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Prevalence of *Ichthyophonus sp.* in yellowtail flounder sampled during the seasonal bycatch survey on Georges Bank

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ABSTRACT

The parasitic protozoan, *Ichthyophonus sp,* is a growing concern for the Georges Bank stock of yellowtail flounder (*Limanda ferruginea*), due to the vulnerable condition of this stock. Yellowtail biomass estimates for Georges Bank continue to decrease resulting in stringent regulations in the scallop industry that reduces yearly allowable catch. Recent stock assessments have identified high natural mortality estimates with no known cause. *Ichthyophonus sp.* has been identified as the causative agent in mass mortality for several fish species in Atlantic waters over the past century. Yellowtail flounder were randomly selected for at-sea examination during a seasonal bycatch survey aboard scallop fishing vessels on Georges Bank every six weeks starting June 2012. After capture, the peritoneal and pericardial cavities of each fish were opened and examined macroscopically for any observable abnormalities. Each abnormality was noted, imaged and preserved in 10% neutral buffered formalin for histological evaluation. The majority of yellowtail flounder sampled in this study hosted a variety of parasites including *Ichthyophonus* species as well as nematodes and cestodes. The individual fish with macroscopic signs of *Ichthyophonus* were severely infected with multifocal organ involvement characterized by abundant organisms causing necrosis and surrounded by granulomatous inflammation. Histological evaluations of tissues suggest that *Ichthyophonus* may spread quickly through tissues causing significant damage and resulting in direct mortality or indirect mortality due to dehabilitation. During this study yellowtail flounder had a prevalence of 2.55%. The work will continue to monitor changes and identify potential epizootic events.

Introduction

Ichthyophonus hoferi, a protozoan parasite, has been identified as a cause of disease in over 100 species of marine, fresh, and brackish teleost fish as well as reptiles and crustaceans since its original classification in 1911(Rahimian 1998, Rand 1992). *Ichthyophonus hoferi* causes systematic infection in Atlantic herring and other fish resulting in tissue granulomatosis and necrosis of vital organs (Fish 1934, McVicar 1984, McVicar & Mclay 1985, Rahimian 1998). *Ichthyophonus sp.* was first observed in yellowtail flounder (*Limanda ferruginea)* sampled near Western Sable Island, off Nova Scotia in 1966 (Powles *et al*. 1968). The samples of yellowtail flounder collected off of Canada showed prevalence of *Ichthyophonus* ranging from 2.8% to 57.4% however the locations of high infections were relatively small and limited to the area off of Sable Island (Ruggieri *et al.* 1970) Molecular analysis of infected tissues from yellowtail flounder collected on Brown's Bank, Nova Scotia in 1987 showed the infection was caused by a new species, *Ichthyophonus irregularis* which has only been identified in yellowtail flounder (Rand 1994, Rand *et al.* 2000). *Ichthyophonus sp.* infection is known to be either lethal or debilitating in many fish species (Rahimian 1998, Vollenweider *et al.* 2011). Laboratory studies have observed weakened responses, swimming abnormalities, loss of pigment, and swelling of the abdomen in herring, salmon and trout with *Ichthyophonus* (Kocan *et al.* 2009. McVicar 1984, Vollenweider *et al.* 2011).

Ichthyophonus outbreaks have resulted in epizootics of several fish species in the northwest Atlantic, but the cause resulting in enzootic levels to progress to epizootic levels is uncertain (Kramer-Schadt et al 2010, Lauckner 1984, McVicar 1981, Petterson 1996 Sindermann 1958,). The infection can present itself either as chronic or acute with no clear understanding of the differences that resulted in either appearance (Sindermann 1966). Chronic infection was associated with levels of enzootic prevalence less than 1% with progressive connective tissue encapsulation of the *Ichthyophonus* spores. Even in the chronic condition, infection was rarely fully controlled by the host and deaths occurred within six months. Acute infections were noted as severe tissue invasion and necrosis leading to death within 30 days and was usually associated with epizootic mortality events (Sindermann 1966). Low prevalence rates in a population can also have significant effect on a fish stock. Rahimian and Thulin (1996) reported a prevalence of 2.4% in Atlantic herring (*Clupea harengus*) by *I. hoerferi* resulting in the estimated mortality of 220 million herring off of Norway. Even low prevalence has been suggested to lead to high fish mortality due the quick progression of the infection (Mellengaard & Spanggaard 1997). Pathogenicity is believed to vary according to host species, environmental conditions and the species of *Ichthyophonus* (Kocan *et al* 2009, Mellergaar & Spanggaard 1997).

Yellowtail flounder is a commercially important species in New England waters and is a limiting bycatch species in many fisheries including the sea scallop fishery (O'Keefe & DeCelles 2013). Yellowtail flounder are distributed from the Chesapeake Bay to the Gulf of St. Lawrence (Bigelow & Schroeder 1953). The estimated spawning stock biomass of Georges Bank yellowtail flounder has significantly decreased from 21,000 mt in 1973 to 869 mt in 2012 (Legault *et al.* 2013). Fishing pressure has lessened over the past decade, however total mortality continues to increase (Legault *et al.* 2013). Natural mortality is one potential explanation of increased total mortality. This study investigates a potential new source of natural mortality in this stock.

Methods/Results

Yellowtail flounder were sampled during a seasonal bycatch survey aboard commercial scallop vessels on Georges Bank every six weeks starting June 2012. The results presented in this report used data collected from June 2012 to January 2014 (n=1256). Samples were collected by two standardized 4.57m (15ft) wide scallop dredges towed simultaneously at a target speed of 4.8 knots at fixed stations on Georges Bank (Figure 1). Yellowtail flounder were randomly selected from the catch throughout the survey area for at-sea examination. After the yellowtail were sorted from the catch they were weighed and measured. The peritoneal and pericardial cavities of each fish were opened and macroscopically examined for abnormalities. Each abnormality was noted, photographed and tissues were fixed in 10% neutral buffered formalin for histological evaluation.

The fish were classified into three groups based on macroscopic appearance: no observable abnormalities, macroscopic signs of *Ichthyophonus*, and lesions not identified as *Ichthyophonus*. Presence of *Ichthyophonus* was identified by characteristic off-white cysts and a whitish sheen to the serosal surface of the peritoneal organs or heart (Figure 2; Fish 1934, McVicar 1982). Other lesions not identified as *Ichthyophonus* were also collected for histological examination. Tissues from animals with no observable lesions were not collected.

Fixed tissues were trimmed and transferred into 70% ethanol solution, embedded in paraffin, and 6µm-sections were cut and stained with hematoxylin and eosin. Resulting slides were evaluated histopathologically.

Results

Macroscopic examination (n = 1257) and histological (n=380) evaluation indicates that 26.7% of the animals examined were free of all tissue abnormities. 70.7% of all fish sampled had lesions, primarily due to other types of parasitic infection, but no signs of *Ichthyophonus*. Macroscopic signs of *Ichthyophonus* were seen in 2.55% of the fish sampled with no clear indication of differences in infection rate between sizes (Figure 3)

Ichthyophonus infection was apparent upon opening the peritoneal cavity and, in many cases, there was peritonitis characterized by flocculent to fibrillar white material overlying organs and the serosal surfaces and by loose fluid that had enlarged the abdominal cavity. In all cases where macroscopic signs of *Ichthyophonus* infection were observed, *Ichthyophonus* nodules were embedded in the surface of the liver and in the parenchyma and were characterized by small (1mm) firm white, clear, or yellowish nodules. Even in low to moderate infections, the individual foci were distinct in appearance and were identifiable) as *Ichthyophonus sp*. infections (Figure 4). In severe cases of infection, *Ichthyophonus* had resulted in confluent nodular inflammation on the serosal surface and in the parenchyma leaving little intact hepatic tissue (Figure 5).

Histological examination showed the most likely route of infection is through the intestinal wall probably after the spores are activated by the low pH of the stomach. *Ichthyophonus* was noted in a vessel in the intestinal wall. Other organisms were noted in vessels of the attached mesenteries which drain into the hepatic portal veins. *Ichthyophonus* is first identified histologically in the liver parehchyma and serosa. The organism most likely travels though the blood to the heart from the liver since if the heart was infected so was the liver. Severe necrotizing granulomatous myocarditis accompanied by severe diffuse granulomatous epicarditis and pericarditis (Figure 6) was noted in almost all such individuals and was consistently associated with abundant, often budding, *Ichthyophonus* cells occasionally leading to such severe lesions that it became difficult to identify the heart tissue (Figure 7). In some cases, the parasite caused severe infections resulting in diffuse filling of the pericardial sac with granulomatous inflammation and parasites restricting the heart function

In fish with severe systematic *Ichthyophonus* infection, the kidneys were swollen and the parenchyma contained many small, white, firm nodules (Figure 8). Nodules were present in the lumen and leaflets of the gonads. Testes often had a higher abundance of nodules than ovaries. *Ichthyophonus* was identified essentially in all remaining tissues of severely affected animals including the stomach, intestine, spleen, muscle, and the brain. Macroscopic identification of *Ichthyophonus* in the brain was nearly impossible due to the similarity between the appearance of nodules and the cerebral tissue.

In almost all animals, mature adult *Anascaridoidoiea* worms were commonly visible on the serosal surface of the liver (Figure 9). *Anascaridoidoiea* larvae were macroscopically identified in small (1mm) white or clear nodules attached to the serosal surface of the liver, intestine and throughout the mesentery and were confirmed with histology, (Figure 10). In some cases localized to extensive subacute peritonitis was associated with infection.

