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***Contracaecum osculatum* and other anisakid nematodes in grey seal and cod in
the Baltic Sea: molecular and ecological links**

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Abstract

Populations of grey seal *Halichoerus grypus*, sprat *Sprattus sprattus* and cod *Gadus morhua* populations in the Baltic Sea are relatively stationary. The present work, applying classical and molecular helminthological techniques, documents that seals and cod also share a common parasite, the anisakid nematode *Contracaecum osculatum* which uses seals as final host and fish as transport hosts. Sequencing mitochondrial genes (COX1 and COX2) in adult worms from seals and third stage larvae from livers of the Baltic fishes (sprat and cod), showed that all gene variants occur in both seals and fish. Other anisakid nematodes *Pseudoterranova decipiens* and *Anisakis simplex* are also found in both seal and cod in the Baltic Sea but at much lower rates. The Baltic grey seal population was left at a critical low level (comprising a few hundred individuals) during the last part of the 20th century but from the year 2000 a marked increase of the population, reaching more than 40,000 individuals at present, has been observed. Ecological consequences of the increased seal abundance may result from increased predation on fish stocks but recent evidence also point to influence of elevated parasitism on fish performance. *C. osculatum* larvae preferentially infect Baltic cod liver, considered a vital organ of the host, but where low prevalences and intensities in cod were reported during the 1980s and 1990s the present study documents 100% prevalence and a mean intensity above 80 worms per fish at present. Recent studies have also indicated the zoonotic potential of *C. osculatum* larvae in fish following consumption of raw or under-cooked fish. Therefore the present work discusses the impact of parasitism on the cod stock, the increasing risk for consumer health and list possible solutions for control.

Introduction

The Baltic Sea is a semi-enclosed brackish water sea receiving high salinity water through the Danish straits from the North Sea. It is the largest brackish water system in the World and covers regions north of the polar circle to temperate areas. Precipitation and river inflows from catchment areas (Denmark, Sweden, Finland, Russia, Baltic republics, Poland and Germany) around the sea secure a continuous freshwater dilution whereby a salinity gradient is formed through the Baltic with high salinity water in west and low salinity water in the Northern and Eastern part. This brackish water zone is populated by local stocks of various species of teleosts and marine mammals including Atlantic cod (*Gadus morhua*) and grey seal (*Halichoerus grypus*). The pinniped population increased markedly since the year 2000 and reached between 30,000 and 40,000 individuals in 2014 (Helcom, 2016). During recent years also the main spawning ground of the local cod population, located immediately east of the island Bornholm, has been affected by the seals. The small islets Ertholmene, located next to the spawning zone, have been taken into use as haul out area for the grey seals and counts have increased from 1 seal in the year 2001 to 440 in 2014 which merely reflects the general population surge in the Baltic. The cod stock is influenced by a series of abiotic and biotic factors, including oxygen levels and food availability (Bagge *et al.*, 1994; Hüseyin *et al.*, 2016), but it is interesting that a fish catch decline has been observed concomitant with the marked seal population increase which suggests that the cod population also may be negatively affected by seals (Fig. 1). Foraging activities exerted by these marine mammals may play a role for the stock size (Chouinard *et al.*, 2005) as the daily fish intake for each seal may reach several kg. It is well known that seals do not merely target freely swimming fish but also fish immobilized in fishing gear. This has caused concerns of local fishermen as often 30-60% of the fish recovered from fishermen's gear carry marked damages. Thus, seals often peel of the skin, remove entrails partially or eat the entire fish body except the head. The most valuable fish species

