

## Research Article

## Spatial and temporal monitoring of invasive *Hydroides dianthus* (Verrill, 1873) (Annelida, Serpulidae) in Eel Lake, Argyle, Nova Scotia using a species-specific molecular assay

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### Abstract

In 2012, an invasion by a serpulid tubeworm, unknown to the Atlantic Canada region, caused heavy biofouling on an oyster lease in Eel Lake, Argyle, Nova Scotia. The species was identified as *Hydroides dianthus* (Verrill, 1873), and this morphological identification was confirmed by comparing a newly sequenced COI gene fragment with *H. dianthus* sequences available in GenBank. Heavy biofouling on the oyster lease resulted in the need to develop mitigation strategies. It was hypothesized that by understanding the spawning behaviour and spatial/temporal patterns of *H. dianthus* larvae in Eel Lake, biofouling could be mitigated by determining an optimal depth that oyster cages should be maintained at during its active spawning period. To monitor *H. dianthus* in Eel Lake, species-specific primers associated with the COI gene were developed. Specificity and sensitivity of primers were tested, and the primer set *H. dianthus* COI4F/R was chosen for monitoring. Water samples were collected from Eel Lake from June to August 2013, and eDNA was extracted. Spatial and temporal monitoring of *H. dianthus* using eDNA was completed at four sites (three fouled, one non-fouled), and at three depths (0.3 m, 1.5 m, and 3.0 m) in Eel Lake. Water samples tested positive for *H. dianthus* in most sites and depths on June 20<sup>th</sup>, indicating a spawning event had occurred. Subsequently, no significant differences between sites and depths were found and *H. dianthus* was detected throughout the summer in all locations and depths monitored. The mean % of positive PCR results significantly increased from 18.7 to 65.0% from June to August. Results suggest that *H. dianthus* spawned frequently during a reproductive season ranging from at least June 20 to August 30 in Eel Lake at temperatures ~ 18–22 °C. The results of this study indicated that an optimal oyster cage depth for biofouling mitigation could not be determined and highlighted the challenges to aquaculture associated with biofouling of *H. dianthus*.

**Key words:** biofouling, environmental DNA (eDNA), cytochrome *c* oxidase gene (COI), invasive species

### Introduction

The shellfish aquaculture industry in Atlantic Canada is facing significant challenges with biofouling organisms, especially invasive ascidians (tunicates) such as *Botrylloides violaceus* Oka, 1927, *Styela clava* Herdman,

1881, *Botryllus schlosseri* Pallas, 1766, and *Ciona intestinalis* Linnaeus, 1776 (Locke et al. 2007; Ramsay et al. 2009; Sephton et al. 2011, 2016; Simard et al. 2013; McKenzie et al. 2016). Polychaetes in the family Serpulidae Rafinesque, 1815 belong to another important group of biofouling organisms that impact the aquaculture industry (Yan et al. 2008; Ma et al. 2009). Despite 20 species of Serpulidae being documented in Atlantic Canada (Carr 2012), none have been a source of significant biofouling on aquaculture leases in Atlantic Canada. However, in the summer (June to August) of 2012, a serpulid heavily fouled Eel Lake, Argyle, Nova Scotia (NS), Canada. Eel Lake contains brackish water (~ 12–22 ‰), with its saltwater supply in-flowing from strong tides of the Bay of Fundy. The serpulid species responsible for the biofouling was not reported before in the Atlantic Canada region. Taxonomists from Fisheries and Oceans Canada identified the serpulid as a *Hydroides* spp. and consulted Dr. Harry ten Hove (Naturalis, Leiden, the Netherlands), who confirmed the serpulid as *Hydroides dianthus* (Verrill, 1873) (*pers. comm.* between Dawn Sephton and Sarah Stewart-Clark 2013).

*Hydroides dianthus* belongs to the genus *Hydroides* Gunnerus, 1768 with over 100 species, five of which are confirmed invasive biofoulers, including *H. dianthus*, *Hydroides dirampha* Mörch, 1863, *Hydroides elegans* (Haswell, 1883), *Hydroides ezonesis* Okuda, 1934, and *Hydroides sanctaecrusis* Krøyer [in] Mörch, 1863 (Capa et al. 2021). The reported range for *H. dianthus* spans as far north as Maine, USA and as far south as the coast of Florida and Texas (Hedgpeth 1950; Bastida-Zavala and Salazar-Vallejo 2000; Bastida-Zavala and ten Hove 2002; Trott 2004). However, *H. dianthus* has been documented in many geographical locations globally (Sun et al. 2017). The introduction of invasive biofouling species globally has been accredited to shellfish transfers (Mckindsey et al. 2007), shipping vectors (ballast water systems and hull fouling) (Ruiz et al. 2011; Sylvester et al. 2011; Briski et al. 2012) and recreational boats with hull fouling (Darbyson et al. 2009; Davidson et al. 2010). These invasions can be facilitated by propagule pressure and anthropogenic changes in environmental conditions (Locke et al. 2007).

Heavy biofouling on shellfish aquaculture leases causes equipment and gear to be weighed down and can negatively affect growth and condition in shellfish (Braithwaite and McEvoy 2005). This ultimately leads to economic losses to the shellfish aquaculture industry through increased cost of maintenance through mechanical/chemical removal, waste removal, and labor of harvesting, transporting, and processing (Fisheries and Oceans 2006). The cost of chemical and physical removal of biofouling is estimated to be between 5–10% of industry labor costs, or between US \$1.5–3 billion per year for the industry globally (Fitridge et al. 2012). *Hydroides dianthus* has been found to settle on aquaculture infrastructure and shellfish, and secrete calcium carbonate tubes that the worms reside in for the remainder of its lifecycle (Wisely 1958; Scheltema et al. 1981). The removal of the

calcium carbonate tube from the shellfish and equipment increases the labour costs for shellfish growers (Fitridge et al. 2012). Even if the worms are successfully removed, they may have impacts on the aesthetics of the shell which can result in reduced shellfish value (Watson et al. 2009; Dürr and Watson 2010).