In many cases multiple large cestode cysts characterized by round white 2-3mm capsules were observed in the mesenteries, on the serosa and embedded in the intestinal walls and stomach. Cestodes were noted histologically to be in the tunica muscularis or submucosa) of the organs and occasionally appeared to cause obstruction or focal enteritis (Figure 11. Cestodes in these capsules are most likely the previously reported *Rhynchobothrium imparispine* (Linton 1901). Trematodes were also identified microscopically throughout the mesentery and in cysts attached to the serosal surfaces of the liver and intestine (Figure 12). These cysts were characterized macroscopically by white gelatinous lesions with irregular attachments sometimes embedded deeper in the organs' tissues underlying the serosal surfaces. Small nematode larvae of unknown type were noted microscopically primarily in stomach tissues and resulted in subacute to chronic granulomatous mild as well as severe focal enteritis.

Discussion/Conclusions/Summary/Recommendations

Ichthyophonus was first described in yellowtail flounder in 1968 off of Canada and has only been reported in Canadian waters since then (Powels et al. 1968, Rand et al. 2000). It is unknown if this disease was present, but unreported in U.S. waters before this study, or if *Ichthyophonus* has recently become more prevalent in Georges Bank yellowtail flounder. During this study we observed 2.55% of sampled yellowtail to be infected with *Ichthyophonus* with monthly averages ranging from 0 to 8.4%. Continued sampling in 2014 will determine whether prevalence of infection is changing over time.

The focus of this study was to estimate the prevalence of *Ichthyophonus* in yellowtail flounder within the sample area on Georges Bank and to observe its pathological effect on yellowtail tissues. Genetic analysis is underway to determine if the observed species of *Ichthyophonus* is the ubiquitous species*, I. hoferi, or* the relatively recently described species *I. irregularis,* or a new species yet to be classified. *Ichthyophonus irregularis* has not been identified in any host other than yellowtail flounder. With molecular tools and more current genetic information, it is possible that many species previously identified as *I. hoferi* could have been misidentified as a different species of *Ichthyophonus* which could explain the variability of *Ichthyophonus* reported between hosts and environmental conditions (Rand et al. 2000).

Past studies have demonstrated that the pathogenicity of *Ichthyophonus* is highly variable depending on different hosts and environmental conditions (McVicar 1981, Kocan et al 2009, Mallergaar & Spanggaard 1997). Further research is needed to determine whether *Ichthyophonus* invades yellowtail flounder though consumption of an intermediate species or through ingestion of substrate. *Ichthyophonus* has been observed in many intermediate prey species including crustaceans and copepods, all which are part of the diet of yellowtail flounder (Rahimian and Thulin 1996). The data collected is not appropriate to identify the source of *Ichthyophonus* nor the abundance of the source. The infection had no apparent spatial pattern. The infection rates seemed to randomly vary between trips from 0 to 8.43%, but more data is needed to determine whether there is a seasonal pattern in infection rates (Table 1). Infection does not appear to be spatially specific, since it was randomly detected throughout the study area (Images 13-20).

The level of tissue damage observed in combination with no visual evidence of recovery from *Ichthyophonus* leaves little doubt that this infection is lethal in yellowtail flounder. However time until death and the timeline for disease progression have not been demonstrated for yellowtail flounder. During a trial experiment five yellowtail flounder were necropsied 127 days after being injected with 1000 spores of *Ichthyophonus hoferi* collected from diseased wild yellowtail flounder (Rand *pers com*). All fish showed lesions on the liver, heart, spleen kidney and intestine identical to lesions in infected wild-yellowtail (Rand *pers com*). This trial study was done with a small sample size from fish collected in the early 90's before *Ichthyophonus irregularis* was identified in yellowtail flounder. It has not been repeated with *I. irregularis* which is believed to have different pathogenicity in yellowtail flounder (Rand 1994, Rand *et al.* 2000). The reported time until death is rapid in other host species with time until death ranging from 7 days to a few months (Table 2; Kocan et al. 2009, Mellergaard & Spanggaard 1997, Oskarsson & Palsson 2011).

The timeline for disease progression is necessary to determine the overall effect of the infection. During years when *Ichthyophonus hoferi* was identified in herring off of Iceland, an estimated time of death of 105 days was used to calculate increased natural mortality due to infection, (Anonymous 1993, Mellergaard & Spanggaard 1997, Oskarsson & Palsson 2011). Estimated annual mortality can be calculated where;

$$\textbf{Annual mortality} = \frac{(\textbf{Prevelance})(\textbf{Days in a year})}{\textbf{Time Until Mortality}}$$

This formula is dependent on an accurate value for the disease progression and assumes that infection rate and time until death are spatially and temporally constant. The variability of infection rate between species and environmental conditions confirms that it is important to study the progression of infection on a case by case basis (Mellergaard & Spanggaard 1997). Since dependable information on disease progression is missing for yellowtail flounder, it is difficult to determine the implications of the observed prevalence level.

Identification of infected individuals using visual and histological identification, could underestimate the number of samples with low infection levels (Kocan & Hershberger 2011). To account for the possibility of misidentifying low infections during this study each tissue abnormality was processed for histology. However, if no tissue abnormalities were visible then there would be a very slim chance that *Ichthyophonus* would be identified if present. Low infections with few *Ichthyophonus* nodules on the liver were identified macroscopically and collected for confirmation by histology, but during the early stages of *Ichthyophonus* the infection can be cryptic. Extensive culturing of tissue would have to be completed to detect prevalence of very early stages of infection. However, early infection stages may be very brief. In other species of fish, *Ichthyophonus* was microscopically identified penetrating the stomach epithelium after two days and caused massive secondary infection after 8 days, resulting in visible nodules (McVicar 1982).

Preliminary analysis indicates a 2.55% infection level of *Ichthyophonus sp.* in yellowtail flounder within our sampling location. Further research is needed in order to identify the species as well as to estimate progression rate of the infection and possible implications for Georges Bank yellowtail flounder.

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Tables

Table 1 Rates of infection for *Ichthyophonus* by month (number of infected fish per number of fish sampled)

	2012	2013	2014
Jan		2.74% (2/73)	0.93% (1/108)
Feb			
Mar			
Apr		1.28% (1/78)	
May			
Jun	6.02% (5/83)	2.94% (3/102)	
Jul		0.95% (1/105)	
Aug	1.32% (1/76)	2.10% (2/95)	
Sep	2.20% (2/91)	4.95% (5/101)	
Oct		2.35% (2/85)	
Nov	8.43% (7/83)		
Dec	0.00% (0/78)	0.00% (0/97)	

Table 2 Summary table of effects of *Ichthyophonus hoferi* on various host species from literature. These studies were done using *I. hoferi* in various hosts under different conditions, the parasite observed in this study was likely a different species of *Ichthyophonus*

Host species	Fastest time until mortality	Average Time until mortality	Comments	Citation
Yellowtail Flounder	Unknown	Unknown	5 fish Injected with *I hoferi,* necropsied after 127 days showing significant lesions	Rand *pers com*
Pacific Herring	7 days	36 days	80% Killed after 2 months	Kocan *et al.* 1999
Rainbow trout	10 days	13.4 days	Higher water temp results in faster disease progression	Kocan et *al* 2009
Atlantic Herring		105 days	3.6% prevalence resulting in 12.5% annual mortality	Anonymous 1993
Atlantic Herring	~30 days	~180 days	Difference in time until death between acute infection and chronic infection	Sindermann 1966

Figures

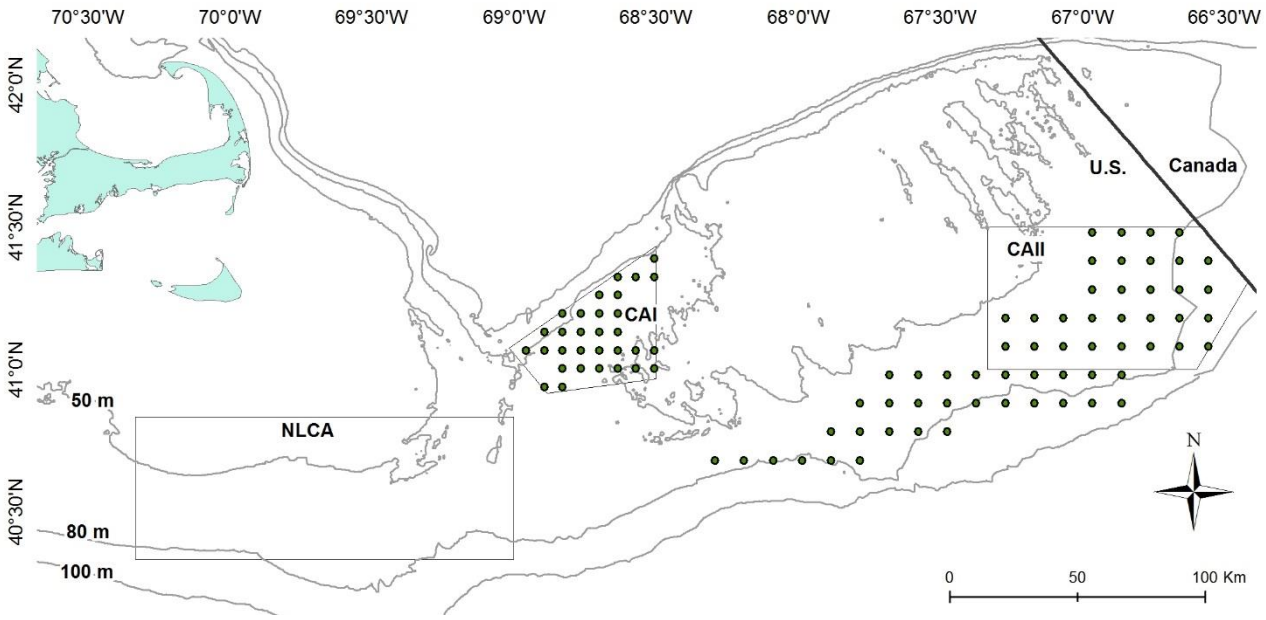

Figure 1 Map of sampling locations for the Bycatch survey. During the 2012 funding year 16 stations in the open area were not sampled.

