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Article

Zoonotic Anisakidae Nematodes - Emerging Pathogens in Salmon and Other Commercially Important Fish Species from Polish Marine Waters

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Abstract: The zoonotic Anisakidae nematodes *Anisakis* spp., *Pseudoterranova* spp., *Contracaecum* sp. have been found in Baltic Sea fish species. In particular, there has been an alarming increase in the dispersion of *Contracaecum* sp. over the last decade, due to the growing number of grey seal (*Halichoerus grypus*) in the area. This marine mammal is also the final host in the *Pseudoterranova* spp. life cycle. The aim of our study was to summarize the presence of Anisakidae nematodes in the salmon (*Salmo salar*) and other commercially important fish species (cod, herring, sprat and flounder) from Polish marine waters. This was evaluated by parasitological inspection of the internal organs and body cavities of 89 individual salmon and the results were compared with infection levels in cod, herring, sprat and flounder. The presence of *Pseudoterranova* sp. is reported for the first time here in the muscle tissue of salmon from the Baltic Sea. In addition, we highlight the elevated levels of cod and sprat infection with *Contracaecum* sp. over the last decade. The presence of Anisakidae nematodes in commercially important fish species may have implications for human health since these parasites pose a risk of anisakidosis.

Keywords: Anisakidae; salmon; zoonotic; southern Baltic

1. Introduction

The sustained growth in overall production and consumption have been observed in the global fishery and aquaculture sectors [1]. Although seafood is part of a healthy diet, in particular cases its consumption may cause health problems [2,3]. Presence of parasites in wild fish is rather rule than exception. Most of the endoparasites present in visceral organs of fish are removed during the fish processing. However, there is a group of endoparasites able to migrate into edible parts of the fish (like muscle tissue, liver). These parasites may be a source of zoonosis (zoonotic diseases), therefore diseases that are transmitted from animal species to humans [4]. Zoonotic Anisakidae nematodes of the genera *Anisakis*, *Pseudoterranova* and *Contracaecum* have been found in a variety of Baltic Sea fish species, also these of consumers interest. The list of the most important Baltic Sea fish species intended for human consumption contains of: herring *Clupea harengus*, cod *Gadus morhua*, sprat *Sprattus sprattus*, salmon *Salmo salar*, flatfishes like flounder *Platichthys flesus*. Numerous studies are describing the presence of *Anisakis simplex* (called also herring worm or whale worm) in herring [5–7]. This parasite has been noted also in cod [8]; stickleback *Gasterosteus aculeatus* [9] and flatfishes: flounder [9], dab *Limanda limanda* [10], plaice *Pleuronectes platessa* [10] or turbot *Psetta maxima* [10]. However, during last decade the most abundant nematode in these fish species in the Baltic Sea is *Contracaecum osculatatum* so called liver worm [8,11–13], reported also in sprat [14], great sandeel [15] and salmon [16] *Pseudoterranova decipiens* (named also codworm or seal worm) the rarest among listed nematode species, was found in cod [8] and shorthorn sculpin [17] from the Baltic Sea.

The life cycles of these nematodes are very similar, with marine mammals playing the role of final hosts [18,19], but there is clear host specificity: particular species of marine mammal are responsible for closing the life cycle of a particular parasite species. Thus, the final hosts for *A. simplex* are cetaceans, in the Baltic Sea represented by the harbor porpoise, *Phocoena phocoena* [20]; for *C. osculatum*, the final host is the grey seal, *Halichoerus grypus* [21]; and for *P. decipiens*, both the harbor seal, *Phoca vitulina* [22] and the grey seal [23] can adopt this role. In the final host, larval stage L4 transforms to the dioecious mature stage, which is capable of producing fertilized eggs. Parasite eggs are transferred into the water environment via the feces of their final hosts. Transformation to the larval stages L1 and L2 (or even L3) occurs within the egg [24]. Crustaceans (intermediate hosts) take up L2 (or L3) larvae and transformation from L2 to L3 can take place in these intermediates. Such infected crustaceans are eaten by pelagic fish (e.g. sprat, herring), which in turn are eaten by predatory fish (e.g. cod, salmon). Marine mammals represent the last link in the trophic chain: they feed on infected fish and thus become the definitive, i.e. final, host and subsequently the life cycle is complete [24–26].

