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Increased prevalence and severity of *Kudoa thyrsites* (Cnidaria: Myxosporea) in Atlantic salmon *Salmo salar* exposed to deeper seawater

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ABSTRACT: *Kudoa thyrsites* is a myxozoan parasite of marine fish with a global distribution. In British Columbia (BC), Canada, severe infections are associated with an economically significant degradation of fillet quality in farmed Atlantic salmon. Exposures to naturally occurring actinospores at a coastal research laboratory were used to test the hypothesis that the prevalence and severity of *K. thyrsites* infections acquired by exposure of Atlantic salmon to seawater (SW) of various depths are not different. In Expt 1, fish were exposed to SW from 1, 7 or 13 m below the surface. Following exposure to deeper-sourced SW, the prevalence of *K. thyrsites*, determined from microscopic examination of muscle histology sections, was greater in all 4 trials and the severity of infection was greater in 2 trials. In Expt 2, infections were compared over time among salmon held in tanks supplied with deep-sourced SW (raw or UV-irradiated) or in a surface net-pen. The infection was observed in 35 of 40 fish sampled between 3 and 6 mo after tank exposure to raw SW. Coincidentally, the parasite was observed in 4 of 40 fish maintained in the net-pen. No consistent association of the parasite infection was observed with temperature; however, reduced salinity and solar radiation were not ruled out as factors which may reduce the risk of infection from surface SW. These findings require verification at commercial aquaculture sites in BC, as they will inform considerations related to farm siting and net-pen configuration.

KEY WORDS: *Kudoa thyrsites* · Myxozoa · Atlantic salmon · Depth · Infection

1. INTRODUCTION

Kudoa thyrsites is a multivalvulid myxozoan parasite that sporulates within plasmodia, occupying skeletal myocytes in several species of marine fish (Moran & Kent 1999, Moran et al. 1999a). Postmortem myoliquefaction and subsequent degradation of fillet quality is caused by proteolytic secretions from the plasmodium (Funk et al. 2008) and is associated with more severe infections in cultured Atlantic salmon *Salmo salar* in British Columbia (BC) (Whitaker & Kent 1991, Dawson-Coates et al. 2003).

The life cycle of *K. thyrsites* has not been fully elucidated. However, failure to transmit the infection to naïve salmon by oral intubation with a homogenate of infected muscle tissue (Moran et al. 1999b) suggests that, like other myxozoans, *K. thyrsites* requires an invertebrate host for the development and dissemination of fish-infective actinospores. The presence of actinospores in seawater (SW), defined here as infective SW, is indicated from controlled studies in which naïve salmon became infected following exposure to infective SW (Moran et al. 1999b, Jones et al. 2012). Variation both in the prevalence and severity of infections among aquaculture production sites in BC suggests spatial heterogeneity in the infectivity of SW (Marshall et al. 2016). Similarly, a comparison of seasonally acquired infections in

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salmon following controlled exposures to SW from Departure Bay, BC, indicates that SW infectivity is greatest during summer months (Moran et al. 1999a). More recent work supported Moran et al. (1999a) by showing that concentrations of *K. thyrsites* environmental DNA (eDNA) were elevated in the summer months in Departure Bay and at a salmon production site approximately 150 km to the northwest (Jones et al. 2016, Marshall et al. 2022).

In the present study, we sought to clarify evidence of a greater prevalence of *K. thyrsites* in salmon held in an enclosed bag-pen rearing system into which deeper SW was pumped, relative to that in an adjacent and relatively shallow conventional flow-through net-pen (Kreiberg & Cooke 2000). We describe the prevalence and severity of *K. thyrsites* in Atlantic salmon smolts following exposure to potentially infective SW from various depths in Departure Bay and test the null hypothesis that in Departure Bay, the prevalence and severity of *K. thyrsites* infections acquired from various depths are not different.

