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A case study on farmed European seabass and gilthead seabream in Central Italy: the negligible parasitological risk of nematode larvae paves the way for the freezing derogation.

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Abstract:	Gilthead seabream and European seabass are among the most appreciated farmed fish species in the European Union. This case study analysed the self-control plan procedures adopted in an offshore cage farm in Central Italy to prevent anisakids infection, in the light of the Anisakis contamination pathways previously proposed for farmed Atlantic salmon (Salmo salar), and of the criteria recommended by the European Food Safety Authority. Moreover, the results of the visual parasitological examination conducted by the Food Business Operator, as part of the self-control plan, on 5% of the total specimens with commercial size (2016-2020 period) were also considered. Results show an extremely low to negligible risk for the introduction of ascaridoid larvae, confirming the absence of these parasites in farmed specimens of both species. However, few implementations to the self-control plan are suggested for obtaining the derogation to preventive freezing, as established by the European legislation. These include the parasitological examination of a statistically significant sample of the farmed specimens (commercial sizes) conducted by trained personnel, as well as of farmed specimens found dead or underdeveloped (runts) and of wild specimens of other species which may enter the cages. The proposed approach can be adapted by other farms by adjusting the sample size based on the production volume and risk categorization. The exemption from the preventive freezing would represent an additional market opportunity for Italian aquaculture plants.

Pisa, 17th December 2020

Dear Editor,

please find enclosed the manuscript entitled "**A case study on farmed European seabass and gilthead seabream in Central Italy: the low parasitological risk of nematode larvae paves the way for the freezing derogation**" to be considered for publication in Food Control.

Gilthead seabream and European seabass are among the most appreciated farmed fish species in the European Union, where Italy represents the largest market. New food fads have led to an increase in the consumption of these two species in the form of raw products (*sushi, sashimi, carpacci, tartare*) exposing consumers to risks associated with the presence of anisakid nematodes, responsible for severe human illnesses. In addition, the presence of larvae (dead and alive) can provoke consumers disgust and also negatively affect the fish market.

This case study, conducted in the framework of the project "*Anisakids infection in European seabass and gilthead seabream in the Tyrrhenian Sea and prospects for the risk management through the application of "Anisakis free" production methods*" funded by the Italian Ministry of Health, analysed the production system and the level of infection of ascaridoid nematodes in an offshore cage farm in Central Italy. The self-control plan procedures adopted by the FBO before the capture to reduce or prevent anisakids infection were analysed in the light of the *Anisakis* contamination pathways proposed by Crotta et al., Food Control 69 (2016) 275e284 for farmed Atlantic salmon (*Salmo salar*) and also taking into consideration the criteria recommended by EFSA (EFSA Journal 2010; 8(4):1543) for assessing when fishery products from aquaculture do not present health hazards related to the presence of parasites. In addition, the results of the visual parasitological examination conducted by the FBO, as part of the self-control plan, on 5% of the total specimens with commercial size (2016-2020 period) were also considered.

The results show that the examined plant can be considered at low to negligible risk for the presence of ascaridoid larvae in the farmed gilthead seabream and European seabass and confirm the absence of ascaridoid nematodes in these farmed species, as already reported in the available literature data. Few implementations to the self-control plan are suggested for obtaining and maintaining the derogation to preventive freezing, as established by the European legislation: i) the parasitological examination of a statistically significant sample of the farmed specimens conducted by trained personnel, with detailed record of the results; ii) the systematic collection, record and parasitological examination of farmed specimens found dead or underdeveloped (runts); iii) the systematic collection, record and parasitological examination of wild specimens of other species which may enter the cages. This approach can be adapted by other farms by adjusting the sample size based on the production volume and risk categorization.

Data arising from this study would be of interest considering that although wild gilthead seabream and European seabass have a higher market value, aquaculture represents the main production method of both species at community and global level and the largest part of products deriving from this species on the Italian market is of farmed origin.

The possibility to obtain an exemption from the preventive freezing of farmed fish species by the implementation of a correct management system in offshore plants, validated by the Official Authority, as established by the current legislation, would represents a new market opportunity for Italian aquaculture plants. This is particularly true considering that farmed Atlantic salmon, for which an exemption from the preventive freezing already exists, are currently arriving on the Italian market (Ministry of Health note on Official control on parasites in seafood - DGISAN 0043259-P-03/12/2020).

The manuscript has not been published elsewhere nor is it being considered for publication elsewhere. All authors have approved this manuscript, agree to the order in which their names are listed, declare that no conflict of interests exists and disclose any commercial affiliation.

Yours sincerely,
Andrea Armani and co-authors

Dear Editor and reviewers,

Thank you for your positive feedbacks. We have revised the manuscript addressing all the reviewers' suggestions. Please find a detailed response below.

Reviewer #1: The manuscript is well written, fluid and logical. References and methods are appropriate; I only have some minor comments mainly to improve the transparency of the methods.

Line 54, probably better "Humans" rather than "Man"

Done

Figure 2 is not necessary in my view

Figure 2 has been deleted

The section "3.2 Analysis of the parasitological risk procedures adopted in the self-control plan." Looks very much like a sort of qualitative risk assessment, indeed likelihoods are used. I strongly recommend to include a table with the definition of the likelihoods, for example at line 306 reads "low to negligible risk", but what "Low" means and why this could not be "Very Low"? As part of the M&M it should be specified the likelihood scale that is used and the description of the likelihood terms.

