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METHODS AND RESOURCES

A validated targeted assay for environmental DNA detections of the Atlantic wolffish (*Anarhichas lupus*)

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Abstract

The Atlantic wolffish (*Anarhichas lupus*) was assessed as a species of Special Concern by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) under the Canadian Species-At-Risk Act (SARA) in 2001, and by the National Marine Fisheries Service in USA in 2004. Monitoring of marine Species-At-Risk would rely ideally on non-destructive methods. However, most monitoring of marine fish at-risk rely on trawl surveys that are potentially destructive of the environment. Inferring a species presence using environmental DNA (eDNA) detections offers an attractive alternative for Species-At-Risk monitoring, because it is non-destructive, specific, and sensitive. We developed and optimized a real-time quantitative PCR probe-based (qPCR) detection protocol that targets the eDNA of Atlantic wolffish, *A. lupus*. The qPCR protocol was validated *in silico*, *in vitro*, and *in situ*. Species-specificity was assessed *in vitro* by testing against the two other species of *Anarhichas* present in the northwest Atlantic. We did not observe DNA amplification for either of these two species. The assay was highly sensitive, with a limit of detection (95% confidence level) of 1.5 DNA copies per qPCR reaction. *In situ* tests showed that *A. lupus* eDNA is detected from expected depth strata in areas of known wolffish abundance. This study provides a proof-of-concept experiment that offers a robust, targeted, and non destructive protocol for detection eDNA of the Atlantic wolffish.

Keywords *Anarhichas lupus* · Environmental DNA · qPCR · Targeted detection · Species-At-Risk

Introduction

Environmental DNA (eDNA) is "DNA collected from an environmental sample without any attempt to isolate the organism(s) from which the DNA derives" (Darling 2019). Detection methods of eDNA are used to infer the presence of a species with a non-destructive approach in terrestrial and aquatic environments (Moran et al. 2019; Antognazza et al. 2019; Leempoel et al. 2020). Furthermore, a targeted detection method of eDNA by quantitative polymerase chain reaction (qPCR) can be more sensitive than traditional methods for low-abundance species (Beng and Corlett 2020; Keller et al. 2022). Such non negligeable advantage most likely underlies the gain in popularity of eDNA detections from water samples during the last decade for the monitoring of rare species from freshwater (Thomsen et al. 2012; Wilcox et al. 2013; Rees et al. 2014; Davy et al. 2015; Veldhoen et al. 2016; Roux et al. 2020) and marine environments (Gargan et al. 2017; Weltz et al. 2017; Stoeckle et al. 2018; Wood et al. 2019; Hansen et al. 2020; Chevrinais et al. 2023).

Three species of wolffish, in the genus *Anarhichas,* occur in the northwest Atlantic Ocean (Kulka et al. 2007). These are *A. denticulatus* (northern or broadhead wolffish), *A. minor* (spotted wolffish), and *A. lupus* (Atlantic or striped wolffish). The three species inhabit shelves from the southern Grand Banks to the Davis Strait (Kulka and DeBlois 1996). *A. lupus* differed from the two other species by being densely concentrated on the shallow part of the southern Grand Banks (Kulka and DeBlois 1996). This species is also present along the Labrador Shelf, in deep parts of the Gulf of St. Lawrence, on the Scotian Shelf, in the Bay of Fundy (McRuer et al. 2000), along the coast of Maine and on Georges Bank (Nelson and Ross 1992). Higher densities

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of *A. lupus* are observed between 150 and 350 m at temperatures between 1.5 and 4 °C (Kulka et al. 2007), and lower densities are also surveyed in shallow waters (< 22 m) (Novaczek et al. 2017). Atlantic wolffish are largely sedentary, remaining in a 4–8 km area during their adult stage (Templeman 1984; Simpson et al. 2014). Adults may undergo seasonal migration from shallow water (< 120 m) in spring to deeper water in autumn (Nelson and Ross 1992). Those fish inhabit caves in boulder bedrocks (Novaczek et al. 2017). In the northwest Atlantic, juveniles of the Atlantic wolffish occur in deeper offshore waters (> 30 m) than adults and adults seem to operate a migration to shallow waters (< 30 m) at sexual maturity to begin to spawn in late August (Keats et al. 1986).