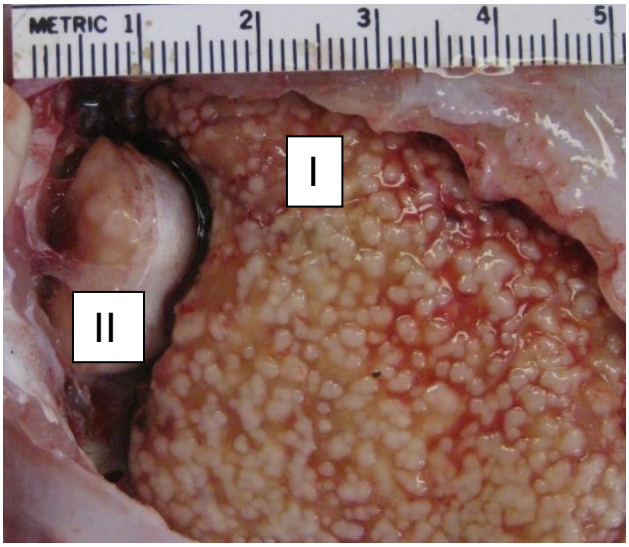

Figure 2 Characteristic macroscopic hepatic lesions of *Ichthyophonus* of a heavily infected yellowtail flounder. The serosal surface of the liver is covered with small firm white, clear or yellowish nodules (I). Also note the severe epicarditis and pericarditis of the heart (II).

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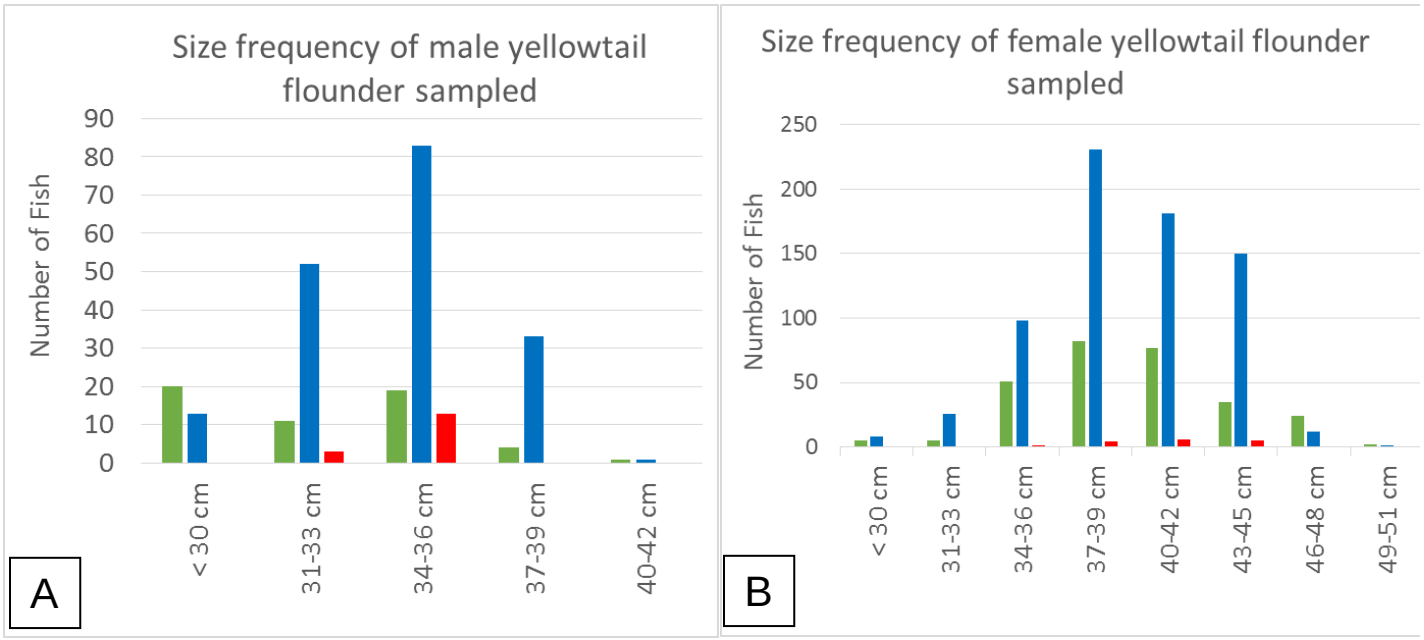

Figure 3 Size frequency of all fish sampled with males (A) and females (B). Fish with no visible lesions are in green (26.7%), Samples which had various parasites not including *Ichthyophonus* are in blue (70.7%) Fish infected with *Ichthyophonus* are in red (2.55%)

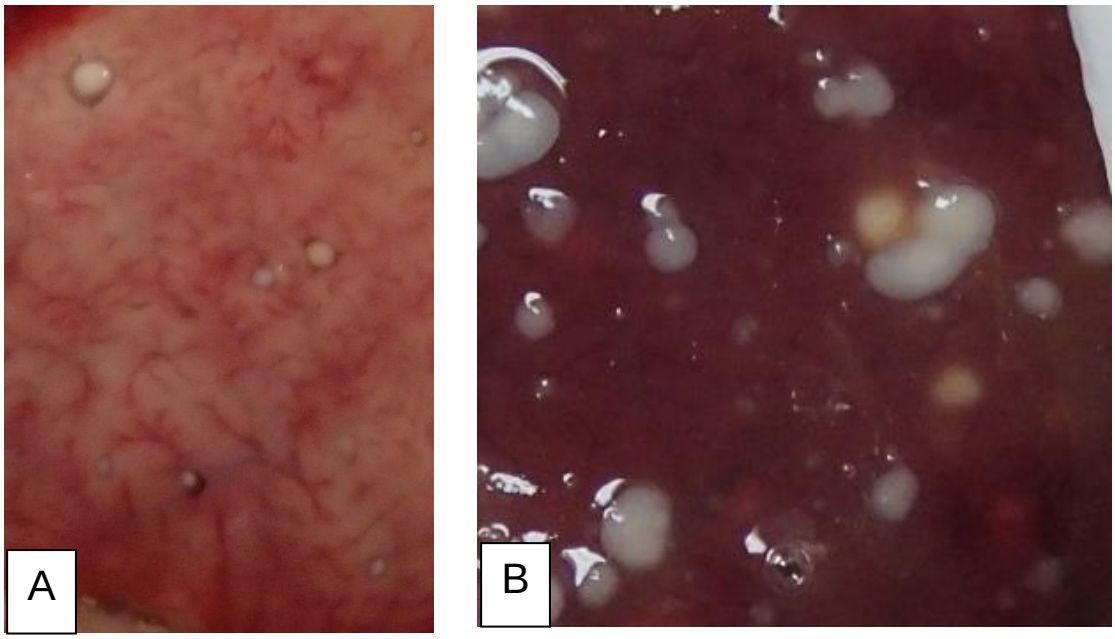

Figure 4 Low (A) and moderate (B) infections of *Ichthyophonus* in the liver of yellowtail flounder. The *Ichthyophonus* nodules are disperse throughout the serosal surface

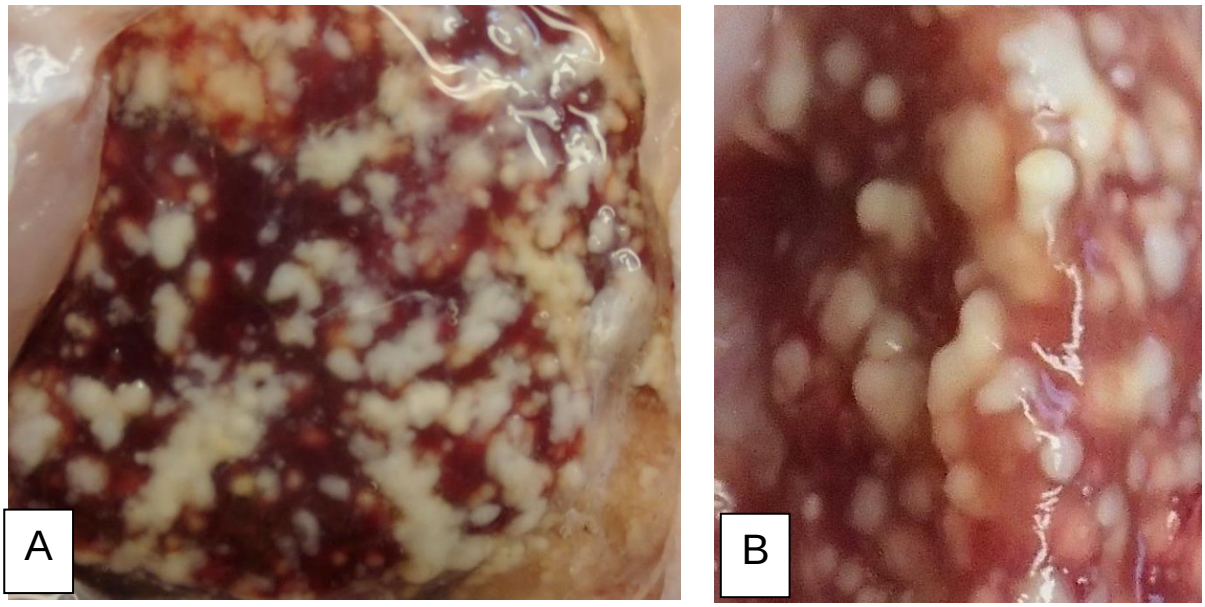

Figure 5 Severe infection of *Ichthyophonus* in the liver of yellowtail flounder. The serosal lesions of *Ichthyophonus* are multilobulated and extensive on the serosal surface (A) and continue into the hepatic parenchyma (B)