I would also suggest to provide a qualitative estimate of the uncertainty that is associated to the likelihoods. This allow the readers understand how strong is the evidence supporting the likelihood estimates.

The authors wish to thank the reviewer for his valuable suggestions. A summarizing table was added, the title and the text were also slightly modified to make the use of the terms consistent throughout the manuscript.

Line 252, Not sure starting a statement with "In agreement" is correct".

In agreement was replaced with "Accordingly"

Line 291; "P" value? Is this a Prevalence? Please replace "P" with "Prevalence" along the manuscript.

Done

Reviewer #2: The manuscript is an interesting retrospective study aimed at evaluating the possibility of obtaining freezing derogation of farmed European seabass and gilthead seabream to be eaten raw or almost raw, regarding the Anisakis risk. The paper is well written and can be published with minor revision.

Introduction:

Lines 62-64: It is good to remember here, that in any case the devitalized larvae, also by biocides, are able in vitro to stimulate inflammation and inhibit apoptosis (Speciale et al. 2017) doi: 10.1007/s00436-017-5551-6.

The information and related reference were integrated in the text.

Results:

Lines 193-194: The intake of krill by adults cannot be completely excluded, in the absence of investigations on the stomach contents. If available this data could be inserted here to confirm this indication.

The sentence was modified.

Lines 266-291: is considered important to remember that in both farmed species it has been experimentally demonstrated that the L3 larvae have the ability to penetrate the gastric mucosa, thus being able to give rise to a re-incistation. See Macrì et al., 2012 (doi:10.1016/j.aquaculture.2012.01.015) and Marino et al., 2013 (doi:10.1155/2013/701828).

The information was integrated in the text for *Sparus aurata*, while for D. labrax we consider it not necessary as natural infections are frequently been reported, especially in the Atlantic Ocean.

A case study on farmed European seabass and gilthead seabream in Central Italy: the ~~low~~ negligible parasitological risk of nematode larvae paves the way for the freezing derogation.

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Abstract

Gilthead seabream and European seabass are among the most appreciated farmed fish species in the European Union. This case study analysed the self-control plan procedures adopted in an offshore cage farm in Central Italy to prevent anisakids infection, in the light of the *Anisakis* contamination pathways previously proposed for farmed Atlantic salmon (*Salmo salar*), and of the criteria recommended by the European Food Safety Authority. Moreover, the results of the visual parasitological examination conducted by the Food Business Operator, as part of the self-control plan, on 5% of the total specimens with commercial size (2016-2020 period) were also considered. Results show an extremely low to negligible risk for the introduction of ~~presence of~~ ascaridoid larvae, confirming the absence of these parasites in farmed specimens of both species. However, few implementations to the self-control plan are suggested for obtaining the derogation to preventive freezing, as established by the European legislation. These include the parasitological examination of a statistically significant sample of the farmed specimens (commercial sizes) conducted by trained personnel, as well as of farmed specimens found dead or underdeveloped (runts) and of wild specimens of other species which may enter the cages. The proposed approach can be adapted by other farms by adjusting the sample size based on the production volume and risk categorization. The exemption from the preventive freezing would represent an additional market opportunity for Italian aquaculture plants.

Keywords: ascaridoid nematodes; *Anisakis* spp.; *Sparus aurata; Dicentrarchus labrax; Anisakis free products*

1. Introduction

One of the major risks associated with the consumption of raw or undercooked seafood is anisakidosis, a parasitic infection sustained by a group of nematodes generically called anisakids (EFSA, 2010). These parasites display an indirect life cycle in aquatic ecosystems, involving marine mammals and fish-eating birds as definitive hosts, crustaceans as first intermediate hosts and fish and cephalopods as intermediate or paratenic hosts (Anderson, 1992; Mattiucci et al., 2008; 2018). ~~Man~~ Human acts as an accidental host when eating raw or undercooked fish and cephalopods carrying the zoonotic infective stage (third stage larva, L3). The ingested live larvae can penetrate the alimentary tract and be responsible for severe gastroenteritis. Almost all episodes of anisakidosis have been attributed to the species *Anisakis simplex*, followed by *Anisakis pegreffii* and *Pseudoterranova* sp., while very sporadic are the infections associated with *Contracaecum* sp., another genus of the same Anisakidae family (Buchmann & Mehrdana, 2016; Shamsi, 2019). Moreover, only *Contracaecum* species infecting marine mammals (e.g. *C. osculatum*) seem to be responsible for anisakidosis (Kanarek and Bohdanowicz 2009; Shamshi, 2019). Crude extract of *Anisakis* spp. can affect intestinal integrity and permeability and also play a role cell growth and death (Carballeda-Sangiao et al., 2020; Speciale et al., 2017). Beside gastrointestinal cases, allergic reactions in sensitized patients have been reported, even after the ingestion of devitalized larvae (Audicana and Kennedy, 2008; Nieuwenhuizen et al., 2006; Smith, 1999). Allergic reactions develop upon exposure to *A. simplex* or *A. pegreffii*, while the allergenic potential of *Pseudoterranova* spp. and *Contracaecum* spp. remains unknown (Kochanowski et al., 2019). The other nematode genus frequently found in seafood is *Hysterothylacium* (Raphidascarididae: Ascaridoidea: Nematoda), which is generally believed to be non-zoonotic (Levsen et al., 2018) and of doubtful allergenic potential (Bao et al., 2020). All these parasites are generically referred to as ascaridoid nematodes.