It is important to emphasize that *Pseudoterranova* spp. are characterized by the demersal life cycle. Eggs settle to the benthos, complete embryonation and hatch [27]. Then second or third-stage larvae (L2 or L3) emerge from the eggs and attach to the substratum, where intake by infaunal and epifaunal benthic copepods (harpacticoids and cyclopoids) may occur [28]. Various benthic macroinvertebrates species have been found infected with *P. decipiens* [27]. Contrary *Anisakis* spp. are considered to follow a pelagic life cycle, involving pelagic invertebrate and vertebrate intermediate hosts [25,29]. Eggs embryonate in the seawater [9]. [30] found larvae surrounded by sheaths with lateral extensions, which may be an adaptation to pelagic conditions. Larvae were able to swim and used mainly pelagic crustacean hosts, such as copepods and euphausiids. According to [24] in case of *C. osculatum* the first two moults occur in the egg, the next and last two moults occur in the final host, the seal. Therefore no moult occurs in the crustacean or fish intermediate hosts and larvae are free-swimming [24].

In the life cycle of these Anisakidae nematodes, fish play the role of paratenic hosts in which larvae do not change their larval stage, but migrate to the fish organs and muscle tissue, where they wait until the host fish is eaten by marine mammals. Organ specificity has been described in particular species of nematode for a particular species of fish. Each of the three listed parasite species have different organ preferences, which may vary depending on the host species. *A. simplex* is often found in herring in the body cavity and on the surfaces of the viscera [31]; in cod it is mostly recorded in muscle [32]; in salmon it has been found in the muscles and viscera [33]. *P. decipiens* prefers muscle tissue in both cod [32] and salmon [34]. *C. osculatum* is found predominantly in the liver of cod [11] and also salmon [16,35]. Fish accumulate these nematodes throughout their lives.

The European Food Safety Authority (EFSA) recommends that all wild-caught fish intended to be eaten raw or almost raw must be considered at risk of containing any viable parasite of concern to human health [36]. Thus, for example, the consumption of raw or unprocessed fish containing live Anisakidae nematodes can lead in humans to anisakidosis or anisakiosis in case of intake of *Anisakis* spp. larvae. The symptoms of acute anisakidosis are non-specific: nausea, diarrhea, vomiting and intense abdominal pain [37,38]. Adequate treatment of infected fish kills the parasites [39], but some allergenic proteins of anisakid nematodes are thermostable [40,41]. Therefore, even dead parasites can be dangerous and can cause allergic symptoms. Accordingly, the level of infection with Anisakidae nematodes should be monitored, at least in commercially important fish species.

For decades, a range of fish species in the Baltic Sea, including cod (*Gadus morhua*), sprat (*Sprattus sprattus*), herring (*Clupea harengus*), salmon (*Salmo salar*), flatfishes have been crucial to Polish fisheries. These fish were mainly intended for human consumption. The National Marine Fisheries Research Institute (NMFRI) has collected data regularly since the 1980s on the level of herring infection with Anisakidae parasites in the body cavity; data on cod-liver infection with these nematodes are also available from 2011. In contrast, information on sprat infection with Anisakids is scarce. Similarly, there is a lack of knowledge about the level of salmon infection with these zoonotic nematodes. Therefore, the aim of our study was to summarize current data on the presence of

zoonotic Anisakidae nematodes in the salmon (*S. salar*) and other commercially important fish species (cod, herring and sprat) from Polish marine waters.

2. Materials and Methods

2.1. Sampling of Salmon and Parasitological Inspection

Salmon were caught in March 2012 in the Polish waters of the southern Baltic Sea (ICES 39G7 and 39G8) during commercial cruises and were gutted on board. Sex of the fish was determined. Basic measurements (length and gutted weight) were conducted and scales were collected for age determination. To evaluate the condition of the fish Clark's condition factor (K_c) was calculated as follows: $K_c = 100 * GW / L^3$, where GW is gutted weight and L is the total length of the individual fish. In total, the body cavities of 89 fish were visually inspected for the presence of Anisakidae nematodes. Descriptions of nematode anatomo-morphological features as described [42–44] were used for preliminary identification of the larva found. Parasitological descriptors were calculated according to the definitions given by [45].