2. MATERIALS AND METHODS

2.1. SW characteristics and fish

The research aquarium at the Pacific Biological Station (PBS-Aq) is supplied with SW pumped from the seabed in Departure Bay, approximately 2 m below the low tide datum, and filtered twice through sand beds (raw SW, RSW). Since 2010, RSW supplied to the PBS-Aq has been UV-irradiated (UVSW; see Jones et al. 2016). In Expt 1 (2001–2002), a separate lot of juvenile Atlantic salmon was used in each of 4 trials. The fish were obtained from commercial hatcheries on Vancouver Island and held at the PBS-Aq in aerated, dechlorinated freshwater (FW) (9–11°C). The fish were fed a daily ration of pelleted salmon feed *ad libitum* and stocked directly into experimental tanks upon smoltification. In Expt 2 (2018–2019), vaccinated Atlantic salmon smolts were obtained from a commercial hatchery on Vancouver Island and transported in aerated FW to the PBS-Aq. Details concerning the origin and history of fish used in this experiment were described earlier (Jones et al. 2020). Salmon were smolted onto UVSW in 6500 l flow-through tanks and acclimated for 2 wk. Fish husbandry followed guidelines of the Canadian Council on Animal Care (Pacific Region Animal Care Committee, AUP#18-010).

2.2. Study design

Expt 1 consisted of 4 trials (Fig. 1, see Table 1) for which 9 fiberglass tanks (300 l) were established in sets of 3 on a floating dock at Brandon Island, Departure Bay. A roofed, wire mesh enclosure reduced direct exposure of the tanks to sunlight and preda-

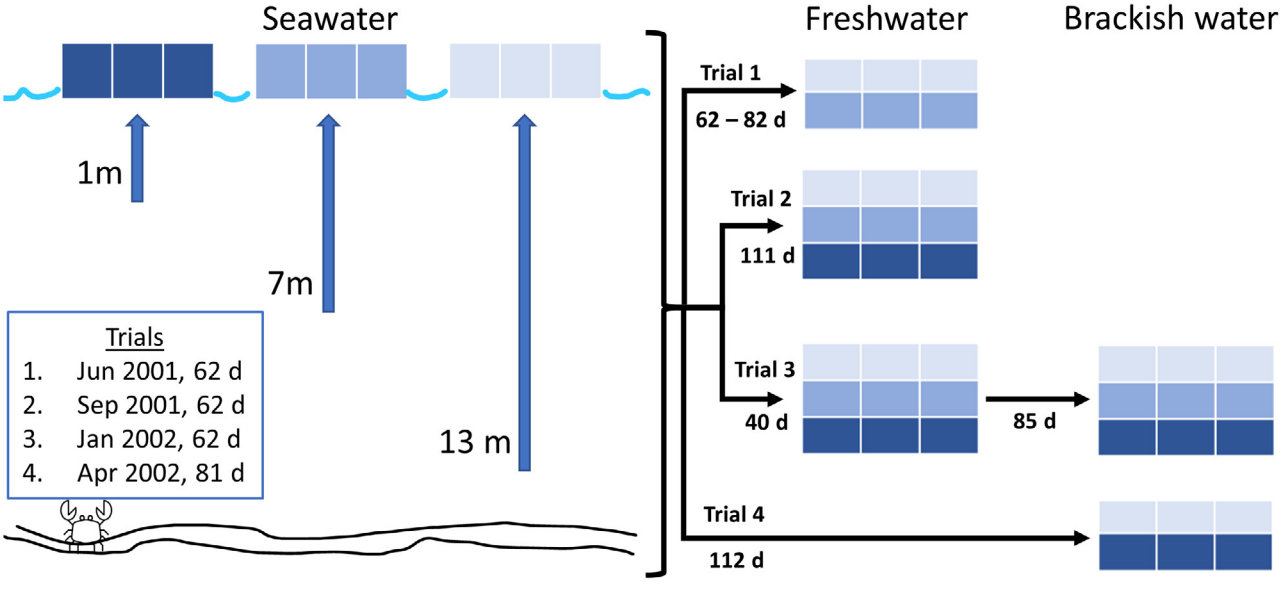

Fig. 1. Schematic of Expt 1. Triplicate tanks of 75 Atlantic salmon smolts were exposed to seawater (SW) pumped from 1, 7 or 13 m beneath the surface (distances not to scale) in 4 trials, with start dates and durations in SW indicated. Fish in each tank were then moved to a corresponding tank of freshwater, brackish water or both for the indicated duration of the trial. See Section 2.2 and Table 1 for further details