A decline in the density of *A. lupus* was recorded between 1980 and 2001 in Canadian waters based on trawl surveys (Kulka et al. 2007). In 2001, this species was assessed by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) as of "Special Concern", i.e., a species that might become Threatened or Endangered if trends continue. It is the subject of a management plan formulated in 2007 to increase its densities and distribution, and thus achieve long-term viability of this species. *Anarhichas lupus* is still vulnerable as bycatch in 20 directed demersal fisheries (Kulka et al. 2007; Bluemel et al. 2022). Trawl survey monitoring is also the primary source of information for monitoring *A. lupus*. However, some *A. lupus* habitats such as boulder bedrocks are unsuitable for trawling and no occurrence information is available in these areas. Environmental DNA monitoring would be a complementary non-destructive method providing occurrence information across the species distribution and limiting mortality due to trawling.

In this study, we developed and optimized a qPCR assay to detect DNA from Atlantic wolffish with *in silico*, *in vitro* and *in situ* validation steps. Our aim was to provide a new molecular and non-destructive tool for the detection (*i.e.*, presence, absence) of this species. A probe-based qPCR assay was developed following the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR experiments, Bustin et al. 2009), the critical considerations for the application of eDNA methods to detect aquatic species (Goldberg et al. 2016), the Fisheries and Oceans Canada (DFO) minimum requirements in eDNA studies (Abbott et al. 2021), and the Canadian Standards Association eDNA reporting requirements and terminology (Gagne et al. 2021). We report a detailed protocol for the qPCR assay, an evaluation of performance characteristics, and an initial field test of the detection protocol as an eDNA biomonitoring tool for a Species-At-Risk.

Materials and methods

In silico design

A partial sequence of the mitochondrial cytochrome B (CYTB) gene was targeted because interspecific variation for the *Anarhichas* genus was previously documented (Johnstone et al. 2007; Lait and Carr 2018). Sequences from west Atlantic specimens were retrieved from NCBI (National Center for Biotechnology Information, nih.gov; Table S1). They were aligned with Geneious Prime 2020.0.4 (https://www.geneious.com) with the ClustalW alignment to identify conserved and variable regions in *A. lupus* and closely related species. Specific primers and TaqMan™ Minor Groove Binding (MGB) probe were designed manually to amplify a region of 92 base pairs (bp). Mitochondrial DNA fragments under 200 bp were targeted to maximize the likelihood of detecting short fragments of eDNA (Jo et al. 2022). Assay specificity was tested *in silico* with Geneious Prime 2020.0.4 with sequences from closely related species and using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table S1, Table 1). The qPCR amplification parameters were optimized using OligoEvaluator (http://www.oligoevaluator.com) and Tm Calculator (http://tmcalculator.neb.com). The qPCR assay was then optimized for an annealing temperature of 60 °C.

In vitro specificity

In vitro specificity of the qPCR assay was tested with DNA extracts from congeneric species of *A. lupus*, *A. denticulatus*, and *A. minor* (Table S2). Genomic DNA was extracted from each specimen using the DNeasy® Blood and Tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol and was stored at -20 °C.

Table 1 Information on qPCR assay targeting *Anarhichas lupus* DNA, primers (forward, F; reverse, R), TaqMan® Minor Groove Binding probes (P), and qPCR assay performance. LOD95% is for Limit Of Detection and 95% confidence.

Primers and probes	Sequence 5'-3'	Amplicon size (bp)	Efficiency (%)	Regression equation	LOD95% (copies/reaction)
Al_CytB_F Al_CytB_R Al_CytB_P	CCCTACTGCTCAAAGAAAGGAGATTTTAACTC TGAATACCATTGAAATGGTTAAAATAAATACATGGTGATA 6FAM-TGCGCGTATGTAACTAA TCATGTATGTACTTAGTGCAT-MGBNFQ	92	103.6	-3.24 (log(x)) + 40.32	1.5

Nonspecific amplification and primer dimer amplification were assessed by specificity testing with TaqMan® probe qPCR. The volumes and concentrations of qPCR assay solution were 12.5 µL of TaqMan™ Gene expression master mix (Thermo Fisher Scientific™, Waltham, USA), 1.2 µL of forward and reverse primers (10 µM), 0.5 µL of the TaqMan™ MGB probe (10 µM, Applied Biosystems®, Foster City, USA), 3 µL of DNA template, 1 µL of 1% Bovine Serum Albumin (Sigma-Aldrich, Saint-Louis, MO, USA), and 5.6 µL of nuclease-free water (Sigma-Aldrich, Saint-Louis, MO, USA) to yield a volume of 25 µL per reaction. Cycling parameters were 2 min. at 50 °C, 10 min. at 95 °C followed by 40 cycles of 30 s. denaturation step at 95 °C, 30 s. annealing step at 60 °C, and 30 s. extension step at 72 °C. All qPCR reactions were conducted on the AriaMX qPCR system (Agilent Technologies, Santa Clara, CA, USA). Fluorescence was measured at the end of each elongation step.

