or anthropogenic stressors. Despite relatively high parasite prevalences and intensities, particularly in *S. aurita*, no severe histopathological damage or negative effects on condition were observed, suggesting that current infection levels do not compromise host health. Likewise, the absence of detectable effects associated with anthropogenic items ingestion and heavy metals accumulation suggests that current exposure levels in the studied area are below levels that would compromise fish health.

These results highlight the importance of health assessments at local scales, to support fisheries management and distinguish natural variability from stress-induced alterations. Seasonal differences in round sardinella condition and nutritional composition reflected the influence of reproduction, food availability and environmental conditions, whereas geographical differences in zoonotic parasite distribution in European hake suggested the role of local environmental conditions and trophic interactions on parasite transmission. The absence of severe lesions and the low contaminant concentrations detected in both species provide a useful baseline for future monitoring under global change scenarios. For example, the presence of melanomacrophage centres and differences in lipid vacuolization observed in both species reflected the high natural variability of histological descriptors in wild populations, which may be influenced by physiological and environmental factors as well as feeding activity and could otherwise be misinterpreted as stress responses. This is especially relevant in a context of global change, where shifts in environmental conditions may alter trophic interactions and ecosystem structure (van der Putten et al., 2004; Coll et al., 2008; Sánchez-Zulueta et al., 2023). However, establishing direct cause-effect relationships in wild populations remains difficult because fish condition is influenced by multiple interacting biological and environmental factors. This complexity was also evident in the

present thesis, where no clear relationships were observed between contaminant exposure and fish condition or histopathological alterations. However, the health indicators applied in this thesis were designed to assess fish health from individual (including tissue alterations) and population levels under natural conditions, and may therefore not detect subtle, sub-lethal or physiological responses, more commonly investigated through experimental studies.

In this context, integrative health assessments provide a valuable framework to capture this complexity and improve the interpretation of fish responses, which would be included in long-term stock monitoring programmes. When combined with parameters reflecting stock productivity (e.g., growth, natural mortality, abundance, reproduction and recruitment), these approaches could enhance the ability to detect vulnerable populations and anticipate potential changes in fish stock (Lloret et al., 2012). For instance, populations showing high parasite burdens together with reduced condition, depleted lipid content and increased histological alterations could indicate compromised health status compared to other areas. These patterns may help identify stocks with limited recovery potential and improve predictions of future trends in growth and mortality rates under adverse environmental scenarios. In contrast, fish with enhanced condition may be indicator of habitat quality suitable for juvenile settlement, stock productivity, yield and quality of fish resources (Lloret et al., 2014).

2. Current health of fish species from the Catalan coast and implications for fisheries

2.1. The European hake

The health assessment of the European hake revealed spatial differences at a local scale. Hakes from Ebro Delta showed an enhanced condition than those from Blanes and Barcelona, suggesting higher food availability and favourable environmental conditions, supported by the results of condition indices, abiotic factors (i.e. low turbidity levels and high oxygen concentration) and higher parasite diversity, indicating a wide range of intermediate hosts (e.g. digeneans). This interpretation is consistent with previous studies indicating that hake condition is strongly influenced by prey availability and environmental conditions (Hidalgo et al., 2008; Cartes et al., 2009; Lloret et al., 2014; Korman et al., 2020). In contrast, reduced condition associated with limited availability of benthopelagic prey has been reported in juvenile hakes from deeper depths in the Gulf of Lions, affecting their density and survival (Ferraton et al., 2007). In this context, the lower condition observed in hakes from Blanes together with reduced digenean richness supports the hypothesis of more limited feeding resources. On the other hand, hakes from Barcelona

showed relatively high parasite richness but lower condition, suggesting that prey availability alone does not fully explain fish condition (Chapter 4). Due to the proximity to the polluted area of Barcelona, it was expected that hakes from this locality were exposed to higher levels of pollutants to explain their reduced condition. Although Barcelona showed higher concentrations of large anthropogenic items (AIs) in water samples, hakes mainly ingested small fibres similar in size to their prey, suggesting passive ingestion related to feeding behaviour rather than environmental AIs concentrations. Moreover, levels of heavy metals detected in their muscle were comparable to those reported in other Mediterranean regions and generally increased with fish size, particularly mercury. Therefore, other pollutants, environmental conditions or trophic competition may also influence hake condition (Chapter 5). Thus, other pollutant sources, environmental conditions and/or intra- or interspecific competition could be influencing their condition (Lloret et al., 2002).