The European legislation provides that all Food Business Operators (FBO) must conduct a non-destructive visual inspection of the fishery products for the detection of "*visible parasites*" ("*which in terms of size, colour or texture is clearly distinguishable in fish* tissues"), to avoid placing "*obviously contaminated products*" on the market (Commission Regulation EC 2074/2005). As the visual examination does not allow to completely remove the hazard (Goffredo et al., 2019; Llarena-Reino *et al.*, 2012), the Commission Regulation (EU) 1276/2011 states that fish and cephalopods intended to be eaten raw or almost raw must be subjected to preventive freezing. This is important considering that reaching 60°C for 1 min in the thermal centre is not sufficient to kill all L3 larve (Sánchez-Alonso et al., 2021). Moreover, the presence of visible parasites (dead or alive) in seafood products not only represents a public health issue, but also affects the product quality, making it unappealing to the consumer, not suitable for sale and thus provoking economic loss (D'Amico et al., 2014; Mattiucci et al., 2018). Currently, preventing the infection of farmed fish is considered a viable alternative to the temperature treatment (heating and freezing), which is capable of killing the larvae but not to fully remove the parasite contamination (Parafishcontrol, 2017). Preventing the infection is possible if farms adopt specific procedures to manage the parasitological risk as provided by the EFSA Scientific Opinion on risk assessment of parasites in fishery products (EFSA, 2010) and by the Commission Regulation EU 1276/2011.

The first farmed species exempted from the preventive freezing treatment was the Atlantic salmon (*Salmo salar*) in the United Kingdom, followed by halibut (*Hipoglossus hipoglossus*) and rainbow trout (*Onchorynchus mykiss*) (Brooker et al., 2016; https://www.food.gov.uk/business-guidance/freezing-fish-and-fishery-products). A similar derogation is present in Norway for rainbow trout and Atlantic salmon (Norwegian Food Safety Authority, 2018; Roiha et al., 2020) and, recently, these products are arriving on the Italian market (DGISAN, 2020). Other studies confirmed a very low risk of carrying nematodes for farmed salmonids species (Roiha et al., 2020 and ref. therein). For other farmed species, such as gilthead seabream (*Sparus aurata*), turbot (*Scophtalmus* spp.), umbrine (*Umbrina cirrosa*) and European seabass (*Dicentrarchus labrax*), the risk of contracting anisakiasis was estimated to be from extremely low to negligible (Asociación Empresarial de Productores de Cultivos Marinos, 2012; Brooker al., 2016; Peñalver et al, 2010; ~~Parafishcontrol, 2017~~Fioravanti et al., 2021). However, to date, derogations from the preventive freezing has not been ~~proposed~~ authorized for these latter species.

As safety is a very important quality pre-requisite and both are interrelated and linked to consumers' confidence, safety control in the aquaculture sector is essential, and the implementation of control plans impact the overall quality conception of the final product (Freitas et al., 2020). This is particularly true for farmed fish where any defects, such as the detection of ascaridoid nematode larvae (especially if still alive), could seriously affect consumers’ confidence in the aquaculture industry (Mattiucci et al., 2018). A recent study in Spain has shown that consumers are willing to pay more for Anisakis-free fish products (Bao et al., 2018).

Gilthead seabream and European seabass are among the most marketed species in the European Union (EU), together accounting for around 20% of the total value of EU aquaculture production. Both species are bred throughout Europe and in the Mediterranean basin using intensive methods, in tanks or ponds on land and more frequently in offshore cages at sea. They are prevalently farmed in Greece and Spain (EUMOFA, 2020) and they are the most important marine species cultured and marketed in Italy, especially in two central regions: Lazio and Tuscany (EUMOFA, 2019; MIPAAF, 2014). In fact, with more than 30 000 tonnes of product sold yearly, Italy plays a leading role within the Mediterranean and European aquaculture market for both species (EUMOFA 2017; 2019).

Following these premises and according to EFSA recommendations (EFSA, 2010), this case study analysed the production system and the level of infection of ascaridoid nematode in an offshore cage farm in Central Italy. By suggesting the implementation of a specific procedure for the management of the parasitological risk, this work proposes an approach for obtaining the freezing derogation.

2. Materials and methods

2.1 Analysis of the breeding plant

The geographical location, the structure of the installation at sea, the environmental context of the plant and the production process were analysed through the examination of the company's self-control plan and by on-site visits. During these visits, meetings were held with various employees of the structure (veterinarian, company director, production manager) and a questionnaire was administered, to collect information on the various stages of production.

2.2 Analysis of the parasitological risk procedures adopted in the self-control plan

The self-control plan procedures adopted by the FBO before the capture to reduce or prevent anisakids infection were analysed in the light of the *Anisakis* contamination pathways of introduction proposed by Crotta et al., (2016) for farmed Atlantic salmon (*S. salar*):

1) through the capture of wild juveniles for subsequent growth on the farm;

2) through the use of feed contaminated with viable larvae of *Anisakis* spp.;

3) through wild European seabass and gilthead seabream accidentally penetrated into the cages;

4) by ingestion of infected hosts that have entered the cages.