In vitro optimization

Optimal primer and probe concentrations were defined as the conditions generating the lowest quantification cycle (Cq value) and were assessed using plasmid DNA (pDNA, Genewiz, South Plainfield, NJ, USA) containing the target CYTB sequence. Primer concentrations were first optimized asymmetrically, testing forward and reverse primer final reaction concentrations with combinations of 200, 400, 600 and 800 nM and a fixed probe concentration of 200 nM (Fig. S1A). Then, probe concentrations of 100, 150, 200, and 250 nM were tested with the best primer concentrations combination (Fig. S1B). A last qPCR test was performed to assess the impact of low template DNA concentrations (20 to 2 DNA copies per qPCR reaction) with the optimal primers and probe concentrations (Fig. S1C).

The optimized volumes and concentrations of qPCR assay solutions were 10 µL of TaqPath™ ProAmp™ 2x master mix (Thermo Fisher Scientific™, Waltham, USA), 1.6 µL of forward and 1.2 µL of reverse primer (10 µM), 0.5 µL of the TaqMan™ MGB probe (10 µM, Applied Biosystems®, Foster City, USA), 3 µL of DNA template, 0.8 µL of 1% Bovine Serum Albumin (Sigma-Aldrich, Saint-Louis, MO, USA), and 2.9 µL of nuclease-free water (Sigma-Aldrich, Saint-Louis, MO, USA) to yield a volume of 20 µL per reaction. Cycling parameters were 10 min. at 95 °C followed by 45 cycles of 15 s. denaturation step at 95 °C, and 1 min. annealing step at 60 °C with the same apparatus as in the previous section.

In vitro sensitivity

Serial dilutions of pDNA were used to determine the *in vitro* sensitivity of the qPCR assay with optimal qPCR conditions from Sect. 2.3. Serial dilutions from 20,000 to 0.25 DNA copies per reaction were tested in six replicates by qPCR to determine the assay efficiency using the equation $E = -1 + 10[-1/slope]$ and to calculate the theoretical limit of detection (LOD). The LOD95% was defined as the lowest standard concentration at which 95% of the replicates had pDNA detected. The LOD95% is determined following the method developed by Klymus et al. (2019).

In situ validation

Samples were processed following a qPCR detection workflow with procedures to limit contamination (Chevrinais et al. 2023, Fig. 1, Appendix 1). Briefly, all samples were processed in an eDNA specific ultraclean laboratory, located at the Maurice Lamontagne Institute (MLI, Fisheries and Oceans Canada, Quebec). All laboratory users were trained to work in clean conditions according to a standard operating procedure.

Sampling and filtration

The qPCR assay was validated *in situ* with water samples from the 2020 DFO annual ecosystem survey in the Estuary and Gulf St. Lawrence. Samples were collected from six stations of the Gulf of St. Lawrence (Fig. 1). Four stations (1–4, Fig. 1) and two stations (5, 6, Fig. 1) were in areas of high and low densities of *A. lupus*, respectively, according to trawl captures with a four-sided Campelen 1800 shrimp trawl equipped with a Rockhopper footgear (McCallum and Walsh 2002; Bourdages et al. 2021). At each station, two 2 L samples were collected from two distinct Niskin bottles, i.e. one at 15 m from the surface and the other at one m from the bottom (Fig. 1, $N_{samples} = 12$). Samples were frozen at – 20 °C until filtration at MLI. Along with field samples, a field negative control was collected each sampling day. It consisted in Milli-Q® water (MilliporeSigma, Darmstadt, Germany) transferred between two 2 L bottles in the vessel sampling room.