It is important to note that this thesis focused on juvenile hakes, the most commercially exploited size class in the study area (ICATMAR, 2025), so the observed patterns may not apply to adults. Several parasites and heavy metals may accumulate with fish size and potentially cause severe effects in adults, affecting liver reserves, reproduction and overall condition. Previous studies in close areas such as the Gulf of Lions reported negative correlations between endoparasite infection and gonadal reserves, particularly for anisakid nematodes, which were associated with reduced energy reserves in both liver and gonads (Ferrer-Maza et al., 2014). However, authors did not find evidence of detrimental effects on overall condition (Ferrer-Maza et al., 2014). Parasites such as *Aporocotyle spinosicanalis* and *Lernaeocera lusci*, detected at low prevalence, could produce more severe pathological effects at higher infection intensities, compromising respiratory or cardiac function and increasing energetic costs. In fact, mobilization of liver energetic reserves could be increased to meet the metabolic demands due to parasite infection, as observed by Hajji et al. (2021) in hakes from the Tunisian coast. Energetic reserves from liver and gonads were also reduced in adult hakes that were infected by two other copepod species from the Lernaeopodidae family (i.e. *Clavella stellata* and *Parabrachiella insidiosa*), probably to redirect part of their energy reserves to repair the damage caused by these parasites (Ferrer-Maza et al., 2014). Hence, heavier infections may force fish to face trade-offs between allocating energy to immune responses and investing energy in gonadal development and reproductive success (Lloret et al., 2014). Moreover, the life cycle of *A. spinosicanalis* is still not known, and it is expected that its intermediate host might be a polychaeta. Thus, further studies on identifying the intermediate host would help to better identify areas with higher probability for hakes to get infected. However, detecting these impacts in wild populations is difficult because severely affected fish may not survive and are likely

underrepresented in field samples. These limitations highlight the need to include different life stages in future health assessments.

The results obtained in this thesis have direct implications for fisheries management. Although parasites and pollutants did not appear to critically affect juvenile hake condition, environmental variability strongly influenced fish health, highlighting the need to integrate ecological context and health indicators into local management strategies to identify high productive or potential vulnerability areas. Productive shallow shelf areas such as the Ebro Delta should be protected as key nursery grounds areas (Maynou et al., 2003; Paradinas et al., 2015). Considering that hake is mainly captured by non-selective bottom trawls, the temporal closure and protection of nursery areas was proposed as the most effective management action for preserving hake stocks, showing increased recruits and large adults after a seven months period (Khoukh & Maynou, 2018; Tuset et al., 2021). In addition, revising the current minimum capture size (20 cm total length) should be considered, as many individuals are still immature at the legal landing size. Considering that females from the Catalan coast reach their sexual maturity at around 31–33 cm and males at 24–26 cm (ICATMAR, 2025), increasing the minimum size to around 30 cm could allow reproduction before capture. Within the ongoing global change, diversification of fishing pressure may help hakes and other vulnerable fish stocks included in the Multi-Annual Plan to recover and achieve a sustainable yield of fish stocks.

2.2. The round sardinella

At the same time, the expansion of thermophilic species in the Mediterranean may create new fishing opportunities, particularly for species such as *S. aurita*. Its generalist and opportunistic feeding behaviour provides high trophic plasticity to food availability (Bachiller et al., 2021), reducing the interspecific trophic competition with sardines and anchovies (Albo-Puigserver et al., 2017, 2019). Although thermophilic species generally contain lower omega-3 levels than those found in cold and temperate-water species (e.g. sardines) (Lloret et al., 2025), round sardinella along with the Atlantic horse mackerel (*Trachurus trachurus*) and the Atlantic mackerel (*Scomber scombrus*), could still represent an additional intake route of these fatty acids (Ollé-Vilanova et al., 2026).