2.2. Identification of Nematode

The single nematode larva found was also identified using molecular methods (ITS-1 rDNA). Genomic DNA was isolated using the Sherlock AX DNA purification kit (A&A Biotechnology) by a modified method that employed mechanical lysis (i.e. mincing tissue with a surgical blade). For ITS-1 analysis, a polymerase chain reaction (PCR) was performed using NC5 (forward) 5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3' and NC13R (reverse) 5'-GCT GCG TTC TTC ATC GAT 3' primers [46,47]. The reaction mixture consisted of 25 μ l 2x PCR Master Mix Plus High (A&A Biotechnology), 0.2 μ l each primer (concentration 100 μ M) and 5 μ l DNA template, supplemented with deionized water up to 50 μ l. The PCR conditions were as follows: 2 min at 94°C (initial denaturation) followed by 35 cycles of denaturation at 94°C for 30 s, annealing of primers at 58°C for 30 s, strand elongation at 72°C for 45 s and a final extension step of 5 min at 72°C. PCR products were sequenced directly using standard procedures and the amplification primers. Sequences were analysed using GeneStudio TM Professional (GeneStudio, Inc., USA) and confirmed by a BLAST search of GenBank. The sequence obtained was deposited in GenBank with the accession number given in the Results.

3. Results

3.1. Salmon

The salmon investigated in this study were between 43 and 102 cm long, but the majority of the fish (63.92 %) belonged to the length class 70-79 cm. Among analysed fish 66.29% were females (66 - 96 cm in length) and 33.71% males (43 - 102 cm in length). Length-weight relationfish was similar for males and females (Figure 1.). Clark's condition factor (K_c) was similar for both sexes: females (min = 0.754; max = 1.128; mean = 0.934) and males (min = 0.780; max = 1.131; mean = 0.944). Regarding age, two-year-old fish predominated (71.91%), one-year-old fish constitutes 15% of analysed individuals, three-year-old 8% and four-year-old 2%.

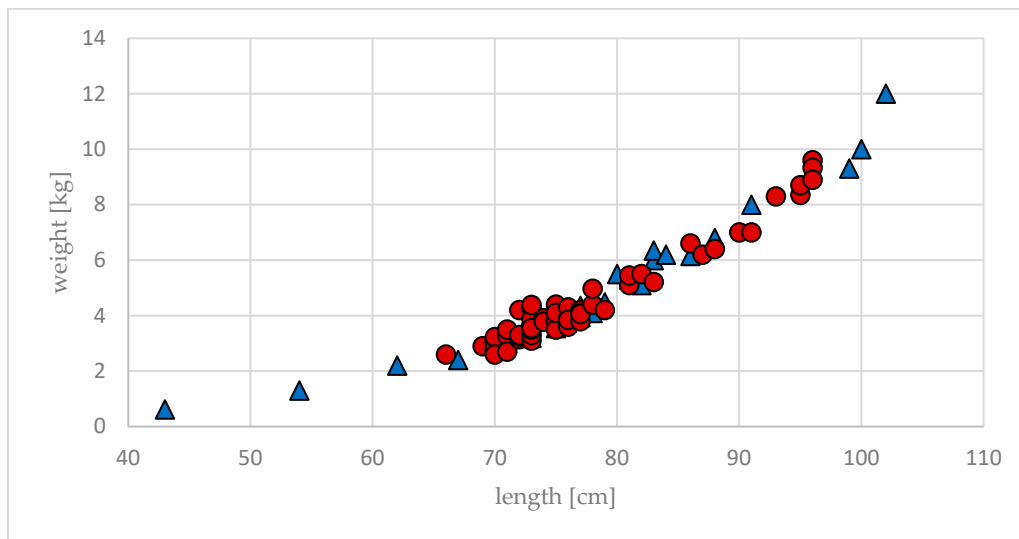


Figure 1. Length-weight relationship of analysed salmon (males - blue dots, females - orange dots).