In the summer of 2012, heavy biofouling of *H. dianthus* on oysters and cages in Eel Lake resulted in increased labour costs associated with removal of tubeworms. Therefore, mitigation strategies to prevent biofouling from *H. dianthus* were sought. In the suspended oyster aquaculture industry, floating bags or OysterGro® cages are flipped periodically to expose biofouling organisms to air which results in desiccation (Mallet et al. 2009). This flipping method has been found effective for removing biofouling of barnacles, mussels, and tunicates (Mallet et al. 2009; Government of New Brunswick 2018). However, initial field observations found *H. dianthus* particularly problematic at Eel Lake, as desiccation alone did not remove biofouling from oysters and cages.

Serpulid larvae are known to exhibit photoresponse. For example, trochophores of *H. ezoensis* have been found to exhibit photopositive behaviour, whereas older larval stages exhibit photonegative responses (Miura and Kajihara 1981; Kupriyanova et al. 2001). Other marine invertebrates also exhibit photoresponse. Thorson (1964) found that out of 141 marine bottom invertebrates studied, 82% of early pelagic larvae migrated to light in surface water (photopositive), while later in their pelagic life-stage they migrated away from light (photonegative). Little is known about *H. dianthus*' photoresponse, other than they have been reported to settle in illuminated areas of containers in laboratory experiments (Zeleny 1905; Kupriyanova et al. 2001), therefore, more research is warranted on this subject to understand their behaviour in a natural setting. In addition to flipping, oyster bags/cages have the ability to be floated on surface waters or sunk during storms or when sea ice is present (Doiron 2008). In this study it was hypothesized that it might be possible to hold oyster cages at an optimal depth when *H. dianthus* spawning was occurring, in order to prevent heavy biofouling. Therefore, knowledge of the frequency of spawning and larval depth dispersal of *H. dianthus* in Eel Lake would be required to determine feasibility of such a depth-based management strategy.

The objective of this study was to monitor *H. dianthus* over the summer of 2013 in Eel Lake, NS to better understand spatial and temporal distribution of larvae at different depths and sites. Distribution of *H. dianthus* in Eel Lake was monitored by development of a molecular assay capable of detecting *H. dianthus* in environmental water samples (eDNA). The use of molecular assay to detect larval aquatic organisms can be optimal, as identifying different species of planktotrophic larvae using visual morphological taxonomy is difficult (Darling and Mahon 2011).

Molecular assays are being used to track and monitor invasive species globally (e.g., Stewart-Clark et al. 2009, 2013; Willis et al. 2011; Egan et al. 2015; Ardura et al. 2015; Dougherty et al. 2016; Thomas et al. 2020; LeBlanc et al. 2020). In Atlantic Canada, this approach has been successfully used to detect invasive tunicates *S. clava*, *C. intestinalis*, *B. schlosseri*, *B. violaceus* and *Diplosoma listerianum* (Milne-Edwards, 1841) in Prince Edward Island (PEI), Canada, in several life history stages such as in eggs, larvae, and adults (Willis et al. 2011; Stewart-Clark et al. 2013; Ma et al. 2016). Target invasive tunicates were also detected in PEI through molecular assay in environmental water samples (Stewart-Clark et al. 2009). This method of water sampling is useful in understanding geographical, spatial, and temporal distribution of invasive species. Therefore, the molecular assay in this study was used to understand *H. dianthus* larval spatial and temporal patterns, to identify peak spawning periods and preferential depths that could be useful as a method of biofouling mitigation.

## Materials and methods

### *Sample collection and DNA extractions*

Samples of adult *H. dianthus* were collected from cages and oysters from Eel Lake, Argyle, NS in March 2013. The worms were preserved in 100% ethanol and extracted from their tubes using a scalpel blade. DNA was extracted from eight abdominal tissue samples (~ 10–15 mg) using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions.

### *Amplification of mitochondrial cytochrome c oxidase subunit I gene*

A fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) was amplified in *H. dianthus* using Hydro-COIF/Hydro-COIR primer set (Sun et al. 2012). Polymerase Chain Reaction (PCR) was performed in a 50 µl reaction, containing 25 µl of AmphiTaq Gold™ 360 Master Mix (Applied Biosystems™, Massachusetts, USA), 2 µl of the forward primer, 2 µl of the reverse primer, 19 µl of sterile molecular grade water, and 1 µl of template DNA. The COI gene was amplified using the following thermocycler protocol: 94 °C for 5 minutes, 5 cycles of 94 °C for 30 seconds, 47 °C for 30 seconds and 72 °C for 40 seconds. After the initial 5 cycles, 30 additional cycles were completed at 94 °C for 30 seconds, 51 °C for 30 seconds and 72 °C for 40 seconds. Then, the PCR amplicons underwent elongation for 7 minutes at 72 °C. PCR products were separated using agarose gel electrophoresis on a 2% agarose gel containing 0.1 µl/ml of SYBR™ Safe Gel Stain (Invitrogen™, Massachusetts, USA). The gel was run at 135 V for 45 minutes, then visualized using ultraviolet light in the Gel Doc™ XR + Molecular Imager® with BIO-RAD Image lab 5.1 software (California, USA).

**Table 1.** COI sequences of *Hydroides* spp. used to align against *Hydroides dianthus* Eel Lake sequence to develop species specific primers.