The criteria for assessing when fishery products from aquaculture do not present health hazards related to the presence of parasites were also considered: information on the prevalence, abundance, as well as species and geographical distributions of the parasites and their hosts, together with monitoring systems; information on the fish species and susceptibility to parasites; origin of the stock; production system; type of feed and feeding methods; time span for growth; and processing method (EFSA, 2010). In this study, possible contamination by other ascaridoid nematodes belonging to the genus *Contracaecum* and *Hysterothylacium* was also considered. *Pseudoterranova* sp. was not included because its presence in the Mediterranean Sea and therefore in the examined farm is unlikely (Alt et al., 2019; Cavallero et al., 2016). The qualitative terms used to describe the likelihood the related levels of uncertainty of events leading to contamination with ascaridoid nematodes as proposed by Crotta et al., (2016) are reported in Table 1.

Table 1 Summary of the likelihood and uncertainty for each pathway of introduction of ascaridoid nematodes into the investigated European seabass and gilthead seabream farm, with definition of the qualitative terms used to describe the likelihoods and the related levels of uncertainty (modified from Crotta et al., 2016).

			Introduction pathways			
	Levels	**Definition**	**Capture of wild juveniles for subsequent growth on the farm**	**Use of feed contaminated with viable larvae of *Anisakis* spp.**	**Wild European seabass and gilthead seabream accidentally penetrated into the cages**	**Ingestion of infected hosts that have entered the cages**
Likelihood	High (H)	Expected to occur	N	N	N	EL-N
	Low (L)	Unlikely to occur				

	Moderate (M)	Occurrence less than 50% probability				
	Very Low (VL)	Rarely occur				
	Extremely Low (EL)	Very rarely occur				
	Negligible (N)	Chance of occurrence so small that can be ignored				
Uncertainty	Low (L)	Estimation strongly supported by data-evidence Agreement by different authors	L	L	L	M
	Medium (M)	Estimation supported by few or incomplete data. Some authors report slightly different conclusions				
	High (H)	Estimation supported only by scarce data or based on hypotheses not yet proved. Strong disagreement from different authors				

2.3. Retrospective analysis of the results of the parasitological examinations conducted in the self-control plan

The results of the parasitological examination conducted by the FBO, as part of the self-control plan, on 5% of the total specimens with commercial size were analysed. Specimens were analysed by visual inspection, according to the current legislation (Commission Regulation EU 2074/2005). The number of examined specimens (n) for each year was calculated considering an average commercial size of 400 gr per fish and the annual production (Table ~~1~~2). The usefulness of n for the estimation of a prevalence of 0.5%, and for the assessment of freedom from a disease, with variable confidence/test sensitivity were evaluated. For the purpose, an online platform providing epidemiological tools for estimating prevalence and freedom from disease in a population was used (https://epitools.ausvet.com.au/). In addition to the abovementioned n, the results of the parasitological examination (visual inspection) conducted on a small part of the gilthead seabream production, which is commercialized as gutted, was also considered, as well as customer complaints due to the presence of ascaridoid nematodes.

Table 2~~1~~. Total production and examined specimens in the 5-years period of the retrospective analysis.

Year	Total production (Kg)	Total n of examined fishes (5% of total prod)	n of examined gilthead seabream	n of examined European seabass
2016	427,305	53,400	32,040	21,360
2017	480,733	60,000	36,000	24,000
2018	473,853	59,200	35,520	23,680
2019	~ 500,000	62,500	37,500	25,000
2020	~ 600,000	75,000	45,000	30,000
2016-2020	**~ 2,481,891**	**310,100**	**186,060**	**124,040**

3. Results and discussion

3.1 Analysis of the breeding plant

3.1.1 Geographical location and structural characteristics of the plant. The mariculture plant is located in the Gulf of Follonica (Grosseto, Tuscany, Fig. 1) where it covers an area of 2000 x 1000 metres. The minimum distance from the coast is 2.25 miles from the east coast and 4.5 miles from the Port of Piombino. The plant consists of 22 circular floating cages with a diameter of 22 metres and distributed in 3 grids. The cages are made of 2 high density polyethylene tubular elements (HDPE DN250) and a synthetic fibre mesh bag (dyneema) of 6-10 metres in height which delimits the volume of livestock farming, making it 1900 m3 for seeding nets and 3800 m3 for the others~~ (Fig. 2)~~. The mesh size of the nets varies from 6-8 mm to 22 mm depending on the size of the fish: sowing nets (6-8 mm in diameter), cycle of about 3-4 months; 15 mm nets, cycle of about 7 months; 22 mm nets, until the end of the production cycle.

Fig. 1. Location of the plant: Gulf of Follonica, Tyrrhenian Sea (FAO 37.1.3) (the red dot indicates the plant location)

~~**Fig. 2.** Overview of part of the cages at sea of the plan analysed in the case study~~

3.1.2 Environmental context. The presence of cetaceans, definitive hosts of *Anisakis* spp. and responsible for expelling parasitic eggs with their faeces, is known and frequent in the Tyrrhenian Sea (www.islepark.it/visitare-il-parco/santuario-dei-cetacei). However, according to the FBO of the plant small groups of dolphins (mainly *Tursiops truncatus*, more rarely *Stenella coeruleoalba*) are found near the cages only sporadically (especially in the period between late winter and early spring), while there are rare sightings of more numerous colonies that generally remain outside of the Gulf. On the contrary, cormorants acting as definitive hosts of some *Contracaecum* species (such as *C. rudolphii*), are frequently recorded around the plant, with increased density above the cages in the winter months (up to groups of 200-300 subjects). As regards *Hysterothylacium* sp., the possibility that its biological cycle may occur near the installation cannot be excluded given the multiplicity of definitive hosts, exclusively cold-blooded animals (both predatory and planktivorous fish) in which the maturation of the larvae may occur (Bao et al., 2020).