During visual inspection of the body cavity of a single salmon, one Anisakidae nematode larva, attached to muscle tissue, was detected. The larva was identified based on anato-morphological features, and the prevalence of infection was 1.1% (intensity of infection = 1). *Pseudoterranova* sp. from Baltic salmon has been presented on Figure 2.

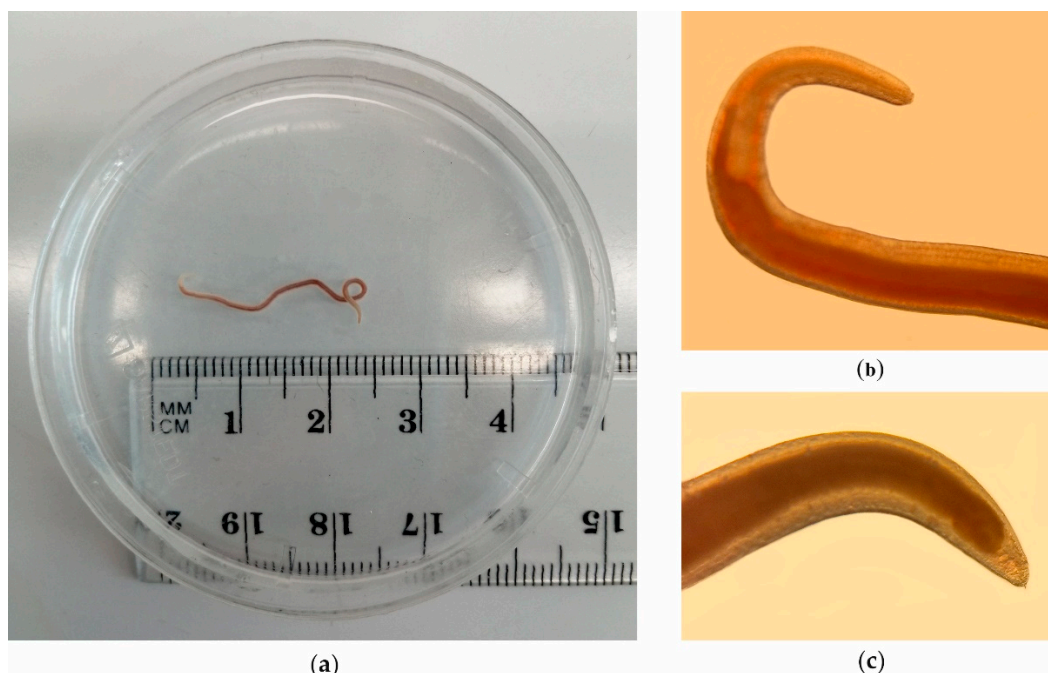


Figure 2. *Pseudoterranova* sp. from Baltic salmon (a) whole parasite; (b) anterior part (head); (c) posterior part (tail).

3.2. Molecular Identification

ITS-1 amplification and sequencing identified the larva as *Pseudoterranova* sp. The sequence has been deposited in GenBank with ID OQ732699. This is the first report of *Pseudoterranova* sp. in the muscle tissue of salmon from the Baltic Sea.

4. Discussion

Parasites, including *A. simplex*, *C. osculatum* and *P. decipiens*, found in fishery products represent a possible public health risk [48]. All the above species of nematode have been detected in

commercially important fish species, after harvesting for human consumption in Polish marine waters.