Species	Reg. #	Accession #	Collection location
<i>Hydroides brachyacantha</i> Rioja, 1941	AM W.40537	JQ885942.1	Mazatlán, Mexico
<i>Hydroides crucigera</i> Mörch, 1863	AM W.40538	JQ885947.1	Mazatlán, Mexico
<i>Hydroides dirampha</i> Mörch, 1863	AM W.40539	JQ885946.1	Hong Kong, China
<i>Hydroides elegans</i> (Haswell, 1883)	AM W.40540	JQ885938.1	Hong Kong, China
<i>Hydroides ezoensis</i> Okuda, 1934	AM W.40544	JQ885951.1	Vladivostok, Russia
<i>Hydroides fusicola</i> Mörch, 1863	AM W.40545	JQ885950.1	Manazuru, Japan
<i>Hydroides operculata</i> (Treadwell, 1929)	AM W.40550	JQ885949.1	Hong Kong, China
<i>Hydroides operculata</i> (Treadwell, 1929)	AM W.40551	JQ885948.1	Hong Kong, China
<i>Hydroides sanctaecrucis</i> (Mörch, 1863)	AM W.40549	JQ885943.1	Mazatlán, Mexico
<i>Hydroides sanctaecrucis</i> Krøyer in Mörch, 1863	AM W.40548	JQ885944.1	Hong Kong, China

### *Sequencing and species confirmation*

The amplified COI gene fragment was sequenced using Guelph Laboratory Services, University of Guelph, Ontario, Canada. The amplicon of one specimen was sequenced in both directions, and a consensus strand was created using ClustalW 2.0 software (Larkin et al. 2007). The consensus sequence was used in a blastn search of the National Center of Biotechnology Information (NCBI) GenBank database to confirm species identity of *H. dianthus*.

### *Species specific molecular assay*

#### Primer development:

Primers were developed from the COI gene fragment isolated from Eel Lake. COI sequences of 10 *Hydroides* species were downloaded from GenBank (Based on May 2013 sequence availability) (Table 1). Eleven sequences were aligned using MEGA6 (Tamura et al. 2013) to determine unique fragments suitable for primer design. Primers were designed using Integrated DNA Technologies (IDT) (Iowa, USA) primer design tool. Each newly developed primer was examined for possible hairpin structures, homodimers, and heterodimers using the built-in quality check within the primer design tool, to ensure its suitability. Primer sequences were searched on NCBI database using the Primer-BLAST tool to check for unintended amplification of non-target organisms.

#### Efficacy, sensitivity, and specificity testing

Each primer set developed was tested against a positive Eel Lake *H. dianthus* DNA sample and a negative control to determine their efficacy using PCR. Primer sensitivity was tested with DNA samples of four concentrations (100 ng/l, 10 ng/l, 1 ng/l, and 0.1 ng/l) to determine the lowest limit of detection. To evaluate primer set specificity, we used ethanol-preserved tissues of specimens belonging to 10 species of *Hydroides* from the collection of the Australian Museum (Sydney, Australia) (Table 2). DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden Germany),

**Table 2.** *Hydroides* spp. specimens donated from the Australian Museum (Sydney, Australia) used to test primer specificity.

Species	Reg. #	Collection location
<i>Hydroides brachyacantha</i> Rioja, 1941	AM W. 45886	Sinaloa, Mexico,
<i>Hydroides dirampha</i> Mörch, 1863	AM W. 43679	Gulf of Mexico, Mexico,
<i>Hydroides elegans</i> (Haswell, 1883)	AM W. 45940	New South Wales, Australia
<i>Hydroides ezoensis</i> Okuda, 1934	AM W.45888	Qingdao, China,
<i>Hydroides heterocera</i> (Grube, 1868)	AM W. 45891	Hadera, Israel,
<i>Hydroides homoceros</i> Pixell, 1913	AM W. 45894	Jaffa, Israel,
<i>Hydroides longispinosa</i> Imajima, 1976	AM W. 45900	Queensland, Australia,
<i>Hydroides minax</i> (Grube, 1978)	AM W. 43286	Hadera, Israel,
<i>Hydroides recurvispina</i> Rioja, 1941	AM W. 45897	Sinaloa, Mexico,
<i>Hydroides trivesiculosa</i> Straughan, 1967	AM W. 45904	Queensland, Australia,

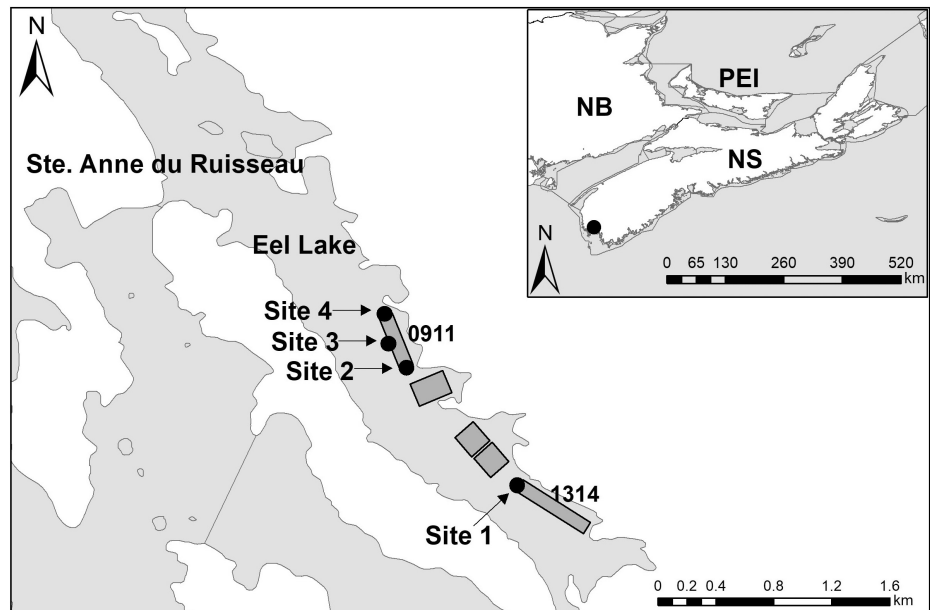
following the manufacturer's instructions. Initially, PCR amplification was completed using Hydro-COIF/Hydro-COIR primer set (Sun et al. 2012) to ensure that DNA extracted from *Hydroides* spp. was amplifiable. Then, PCR was completed using the designed *H. dianthus* primers to validate specificity.