The seasonal assessment conducted in this thesis revealed new insights into the condition, nutritional status, parasite communities and exposure to stressors in round sardinella from the coast of Barcelona. Fish showed an enhanced condition observed in spring, suggesting favourable environmental conditions and increased food availability, and also reflected the accumulation of

energy reserves prior to spawning, consistent with its capital breeding strategy (Albo-Puigserver et al., 2017; Vila-Belmonte et al., 2024; Ollé-Vilanova et al., 2026). In contrast, in summer round sardinellas showed lower condition and reduced lipid content in muscle tissue (Chapter 6), likely associated with reproductive investment, expected in the spawning period. Summer samplings also coincided with extreme marine heatwaves (Martínez et al., 2023; Juza et al., 2024), which may have affected fish energetic reserves, particularly in 2022. Moreover, it was reported that this species seems to accumulate more fat energy in the mesenteric cavity and liver rather than in muscle tissue (Ollé-Vilanova et al., 2026), contributing to explain the reduced observed lipid content. Pre-spawning and spawning periods are particularly demanding for fish because if stored reserves are limited, fish may become more vulnerable to external stressors, such as parasites and pollutants (Lloret et al., 2012). Exposure to anthropogenic pollutants was considered due to the urbanized and industrialized nature of the study area, and to the filter-feeding behaviour of this pelagic fish. Round sardinellas from winter showed the highest abundance of ingested AIs, indicating either a higher concentration of AIs in the environment and/or a higher ingestion due to its filter-feeding behaviour (Albo-Puigserver et al., 2019), but with no apparent impact on their condition (Chapter 5). Regarding parasites, although high abundances of trophically transmitted helminth parasites (i.e. *Aphanurus* sp., *Parahemiurus merus* and Tetraphyllidea cestode larvae) were observed during summer samplings, no apparent harmful effects were detected. However, microsporidian infections deserve special attention because similar xenoma proliferations of the microsporidian *Glugea sardinellensis* have been associated with mortality events in other regions of the Mediterranean, especially in warmer periods (Mansour et al., 2016; Ramdani et al., 2022; López-Verdejo et al., 2024). Thus, further studies of the genetic and morphological identification of these xenomas in the Catalan coast should be performed to confirm their identity due to its potential detrimental impacts on fish health and populations. Likewise, epitheliocystis were found to be more prevalent particularly in autumn, and although only mild alterations were observed in large cysts, further identification studies and monitoring should be undertaken since mortalities in cultured fish caused by respiratory stress have been reported (Blandford et al., 2018).

Therefore, these findings highlight the value of seasonal monitoring studies to identify periods when fish populations are more vulnerable to stressors and in a poorer condition, considering also its reproductive cycle. Detecting these patterns may help refine periods in which fishing effort should be enhanced or reduced to ensure both conservation and fisheries benefits (Lloret et al., 2012). In this case, fishing during summer should be avoided because reproduction occurs and fish condition is reduced. In spring, although fish are at its peak of condition, fishing in this period could contribute to the reduction of spawners while not achieving the maximum product yield

nor the highest nutritional benefits. Instead, autumn may represent a more suitable period for exploitation from both fisheries and nutritional perspectives. However, because most autumn specimens analysed in this thesis were immature individuals (around 13 cm total length), establishing a minimum catch size of at least 15 cm should be considered to avoid juvenile overexploitation.

3. Implications for consumers and seafood safety

Although round sardinella should be classified as a lean species due to its low lipid content compared to sardines and anchovies (Vila-Belmonte et al., 2024; Ollé-Vilanova et al., 2026), it showed high omega-3 fatty acids levels during summer and autumn (46.7 – 53.7% of the total fatty acids). A 100 g portion (equivalent to four individuals) of round sardinellas consumed in autumn provides approximately 290 mg of EPA and DHA, fitting within the recommended daily intake range (250 – 500 mg/day), supporting its nutritional and commercial value. Moreover, promoting its consumption during autumn could also reduce fishing pressure on sardines during their reproductive period, while favouring seasonal diversification of local fish consumption.

Another important aspect for consumers is the transparency regarding the origin of fish products, given that abiotic and biotic conditions from each fishing ground determine the distribution of zoonotic species, such as those of the genus *Anisakis*. In this case, hakes from the Adriatic Sea were mainly infected by *Anisakis pegreffii*, a zoonotic parasite responsible for the fish-borne disease anisakiasis, whereas its dominance decreased westwards across the Mediterranean (Chapter 6). Indeed, the absence of zoonotic parasites (i.e. *Anisakis* spp.) in hakes and round sardinellas from the Catalan coast, together with heavy metals levels below the maximum permissible limits, demonstrate a good quality and traceability of these fish resources. However, most hake consumed in Catalonia (almost 80%) consists of large specimens (> 1 kg) imported from Spanish Atlantic waters, mainly Galicia and Cantabria coasts (Mercabarna, 2025), where infections by anisakid nematodes, particularly *Anisakis simplex*, can reach very high abundances (up to 410 larvae/fish) (Cipriani et al., 2015; Diez et al., 2022). Moreover, this species has a greater tendency to migrate into fish flesh than *A. pegreffii*, increasing the risk for consumers if the fish is not properly processed before consumption (Cipriani et al., 2015). Conversely, round sardinellas from the Adriatic Sea were not infected with *Anisakis*, suggesting lower exposure to these parasites due to their trophic ecology or other intrinsic factors, which adds value to its commercial potential. Therefore, monitoring programs assessing zoonotic risks and the origin of fish products in local markets are needed. In this regard, the UV-press method combined with PCR-RFLP identification,