In this report, we describe a single example of a salmon that was infected with an Anisakidae larva attached to the muscle tissue. It is the first report of *Pseudoterranova* sp. in the muscle tissue of a salmon from the Baltic Sea. Attachment of the parasite to the muscle tissue in the body cavity indicates post-mortem migration of the larva. Presented studies were conducted in 2012, when the dispersion of Anisakidae nematodes in the southern Baltic Sea waters was not as widespread as today. Repeating the study in more recently caught salmon, with a focus on the presence of Anisakidae parasites not only in the body cavity, but also in the muscle tissue, would be justified, due to the high level of infection observed recently in other fish species caught in Polish marine waters. Salmon are diadromous fish, which spawn in the rivers, where they spend the first years of their life. They then undertake a migration to their feeding areas, which in the Baltic are located in the southern part of the sea, eastwards of Bornholm, where seals colonies occur. In the sea, salmon prey intensively on smaller fish, mostly sprat. It is at this time that salmon are exposed to infection with Anisakidae nematodes. Analysis of the livers of 120 salmon caught in Polish marine waters in 2020 revealed that 13% of them were infected with Anisakids, i.e. *C. osculatum* and *Pseudoterranova* sp. [16]. It was the first attempt to describe the level of liver infection in salmon caught in Polish marine waters (southern Baltic Sea).

The reports describing the infection of cod in Polish marine waters with Anisakidae nematodes date back to the 1970s. In 1974–75 the average prevalence of cod infection with *C. osculatum* was 28%, with intensities of 1–24, while the presence of *A. simplex* was recorded in the liver of cod in this area for the first time [49]. The studies conducted by [50] from 1988 to 1993 also indicate that nematodes were mainly found in the liver, predominately *C. osculatum* (the prevalence of infection ranged between 1.8 and 3.2% in cod sampled from several Polish fishing grounds), but also *A. simplex*. [51] investigated cod in the period 1987 to 1994 and revealed the presence of all three zoonotic Anisakids. The vast majority were *C. osculatum* (98% of larvae), but *A. simplex* and *P. decipiens* were also found (in 1 of 3036 fish examined). Overall, 4% of the cod sampled were infected with Anisakidae. [52] recorded the presence of *A. simplex* and *C. osculatum* in the liver of cod sampled in the Gulf of Gdansk and the Pomeranian Bay (the Western Baltic) between 2001–2003: the prevalence of infection with *A. simplex* larvae in cod ranged from 1.9% in the Gulf of Gdansk to 8.9% in the Pomeranian Bay, while with *C. osculatum* the prevalence was 9.8% and 2.5%, respectively. Studies on the presence of Anisakidae nematodes in cod liver conducted in 2011 revealed the presence of *Contracaecum* sp. (95% of collected nematodes), *Anisakis* sp. and *Pseudoterranova* sp.; the prevalence of cod infected with Anisakidae was 11% [11]. Recent results are alarming, indicating that the level of cod infection with *C. osculatum* extends throughout the entire southern Baltic, reaching 90% prevalence in ICES Subdivision 26 for length classes of cod 35 cm and greater, and showing an increasing trend for the period 1987–2019 [53].

In the case of herring from the Baltic Sea, *A. simplex* is the most common nematode parasite recorded. Studies conducted by [5] in 1970s indicated that more than 90% of herring caught in May in the Pomeranian Gulf (western part of Polish marine waters) was infected with this nematode. The highest prevalence of herring infection with *A. simplex* in the Polish EEZ, estimated using a generalized linear model, was in the period 1997–1999 (up to 97% in fish at length of 30 cm) [6]. The estimated year effect decreased markedly during the period 2013–2019, however, although the highest prevalence of infection was still >26% in herring caught in the first and second quarters of 2019 in coastal waters of ICES Subdivision 24 [53]. It is worth to emphasise, that there are a few herring stocks in the Baltic Sea and for decades the scientists stated that only western herring migrating out off the Baltic Sea might get infected with *A. simplex* larvae [5]. It was believed that herring can not get infected with that parasite in the Baltic Sea due to the lack of Euphasiacean intermediate hosts, lack of cetacean final hosts responsible for dispersion of the eggs of parasites in the water, too low salinity [5]. The results obtained by [54] indicated that infected fish represented a mixed group of western and central Baltic herring. Experiments conducted by [9] revealed that intermediate hosts are not indispensable in the life cycle of *A. simplex*. Moreover, nowadays due to the

presence of harbour porpoise and appearance of other visiting cetacean species in the western area of the Baltic Sea it is likely that fish might get infected with *A. simplex* also in these sea waters. Such possibility has been already suggested by [55], despite of the fact, that so far there was no evidence that *A. simplex* can complete the life cycle in the Baltic Sea.