tion. Each set of 3 tanks was supplied with SW pumped by using entrained air from 1, 7 or 13 m below the surface. For each trial, with start dates in June 2001, September 2001, January 2002 and April 2002, the 9 tanks were each stocked with 75 salmon directly from FW. The SW temperature in all tanks was measured daily. In Trials 1–3, fish were exposed to SW for 62 d and then, to preclude further exposure to *K. thyrsites*, returned to FW at 10 ± 1°C (see Table 1) in 500 l tanks within the PBS-Aq. In Trial 3, mortality associated with *Saprolegnia* after approximately 40 d in FW was mitigated by transferring fish to a brackish water (BW) mixture of FW and RSW for the remaining 862 degree-days (DD) of the trial. In Trial 4, following an 81 d SW exposure, fish were transferred directly to BW in which they were maintained for 1132 DD. The duration in FW or BW was adjusted to achieve a minimum of 1500 DD since onset of the SW exposure. Daily salinity measurements of BW were made with a refractometer. At the end of all trials, fish were killed by immersion in 200 mg l^{-1} tricaine methane sulphonate (TMS; Syndel). Individual weight was recorded, and 3 samples of skeletal muscle were collected as described earlier (Jones et al. 2012) and fixed in Davidson's solution.

Expt 2 (June–November 2018) consisted of 1 trial with 7 sample points. Smolts were allocated to 2 flow-through tanks (4300 l) of either RSW (n = 300 $tank^{-1}$) or UVSW (n = 145 $tank^{-1}$) at the PBS-Aq and to an open net-pen (4.6 × 4.9 × 4.6 m) (n = 700) in Departure Bay (see Jones et al. 2020). Fish were fed a commercial pelleted diet at a daily rate of 1% biomass during acclimation and experimentation. Daily measurements were taken of dissolved oxygen, water temperature and salinity as well as feeding performance and mortality. Prior to the study and at approximately 4 wk intervals following transfer, fish were netted from the 2 tanks (n = 10 $tank^{-1}$) and from the net-pen (n = 20) and killed in 200 mg l^{-1} TMS. Individual weight was recorded, and 3 samples of skeletal muscle were collected and preserved in 10% neutral buffered formalin.

2.3. Histological assessment

Fixed muscle samples were processed for routine histology and stained with haematoxylin and eosin stains. Infection severity for each fish was estimated from the average number of plasmodia mm^{-2} determined from microscopic examination of the 3 sections $fish^{-1}$ (Jones et al. 2012). The percentage of fish with histological evidence of infection (prevalence) and median severity of infected fish were calculated for each exposure group.

2.4. Statistical analysis

All analyses were done in R version 4.1.1 (R Core Team 2021), and results were considered significant if $p \leq 0.05$. To determine the statistical significance of differences in the proportion of infected fish at varying depths, Pearson's chi-squared test of independence was done. For post hoc analyses, pairwise chi-squared tests using Fisher's exact test were employed and Bonferroni-adjusted p-values were generated using the R package 'rcompanion' (Mangiafico 2021).

For all severity analyses described herein, samples with a severity score of 0 were removed prior to analysis. Data were not normally distributed (Shapiro-Wilks normality test, $p \leq 0.05$) and variances were not equal (Bartlett's test for homogeneity of variances, $p \leq 0.05$), even following log-transformation of the data. As such, non-parametric analyses were necessary. To confirm that between-tank variation was not significant for any of the treatments, a Kruskal-Wallis test was used to analyze differences in severity between tanks for an individual depth. To test for differences in median severity between depth treatments in Expt 1, Kruskal-Wallis (Trials 2 and 3) or Mann-Whitney (Trials 1 and 4) tests were used. If differences in severity were significant, a Dunn's test for multiple comparisons was used and Benjamini-Hochberg-adjusted p-values were generated. The same set of analyses were used to test for differences in temperature between depth treatments.