proved to be a rapid and effective technique when working with large samples, and could be implemented in routine monitoring programmes. These findings highlight the importance of studies evaluating zoonotic risk, as well as communicating the low risk associated with local fish products to improve consumer awareness and campaigns that promote sustainable local fisheries.

Overall, fisheries resources from the Catalan coast are diverse, healthy and safe for human consumption, supporting the nutritional value of the Mediterranean diet (Lloret et al., 2024). However, a recent report indicated a decline in the consumption of local fresh fish, alongside an increasing incorporation of other species, mainly from aquaculture such as salmon, tuna and gilthead seabream, into consumers' diets. This shift observed over the past 25 years has been mainly attributed to several factors such as the price, changing dietary habits, reduced time spent on cooking and a growing preference for ready-to-eat or easy to prepare products (AECOC, 2020; Ortega et al., 2025). As a result, fresh fish supplied by the Catalan fleet currently represents less than 25% of the total fish demand (Ortega et al., 2025). Studies such as the present thesis are therefore important to improve the traceability, knowledge and quality of local fishery products, helping consumers better understand the origin, health status and nutritional value of the fish they consume. Accordingly, it is crucial that these outcomes are transferred to stakeholders including the fishing sector, administration and consumers to facilitate the collaboration in the development of future co-management strategies to improve the sustainability of these fisheries (Lloret et al., 2012). Particularly, the results obtained on the nutritional status and related safety risks of *S. aurita* (Chapter 6) were communicated to the fishing sector and general public to promote the consumption of round sardinella by highlighting its nutritional qualities and culinary potential through sample dishes. This initiative received positive feedback and acted as a first step towards promoting more sustainable exploitation practices, while reinforcing the role of local fisheries within the Mediterranean diet.

Finally, future research should move towards the development of long-term monitoring programmes integrating fish health indicators with stock productivity parameters to better understand fish population dynamics. Expanding this approach across broader spatial scales and life stages, would improve the assessment of stressors throughout fish life cycle. In parallel, continued attention should be given to emerging pressures linked to pollution, climate variability, such as marine heatwaves and other extreme events, which may alter fish condition, trophic interactions, and disease susceptibility. Thus, further efforts are also needed to identify and monitor parasite species with zoonotic or pathological importance. Overall, integrative studies of this kind are essential to understand fish responses to global change and to support sustainable fisheries management strategies within a One Health perspective.

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CHAPTER 8

Conclusions

1. The multidisciplinary assessment of *Merluccius merluccius* and *Sardinella aurita*, integrating pathology, histopathology, parasitology and condition indices, showed no substantial impacts of parasites, heavy metals or anthropogenic items on fish health (**Chapter 4 and 5**).
2. The parasite community of *M. merluccius* comprised 22 species, with digeneans contributing most to species richness, while the nematode *Hysterothylacium aduncum* and the cestode *Cleistobothrium crassiceps* were the most prevalent species. Low infracommunity richness suggested heterogeneous prey consumption and spatial variation in invertebrate intermediate hosts (**Chapter 4**).
3. The pelagic *S. aurita* harboured eight parasite species, mainly digeneans (*Aphanurus* sp., *Parahemiurus merus*) and Tetraphyllidean cestode larvae, with higher summer intensities likely linked to seasonal dietary shifts towards large zooplankton acting as intermediate hosts (**Chapter 4**).
4. Most histopathological alterations were mild and focal, and parasite infections generally showed low intensities, suggesting balanced host-parasite relationships without evident impairment of fish condition or organ functionality (**Chapter 4**).
5. The gills were the organ with the highest prevalence of histopathological alterations in both species. Specific pathologies such as *Aporocotyle spinosicanalis* egg infestation in *M. merluccius* and epitheliocystis in *S. aurita*, as well as microsporidian infections deserve particular attention due to their potential impact of fish health and fisheries (**Chapter 4**).
6. *Aporocotyle spinosicanalis* eggs found at low prevalence (<10%) induced mild inflammatory responses in *M. merluccius* gills, with no significant alteration to the respiratory structure. Adults in the heart caused marked inflammation and fibrinoid necrosis probably caused by the mechanical damage of spines in their tegument, highlighting its continued monitoring in adult fish for its potential severe pathological effects at higher intensities (**Chapter 4.2**).
7. The histological assessment of *Cleistobothrium crassiceps* attachment to its host revealed only mild effects on the intestinal mucosa of *M. merluccius* (**Chapter 4.1**).
8. The phylogenetic intraspecific variation observed in *C. crassiceps* was comparable to the genetic divergence between congeners *Cleistobothrium cristinae* and *Cleistobothrium splendidum*, supporting the synonymization of *C. cristinae* with *C. splendidum*, previously described as distinct species (**Chapter 4.1**).
9. Anthropogenic items (AIs) were ubiquitous in the environment and ingested by both species, with cellulosic fibres dominating over synthetic ones regardless of environmental availability (**Chapter 5**).
10. For a mean environmental concentration of 0.85 items/m³ of AIs, *M. merluccius* ingested a mean of 1.39 (SD=1.39) items/individual, mainly small fibres (< 6 mm size), likely related to