As regards the first intermediate hosts (euphasids), their presence in the water surrounding the establishment at sea cannot be excluded. In fact, these microorganisms are normally present in the marine environment and they cannot be constantly monitored. However, the fishes ~~fry~~ of the establishment, ~~the category of fish most prone to krill ingestion~~at all development stages, are fed with compound feed (pellets) (see section 3.2.2).

The Tyrrhenian Sea is home to more than 420 species of fish, most of them belonging to the families Gobiidae, Sparidae, Labridae and Bennidae (Psomadikis et al., 2012). Personnel involved in the management and maintenance of the cages at sea reported sardines (*Sardina pilchardus*), bogues (*Boops boops*) and horse mackerel (*Trachurus trachurus*) as the species most frequently observed around and accidentally recorded inside the cages. These species can harbour ascaridoid nematodes and thus the specific risk posed by their presence will be discussed below (see section 3.2.4).

3.1.3 Production flow. The production flow of the plant starts with the sowing of fry (size 3-5 gr) purchased from an Italian plant where they are fed with microalgae and zooplankton. The juveniles are transported to the offshore cages in compliance with animal welfare standards (Commission

Regulation EU 1/2005). The next phase consists in the growth of the fish through the administration of pelleted feed. The amount of feed supplied daily varies according to the biomass reared and the water temperature, while the meals frequency is influenced by light hours, temperature and size of the fish. Unintentional fasting periods of up to four days may occur only if severe bad weather conditions do not allow the staff to go out to the sea plant for safety reasons. The average commercial size (400 gr.) is generally reached in 12-14 months for gilthead seabream and 18-20 months for European seabass. During farming, the monitoring of the biological function (fortnight sampling of 5 individuals in rotation in the different cages checking the weight, lesions, or malformations) favour the homogeneous growth preventing the presence of runts (undersized specimens).

The catches (preceded by 24 hours of fasting) are periodic, according to commercial needs; with the aid of a purse seine fish are loaded into the boat and immersed in water and ice (self-produced with drinking water) contained in closed bins of 600-1000 liters, to speed up the suppression (less than 1 hour) and minimize the suffering and stress of the captured specimens. In the processing room the fish is kept constantly at a temperature not exceeding 3°C using ice. The fishes are then selected according to the size and chilled with ice. Part of them is commercialized as gutted.

3.2 Analysis of the parasitological risk procedures adopted in the self-control plan.

3.2.1 Introduction through the capture of wild juveniles for subsequent growth on the farm. The breeding of wild fry could be a potential route of infestation for the farmed products. However, the practice of catching young specimens that are then reared up to commercial size, which occurs for other species (Huech et al., 2011; Mladineo and Poljak, 2014; Smrzli et al., 2012), is not used for European seabass and gilthead seabream. These species are bred in intensive offshore farms, as the one analysed in this study, involving the growth of juvenile fish produced in hatcheries, fed on controlled and often self-produced diet (based on rotifers and *Artemia*) (EFSA, 2010). Therefore, this pathway is negligible with a low level of uncertainty as a source of parasitic contamination.

3.2.2 Introduction through the use of feed contaminated with viable larvae of ascaridoid nematodes. The likelihood of introducing ascaridoid parasites through food depends on the origin and nature of the raw material used for food as well as any physical and thermal treatments it has undergone. As explained by Wootten and Smith (1975), the use of live feed considerably increases the risk of introducing parasites into the farms; however, current European seabass and gilthead seabream farming techniques only involve the use of compound feed (pellets) produced by extrusion at high temperatures capable of devitalising any parasites present (Cataudella and Bonzi, 2001; Crotta et al., 2016; EFSA, 2010). A feed subject to a similar treatment is also used in the farm analysed in this study (see section 3.1.3). Therefore, the risk of infection linked to this pathway is negligible with a low level of uncertainty.

3.2.3 Introduction through wild European seabass and gilthead seabream accidentally penetrated into the cages. The possibility of infection through this pathway needs two conditions: i) that wild European seabass or gilthead seabream (for other fish species, see point 3.2.4) enter the cages and ii) that these specimens are infected. The first condition is linked to the presence of breaches, which is controlled by a daily monitoring of the nets and constant repairing. Furthermore, the tendency of wild specimens to enter the cages is modest given the preference for low population density habitats (Crotta et al., 2016; Kapota; 2012; Skov et al., 2009). In the case some wild specimens enter the cages, the level of ascaridoid infection in wild specimens must be considered. The analysis of the literature has shown that, in general, a larger set of data is available for European seabass than for gilthead seabream (see table 1 in Guardone et al., 2020). With regard to the European seabass, one study only examining 6 specimens found *A. pegreffii* with a 50% prevalence (Culurgioni et al., 2011), while another study, conducted on 100 specimens, found a prevalence value of about 13% (Zaid *et al*., 2018); the localization of the parasites was exclusively visceral. Despite gilthead seabream was found to be sensitive to experimental infection with *Anisakis* spp. (Marino et al., 2013), ~~For wild gilthead seabream,~~ the finding of *Anisakis* spp. has never been reported in the literature for wild specimens. This could be due to the feeding behaviour of this species (see section 3.2.4). ~~In agreement~~Accordingly, also in a previous study conducted on 40 gilthead seabream and 47 European seabass collected in the area surrounding the analysed farm, no larvae belonging to *Anisakis* sp. were found, but only larvae of *Hystherothylacium* sp. and *C. rudolphii* (Guardone et al., 2020). Unlike European seabass in the Mediterranean Sea, high prevalence values of *Anisakis* spp. (visceral up to 95%, muscular up to 42.5%) were found in wild specimens caught in the northeast Atlantic (FAO 27) (Bernardi 2009; Bernardi et al., 2011). The different infection levels observed in the Atlantic specimens compared to the Mediterranean ones could be due to different growth pattern, as sexual maturity is reached later in the former (males between 4-7 years and females between 5-8 years) than in the latter (generally between 2 and 4 years of age) (https://www.fishbase.in/summary/Dicentrarchus-labrax.html). Therefore, considering the location of the plant, the available epidemiological data, the cage management and the monitoring of the biological functions, the risk of infection linked to this pathway is negligible with a low level of uncertainty. This is particularly true for the products commercialized as gutted.