Sprat from Polish marine waters was investigated between 1987 and 1990 for the presence of Anisakidae nematodes (3511 individuals analysed), but parasites were not detected [14]. In 2009 and 2011 in the Gulf of Gdańsk [56] found 2 of 187 sprats to be infected with a single *C. osculatum* larva each (prevalence 1.1%, mean intensity 1.0, abundance -0.5). In 2016, 2 of 30 sprats from the central offshore area of Polish marine waters were infected with *C. osculatum* (prevalence of infection in that specific area was 6.67%, and 2.22% across Polish marine waters as a whole; mean intensity was 1.0) [14].

Pilot studies conducted in NMFRI in 2012 on the presence of Anisakidae nematodes in flounder from the Gulf of Gdańsk did not reveal the presence of nematodes. Similar results have been obtained during studies conducted in 2016 on flounders from western, central and eastern parts of the Polish marine waters. In 2019 Anisakidae nematode *C. osculatum* has been detected in the livers of flounder [57].

The dispersion of zoonotic Anisakidae nematodes among marine organisms of the Baltic Sea has been reported over the last decade [8,13,16] and is also observed in fish caught in Polish waters. It is in line with the growing population of the grey seal, which is the final host in the life cycle of *C. osculatum* [21] and *P. decipiens* [23], to over 40,000 individuals in 2020 for the whole Baltic Sea [58]. Studies on the presence of nematodes in Baltic Sea seals (*H. grypus*, *P. vitulina* and *P. hispida*) reported that each seal was infected with Anisakidae nematodes, with one grey seal showing an intensity of infection of up to 590 nematodes [59]. It is worth emphasizing that *C. osculatum* and *P. decipiens* females are very productive: one female *P. decipiens* produces on average about 60,000 eggs, while a female *C. osculatum* produces on average about 9,000 eggs [60]. If we multiply the number of grey seals by the number of nematodes in each seal and by the number of eggs that might be produced by each mature female parasite, we end up with a extremely high load of nematodes shed into the environment. The presence of Anisakidae parasites in the food web of the Baltic Sea is not surprising, therefore.

However, if in the Baltic Sea the grey seal is the definitive host for *Contracaecum* and *Pseudoterranova* genera, the question arises why dispersion of only one parasite species - *C. osculatum* is currently reported in the area. It might be explained by several factors. First of all the demersal life cycle of *Pseudoterranova* spp. may not be conducive to dispersion of that genera in the Baltic Sea, that may be related with the lack of particular invertebrate species or limited number of intermediate hosts in particular area, needed in the life cycle of that parasite [17]. Dispersion of the *C. osculatum* might be related with the fact that free-swimming larvae do not need intermediate hosts to complete the life cycle, that has been proven by [24]. Moreover, *Pseudoterranova* spp. prefer water with high salinity [17], because low salinity limits the survival of the sealworm larvae [61] and occurrence of its the intermediate hosts [17]. Contrary, *C. osculatum* has adapted to the prevailing even in brackish water conditions of the northern Baltic Sea - Bothnian Bay [62]. Due to the mentioned reasons, the range of *Pseudoterranova* occurrence is limited mainly by low salinity in the Eastern Baltic Sea [63].

In case of representatives of *Anisakis* genera in the Baltic Sea low number of cetaceans - indispensable final hosts and low water salinity are pointed out as the most important factors limiting the dispersion [64]. According to [65] distribution of this parasite is restricted to south-western areas of the Baltic Sea.

The main source of fish infection with Anisakidae nematodes is infected prey. Studies conducted at the NMFRI revealed that cod might become infected with *A. simplex* via *Crangon crangon* and with *C. osculatum* via *Gammarus* sp. [66]. Fish feed on invertebrates, and fish like sprat are important elements in the diet of cod [67,68] and salmon [16]. Therefore, sprat might be a source of infection for predatory fish.