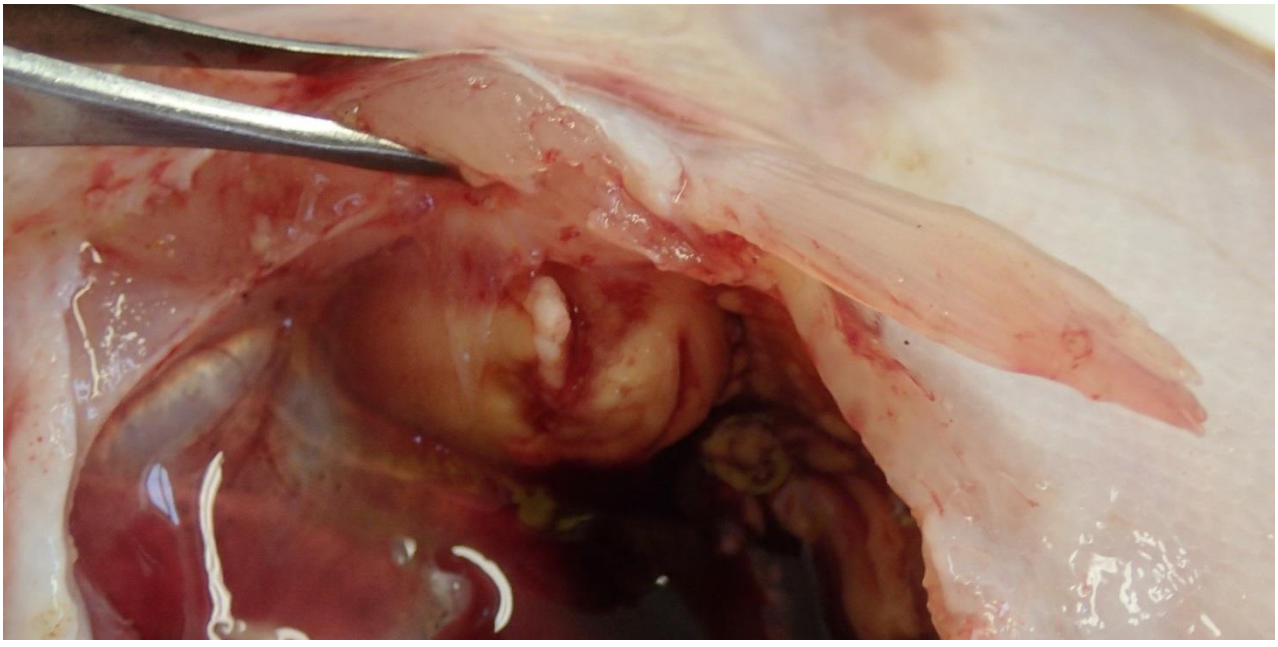

Figure 6 Severe infection of *Ichthyophonus* in the heart of yellowtail flounder resulting in severe epicarditous restricting normal heart functions

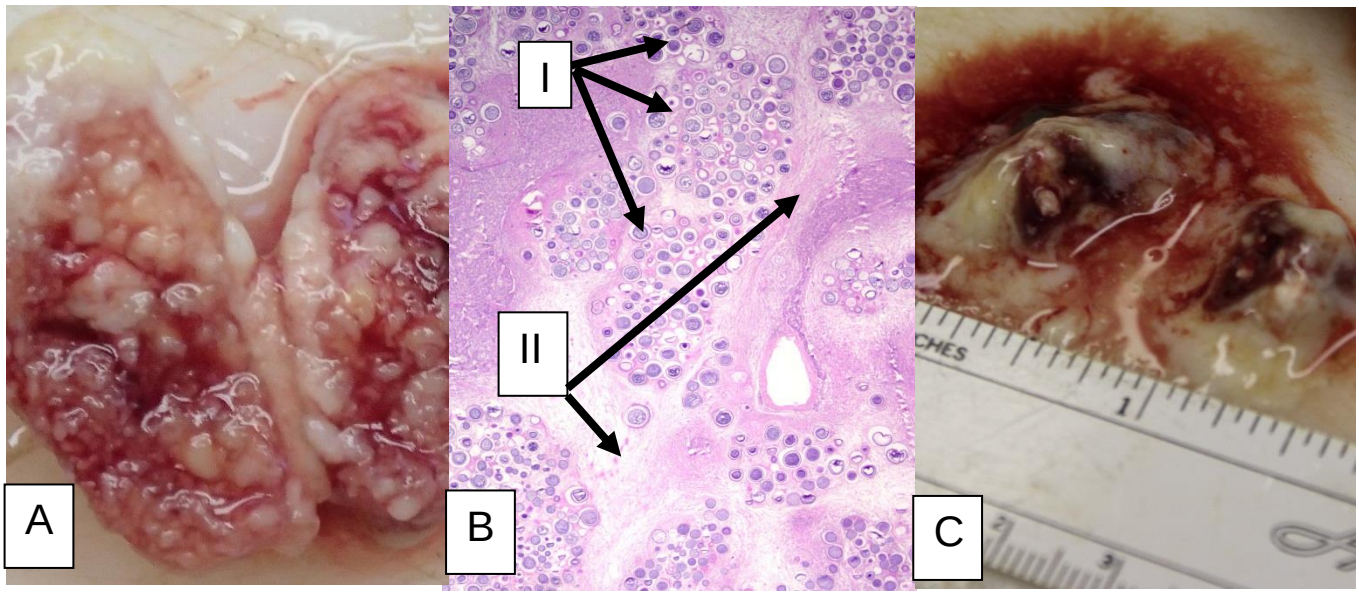

Figure 7 Severe infection of *Ichthyophonus* in the heart of yellowtail flounder (A) *Ichthyophonus* cells are identified throughout the heart (I) and are associated with severe myocarditis, edema and necrosis (II) (B; photomicrograph of a 6 µm paraffin embedded section stained with hematoxylin and eosin, 200x). Extreme infection resulted in diffuse myocardial necrosis (C)

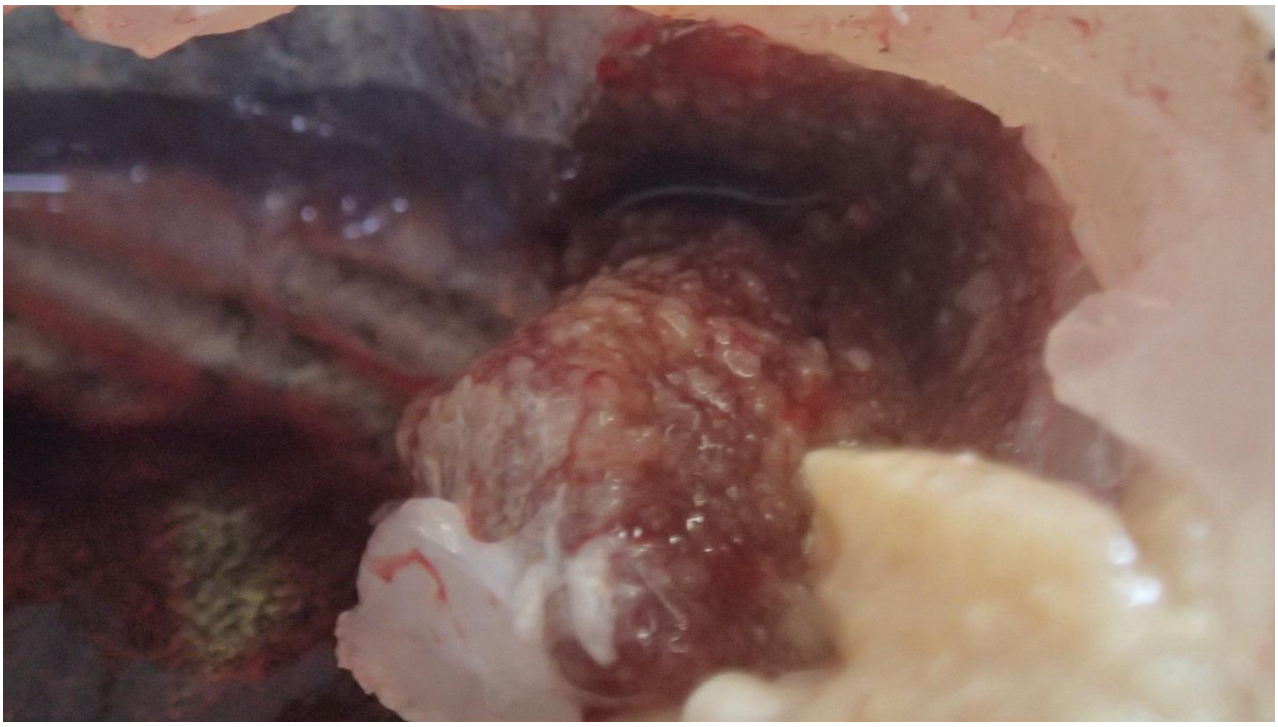

Figure 8 Severe infection of *Ichthyophonus* in the kidney of yellowtail flounder resulting in an enlarged kidney