PCR was performed with 50 µl reactions, using 25 µl of AmphiTaq Gold™ 360 Master Mix (Applied Biosystems™, Massachusetts, USA), 2 µl of the forward primer, 2 µl of the reverse primer, 19 µl of sterile molecular grade water, and 1 µl of template DNA. PCR was completed using the following protocol: 95 °C for 5 minutes; 35 cycles of: 95 °C for 30 seconds, annealing (50–60 °C) for 30 seconds, and 72 °C for 60 seconds; followed by a final extension of 72 °C for 7 minutes. PCR products were separated using agarose gel electrophoresis on a 2% agarose gel containing 0.1 µl/ml of SYBR™ Safe Gel Stain (Invitrogen™, Massachusetts, USA). The gels were run at 135 V for 45 minutes and were visualized using ultraviolet light in the Gel Doc™ XR + Molecular Imager® with BIO-RAD Image lab 5.1 software (California, USA).

### *Spatial and temporal monitoring*

#### Eel Lake sites and sampling procedures

The study site location was Eel Lake, NS (43°49'39.2"N; 65°54'31.2"W), where the invasion of the serpulid occurred (Figure 1). Eel Lake contains brackish water, with its saltwater supplied through a brook in Ste. Anne du Ruisseau. The lake experiences semi-diurnal tidal influence from the Bay of Fundy. The exact tidal influence and current speeds are unknown in the lake; however, data collected in Salt Bay, NS (43°49'06"N; 65°55'06"W, adjacent to Eel Lake) was found to have an average current speed 10 cm/s (NS Department of Fisheries and Aquaculture data 2017) and the closest Canadian Hydrological Service (CHS) tidal station in Abram's River, NS (43°49'57"N; 65°56'20"W) show tidal highs of up to ~ 3.8 m. It can be assumed that the currents and tidal influence in the lake are smaller due to its enclosed nature. Salinity in the lake fluctuates depending on environmental factors such as weather, tidal cycle, depth, etc. Water parameters were monitored in the lake at a fouled oyster lease (43°49'39.263"N; 65°54'31.226"W)



**Figure 1.** Site map of Eel Lake, NS, with sampling locations in summer of 2013. Site 1 – Non-fouled (43°49'3.569"N; 65°53'59.694"W), Site 2 – Fouled (43°49'31.436"N; 65°54'25.65"W), Site 3 – Fouled (43°49'35.67"N; 65°54'29.048"W), Site 4 – Fouled (43°49'41.243"N; 65°54'29.048"W). NS – Nova Scotia; NB – New Brunswick, PEI – Prince Edward Island.

on three dates (June 27, July 8, and September 10) by Fisheries and Oceans Canada. Water parameters were measured in the water column up to 9.1 m and found that salinity fluctuated between 12–22‰, with an apparent vertical salinity gradient. Temperature data was also recorded in the summer of 2013 by Fisheries and Oceans Canada. A HOBO® temperature data logger was deployed on the fouled aquaculture lease in the summer of 2013 into Eel Lake from June 27<sup>th</sup>–October 13<sup>th</sup>, 2013, which recorded the temperature data every hour.

Water samples were collected weekly from June to the end of August in 2013. Sampling occurred at four locations within the lake three fouled sites that were ~ 0.15 km apart and one non-fouled site which was ~ 1 km from the closest fouled site (Figure 1). The fouled sites (2–4) were in close proximity to where water parameters were collected. At each site, one water sample was obtained from three depths (0.3 m, 1.5 m, and 3.0 m) in the water column (three samples total). At each site and depth, the submersible pump was placed in the water and 150 l of water was pumped through a 60-micron sieve creating a highly concentrated sample. The sample was placed in a 50 ml test tube and was immediately frozen.

#### eDNA sample processing

Water samples were thawed and centrifuged at 3000 g for five minutes until a pellet formed at the bottom of the 50 ml test tube. The pellet was pipetted out of the sample and placed into a 1.5 ml microcentrifuge tube. DNA was extracted as above. Optimized species-specific primers described above were used to amplify the DNA extracted from the water samples

using PCR, then agarose gel electrophoresis was performed to determine if *H. dianthus* DNA was present in the water sample collected. Technical replicates were not obtained at each site and depth, and therefore the presence/absence of amplification of one sample was used to determine if the sample was positive for *H. dianthus* for a particular site and depth.

#### Statistical analysis

Three separate statistical analysis were completed in this study to determine if there were significant differences between the sites (site 1 (non-fouled), site 2 (fouled), site 3 (fouled), site 4 (fouled), depths (0.5 m, 1.5 m, 3.0 m), and sampling months (June, July, August). Differences between sites and depths were evaluated based on the overall % positive PCR results over the entire sampling period using a one-way ANOVA. The differences in % positive PCR samples between months was also evaluated using a one-way ANOVA. The interactions between sites, depths, and months could not be evaluated due to lack of replication. The assumptions of normality and equal variance were assessed using Anderson-Darling test of normality and Levene's test of equal variance. All estimates are reported as mean  $\pm$  standard error (SE), unless otherwise specified.

## **Results**

### *Sequencing and species confirmation*

Sequencing resulted in only one 416-bp sequence of the mitochondrial COI gene from a *H. dianthus* specimen collected in Eel Lake, NS (Accession #: ON003460.1). Species confirmation using blastn search resulted in the segment of COI gene definitively identified as *H. dianthus* with the top blast hit associated with two specimens KY605381.1 from China and MT044497.1 from Majorca, Mediterranean, both hits receiving a query coverage score of 100%, e-value of  $\sim$  0.0 and percent identity of 99.28%.

### *Species specific molecular assay*

#### Primer development

Comparison of *H. dianthus* Eel Lake specimen with 10 *Hydroides* spp. COI sequences (Table 1) resulted in the development of five species-specific primers (Table 3). Primer amplicon length was 72–125-bp with optimal annealing temperatures ranging from 51 to 53.2 °C. Each primer was verified with the NCBI database using the Primer-BLAST tool to ensure primers would not result in amplification of non-target organisms.