3.2.4 Introduction by ingestion of infected hosts that have entered the cages. Again, the possibility of infection through this pathway needs two conditions: i) that other hosts enter the cages and ii) that these hosts are infected. The introduction of infected fish hosts into the floating cages depends upon their size, in relation to the mesh size of the nets. As reported in section 3.1.2, only sporadically indigenous species like sardines, bogues and horse mackerel, were found near or inside the cages. In particular, following the opening of other aquaculture facilities (located in a more external position compared to the Gulf Coast) the presence of specimens of these species around the cages has been significantly reduced. Their presence is now sporadic and limited to a few specimens per caught, only in certain periods of the year. Although these are potential vehicle of *Anisakis* sp., and also of *Contracaecum* sp. and *Hysterothylacium* sp., the level of infection in these fish species vary and is generally higher in larger specimens (Angelucci et al., 2011; Bušelić et al., 2018; Cavallero et al., 2012; Ichalal et al., 2015; Piras et al., 2014; Salati et al., 2013; Serracca et al., 2013). All wild caught seawater fish must be in fact considered at risk of containing viable parasites and no sea fishing grounds can be considered free of *A. simplex* (EFSA, 2010). However, the small size of the nets (from 6-8 mm to 22mm) could prevent the introduction of adult specimens of the aforementioned small fish species. Moreover, it is also important to consider the different feeding behaviour of the two farmed species taken into consideration. The gilthead sea bream prefers to feed on gastropods and bivalves (in particular mussels and oyster) while a less frequent consumption of teleost is reported (Guardone et al., 2020; Pita et al., 2002). On the contrary, the European seabass shows a predatory feeding attitude towards invertebrates during the juvenile phase and towards other fish during adulthood. An increase in the size of prey fish has been observed with the increase in the size of the European sea bass, promoting a progressive accumulation of nematode larvae during host's life. This trend is clearly shown in a study in which increasing prevalence values were reported in categories of European seabass of different weights caught in the Atlantic Ocean (Bernardi et al., 2011), while it has not been observed in the Mediterranean Sea, where most of the specimens analysed in the various studies weighed less than 1 kg and had prevalence~~P~~ values ranging from 0 to 13%.

The bioaccumulation of parasites in runts was observed in Atlantic salmon and in rainbow trout (Levsen and Maage; 2016; Mo et al., 2014; Roiha et al., 2020). Runts seem therefore to be the most exposed to contamination as they are pushed, to survive, to the opportunistic predation of the wild species that have penetrated the cages (Fioravanti et al., 2021).

The presence of the first intermediate hosts (euphasids) acting as a potential L3 host for *Anisakis*, *Contracaecum* and *Hysterothylacium* species, within the establishment at sea cannot be controlled. However, the use of farmed rather than wild juveniles fed on pelleted exclude at this stage the risk of infection with anisakid nematodes (Crotta et al., 2016). In fact, the juveniles are kept separate from the surrounding environment until the sowing and then the artificial feeding prevents the predation of potential first intermediate hosts that have entered the cages (EFSA, 2010; Klimpel et al., 2004; www.fao.org/fishery/species/2384/en). The only study on European seabass fry (<50 g, n=50) shows that all the tested samples were negative (Peñalver et al., 2010).

Therefore, the constant monitoring of the nets, of the fish density and health and of the conversion index suggest this pathway, although the less controllable, is linked to a extremely low to negligible risk with a medium level of uncertainty of parasitic infection.

3.3. Retrospective analysis of the parasitological examination conducted in previous years.

In the considered period (2016-2020) 310.236,4 specimens were analysed (Table 2~~1~~). In addition, 1-2% of the total gilthead seabream production (overall ~55842 specimens) were commercialized as gutted. All the fish self-tested were negative for ascaridoid nematodes. In addition, no complaints were received from customers (mainly GDO with quality control systems) due to parasitic contamination.