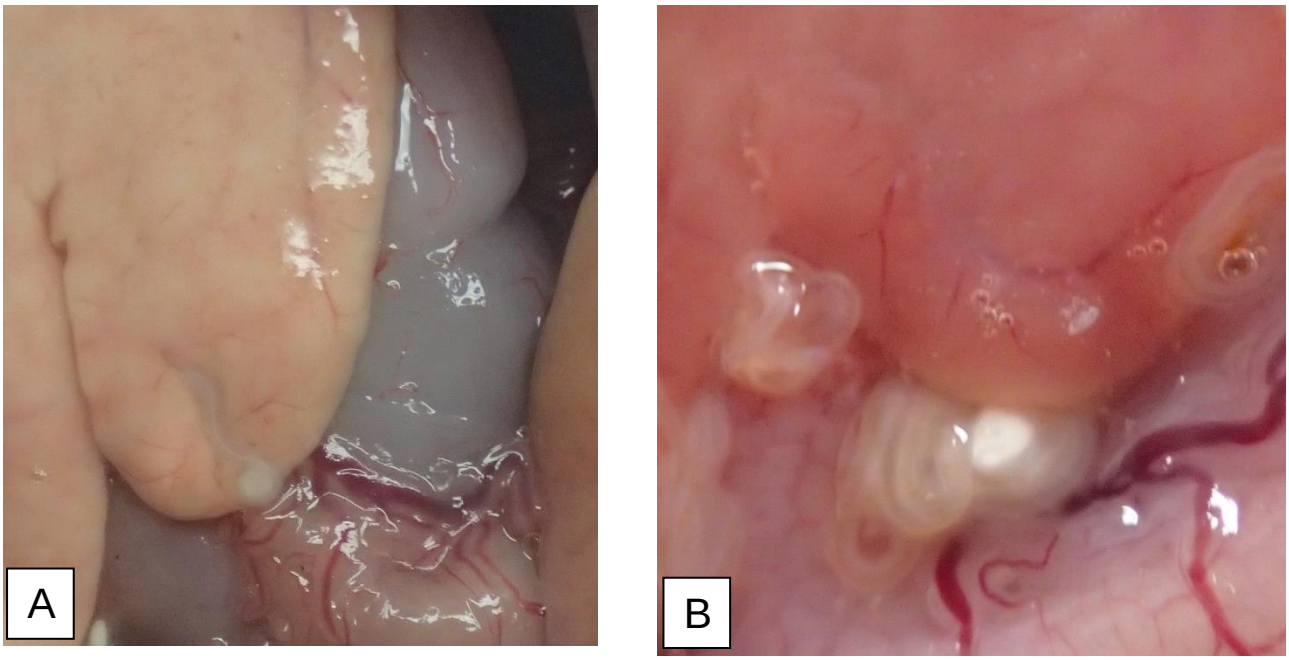

Figure 9 Encysted *Anascaridoidoiea* worms on the serosal surface of the liver (A) and attached to the mesentery around the liver and intestine (B)

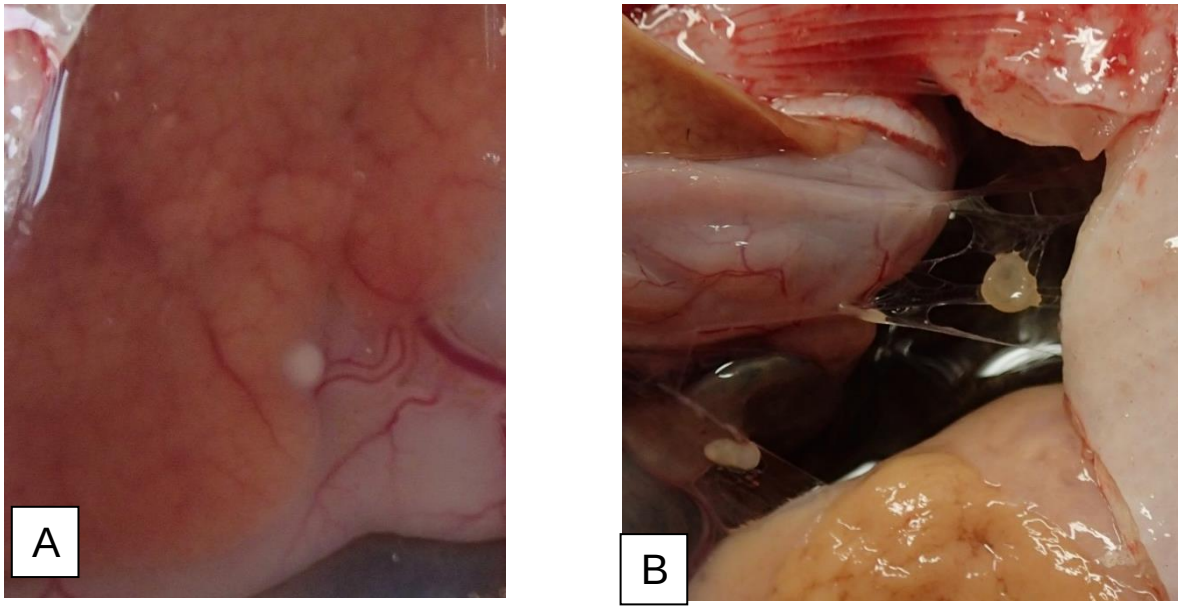

Figure 10 Encysted larval *Anascaridoidoiea* worms on the serosal surface of the liver (A) and attached to the mesentery around the liver and intestine (B)

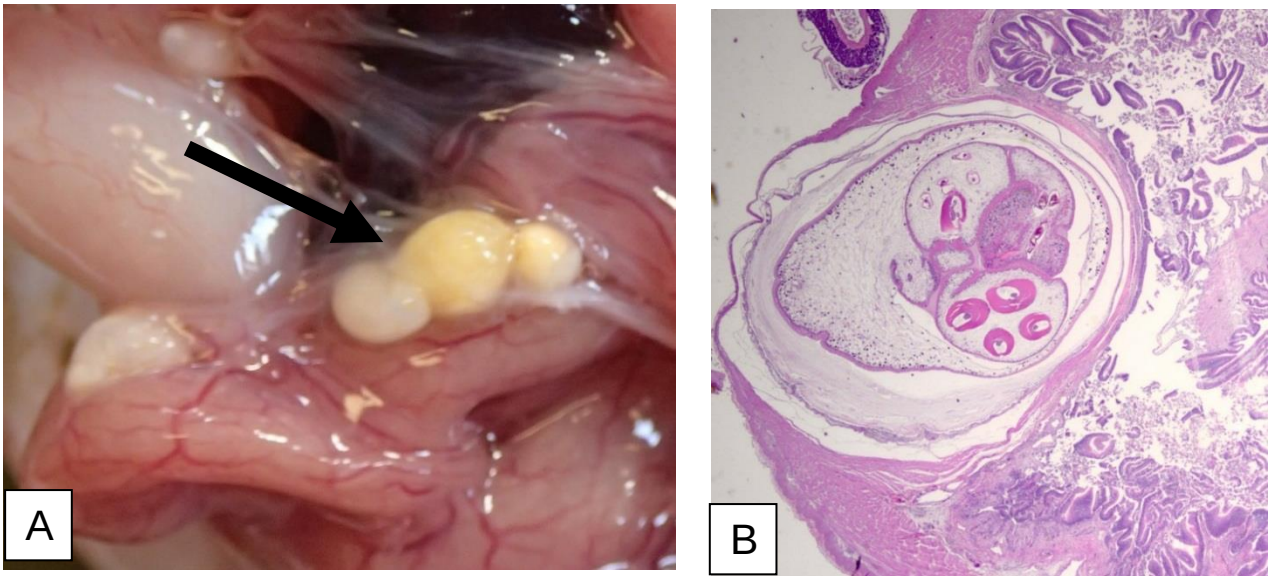

Figure 11 Cysts attached to the serosal surface of the intestine containing cestodes appeared macroscopically as 2-3 mm round white capsules (A). Cestodes were observed histologically to occasionally result in localized enteritis and potential lumenal restriction of the intestinal tract (B) (photomicrograph of a 6 µm section of paraffin embedded tissue stained with hematoxylin and eosin stain; 200x