#### Efficacy, sensitivity, and specificity tests

Amplification of *H. dianthus* was successful for all COI primer sets for the two positive controls and did not result in amplification in negative control



**Table 3.** Species specific primers developed for *Hydroides dianthus* Eel Lake specimens from the COI gene.

Primer	Sequence 5'-3'	Location	Length	Annealing
H. dianthusCOI1F	GGGTCGGTTGGATCGTATAGC	275–295		
H. dianthusCOI1R	AGTCAACCCACCAGCCAAC	371–353	97	53 °C
H. dianthusCOI2F	GTCCAGAGGGTCGGTTGGA	268–286		
H. dianthusCOI2R	GTCAACCCACCAGCCAACA	352–370	103	53.2 °C
H. dianthusCOI3F	GCGATTGGGGTGACAAC	308–326		
H. dianthusCOI3R	AAAAGCATAGTCAACCCACCA	359–379	72	53 °C
H. dianthusCOI4F	AATCGGGGCATCAGACATAAA	8–28		
H. dianthusCOI4R	AAGTTCACCCCGTTGAAGC	132–113	125	51 °C
H. dianthusCOI5F	ATCGGGGCATCAGACATAAAT	9–29		
H. dianthusCOI5R	AAAGTTCACCCCGTTGAAGC	113–133	125	51.2 °C

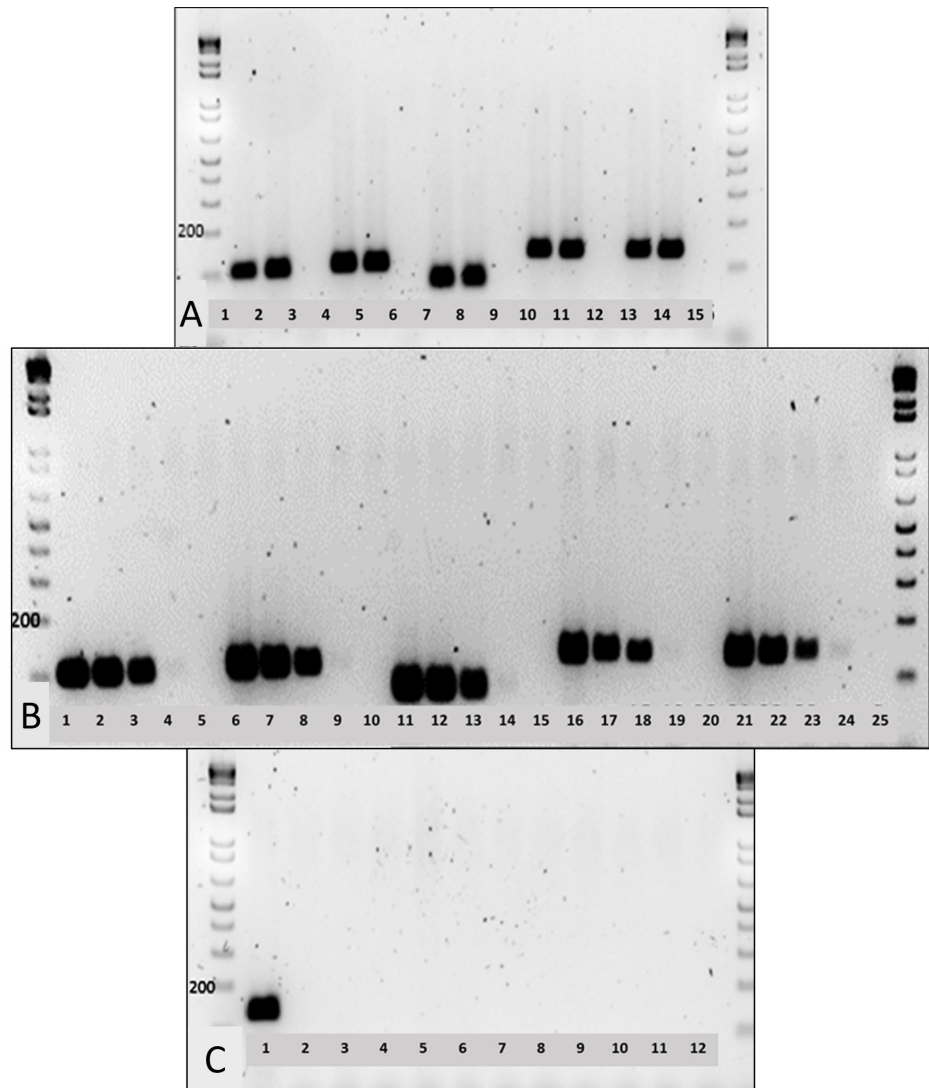
(Figure 2A). All primers could detect down to the lowest concentration (0.1 ng/l) (Figure 2B) and all primers were specific and did not amplify any other *Hydroides* spp. other than the target species (Figure 2C). This indicated that all primer sets were optimal for this research. H.dianthusCOI4F/R was chosen as the best candidate as it resulted in high quality amplification and had a large amplicon size, therefore, was used for spatial and temporal monitoring in this study.