affected are Baltic salmon (*Salmo salar*), Baltic cod (*Gadus morhua*) and sea trout (*Salmo trutta*) (Fig. 2 a-c). The grey seals have also been suggested to affect the local population of cod indirectly by increasing the infection pressure from anisakid nematodes which use seals as final hosts. Thus, two nematode species with suspected origin in seals, *Pseudoterranova decipiens* and *Contracaecum osculatum*, were recently found to infect Baltic cod at a surprisingly high infection rate (Buchmann & Kania, 2012; Haarder *et al.*, 2014). It is especially noteworthy that prevalence and intensity of *C. osculatum* larvae in cod have increased markedly since a low level in the 1980s (Haarder *et al.*, 2014; Szostakowska *et al.*, 2005) and the 1990s (Perdiquero-Alonzo *et al.* 2008) to high levels since 2010 (Mehrdana *et al.*, 2014, Nadolna & Podolska, 2014; Horbowy *et al.*, 2016) (Table 1). Parasite eggs from the adult nematodes in seal (Lunneryd *et al.*, 2015) are released to the sea with seal feces and following hatching larvae are believed to infect copepods. Fish such as sprat, which are feeding on copepods, obtain infection and cod may then take over the third stage larvae when ingesting sprat (Zuo *et al.*, 2016). A third species, *Anisakis simplex*, can also be found in Baltic seals but merely as immature individuals as the final hosts are cetaceans and not seals. Associations between seal abundance and anisakine infections of local fish stocks have been widely studied in other areas including Icelandic (Olafsdottir & Hauksson, 1997; 1998; Hauksson, 2002; 2011), Canadian (McClelland, 2002) and Norwegian (Jensen & Idås, 1992; Aspholm *et al.*, 1995) fish populations but precise estimates of the parasitic impact on the fish are missing. The negative associations between grey seal occurrence, *C. osculatum* infections and the Baltic cod population size are also weakly elucidated but the present study provides data supporting the notion that the recent increase of infection levels in Baltic cod by third stage larvae of *C. osculatum* is connected to presence of infected *H. grypus* in the area. Previous studies have applied molecular methods to establish connections between worms in seals and worm larvae in fish but the target sequences addressed were rDNA with lower resolution (Haarder *et al.*, 2014; Mehrdana *et al.*, 2014; Zuo *et al.*, 2016). In

the present study nematodes from seals, cod and sprat have been recovered, their DNA isolated and subsequently mitochondrial genes (COX1 and COX2) have been sequenced and compared in order to investigate the parasitic link between these hosts with higher resolution. Further, the impact of parasitism on the cod stock, including parasite-induced host mortality, and problems associated with the zoonotic potential of the nematodes are discussed based on recent literature.

Materials and methods

Fish

Baltic cod with total body lengths between 35 and 45 cm were captured by a local trawler in the Baltic Sea immediately east of the island Bornholm during January (n=20) and February (n=20) 2016. Fish were dissected within 1 h post-capture, livers were removed, placed in plastic bags and kept at <5 °C during transportation to the laboratory. Sprat (n=289) were captured by trawl in SD 25 as previously described (Zuo *et al.* 2016). Fish were frozen immediately after catch and brought to the laboratory where individual fish were dissected following thawing.

Seals

Two juvenile grey seals (110 and 165 kg body weight, respectively) were recovered in June and November 2014 by local fishermen in the western Baltic Sea. They were brought to the laboratory and kept frozen until autopsy.

Larval worm recovery from fish

Cod livers were individually incubated at 37 °C under constant stirring in artificial digestion fluid containing water, NaCl, HCl and pepsin prepared according to Skov *et al.* (2010). Following full digestion of fish tissue (2-3 h) the digest was poured through a sieve (mesh size 300 µm) whereafter live worm larvae were removed and placed in PBS for enumeration. Sprats were dissected and

viscera, including livers, removed whereupon all tissues were compressed in plastic bags and scrutinized under a dissection microscope. Nematode larvae, if present, were then isolated by forceps. All nematodes were rinsed in physiological saline and conserved in 96 % ethanol until processing for molecular work.

Adult worm recovery from seals

Autopsy of seals included a longitudinal section in the ventro-medial line whereby the stomach was exposed. Opening the stomach revealed numerous nematodes which were removed by forceps, rinsed in physiological saline and transferred to 96 % ethanol for further molecular identification.

Morphological identification

Frontal and caudal parts of the larval and adult nematodes were placed in clearing agent (Amann lactophenol, VWR, Denmark) for 5 days for clearing and were subsequently mounted in Aquatex® (Merck, Germany) on microscope slides. The nematodes were examined under the light microscope (Leica DM 5000 B, Germany) for genus determination (Mehrdana *et al.*, 2014). In addition, scanning electron microscopy was conducted on both larval and adult worms according to standard techniques. In brief, samples were dehydrated in series of graded ethanol (including 96% for 2x20 and 100% for 2x30 min). Samples were placed in 100% hexamethyl-disilazane (HMDS) for 15 min, transferred to a filter paper and allowed to dry overnight. The samples were then mounted on aluminum stubs, sputter-coated with gold-palladium in Polaron SC7640 Sputter coater (Quorum technologies, UK) and studied with SEM Quanta 200 (FEI, USA).