ontogenetic feeding behaviour, supporting its suitability as a bioindicator of near-bottom micro-debris (**Chapter 5**).

11. *S. aurita* ingested a mean of 2.27 (SD = 2.13) AIs/individual, with seasonal variability likely driven by vertical water mixing, rainfall and feeding strategy, with higher AIs uptake during winter associated with filter-feeding activity (**Chapter 5**).
12. Trace metal concentrations in both species remained within European regulatory limits. Mercury showed size-related bioaccumulation in *M. merluccius*, whereas *S. aurita* exhibited lower concentrations (**Chapter 5**). Methylmercury exposure through consumption of both species could exceed recommended weekly intake thresholds in infants if consumed more than three times per week (**Chapter 6**).
13. No clear relationships were found between contaminant levels (AIs or metals) and fish condition, histopathology or parasite descriptors, suggesting limited impact on fish health under current conditions (**Chapter 5**).
14. *S. aurita* showed seasonal variability in lipid content and fatty acid composition, likely driven by its reproductive cycle, capital breeding strategy and diet composition. Despite its low lipid content (<2%), autumn specimens showed high omega-3 levels, in line with the recommended levels of EPA+DHA for a meal portion of 100g of round sardinella/day, supporting its nutritional value for human consumption (**Chapter 6**).
15. The absence of zoonotic nematodes in both species from the Catalan coast suggests a low anisakiasis risk, in contrast with the Adriatic Sea, where large *M. merluccius* were predominantly infected by *Anisakis pegreffii*, highlighting fish origin and size as key food safety factors (**Chapter 6**).
16. *S. aurita* represents a healthy and safe valuable alternative pelagic resource for human consumption, particularly in autumn. Promoting its consumption during this season could support more sustainable fisheries by redistributing fishing pressure across various species and seasons (**Chapter 6**).

Key conclusions

1. The multidisciplinary assessment of *Merluccius merluccius* and *Sardinella aurita* indicated that both species generally showed a good health status, with limited impacts associated with parasites, anthropogenic items and heavy metals.
2. Parasite communities mainly reflected the trophic ecology and environmental variability of each species. Although some parasites showed high prevalence, infections were generally mild and did not significantly impair fish condition or organ functionality.
3. Gills were the organ most affected by histopathological alterations, particularly due to *Aporocotyle spinosicanalis* in *M. merluccius* and epitheliocystis in *S. aurita*. Despite their low severity, these pathologies deserve continued monitoring because of their potential implications for fish health and fisheries.
4. Anthropogenic items, especially cellulosic fibres, were ingested by both species, likely influenced by feeding behaviour and seasonal environmental processes. However, no clear negative effects on fish health were detected under current conditions.
5. Trace metal concentrations remained within European regulatory limits. Mercury bioaccumulation was observed in larger *M. merluccius* individuals, suggesting a potential exposure risk for infants in cases of frequent consumption.
6. *Sardinella aurita* showed high nutritional value, particularly in autumn due to its elevated omega-3 fatty acid content. Promoting its consumption could support more sustainable and diversified fisheries.
7. The absence of zoonotic nematodes in samples from the Catalan coast suggests a low anisakiasis risk in both species, although this risk may vary depending on fish origin and size.