### *Spatial and temporal monitoring and analysis*

#### Eel Lake depths, sites, and temperature recording

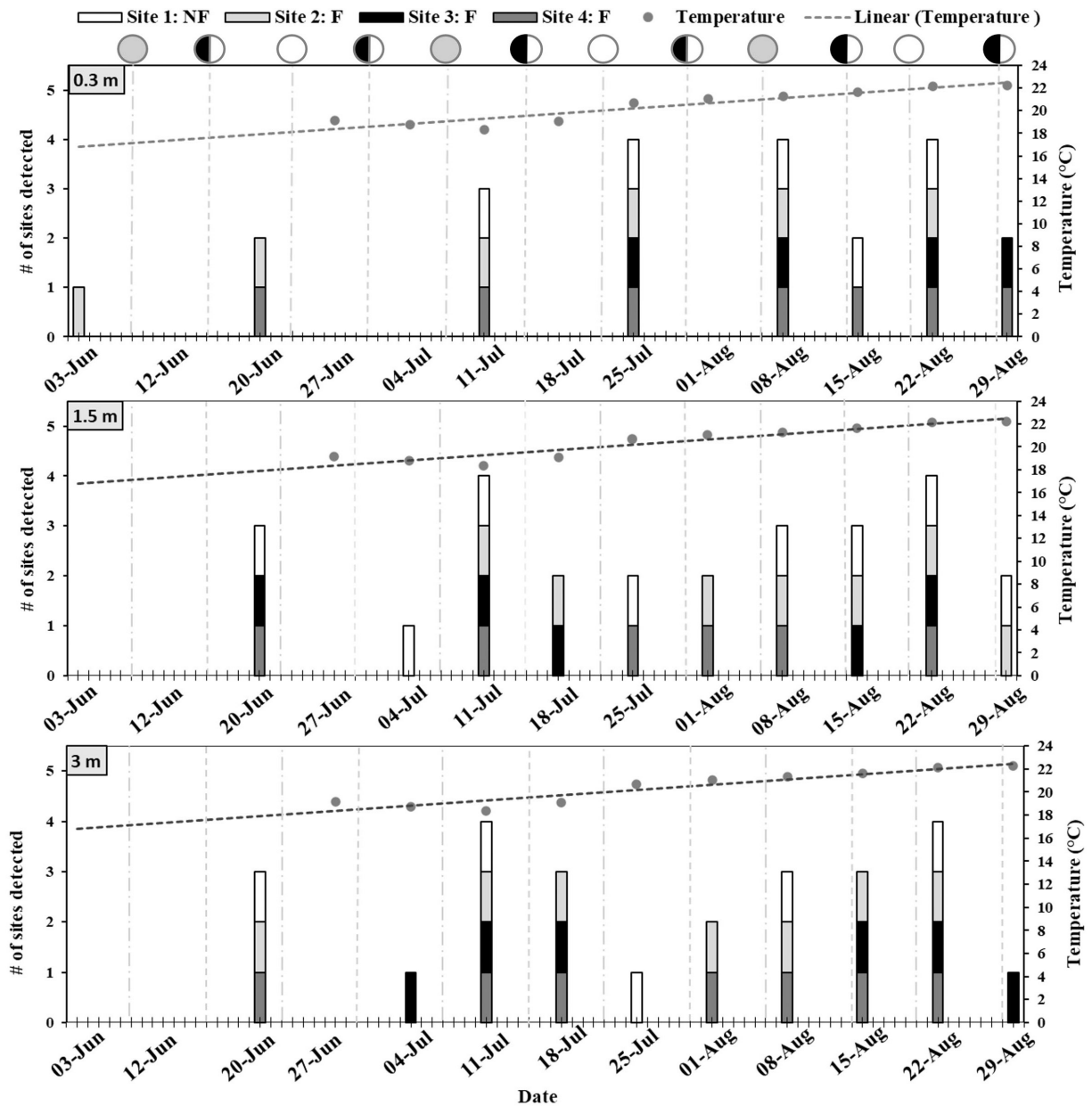
There were significant differences in the % positive PCR samples between months (June to August) ( $p$ -value < 0.001). Each month had significantly different % positive PCR samples with June having a mean of  $18.7 \pm 0.1\%$ , July having a mean of  $52.7 \pm 4.1\%$ , and August having a mean of  $65.0 \pm 2.9\%$ . Figure 3 shows the trend in positive PCR results over the sampling period at each site and depth. On the first week of sampling (June 3), only one water sample tested positive for *H. dianthus*, site 2 (fouled) at depth 0.3 m. No water samples were found positive again until the week of June 20 when all sites, including the non-fouled site, tested positive in at least one depth. After this, there were no positive results until July 4 when site 1 tested positive at the 1.5 m depth and site 3 tested positive at 3.0 m depth. Then, on July 11, all sites and depths tested positive for *H. dianthus*, with exception of site 3 at depth 0.3 m. From this date, positive PCR results occurred more frequently, as seen with higher mean % positive water samples for the months July and August.

The effects of depths were not significantly different ( $p$ -value = 0.5) and *H. dianthus* was detected in all levels. Overall, it was found that the mean % positive PCR result were  $45.8 \pm 5.4\%$  in 0.3 m depth,  $54.2 \pm 5.4\%$  in 1.5 m depth, and  $52.1 \pm 4.0\%$  in 3 m depth. Despite this, an oscillating trend was observed after July 11 in the 0.3 m depth, with one week testing positive for *H. dianthus* in most sites (July 11, July 25, August 8, August 22) followed by a week of negative detection of *H. dianthus* in all sites (or most) (July 18, August 1, August 15). The positive PCR results in the 0.3 m depth occurred



**Figure 2.** Agarose gel of PCR amplicons created using all newly designed primer assays for: A – Efficacy testing for assay developed for COI gene: Ln 1–3 = *H.dianthus*COI1F/R; Ln 4–6 = *H.dianthus*COI2F/R; Ln 7–9 = *H.dianthus*COI3F/R; Ln 10–12 = *H.dianthus*COI4F/R; Ln 13–15 = *H.dianthus*COI5F/R. Positive controls are located in Ln 1, 2, 4, 5, 7, 8, 10, 11, 13, 14. Negative controls are located in: Ln 3, 6, 9, 12, 15. B – Sensitivity test for primer assays developed: Ln1–5 = *H.dianthus*COI1F/R; Ln6–10 = *H.dianthus*COI2F/R; Ln11–15 = *H.dianthus*COI3F/R; Ln16–20 = *H.dianthus*COI4F/R; Ln21–25 = *H.dianthus*COI5F/R. Each primer set was tested for sensitivity using a series of dilutions on *H. dianthus* positive controls: 100 ng/l = Ln 1, 6, 11, 16, 21; 10 ng/l = Ln 2, 7, 12, 17, 22; 1 ng/l = Ln 3, 8, 13, 18, 23; 0.1 ng/l = Ln 4, 9, 14, 19, 24. Negative controls are located in: Ln 5, 10, 15, 20, 25. C – Specificity test using *H.dianthus*COI4F/R assay: Ln 1 = *Hydroides dianthus* positive control; Ln 2 = negative control; Ln 3 = *Hydroides brachyacantha*; Ln 4 = *Hydroides dirampha*; Ln 5 = *Hydroides elegans*; Ln 6 = *Hydroides ezoensis*; Ln 7 = *Hydroides heterocera*; Ln 8 = *Hydroides homoceros*; Ln 9 = *Hydroides longispinosa*; Ln 10 = *Hydroides minax*; Ln 11 = *Hydroides recurvispina*; Ln 12 = *Hydroides trivesiculosa*.