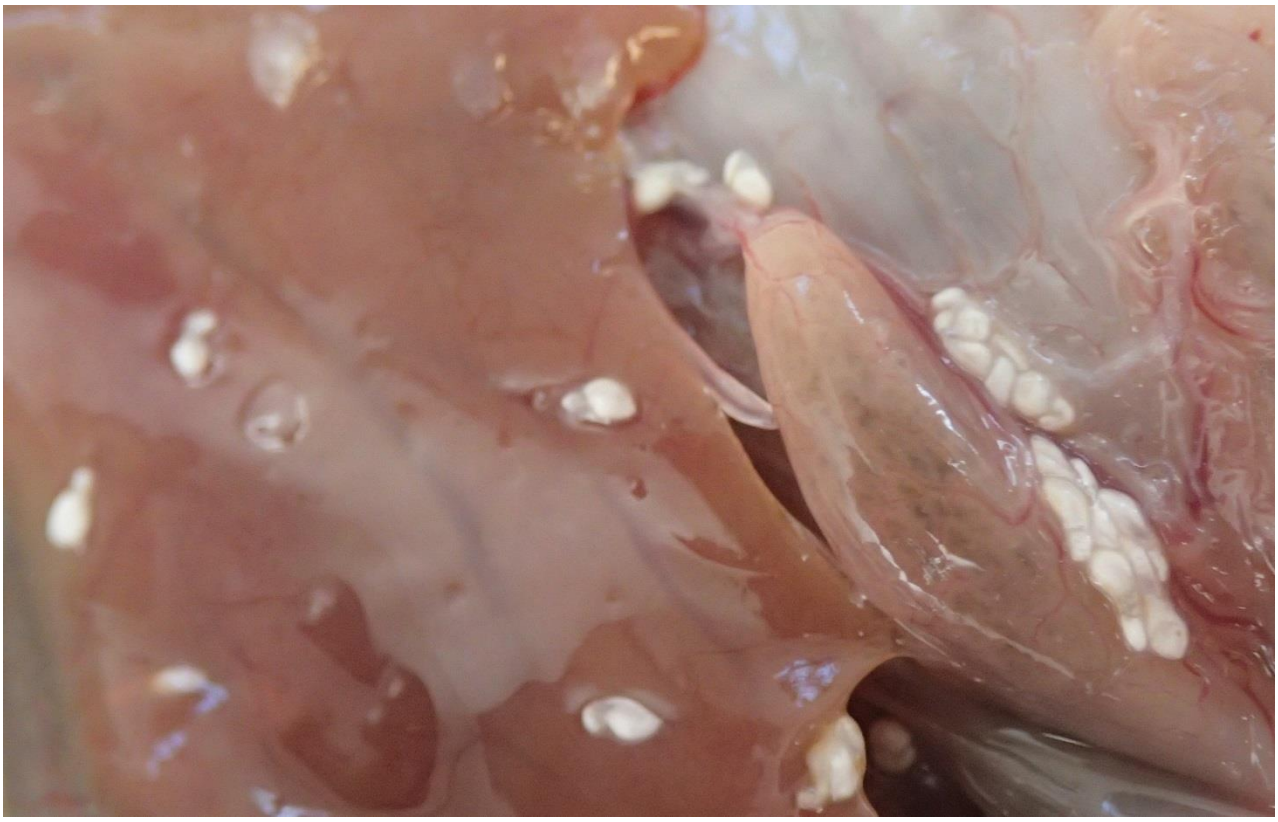

Figure 12 Cysts attached to the serosal surface of the intestine containing trematodes

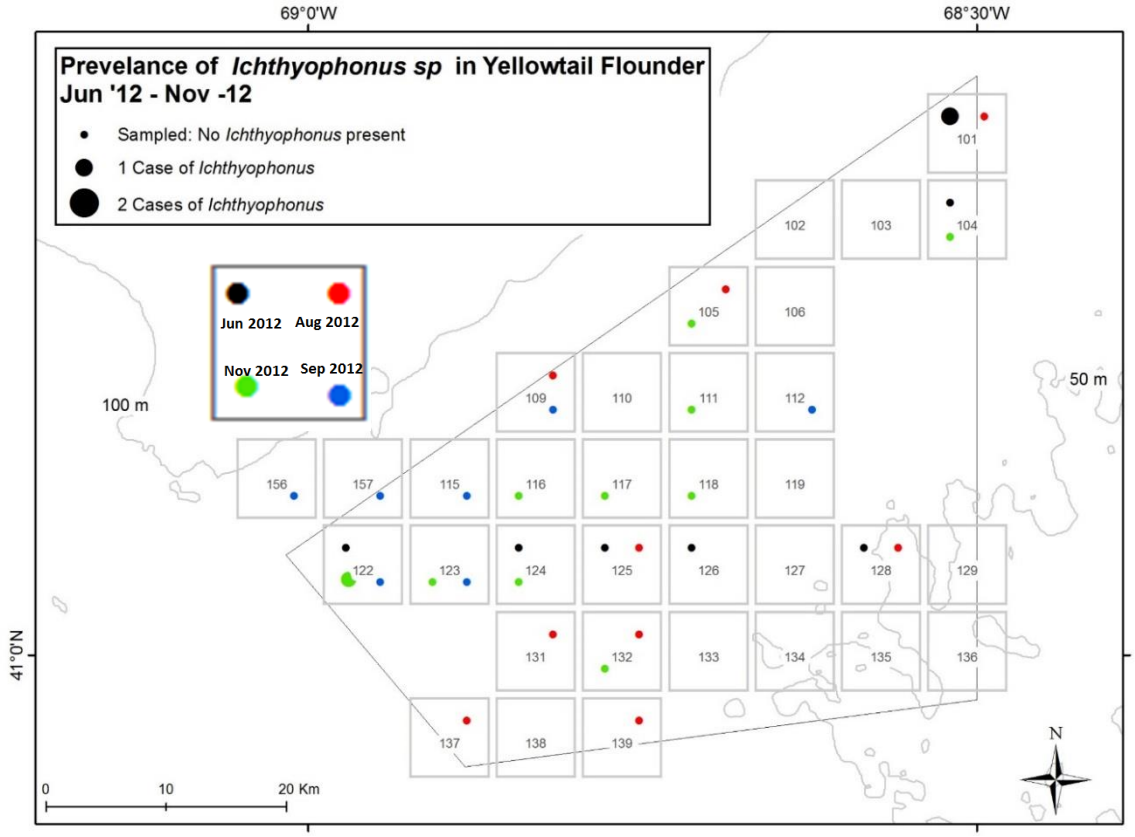

Figure 13 Prevalence of *Ichthyophonus* in closed area I for June 2012 (Black), August 2012 (Red), September 2012 (Blue), and November 2012 (Green)

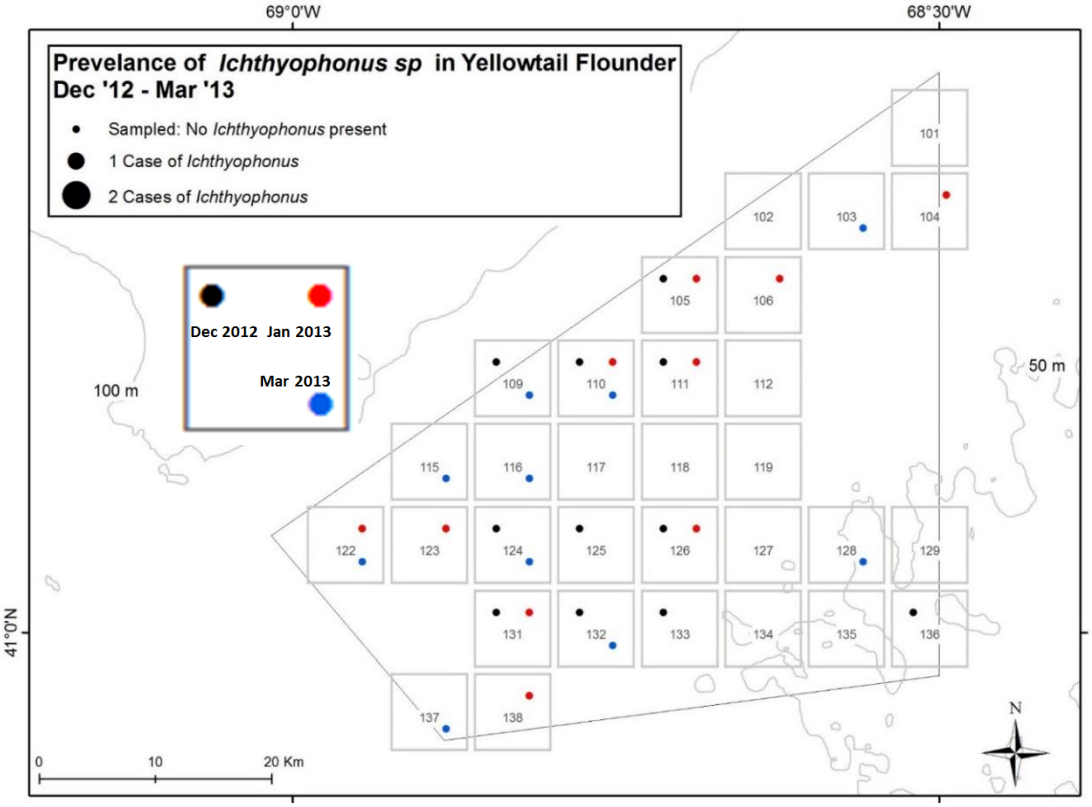

Figure 14 Prevalence of *Ichthyophonus* in closed area I for December 2012 (Black), January 2013 (Red), and March 2013 (Blue)