Molecular identification

Part of the middle section of each nematode specimen was transferred to 100 µl lysis buffer [Tween 20 (0.45%), Proteinase K (60 µl/ml), 10 mM Tris and 1 mM EDTA] at 55°C (450 rpm) in an

Eppendorf Thermomixer Comfort (Eppendorf AG, Hamburg, Germany). Incubation time varied but continued until complete digestion of nematode parts was achieved (confirmed by microscopy). Proteinase K was then deactivated at 95°C for 10 min where after the lysate was used for PCR amplification. PCR was performed in a Biometra T3 Thermocycler (Fisher Scientific, Denmark) using 60 µl reaction volumes. The reaction mixtures consisted of 6 µl lysate as template, 1 unit of BioTaq DNA polymerase (DNA-Technology), 1mM dNTP, 1.5 mM MgCl₂ and 1µM of the two primers. The primers for amplifying COX1 were CoOs_Mith_F3 (5' CTG TTA TTA CTG CTC ATG C -3') (this study) and CO2R1r (5' GCC GCA GTA AAA TAA GCA CGA GA-3') (Dzido *et al.*, 2012). The primers for amplifying COX2 were 211F (5'TTT TCT A TTA TAT AGA TTG RTT YA T-3') and 210R (5'CAC CAA CTC TTA AAA TTA TC-3') (Nadler & Hudspeth, 2000). PCR conditions for COX1 were 2 min of pre-denaturation at 94°C followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 15 sec, elongation at 72°C for 1 min, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 47° for 15 sec, elongation at 72°C for 1 min. PCR conditions for COX2 were 2 min of pre-denaturation at 94°C followed by 36 cycles of denaturation at 94°C for 30 sec, annealing at 46°C for 1 min, elongation at 72°C for 1 min 30 sec. A post-elongation step was performed for both COX1 and COX2 at 72°C for 10 min. Products were analysed by 2% ethidium bromide stained agarose gels. PCR products were purified using Illustra GFX™ PCR DNA and Gel Band Purification kit (VWR, cat. no. 28-9034-71) prior to sequencing at Macrogen Inc. (South Korea). Species identification was based on the sequences encoding COX1 and COX2.

Data analysis

Prevalence (percentage of the cod population infected), mean intensity (mean number of worms per infected fish) were calculated according to Bush *et al.* (1997). Differences between mean intensities in different size groups were evaluated by the Mann–Whitney U-test. Microsoft Excel 2007 and

SigmaPlot 12.5 were used for statistical calculations and a probability level of 5% was used for all analyses.

Phylogenetic analysis. In general the resources of the software package CLC Main Workbench v 7.7.2 were used. The sequences excluding the primer binding sites were aligned using Clustal W. In order to trim the alignment the web-based software Gblocks (Castresana Lab) was used but no matter which stringency levels that were used no blocks to be omit was identified. Four different methods (hierarchical Likelihood Ratio Tests (hLRT), Bayesian Information Criterion (BIC), Akaike Information Criterion (AIC) and Akaike corrected Information Criterion (AICc)) were used to test for the best model for the construction of phylogenetic trees. The tested models were Jukes-Cantor (JC), Felsenstein 81 (F81), Kimura 80 (K80), Hasegawa-Kishino-Yano (HKY) and General Time Reversible (GTR). The phylogenetic tree was achieved by using the model GTR+G+T as all four methods recommended it as the best choice. Bootstrap analysis was performed with 1,000 replicates.

Results

The investigated cod were infected by *C. osculatum* 3rd stage larvae both in January and February 2016. Prevalence was 100% in both months and the mean intensity 82.5 (SD 59.1) with a range from 5 to 377 worm larvae per fish. Sprats were less infected as merely 16% were infected and the intensity range was 1-13 *C. osculatum* larvae. The two seals were infected with 510 and 1100 specimens of nematodes in the stomach, respectively. Three species of nematodes *C. osculatum* (92%), *P. decipiens* (6%) and *A. simplex* (2%) were found but only *C. osculatum* was further treated in this work due to its dominance. Third stage *C. osculatum* larvae from cod livers showed morphological characters described by Fagerholm (1982). The frontal part contained an excretory pore anterior to the nerve-ring, an intestinal caecum and a ventricular appendix. Using scanning electron microscopy the frontal boring tooth and the tapering caudal end without appendages