a few days after the full or new moon (spring tide) (Figure 3). Statistical analysis associated with this oscillating trend could not be completed as more replicates would be necessary. Sites were also not found significantly different ( $p$ -value = 0.8) with all locations having positive PCR results. Overall, mean % positive PCR results in site 1 (non-fouled) was  $50.0 \pm 4.8\%$ , site 2 (fouled) was  $47.2 \pm 2.8\%$ , site 3 (fouled) was  $50.0 \pm 8.3\%$ , and site 4 (fouled) was  $55.6 \pm 7.3\%$ .



**Figure 3.** Bar chart showing summary of spatial and temporal monitoring in Eel Lake, NS from June to August 2013. Each chart represents a different depth (0.3 m, 1.5 m, 3 m). Each Bar show the site(s) at a particular date when a positive PCR result was obtained. Average temperature and trend is shown in relation to each week sampled. NF = Non-fouled; F = fouled. Approximate date of moon phases is shown above the graph: shaded circle = new moon; circle (half shaded) = quarter moon; white circle = full moon.

Temperature recording occurred in the lake starting on the week of June 27, therefore, temperatures for the first week when *H. dianthus* was detected (June 20) could not be determined. However, during the second major peak of the summer (July 11), the temperature in the water column was ~ 18.3 °C. From this point on, water temperature increased until the end of the sampling period when the temperatures were ~ 22.2 °C, and detection of *H. dianthus* occurred frequently (Figure 3).

### Discussion

The frequent positive PCR results in all sites and depths over the summer of 2013 indicate that *H. dianthus* spawned multiple times. This finding is

consistent with the patterns demonstrated for other members of the genus *Hydroides*, which spawn several times over their lifetime, typically, continuously over their reproductive season (Kupriyanova et al. 2001). Grave (1933) documented that *H. dianthus* (as *Hydroides hexagonus* (see Read et al. 2017)) spawned from June to late October in Woods Hole, Massachusetts, and Zuraw and Leone (1968) found that *H. dianthus* could spawn every two to four weeks at 23 °C during their reproductive season. The temperatures in Eel Lake within the study period was between ~ 18–22 °C, indicating that *H. dianthus* was able to spawn at least once but likely multiple times within this temperature range, as the prevalence of positive PCR results significantly increased from June to August. A longer study period, along with visual observation of larvae through sampling would be necessary to understand whether spawning took place in Eel Lake at temperatures above or below this range.

This study hypothesized that heavy biofouling of *H. dianthus* could be mitigated by identifying an optimal depth to hold oyster cages when spawning was occurring. However, there were no significant differences between depths and positive PCR results were found in all examined depths through out the summer. Interestingly, an oscillating trend was found within the 0.3 m depth, where positive PCR results were obtained on a biweekly basis after July 4<sup>th</sup>. It is known that *H. dianthus* larvae exhibits photopositivity in laboratory setting (Zeleny 1905; Kupriyanova et al. 2001), but nothing is known about its behaviour in a natural environmental setting. This oscillating trend could be an indicator of photopositive behaviour when positive PCR results were obtained in surface water. On the weeks where *H. dianthus* tested negative in surface water, it was found positive frequently at the 1.5 m and 3 m depth and could be indicator of photonegative behaviour. While it is not known if this trend was produced by coincidence, it is also not uncommon for pelagic larvae to exhibit this photopositive response followed by photonegative behaviour (Thorson 1964) and has been found in other serpulids (Miura and Kajihara 1981; Kupriyanova et al. 2001). In theory, if *H. dianthus* is found less frequently in the surface waters, then the optimal area to maintain cages would be in the surface water. Unfortunately, oyster bags/cages in Eel Lake were already maintained at the surface, but biofouling from *H. dianthus* persisted. It is likely that important settlement cues, such as an optimal biofilm and presence of conspecifics on bags/cages triggered the associative gregarious settling well-documented for *H. dianthus* (Toonen and Pawlik 1994, 2001). Therefore, there was not an optimal depth to maintain oyster cages during *H. dianthus*' reproductive season.

In Atlantic Canada, prevention of biofouling on oyster aquaculture leases by barnacles (*Balanus improvisus*) and mussels (*Mytilus edulis*) can be mitigated by completing a mid-August flip and early October flip during settlement periods of these species (Mallet et al. 2009). However, in areas

with biofouling from invasive solitary tunicates such as *Ciona intestinalis*, treatment may be needed in mid-July and mid-August (Ramsay et al. 2009), or as frequent as biweekly during times of heavy biofouling for invasive colonial tunicates such as *Botryllus schlosseri* and *Botrylloides violaceus* (Government of New Brunswick 2018). Biofouling by *H. dianthus* results in an additional challenge, as their calcium carbonate tube is difficult to remove. Other biofouling organisms that are difficult to remove, such as barnacles (*Balanus improvisus*) and oysters (*Crassostrea virginica*), can be controlled through desiccation during their initial settlement period (Adams 1991), which is easier to time properly because barnacles and oysters only have one major spawning event per reproductive period. In contrast, frequent spawning events documented for *H. dianthus* in this study indicate that this flipping would have to be done on a weekly or biweekly basis to remove newly settled larvae, as *H. dianthus* has been found ready to settle and undergo metamorphosis as early as 5 days post-fertilization at 24 °C (Scheltema et al. 1981), or 10–14 days (no temperature specified, likely < 24 °C) (Grave 1933, 1937). Biweekly flipping was already used in Eel Lake and was not successful at preventing *H. dianthus* biofouling, indicating that weekly flipping of cages may be necessary to mitigate fouling. The persistence of fouling despite biweekly flipping might be the result of fast growth rate and strong gregarious settlement response. Once settled, the tube of *H. dianthus* can grow up to 54 mm in their first three months (Grave 1933), indicating that biofouling can rapidly accumulate through gregarious settling behaviour (Toonen and Pawlik 1994, 2001). Therefore, integration of weekly flipping of cages during peak spawning/settling activity and frequent cleaning of cages may be the only method to mitigate biofouling of *H. dianthus* on the oyster lease.