After overnight thawing, samples and field negative controls were filtered with 1.2 µm glass fibre filters using a vacuum pump filtering system in the ultraclean laboratory (Chevrinais et al. 2023, Appendix 4). A filtration negative control using Milli-Q® water was included on each filtration day. Filters were preserved at -20 °C until extraction.

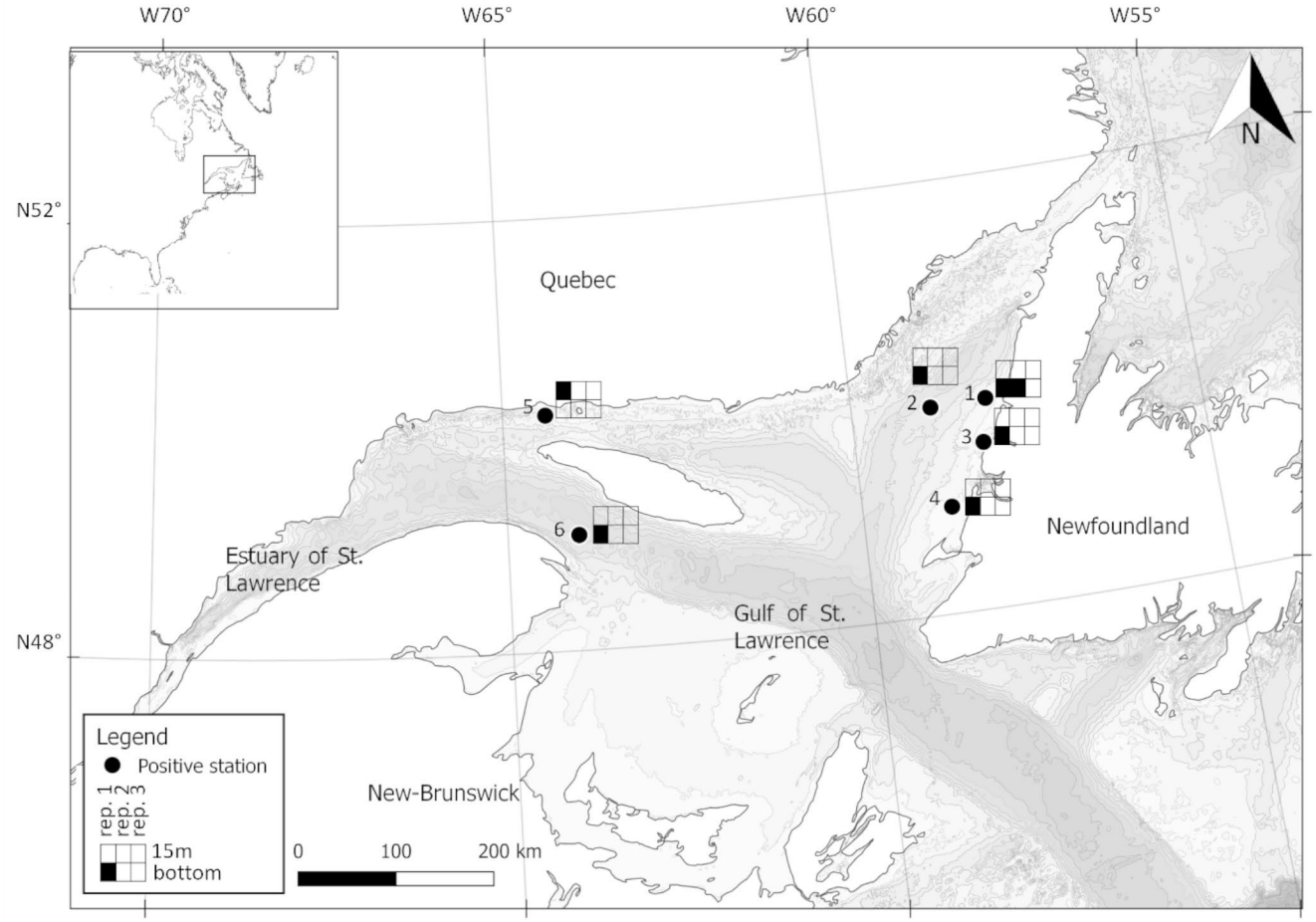

Fig. 1 Sampling stations in the Gulf of St. Lawrence (Canada) and qPCR replicate results for *Anarhichas lupus* qPCR assay. Results from the three qPCR replicates per depth are presented (15 m: upper squares; bottom: lower squares). Black squares indicate positive qPCR replicates, while empty squares indicate negative qPCR replicates (WGS 84/Pseudo-Mercator EPSG:3857). Stations GPS coordinates are provided in Table S3.