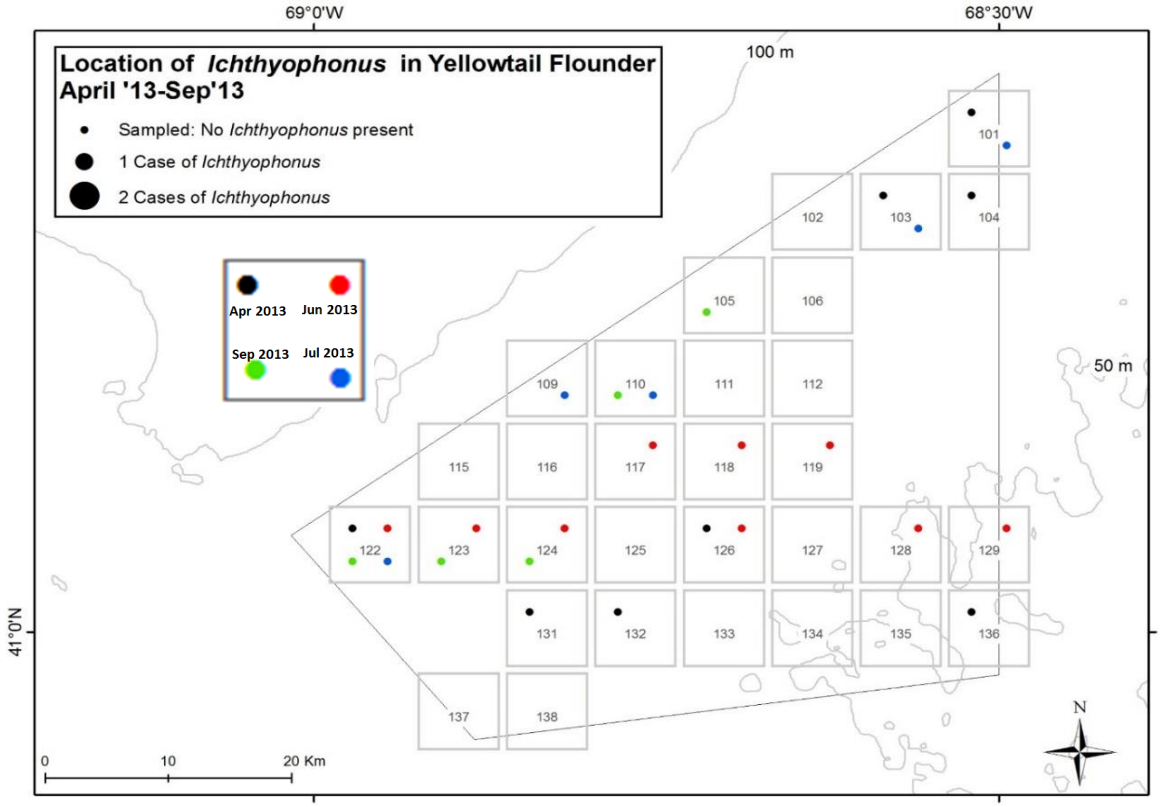

Figure 15 Prevalence of *Ichthyophonus* in closed area I for April 2013 (Black), June 2013 (Red), July 2013 (Blue) and September 2013 (Green)

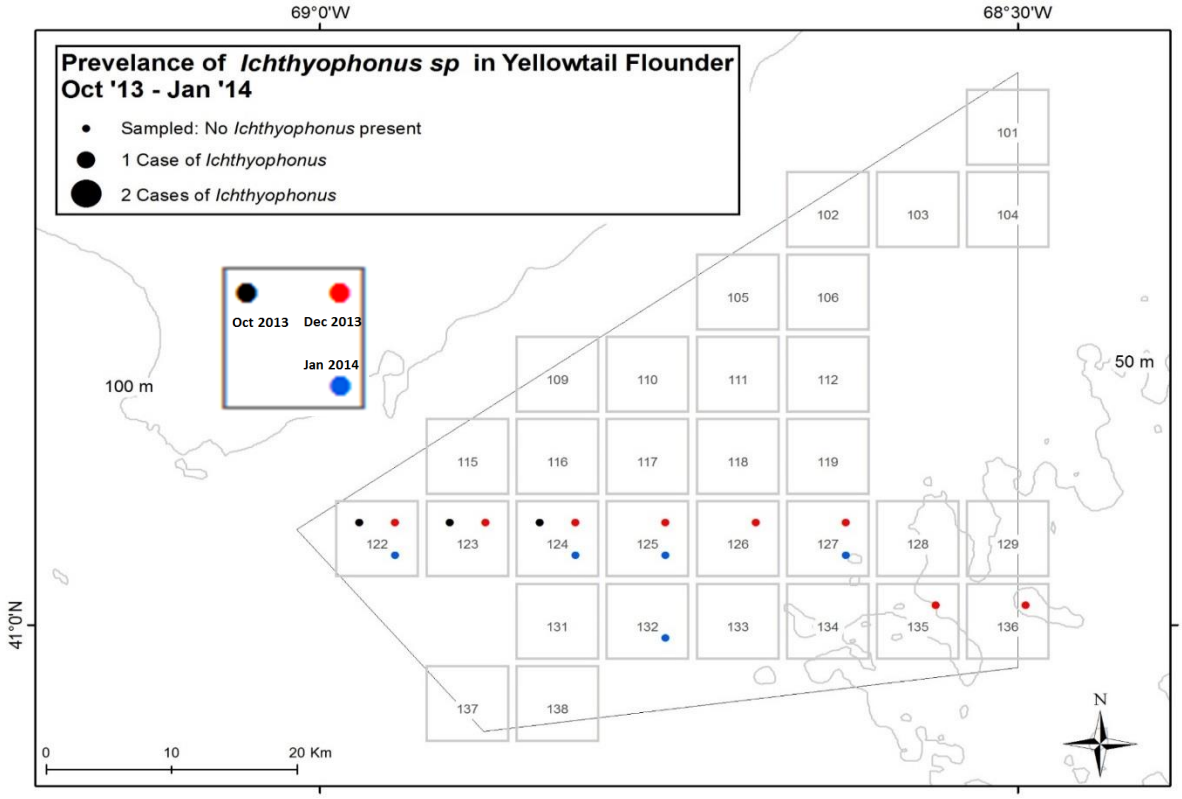

Figure 16 Prevalence of *Ichthyophonus* in closed area I for October 2013 (Black), December 2013 (Red), and January 2014 (Blue)

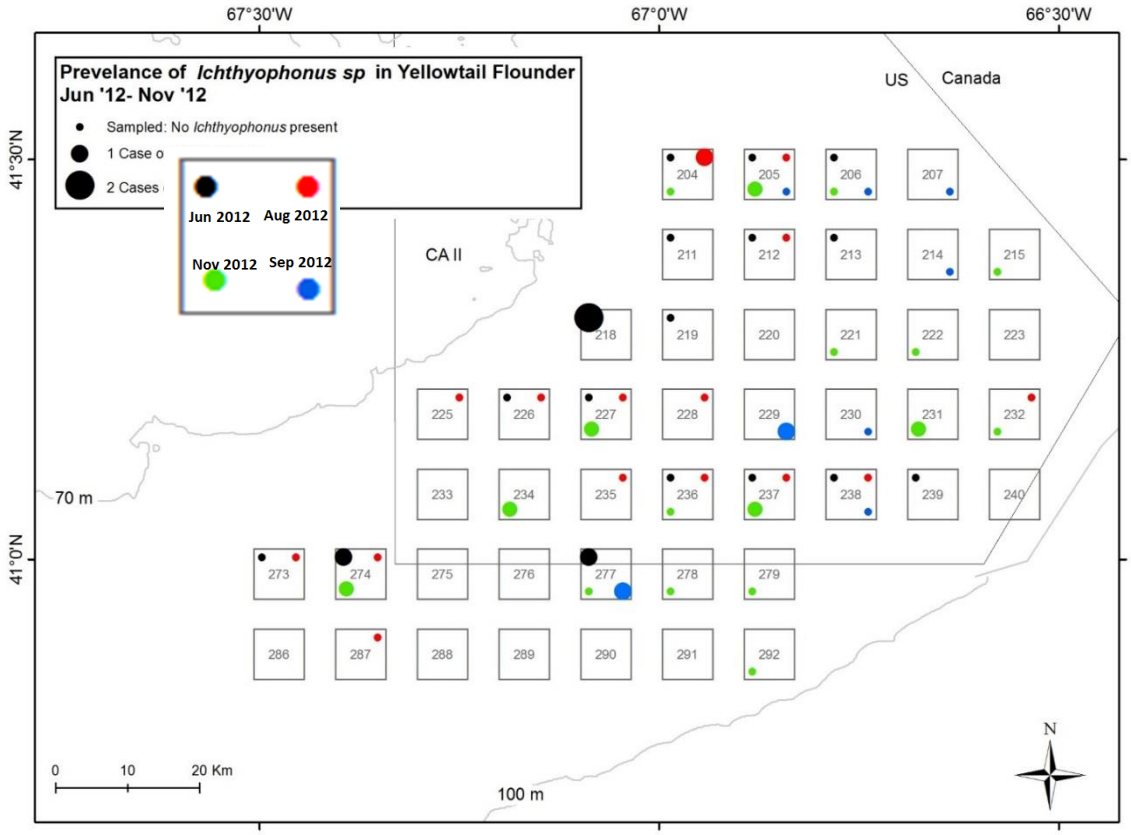

Figure 17 Prevalence of *Ichthyophonus* in closed area II for June 2012 (Black), August 2012 (Red), September 2013 (Blue) and November 2013 (Green)