Interestingly, positive PCR results obtained in the 0.3 m depth on a biweekly basis also appear to occur a few days after a spring tide (i.e. after full or new moon) (Figure 3). Marine organisms have been found to exhibit semilunar rhythmicity (Naylor 2013). Some serpulids from the genus *Spirorbis* synchronize their release of larvae at the neap tide (Garbarini 1933; Knight-Jones 1951; Rothlisberg 1974). For example, *Spirorbis marioni* Caullery and Mesnil, 1897 synchronizes its spawning to the spring tide but release of larvae occurred during the neap tide (Rothlisberg 1974). Release of larvae on neap tides may have benefits to marine organisms as reduced tidal influence mitigates the risk of larvae being carried away to more hostile environments (Naylor 2010). In contrast, some species of corals spawn on spring tides so that their larvae are swept away and allow for wider dispersal (Naylor 2010). *Hydroides dianthus* is a broadcast spawner with external fertilization and planktotrophic development in the water column (Scheltema et al. 1981), therefore the breeding behaviour of this species differs from that found in the genus *Spirorbis* that brood lecithotrophic larvae (Rothlisberg 1974). However, if positive PCR results at 0.3 m depth

are associated with early-stage photopositive larvae it could indicate that spawning occurs closer to a spring tide, where *H. dianthus* could benefit from dispersal during stronger tides to establish new populations, but this hypothesis needs further confirmation. This study also found that there were no significant differences between sites and all sites had positive PCR results for *H. dianthus* throughout the summer, including the non-fouled site. Unfortunately, this could be indicative that the site was recently fouled or that larvae were dispersing toward that area of the lake. Once in a new location, *H. dianthus* colonises new substrates and give off chemical cues to other conspecifics, to settle and form new populations (Toonen and Pawlik 1994).

It is unknown how *H. dianthus* spread to Eel Lake, NS. Aquatic invasions are usually attributed to ballast water systems, shellfish transfers, ship hulls, recreational boating, floating marine infrastructure and debris (Mckindsey et al. 2007; Darbyson et al. 2009; Davidson et al. 2010; Ruiz et al. 2011; Sylvester et al. 2011; Briski et al. 2012). However, ballast water systems have never been a proven method of transport of serpulid larvae (Capa et al. 2021), and international shellfish transfers of oysters are not permitted due to presence of reportable diseases such as MSX (*Haplosporidium nelsoni* (Haskin et al. 1966)) and Dermo disease (*Perkinsus marinus* (Mackin et al. 1950)). It can't be completely ruled out that the invasion was through a natural range extension, as Eel Lake is adjacent to its northern most point of distribution in Cobscook Bay, Maine, USA (Trott 2004) (~150 km away from Eel Lake) which could have been facilitated by floating marine debris that made its way into the lake. However, it is unlikely, as the major currents that circulate around the Bay of Fundy are typically counter-clockwise including the Scotian Shelf Coastal Current and Bay of Fundy Gyre (Aretxabaleta et al. 2009) that would make it difficult for natural currents to transport marine debris from Cobscook Bay to Eel Lake. It is therefore more feasible that *H. dianthus* was transported by anthropogenic means such as by recreational boating with attached fouling.

*Hydroides* spp. biofouling has been known to have consequences to the aquatic ecosystem, aquaculture leases and marine infrastructure. *Hydroides dianthus* has been found to smother juvenile oysters from biofouling (Eno et al. 1997) and is concerning for shellfish growers that rely on wild spat collection. Removal of biofouling can also lead to scars that impacts the aesthetics of shells, which can impact market value of shellfish (Watson et al. 2009; Dürr and Watson 2010). Therefore, mitigating the spread of *H. dianthus* in Atlantic Canada, outside of Eel Lake will be important in the future, and the use of molecular assay using eDNA shows promise in monitoring programs (LeBlanc et al. 2020). The molecular assay developed and applied in this study was useful in understanding the spatial and temporal patterns of *H. dianthus* larvae in eDNA water samples in the summer of 2013; however, it has the potential to be used in the future to monitor spread elsewhere in Atlantic Canada. Future studies should also

include visual observation along side this method of detection to ensure that the detection of *H. dianthus* was from larvae and not other tissues from *H. dianthus* such as sloughed cells.

## Conclusions

Monitoring of *H. dianthus* in eDNA samples from Eel Lake reiterated the challenges associated with serpulid biofouling. Frequent spawning and presence of *H. dianthus* in all depths indicated that there was no optimal depth for oyster cages to be sunk to, and that mitigation of recruitment is likely to be highly challenging. More research in the aquaculture industry is needed to find solutions for biofouling by serpulids. The potential impacts to other shellfish leases in Atlantic Canada warrant the need to mitigate and monitor the spread of this species in the region and the molecular assay developed in this study may be an excellent tool to assist with this.

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## Authors' contribution

Stephanie Hall: investigation and data collection; data analysis and interpretation; writing – original draft; writing – review and editing; Sarah Stewart Clark: research conceptualization; sample design and methodology; investigation and data collection; data analysis and interpretation; writing – review and editing; Elena Kupriyanova: samples and methodology; writing – review and editing.

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