Furthermore, sprat, herring, cod and salmon are consumed by humans, when the presence of zoonotic nematodes in a raw or inadequately prepared fish meal might lead to acute anisakidosis or

an allergic response. Treatment to kill viable parasites in fishery products intended for human consumption is mandatory in many countries. Studies conducted at the NMFRI have shown that Anisakidae may survive freezing if it is performed incorrectly [69]; therefore, compliance with legal regulations regarding marine food treatment is crucial to avoid consumers suffering health problems.

In fact, most reported human case studies are related to the consumption of *A. simplex* larvae [12]. However, there are some examples of anisakidosis caused by *P. decipiens* [12,70,71]. Both nematode species prefer to localise to muscle tissue in their fish hosts, thus increasing the risk of infection with these nematodes. However, *C. osculatum* larvae, which prefer the liver of fish, also have zoonotic potential [12,72] and infections with this species have been recorded, possibly because fish liver might be consumed as well as muscle. In the past, most of the anisakidosis cases have been noted in countries where consumption of raw fish is common, therefore Asian countries especially Japan [12]. Recent reviews prepared by [73,74] show that allergic anisakidosis has been reported almost all over the world with the hot spot in northeastern Atlantic, where during nearly 40 years the average *Anisakis* spp. abundance increased more than 100 [64]. It is noteworthy that, although information on salmon infection with Anisakidae nematodes is scarce and the level of infection relatively low, there was a recently reported case of human anisakidosis in Poland caused by ingestion of viable *A. simplex* present in a meal of raw salmon [75]. Japanese cuisine is becoming more popular in many European countries, including Poland. With the increasing popularity of sushi and sashimi the incidence of cases of anisakidosis is expected to increase [12,76]. Moreover, [77] drew attention to the issue that lack of awareness related with parasitic diseases is associated also with developed countries. It was suggested that crucial for prevention of seafood-borne parasitic diseases are raising awareness, supporting research and the training of all stakeholders [77].

Presence of the parasite has negative impact not only on the consumers, but above all on the fish host. Numerous *A. simplex* larvae reported in Atlantic cod were responsible for severe inflammatory reactions with tissue deformation, including marked cellular infiltration in the stomach wall and mucosa [78] and the term "stomach crater syndrome" was applied for this pathological reaction [12]. High number of *A. simplex* larvae causing so called "red vent syndrome" (haemorrhages and inflammation around the vent) was reported in Atlantic salmon and sea trout returning to rivers in Scotland, England and Wales [79,80]. It was proven, that this nematode species provokes an inflammatory reaction in salmonids [81]. In case of *P. decipiens* encapsulation by host cells has been observed in case of cod muscle tissue [12]. However, its presence may affect the physiological state, health and survival of the host [12]. *P. decipiens* excretes substances that might be responsible for muscle contractility of fish, therefore reduce its swimming abilities and make easier the predation by marine mammals [12].

Negative impact of parasites on fish individuals may have consequences for the entire population. The negative effect of the presence of Anisakidae nematodes in commercially important fish stocks is most visible in the case of Baltic cod. Thus, a study conducted at the NMFRI [82] revealed that Anisakidae nematodes in the liver of cod negatively affects the condition of the fish, may increase natural mortality, and may be one of the reasons for the critical condition of the Baltic cod stocks.

5. Conclusions

The facts described above show that zoonotic Anisakidae nematodes are emerging pathogens for Baltic sea fish species and the humans that eat them. The level of infection with Anisakidae nematodes should be monitored, at least in commercially important fish species. It is also important to focus future studies on the presence of the parasites in edible parts of the salmon, due to the implications for the human health.

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Institutional Review Board Statement: The current study was carried out in strict accordance with the recommendations in the Polish ACT of 21 January 2005 of Animal Experiments (Dz. U. of. 2005 No 33, item 289) and the special permission is not required by local law for the carried-out experiments.

Informed Consent Statement: Not applicable.

Data Availability Statement: The presented in the current manuscript data are available from the corresponding author upon request. The sequences of the parasite is deposited in GenBank with ID OQ732699.

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