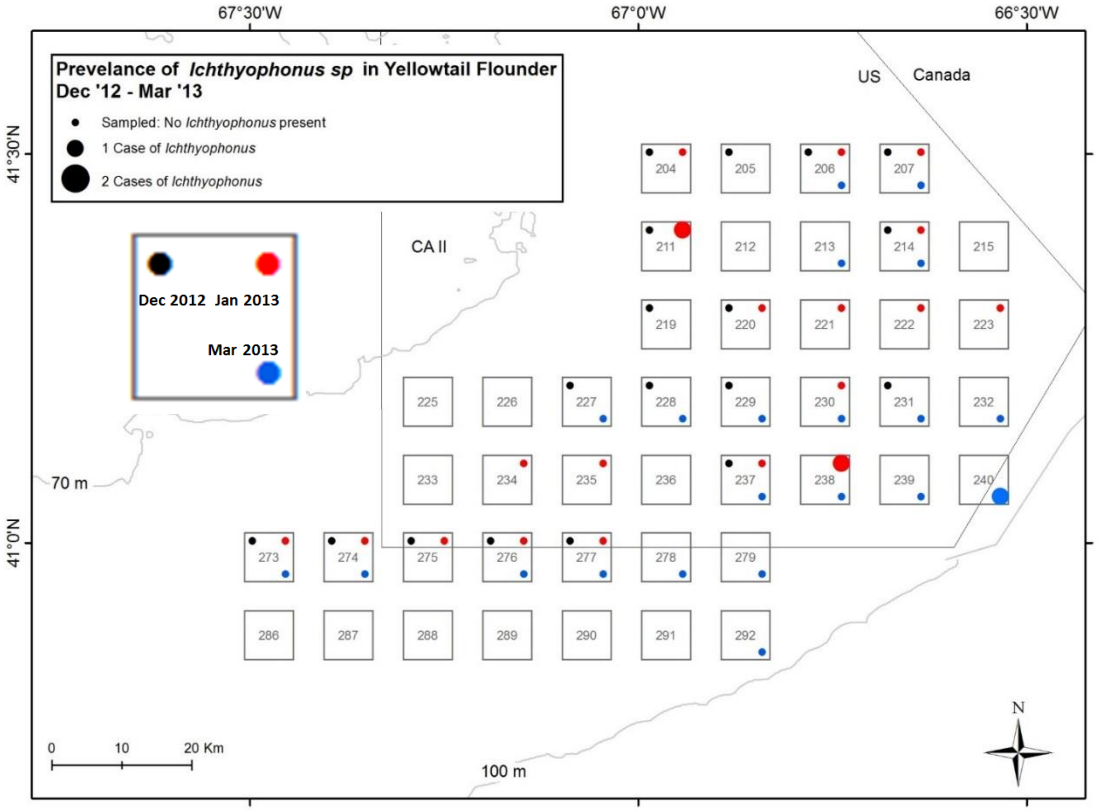

Figure 18 Prevalence of *Ichthyophonus* in closed area II December 2012 (Black), January 2013 (Red), and March 2013 (Blue)

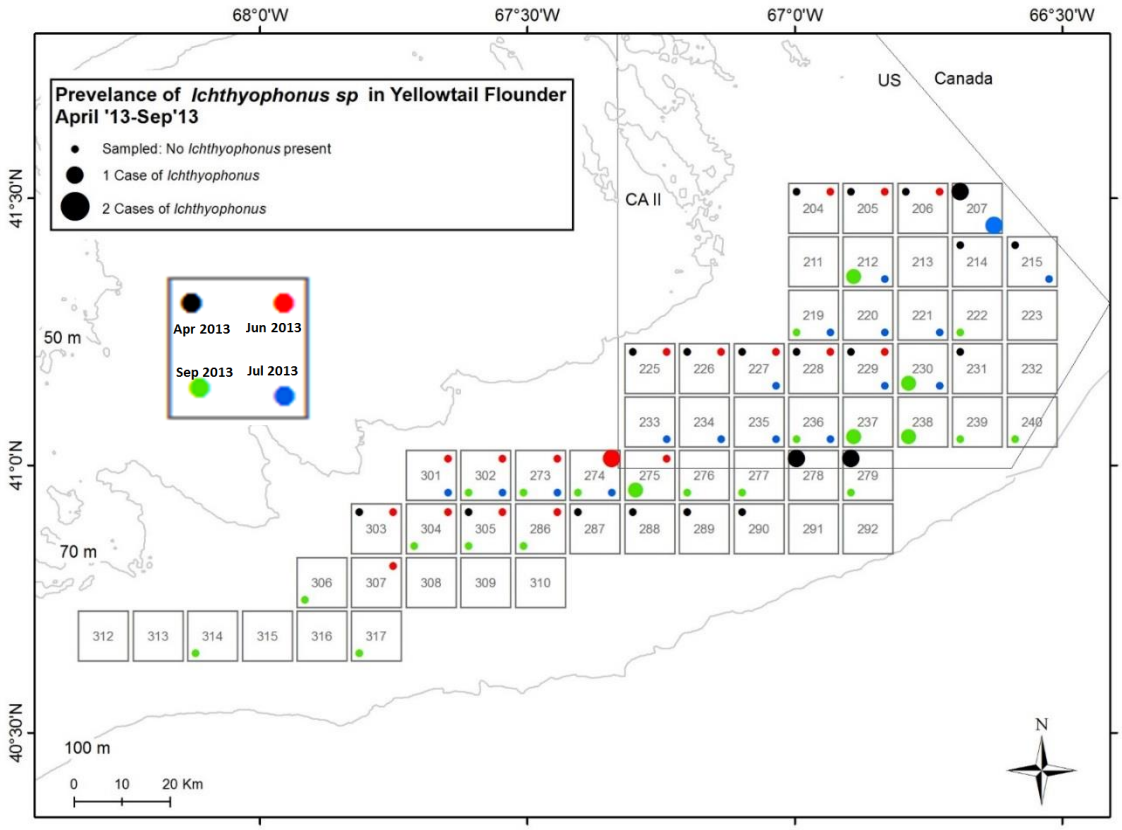

Figure 19 Prevalence of *Ichthyophonus* in closed area II for April 2013 (Black), June 2013 (Red), July 2013 (Blue) and September 2013 (Green)

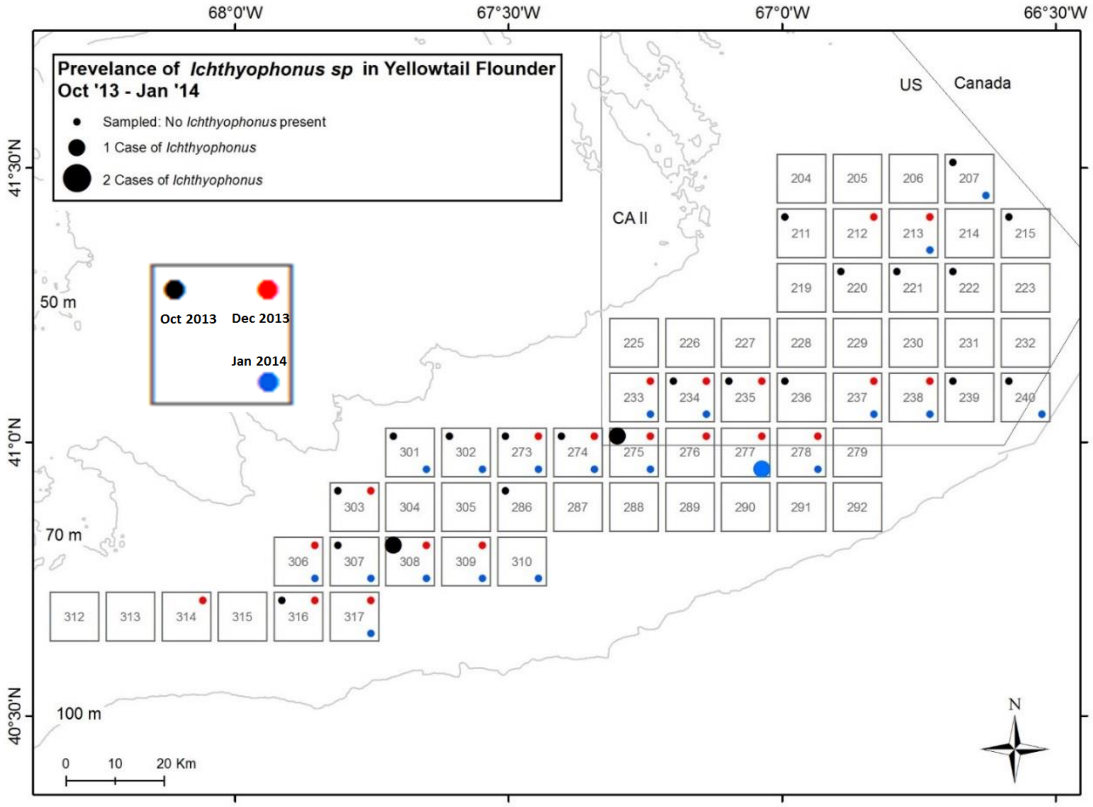

Figure 20 Prevalence of *Ichthyophonus* in closed area II for October 2013 (Black), December 2013 (Red), and January 2014 (Blue)