(mucron) were evident (Fig. 3A and B). Adult *C. osculatum* nematodes exhibited in SEM the characteristic labia in the frontal part and a tapering caudal as described by Krabbe (1878) in his line drawings (Fig. 3C and D). Representative worm samples from all hosts were treated. Of adult *C. osculatum* nematodes recovered from seals 11 were analyzed for COX1 and 19 for COX2. Of third stage larvae from cod 10 specimens were analyzed for COX1 and 18 for COX2. A total of 11 larvae from sprat was analyzed for COX1 and 23 for COX2. Sequencing mitochondrial genes in *C. osculatum* larvae obtained from both cod and sprat and corresponding genes from adult worms recovered from grey seal stomachs demonstrated that the same genetic variations of COX1 and COX2 were found in all three hosts (Fig. 4).

Discussion

A series of studies based on classical methodology have previously reported that grey seals and Baltic fishes are parasitized by the anisakid nematode *C. osculatum* (Fagerholm, 1982; Valtonen *et al.*, 1988). However, morphological identification of *C. osculatum* adults and larvae is not adequate for a full linkage of the different stages in the life cycle and molecular tools may be a necessary supplement (Mattiucci *et al.*, 1998; Mattiucci & Nascetti, 2007; 2008). Some target sequences may be more informative than other. Previous studies on rDNA (ITS region sequences) of *C. osculatum* larvae isolated from cod and sprat (Haarder *et al.*, 2014; Mehrdana *et al.*, 2014; Zuo *et al.*, 2016) showed full similarity to corresponding ITS sequences presented from seals by Skrzypczak *et al.* (2014) but the variability within this region may be too low to obtain the needed differentiation. Mitochondrial gene variations have to be analyzed to obtain higher resolution (Nadler & Hudspeth, 2000; Dzido *et al.*, 2012). The present investigation on worm samples recovered during recent years from grey seals, cod and sprat revealed that *C. osculatum* third stage nematode larvae in Baltic fish livers carry the same genetic variations of mitochondrial genes as adult nematodes found in the stomach of grey seal from the Baltic. We included sequencing of mitochondrial genes (COX1 and

COX2) showing higher variability and suitability for differentiation of subpopulations. All molecular variants occurred in all three host species with the same frequency. This strongly supports the notion that the life cycle includes seals as final hosts with cod and sprat as transport hosts as suggested by recent work (Haarder *et al.* 2014, Nadolna & Podolska 2014, Mehrdana *et al.* 2014; Horbowy *et al.* 2016; Zuo *et al.* 2016). It will further support the impression that the recent build-up of *C. osculatum* infection in Baltic cod is caused by the massive grey seal population increase recognized during the latest two decades. Laboratory life cycle studies on *C. osculatum* performed by Kjøie & Fagerholm (1995) suggested a series of invertebrates and vertebrates to serve as hosts in the life cycle. The present study, based on local samples from the Baltic Sea, demonstrates that Grey seal acts as final host with stomach location of adult nematodes which is in line with earlier investigations (Lunneryd *et al.*, 2015; Skrzypczak *et al.*, 2014). Sprat can serve as transport host and may play an important role in transmission of worms to cod. This is emphasized by the rather high prevalence and intensity recorded in this fish species which is taken as prey by larger cod (Zuo *et al.*, 2016). Sprat itself probably achieves infection during feeding on various species of copepods and cladocerans as these crustaceans are the main food items of sprat (Casini *et al.*, 2004). It was recently shown that cod with body lengths below 30 cm merely have light or no *C. osculatum* infection whereas cod larger than 30 cm become significantly infected probably due to sprat feeding (Zuo *et al.*, 2016). The present study indicated that the infection level of cod with this worm species is increasing compared to recent studies (Haarder *et al.*, 2014; Mehrdana *et al.*, 2014) – and even in relatively small cod with body size between 35 and 45 cm. The lack of infection in very small cod with body lengths below 30 cm (Zuo *et al.*, 2016) is noteworthy due to the fact that cod below 30 cm have a high survival whereas cod above 38cm seems to have a low survival (Eero *et al.*, 2015). Thus, the over-all population size of eastern Baltic cod population has been under pressure during the latest decade. Disappearance of larger cod with body lengths above 38 cm -