3. RESULTS

3.1. Expt 1

In Trials 1–4, the prevalence of *Kudoa thyrsites* was significantly higher in fish reared in SW pumped from 13 m depth compared with those reared in SW from 7 or 1 m depth (Table 1). In Trials 2 and 3, all 3 depths were represented, and in both trials, prevalence in the 13 m group was significantly greater than in the 1 m group ($\chi^2 = 11.95$, $p = 0.003$; $\chi^2 = 6.84$, $p = 0.041$) (Table 1). In all trials, tank-to-tank differences in median severity for each depth were not statistically significant ($p > 0.05$), and data from all tanks were pooled for analysis by depth. Median severities were greater in the 13 m versus the 7 m group in Trial 1 ($p < 0.001$) and greater in the 13 m versus the 1 m

Table 1. Study parameters and the prevalence and severity of *Kudoa thyrsites* in Atlantic salmon smolts following exposure to seawater pumped from various depths in Departure Bay (Expt 1). D: depth of water intake beneath surface; temp: median (interquartile range [IQR]) temperature during seawater exposure; degree-days: total accrued in seawater, freshwater and/or brackish water (number of days in SW in parentheses); weight: median (IQR) salmon weight at termination of the experiment; exam: number of fish examined; no. (%) pos.: number (percent) of fish infected; severity: median (IQR) infection severity. Different superscripts (x, y, z) in each column denote statistical significance ($p \leq 0.05$) within each trial as determined by Mann-Whitney (Trials 1 and 4) and Kruskal-Wallis (Trials 2 and 3) tests for temp and severity and by Pearson's χ^2 for number of fish infected. (–) no data

Trial	Start date (mm/yyyy)	D (m)	Temp (°C)	Degree-days	Weight (g)	Exam	No. (%) pos.	Severity (plasmodia mm^{-2})
1	06/2001	13	10.9 (10.5–12.4)^{x}	1515 (62)	79.6 (74.4–89.8)	103	93 (90.3)	0.82 (0.18–2.11)^{x}
		7	15.4 (13.8–16.9)^{y}	1557 (62)	66.8 (59.1–76.9)	192	130 (67.7)	0.26 (0.04–0.89)^{y}
		1	–	–	–	–	–	–
2	09/2001	13	10.8 (10.2–11.6)^{x}	1585 (62)	60.8 (45.2–149.2)	95	54 (56.8)^{x}	0.21 (0.03–0.73)^{x}
		7	11.0 (10.3–12.5)^{x}	1616 (62)	89.3 (53.1–185.6)	91	39 (42.9)^{xy}	0.11 (0.03–0.23)^{x}
		1	11.1 (10.3–13.3)^{x}	1639 (62)	123.5 (56.6–188.2)	62	18 (29.0)^{y}	0.10 (0.03–0.24)^{x}
3	01/2002	13	8.0 (7.6–8.3)^{x}	1743 (62)	63.7 (55.2–71.2)	104	86 (82.7)^{x}	0.49 (0.10–2.07)^{x}
		7	7.7 (7.2–8.1)^{y}	1727 (62)	57.6 (50.3–63.6)	194	143 (73.7)^{xy}	0.24 (0.07–1.36)^{xy}
		1	7.3 (6.9–7.8)^{z}	1707 (62)	59.4 (51.9–64.1)	106	71 (67.0)^{y}	0.21 (0.05–0.60)^{y}
4	04/2002	13	10.5 (9.7–12.5)^{x}	2037 (81)	330.1 (294.6–365.8)	50	31 (62.0)^{x}	0.08 (0.03–0.39)^{x}
		7	–	–	–	–	–	–
		1	13.1 (10.7–14.5)^{y}	2127 (81)	309.4 (274.6–354.8)	57	16 (28.1)^{y}	0.04 (0.01–0.19)^{x}

group in Trial 3 ($p = 0.023$). There was no effect of depth on the median severity in Trials 2 ($p = 0.12$) and 4 ($p = 0.17$) (Table 1).

SW pumped from 13 m was significantly cooler than SW from 7 m (Trial 1, $p < 0.001$) and 1 m (Trial 4, $p < 0.001$; Table 1). In Trial 3, SW from 13 m was significantly warmer than from 7 or 1 m, and SW from 7 m was warmer than from 1 m ($p < 0.05$; Table 1). There was no effect of depth on SW temperature in Trial 2. Salinity was not measured during exposure to pumped SW. However, the duration of exposure to BW and the median salinity values of the BW in Trials 3 and 4 were 862 DD, and 18.0 parts per thousand (ppt) and 1132 DD and 18.0 ppt, respectively. Salmon weight at the termination of each trial is shown in Table 1.