The visual inspection used for parasite detection represents the method normally adopted at all levels of the seafood chain (D'Amico et al., 2014) in accordance with the current legislation, which is aimed at the detection of "visible parasites". However, small larvae could be overlooked. In fact, the accuracy of visual inspection depends primarily on the parasite appearance and size but also on fish species (fillet thickness, size, texture, presence of pigmentation) as well as on the level of FBO training and experience (Chalmers 2020; Levsen et al., 2005; Pozio, 2005; Shamsi & Suthar, 2016). Beside *Anisakis* sp. L3 larvae, which are visible (14–44 mm in length and 0.4–0.9 mm in diameter) (Murata et al. 2011; Pardo-Gandarillas et al. 2009; Shamsi et al. 2011a), the larval dimension of the various *Contracaceum* sp. species varies from a few millimetres to around two centimetres (Garbin et al., 2013; Shamsi et al., 2011b; 2019). In some studies larvae very minute in size (2-5 mm) have been reported (Garbin et al., 2013; Salati et al., 2013; Shamsi et al., 2011b), for which the term "visible" is difficult to apply (Salati et al., 2013). In addition, some *Contracaecum* larvae can be deeply embedded in the gastrointestinal tissue of the fish and thus they can only be observed by removing the gastrointestinal tissue and keeping it warm for several hours, allowing the larvae to emerge (Shamsi, 2019). As regards *Hysterothylacium*, a wide range of larval types with different morphological characteristics has been described (Shamsi et al., 2013; 2017). In addition, *Hysterothylacium* sp. may be present in fish species in its adult form, which is larger and clearly visible, thus potentially able to cause client/consumer rejection if present in the final product (Bao et al., 2020).

The absence of ascaridoid nematodes in farmed gilthead seabream and European seabass analysed during self-control procedures from 2016 to 2019 agrees with the results obtained for farmed species from the bibliographic analysis (Table 2). In fact, the only ascaridoid nematodes found in Mediterranean Sea in the examined species were: two larvae of *A. pegreffii* in 68 European seabass from Greek farm (Cammilleri et al., 2018), 1 larva of *H. fabri* in 140 European seabass from Italy (Fioravanti et al., 2021) and *Contracaecum* sp. (prevalence around 15%) in both species from Sardinia (Salati et al., 2013). Muscle localization was observed only for *Contracaecum* sp. in gilthead seabream (P=10.8%). Therefore, the available data suggest a ~~very~~ low to negligible prevalence as regards these parasites confirming what already reported for other farmed species (Asociación Empresarial de Productores de Cultivos Marinos, 2012; Brooker al., 2016; EFSA; 2010; Fioravanti et al., 2021; Peñalver et al, 2010; Roiha et al., 2020;~~ Parafishcontrol, 2017~~). Furthermore, visceral localization in European seabass and gilthead seabream is preferred than muscular tissue (Table 1 in Guardone et al., 2020 and Table 3~~2~~). Thus, migration can be completely prevented by evisceration.

3.4 Final remarks: proposal for obtaining the derogation

From a food safety perspective, the most important ascaridoid nematodes belong to the *Anisakis* genus. *A. simplex* (s.s.) and *A. pegreffii* have been reported as responsible for human infections, with the latter species being the most frequently involved in zoonotic infections in Italy (Guardone et al., 2018). Human infections with *Contracaecum* sp. larvae appear less common (Dei-Cas et al., 1986; Im et al., 1995; Nagasawa, 2012; Shamsi and Butcher, 2011; Schaum & Müller, 1967). Different zoonotic potentials have been hypothesized: the species having marine mammals as definitive hosts, such as *C. osculatum*, are believed to be zoonotic, while those having birds, as *C. rudolphii*, are not considered so (Shamsi, 2019). Although for some authors a controversial issue (Shamsi et al., 2013), the zoonotic potential of *Hysterothylacium* spp. seems negligible (Levsen et al., 2018). Finally, as for the allergenic potential, it is proven for *A. simplex* and *A. pegreffii* (Audicana and Kennedy, 2008), while it is doubtful for *Contracaecum* spp. and *Hysterothylacium* sp. (Bao et al., 2020; Kochanowski, 2019).

Overall, the analysis of the parasitological risk management of the self-control plan shows that the risk of ascaridoid introduction is low to negligible. In particular, the risk for the first three pathways is negligible with a low level of uncertainty, while the possible introduction through infected hosts, although extremely low to negligible, is less controllable being characterized by a medium level of uncertainty. However, since the transmission only occurs following the predation of wild specimens, this may eventually occur in the European seabass, while it is highly unlikely for the gilthead seabream. This extremely low to~~/~~negligible risk is supported by the results of the retrospective analysis of the parasitological examination conducted by the FBO, as well as by the absence of customer' complains. In fact, as shown in Table 2~~1~~, in the last 5 years over 30000 gilthead seabream and over 20000 European seabass per year were visually inspected in the plant: all resulted negative, further supporting the very low risk of the presence of ascaridoid, even taking into account the relatively low sensitivity of the visual inspection. In the ~~very recent~~ work of Rohia et al. (2020), a sample of 1000 specimens were estimated as sufficient for assessing a prevalence of 0.05% in a population of 20 million fish specimens. Similarly, in the work of Fioravanti et al., (2021) a sampling plan with a confidence level of 99% and a margin of error (MoE) of 4–8% was used and a samples of 1032 fish per species (least 258 fish per farm) was considered to be statistically significant for obtaining prevalence estimates. Thus, applying an analogous ~~-~~ approach, the number of specimens analysed in the self-control activities by the FBOs would be largely sufficient to found such very low prevalence.

An alternative approach for the calculation of the sample size to be proposed to acquire and maintain the freezing derogation, would be the assessment of freedom from a disease, as already applied for other fish pathogens (Commission Implementing Decision (EU) 2015/1554). In this case, assuming a population of ~1000000 specimens (year production), the sample (n) to be collected for the estimation of low prevalence, is influenced by the test sensitivity and by the required confidence for the calculation (usually 95%), as reported in Table 4~~3~~ (https://epitools.ausvet.com.au/freedomss). Also using this approach, considering the variability reported in the Table ~~3~~4, the number of specimens analysed by the FBOs during the self-control activities would be largely sufficient to assess such very low prevalence.