despite successful recruitment of young cod (Eero *et al.*, 2015) - has remained un-explained. Interestingly, the considerable decline of populations of larger cod is associated with a rapid build-up of the *C. osculatum* in livers of this size-class of cod. The present study therefore confirms observations by other authors (Haarder *et al.*, 2014; Mehrdana *et al.*, 2014; Horbowy *et al.*, 2016, Zuo *et al.*, 2016) and adds to the notion that parasite induced host mortality may explain the problem. Impact of parasitism on health and survival of wild fishes is on the other hand difficult to prove. Early studies by Petrushevski & Shulman (1955), conducted at a time when the seal population in the Baltic was high in the 1940s, reported heavily *C. osculatum* infected cod in the Baltic associated with decreased physiological performance. Correspondingly, Mehrdana *et al.* (2014), during the recent seal population surge, documented haemorrhagic cod livers with decreased weight related to high *C. osculatum* infections and Horbowy *et al.* (2016) indicated parasite induced host mortality of larger cod due to the high infection rate in these fish. Worm effects on hosts can be exerted through various mechanisms including direct penetration of tissue. The related anisakid *A. simplex* has been associated with severe inflammatory reactions and haemorrhages in salmonids (Beck *et al.*, 2008; Noguera *et al.*, 2009) and cod (Levsen & Berland, 2012). In addition, numerous molecules are released from parasites and the anisakid nematodes. *P. decipiens* produce pentanols and pentanones with a putative anaesthetic effect on cod musculature (Ackman & Gjelstad, 1975). It is therefore noteworthy that field observations have linked this nematode with impaired swimming ability (Sprengel & Lüchtenberg, 1991) and decreased survival in European smelt (Rohlwing *et al.*, 1998). Likewise, Bahloul *et al.* (2013) indicated an immunosuppressive effect of *A. simplex* excretory and secretory products which suggests that worms affect the host through a spectrum of mechanisms. The Baltic food web comprising copepods, sprat and herring is also utilized by other predators including Baltic salmon *Salmo salar*. Therefore it could be expected that the increased *C. osculatum* infection noticed in cod during the

last decade could be found in salmon as well. No thorough recent parasitological investigations have been performed on Baltic salmon during the latest decade and this question remains unsolved. However, controlled experimental *C. osculatum* infections of another salmonid, *Oncorhynchus mykiss*, have been conducted (Smith *et al.*, 1990) but it was shown later that this third stage nematode has a relatively low survivability in this host (Haarder *et al.*, 2013). Although anisakid nematode larvae generally exhibit low host specificity variations with regard to susceptibility may exist. Hence it can be expected that infection pressures exerted on Baltic fishes due to increased spreading of parasite eggs from seals may affect different fish stocks differently. Combined laboratory and field studies should therefore be conducted in order to elucidate these dynamic interactions in the Baltic food web.

C. osculatum larvae in fish products also represent a zoonotic problem if fish products are ingested without prior processing. Larval invasion of the human gastro-intestinal tract may occur corresponding to problems with e.g. cod worm *P. decipiens* (Margolis, 1977; Skirnisson, 2006; Torres *et al.*, 2007). Thus, several reports have shown that *C. osculatum* larvae elicit a severe and painful condition in human consumers following ingestion of raw or under-cooked fish carrying third stage larvae of this species. Cases were described from the Baltic region (Schaum & Müller, 1967), from Australia (Shamsi & Butcher, 2011) and from Japan (Nagasawa, 2012). Controlled experimental infections of pigs confirmed the infectivity of *C. osculatum* larvae from Baltic cod livers to pigs and their ability to penetrate the stomach mucosa and elicit eosinophilic granulomas (Strøm *et al.*, 2015). Therefore the societal problems with worms from seals in the Baltic Sea involve both the fish stock stability and consumer safety. Direct intervention comprising regulation of the seal population by hunting, culling or targeted seal fishery will be a solution but may conflict with the protected status of grey seals. Alternative solutions to reduce the seal abundance could involve prevention of their reproduction by hormonal administration or immunization of seals

against their own reproductive molecules. On a theoretical basis it could be suggested to treat seals with anthelmintics in order to reduce the worm burden and subsequent spreading of infective parasite eggs in the marine environment. The practical implementation of this method may be difficult and its environmental aspects remain questionable due to release of drugs in natural animal populations.