3.2. Expt 2

K. thyrsites was not detected in any salmon sampled prior to the study, at 28 or 56 d post-transfer (dpt) or in any salmon maintained in UVSW. In salmon maintained in RSW, the parasite was first detected at 84 dpt (993 DD) with a prevalence of 60%, which increased to 90% at 112 dpt (1325 DD) and 100% at 140 (1646 DD) and 168 dpt (1874 DD). During this period, median severity in RSW increased from 0.05 to 0.75 plasmodia mm^{-2} (Table 2). In salmon maintained in the net-pen, the prevalence of infection

Table 2. Histological prevalence and severity of *Kudoa thyrsites* in skeletal muscle of Atlantic salmon by days following exposure to shallow seawater in a net-pen (NP), or to deep raw (RSW) or UV-irradiated (UVSW) seawater in tanks (Expt 2). Days: number of days since transfer to seawater; exam: number of salmon examined; no. (%) pos.: number (percent) infected; severity: median (interquartile range; individual values given when number infected <3)

Days	Group	Exam	No. (%) pos.	Severity (plasmodia mm^{-1})
28	NP	20	0	–
	RSW	10	0	–
	UVSW	10	0	–
56	NP	20	0	–
	RSW	10	0	–
	UVSW	10	0	–
84	NP	20	0	–
	RSW	10	6 (60)	0.05 (0.03–0.19)
	UVSW	10	0	–
112	NP	20	0	–
	RSW	10	9 (90)	0.06 (0.03–0.47)
	UVSW	10	0	–
140	NP	20	2 (10)	0.01, 0.62
	RSW	10	10 (100)	0.75 (0.20–2.12)
	UVSW	10	0	–
168	NP	20	2 (10)	0.01, 1.98
	RSW	10	10 (100)	0.52 (0.23–1.14)
	UVSW	10	0	–

was 10% at each of 140 (2142 DD) and 168 dpt (2405 DD). The *K. thyrsites* severities of the 2 infected net-pen salmon at each date were 0.01 and 0.62, and 0.01 and 1.98 plasmodia mm^{-2}, respectively.

4. DISCUSSION

In all trials of Expt 1, conducted between 2001 and 2002, the prevalence of infection with *Kudoa thyrsites* was greatest in salmon held in deeper-sourced SW. Furthermore, severities of the infections acquired in Trials 1 and 3, begun in January and June, were significantly elevated in salmon held in deeper-sourced SW. In Expt 2, conducted between 2018 and 2019, all but 4 infections occurred in salmon held in deep-sourced RSW pumped into the research aquarium. The 4 remaining infections occurred in salmon held in the relatively shallow water net-pen. Together, our results confirm the original observations (Kreiberg & Cooke 2000) by demonstrating that the prevalence and severity of infection with *K. thyrsites* in Departure Bay were consistently greater in Atlantic salmon held in deeper SW and thereby failed to support the null hypothesis. Histological examination was adopted over 2 more sensitive quantitative molecular methods to assess the *K. thyrsites* infections in Atlantic salmon because histology is a better predictor of parasite-induced myoliquefaction associated with more severe infections (Funk et al. 2007), increasing the relevance of the findings to salmon aquaculture. As such, we acknowledge that histology likely failed to detect an unknown number of low-severity infections. Another consideration is an outbreak of piscirickettsiosis which occurred during Expt 2 (Jones et al. 2020). However, given that elevated mortality in the net-pen population (34%) was significantly greater than in the RSW population (12%) and that the most severe *K. thyrsites* infections occurred after the outbreak was treated, interactions between the 2 infections were unlikely. Nevertheless, these findings exemplify the uncertainties surrounding the outcomes of mixed infections resulting from natural exposures to pathogens.