DNA extraction

eDNA was extracted from one half filter with the DNeasy® Blood and Tissue extraction kit with minor modifications to the manufacturer's instructions, *i.e.* filters were digested for 2 h at 56 °C in a mixture of 756 µL AL buffer and 84 µL of proteinase K. The elution step of the DNA extraction protocol was modified; the solution was incubated for five minutes using a TRIS buffer (10 mM) instead of the elution with the AE buffer containing EDTA, that likely inhibits the qPCR reaction (QIAGEN 2017). The elution volume was 80 µL. An extraction negative control, consisting in a half filter immersed in Milli-Q® water, was included with the samples. DNA extracts were preserved at -20 °C until qPCR detection and at -80 °C for long-term preservation.

Inhibition qPCR assay

We used an internal positive control (IPC), to assess for qPCR inhibition in sample DNA extract, following the protocol developed by Chevrinais et al. (2023). We added 220,000 copies of the IPC plasmid to all qPCR replicates from samples and field negative control. Inhibition was present in the sample DNA extract if the Cq value was delayed by 2 or more cycles compared to that of the DNA extract from the field negative control (LeBlanc et al. 2020).

Specific qPCR assay

We tested each DNA extract with three qPCR replicates and the *A. lupus* targeted qPCR assay (optimized qPCR protocol described in "*In vitro* optimization"). Each qPCR plate contained one to five qPCR negative controls, and one qPCR positive control. The qPCR positive control consisted in a concentration of 2,000 copies of a plasmid, including the

partial CYTB gene of *A. lupus* and an insertion of 6-nucleotides. This insertion of 6-nucleotides would enable to identify cross-contamination during qPCR plate preparation. Replicates of qPCR from sample DNA extracts were considered positive if they showed a sigmoidal amplification curve and a Cq ≥ LOD95% (Fig. S2).

All qPCR positive detections were Sanger sequenced to confirm the specificity of the *A. lupus* qPCR assay and the absence of contamination from the qPCR positive control. Products of qPCR were amplified using 1 µL of amplicon, 2 µL of each primer (10 µM), 10 µL of 10% trehalose and 0.5 µL of the Big Dye Terminator, 3.75 µL of the 5x buffer from the Applied Biosystems BigDye™ Terminator v3.1 cycle sequencing kit (Thermo Fischer Scientific, MA, USA) and 3.88 µl of nuclease-free water to yield a 20 µL final reaction. Cycling parameters were 1 min. denaturation step at 96 °C followed by 25 cycles consisting of a 10 s. denaturation step at 96 °C, a 5 s. annealing step at 50 °C and a 4 min. elongation step at 60 °C. Following amplification, PCR products were cleaned using the Applied Biosystems BigDye® Xterminator™ purification kit (Thermo Fischer Scientific, MA, USA) and sequenced on the Applied Biosystems SeqStudio genetic analyzer system (Thermo Fischer Scientific, MA, USA).

The qPCR negative controls were considered contaminated if a sigmoidal amplification curve was observed. A qPCR positive control failed if the expected Cq value was delayed by 2 or more cycles than its Cq value in the standard curve.

Criteria for reporting eDNA results from the qPCR assay

Environmental DNA detection results were reported for each of the three qPCR replicates per sample. We also used a decision tree with minimum criteria to summarize eDNA detection results at the sample and station levels. The minimum criteria for positive samples was that at least two out of three qPCR replicates showed an amplification curve and DNA copies number ≥ LOD95%. A sample was considered as inconclusive if one out of three qPCR replicates showed an amplification curve and DNA copies number ≥ LOD95%. A sample was considered as (1) negative if no amplification curves was observed or as (2) inconclusive if DNA copies < LOD95% were detected in all three qPCR replicates. A station was identified as positive if at least one out of the two samples (at 15 m or bottom) was positive, as inconclusive if at least one sample was inconclusive, and as negative if no sample was positive or inconclusive.

Results

qPCR assay performance

The qPCR assay was specific to *A. lupus* (Fig. S2). The in vitro specificity testing of qPCR assay for *A. lupus* showed no amplification of closely related species belonging to the *Anarhichas* genus (Fig. S2). The LOD95% was of 1.5 copies per qPCR reaction (Table 1).