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Baltic cod (*Gadus morhua*) associated with differential food preferences. *Diseases of Aquatic Organisms* **120**, 69-75.

Figure legends

Fig. 1. Grey seal population increase in the Baltic Sea during 2000 to 2014. No. of seals in thousands. Weight of corresponding annual catches of Baltic cod in the same period are shown (in 1000 metric tonnes). Based on data from Helcom (2016) and ICES (2015). Number of seals: \circ , Cod catches: \diamond

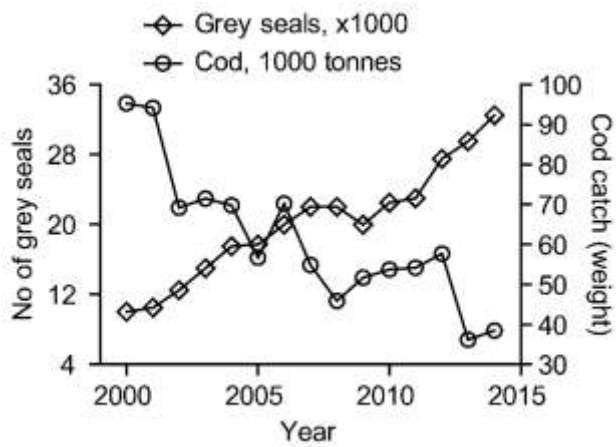


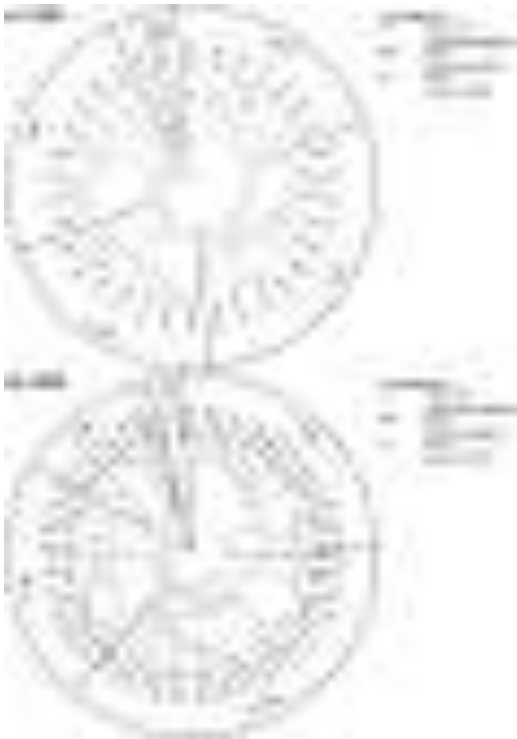
Fig. 2. Fish from the Bornholm Basin on fisherman’s hooks treated by grey seal. A. Baltic salmon (December 2013), B. Baltic cod (October 2013), C. Sea trout (September 2013)



Fig. 3. Scanning electron microscopy (SEM) of *Contracaecum osculatum* third stage larva (A and B) from liver of Baltic cod and adult specimens from grey seal stomach (C and D).



Fig. 4. Cladograms showing similarities of *Contracaecum osculatum* sequences of mitochondrial genes (COX1 and COX2) from adult worms and third stage larvae recovered from seals, cod and sprat, respectively. *P. decipiens* sequences were used as outgroups. The capital letters S, SP and C indicate the hosts. For each gene four clades were defined. Each of these clades contains parasites from all the three hosts.



Tables

Table 1

Temporal and spatial occurrence of *Contracaecum osculatum* in Baltic cod during three decades.

ND: No data

Year	1982	1987-93	2003	2012	2014
Prevalence	22%	ND	53.9%	55.1%	100%
Southern Sweden and Bornholm	Haarder <i>et al.</i> , 2014		Perdiquero-Alonzo <i>et al.</i> , 2008	Haarder <i>et al.</i> , 2014	Mehrdana <i>et al.</i> , 2014
Prevalence Polish EEZ Southern Baltic	ND	2.6% Szostakowska <i>et al.</i> , 2005	ND	Up to 80% Nadolna & Podolska, 2014 Horbowy <i>et al.</i> , 2016	