Table 4~~3~~. Sample size for estimating freedom from a disease depending on the prevalence, test sensitivity and required confidence

Population size (N)	Design prevalence	Test sensitivity	Required confidence	Sample size (n)
500,000-1,000,000	1% (0.01)	0.95	0.95	316
			0.98	412
			0.99	485
		0.8	0.95	375
			0.98	489
			0.99	576
	0.5% (0.005)	0.95	0.95	631
			0.98	824
			0.99	970
		0.8	0.95	749
			0.98	978
			0.99	1151

Beside commercial sizes, the sampling plan should include undersized specimens (runts), fishes eventually found dead or symptomatic, as well as specimens of other species found in the cages. Thus, the total number specimens to be examined will be composed of these different categories. The parasitological examination can be conducted by visual inspection or using more sensitive methods (digestion or UV press) (Gómez-Morales et al., 2018; Guardone et al., 2016). which would reduce the needed sample size. Sampling could be conducted two or four time a year and then, upon continuous negative results, may become annual or biannual, as stated for establishing freedom from other fish diseases (Commission Implementing Decision (EU) 2015/1554).

5. Conclusion

The present case study shows ~~that the examined plant can be considered at~~ an extremely low to ~~/~~negligible risk for the presence of ascaridoid larvae in the farmed gilthead seabream and European seabass of the analysed plant. Nevertheless, a few implementations to the self-control plan are needed for obtaining and maintaining the derogation to preventive freezing (Reg. CE 1276/2011). The proposed approach should include: i) the parasitological examination of the farmed specimens conducted by trained personnel on a statistically significant sample, with detailed record of the results;

the parasitological examination could be conducted by visual inspection or using more sensitive methods (digestion or UV press); ii) the systematic collection, record and parasitological examination of farmed specimens found dead or underdeveloped (runts); iii) the systematic collection, record and parasitological examination of wild specimens of other species which may enter the cages. This approach can be adapted to other farms by adjusting the sample size based on the production volume and risk categorization. The procedure should be evaluated and authorized by the competent authority as required by law. Also considering that farmed Atlantic salmon from Norway, for which an exemption from the preventive freezing already exists, is currently arriving on the Italian market, the freezing derogation would represent a new market opportunity for Italian aquaculture.

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Highlights (for review)

- A case study on ascaridoid larvae in an offshore cage farm in Italy was conducted
- A low/negligible risk for farmed gilthead seabream and European seabass was found
- Management implementations are suggested for the derogation to preventive freezing
- The derogation to preventive freezing represents an additional market opportunity for Italian seafood products

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Table 3 Epidemiological studies (2020-2010) on farmed gilthead seabream and European seabass available in the literature. V: viscera; M: muscle; P%: prevalence.

References	**Geographical area**	**N° examined specimens and species**	**Examined tissue**	**Analythical method**	**Larval identification**	**Larval species (n of larvae if available)**	**V P%**	**M P%**
Fioravanti et al., 2021	5 Italian farms (4 sea cage farms and 1 inland in the Tyrrhenian and Adriatic Sea	1571 sea bass	V and M	Visual inspection (V and M) UV press method (M) Digestion (V)	Morphology PCR-RFLP and PCR and sequencing of ITS region	*Hysterothylacium fabri* (1)	0.04	0
		1563 gilthead seabream				Neg	0	0
	3 Greek sea cage farms (2 in the Aegean and 1 in the Ionian Sea	1125 sea bass				Neg	0	0
		1125 gilthead seabream					0	0
	2 Spain sea cage farms (Mediterranean Sea)	65 sea bass				Neg	0	0
		65 gilthead seabream					0	0
	commercial samples from Greece, Turkey and Croatia	290 sea bass				Neg	0	0
		352 gilthead seabream					0	0
Goffredo *et al*., 2019	2 farms in the Gulf of Manfredonia, 1 farm in the Ionian Sea	75 sea bass 53 gilthead seabream	V and M	Visual inspection Digestion	Morphology PCR-RFLP	Neg	0	0
Cammilleri *et al*., 2018	2 farms in Sicily: Licata and Pachino	83 sea bass from Sicily	Whole fish	Visual inspection Optical microscopy Steromicroscope Digestion	Morphology PCR-RFLP	Neg	0	0
	commercial samples from Greece	68 sea bass from Greece				*A. pegreffii* (2)	0.7	
Pekmezci *et al*., 2014	Black Sea	2 sea bass	V and M	Visual inspection Dissection and candling	Morphology PCR RFLP	0	0	0
	Aegean Sea	6 sea bass						

		6 gilthead seabream						
Salati et al., 2013	Sardinia (land-based tanks)	28 seabass	V and M	Visual inspection Digestion	Morphology PCR (12S)	*Contracaecum* sp.	14.3	0
		38 gilthead seabream					15.8	10.5
Kapota, 2012	32 farms: 12 Greek 20 Italian	926 sea bass 462 sea bream	V and M	Visual inspection Candling	-	-	0	-
Asociación Empresarial de Productores de Cultivos Marinos, 2010	Western Mediterranean occidentale (FAO area 37.1.1)	310 sea bass	V and M	Visual inspection UV candling Digestion	-	0	0	0
		551 gilthead sea bream						