In Expt 1, *K. thyrsites* infections initially acquired by a 62 d exposure to SW continued to develop following subsequent transfer of the fish to FW (Trials 1 and 2), confirming earlier observations (Moran et al. 1999b). In the present study, fish were transferred from SW into FW, FW followed by BW (Trial 3), or BW alone (Trial 4), to allow for the development and sporulation of any infection acquired from SW while halting or limiting further exposure. While this design permitted comparisons in the prevalence and severity of *K. thyrsites* among 4 distinct exposure periods and with respect to BW, improved fish survival compared with a return to FW — the extent to which maintenance in BW halted or limited further exposure to *K. thyrsites* — was not directly measured. However, the durations of the BW exposures of 862 (Trial 3) or 1132 DD (Trial 4) were likely insufficient or barely sufficient to elicit the development of detectable infections. In earlier work, *K. thyrsites* was first detected after 1000 DD (Moran & Kent 1999), and in the present study, the parasite was first detected after 993 DD of RSW exposure (Expt 2). Therefore, the short-duration maintenance of fish in BW in Trials 3 and 4 was unlikely to have affected depth-associated patterns of infection because any infection pressures arising from those exposures would have been uniform among depth groups and of negligible consequence. Whether the abundance or infectivity of the *K. thyrsites* actinospore was further reduced in BW is not known, although earlier studies noted an association between reduced environmental salinity and reduced prevalence and/or severity of *Kudoa* spp. (Pampoulie et al. 2001, Figueiredo dos Santos et al. 2019). Also in the present study (Expt 2), mean salinity in the net-pen (27.9‰) was significantly lower than in the tanks (29.4‰) (Jones et al. 2020). Therefore, hyposalinity cannot be ruled out as a factor limiting the abundance or infectivity of the *K. thyrsites* actinospore in near-surface waters.

Temperature and UV irradiation were also explored as environmental factors which possibly limited the number or infectivity of actinospores in near-surface waters. In Expt 1, seasonal variations were observed in near-surface temperatures in trials initiated in January, April, June and September. In April (Trial 4) and June (Trial 1), the shallower waters were significantly warmer, whereas in January (Trial 3), they were significantly cooler, and in September (Trial 2), water temperature did not vary with depth. In Expt 2, temperature in the tanks was relatively stable with a gradual decline from summer to winter, whereas in the net-pen, there was a marked increase in temperature in the summer followed by a steeper decline from summer to winter (Jones et al. 2020). Overall, water in the tanks (12.0°C) was on average more than 2°C cooler than in the net-pen (14.4°C) (Jones et al. 2020). Thus, although there was a tendency for increased risk of infection in cooler temperatures, this association was not consistently observed. The present study supported our earlier observations that treatment of RSW with UV irradia-

tion abolishes virtually all *K. thyrsites* infectivity (Jones et al. 2016, Jones & Long 2019). Transmission of other myxozoan species, including *K. neurophila*, *K. yasunagai*, *K. amamiensis* and *Myxobolus cerebralis* is reduced or eliminated by UV irradiation of the water (Hedrick et al. 2000, 2007, Cobcroft & Battaglene 2013, Shirakashi et al. 2014). The application of UV irradiation is also efficacious against a range of fish pathogenic viruses, bacteria and fungi (Sako & Sorimachi 1985, Øye & Rimstad 2001, Huber et al. 2010). Similarly, exposure to sunlight inactivates viruses in near-surface waters (Suttle & Chen 1992, Garver et al. 2013) and decreases the infectivity of *Cryptosporidium parvum* oocysts and a bacterial pathogen of the crustacean *Daphnia* sp. (King et al. 2008, Overholt et al. 2020). Historically (1974–1993), seasonal variation of mean daily total insolation in Nanaimo ranged from 2.5–22.3 MJ m^{-2} (Environment and Climate Change Canada 2020). Despite attenuation with depth and turbidity, the contribution of solar radiation to the reduced risk of *K. thyrsites* in near-surface waters cannot be ruled out. Greater clarity on the roles of temperature and UV irradiation in influencing the transmission of *K. thyrsites* will benefit from further research.

The increased prevalence of *K. thyrsites* infection with depth is likely the result of higher actinospore densities in deeper water, as suggested from studies of *Ceratonova shasta* and *Parvicapsula minibicornis*, which showed that the probability of fish exposure is influenced by actinospore concentrations (Bjork & Bartholomew 2009, Hallett & Bartholomew 2009, Hallett et al. 2012). Proximity to source and buoyancy of putative *K. thyrsites* actinospores are potentially important factors influencing their vertical distribution within the water column. Further research is required to identify the *K. thyrsites* actinospore and to test the hypotheses that it is negatively buoyant and shed by a host invertebrate in the benthic community. In the context of the current study, the absence of a *K. thyrsites* eDNA gradient between 5 and 20 m depths at a production farm in BC (Marshall et al. 2022) suggests both caution in interpretating the epidemiological significance of eDNA and a need to better understand the vertical distribution of actinospores at salmon aquaculture sites in BC. Replication of the present findings under field conditions will inform salmon farm siting decisions and net-pen configurations.

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