In situ validation

Negative and positive controls used at various steps of the protocol passed for the *A. lupus* qPCR assay (Fig. S3). Inhibition was not detected in all samples collected in this study, *i.e.*, the IPC assay showed no delayed amplification compared to their field negative control (Fig. S4).

Anarhichas lupus DNA was detected in qPCR replicates from 15 m and bottom samples. The number of copies detected varied between 5 (15 m, station 5) and 16 (bottom, station 3) across positive qPCR replicates (Fig. S5). All qPCR positive replicates were sequenced, which confirmed that the target species was detected and that no contamination from the qPCR positive control occurred.

For bottom samples, station 1 had two positive qPCR replicates, stations 2, 3, 4, and 6 had one positive qPCR replicate, and station 5 had no positive qPCR replicates (Fig. 1). Bottom sample from station 1 was positive whereas those from stations 2, 3, 4, and 6 were inconclusive. For 15 m samples, station 5 had one positive qPCR replicate, all other stations had no positive qPCR replicates. Therefore, 15 m sample from station 5 was inconclusive and all other samples were negative. At the station level, station 1 was positive and all other stations were inconclusive for *A. lupus* eDNA detections.

Discussion

This study provides a specific and sensitive qPCR assay to detect *A. lupus* eDNA. This new powerful tool can be used in trawlable and untrawlable areas for the detection of *A. lupus* eDNA and limit the mortality of this species associated with ongoing survey activities. Future improvements to the *A. lupus* eDNA assay and future studies are discussed.

qPCR assay performance

The *A. lupus* qPCR assay and eDNA protocol were validated to essential-level 3 according minimum criteria from Abbott et al. (2021) and Thalinger et al. (2021). This validation level implies that a qPCR assay and eDNA detection

protocol went through *in vitro* specificity and sensitivity testing, and *in situ* testing. At the level 3, results should be interpreted as eDNA detections indicate that the target is likely present whereas the absence of eDNA detections cannot provide a conclusion whether the target is present or not (Abbott et al. 2021; Thalinger et al. 2021). We also established the LOD95%, a criteria needed for substantial-level 4 of validation (Abbott et al. 2021; Thalinger et al. 2021), at 1.5 copies per qPCR reaction which is adapted to rare species detection and falls into the lower range of the literature (Klymus et al. 2019). This LOD95% could also be improved by increasing the number of qPCR replicates in the standard curve and using binomial detections (Lesperance et al. 2021).

eDNA detections *in situ*

We detected Atlantic wolffish eDNA in qPCR replicates in samples from all six stations in the Gulf of St. Lawrence where the species had been captured in the trawl survey. The majority of qPCR positive detections was from bottom samples which coincide with high densities of juveniles or adults *A. lupus* at depths between 80 and 350 m (Kulka et al. 2007; Bourdages and Ouellet 2011; Dutil et al. 2014). Maximum densities of Atlantic wolffish with trawl data were also observed in the west coast of Newfoundland during the 2020 DFO annual ecosystem survey (Bourdages et al. 2021). The eDNA of *A. lupus* was also detected in shallow waters between 15 and 115 m (stations 1, 3, 4, 5, Table S3). Detections of eDNA in shallow waters are also expected but at lower densities for adults (Novaczek et al. 2017) or during their seasonal migration for spawning (Keats et al. 1986; Nelson and Ross 1992).

Station 1 was positive whereas all other stations were inconclusive for *A. lupus* eDNA detection. This result suggests that a greater number of qPCR replicates should be used to investigate for the presence of rare *A. lupus* eDNA. Theoretically a higher number of replicates would imply lower limits of detection and quantification which could allow an increased sensitivity for the detection of rare eDNA (see Figs 3 and 4 in Lesperance et al. 2021). Higher number of replicates both in the field and in the laboratory (*e.g.*, qPCR replicates) and the sampling of larger volumes would also increase the capacity to detect eDNA, especially for low-abundance species (Sepulveda et al. 2019; Schabacker et al. 2020; Buxton et al. 2021; Govindarajan et al. 2022).

This study provides a monitoring tool for *A. lupus* to survey untrawlable areas (*e.g.*, shoals). Such tool would also be valuable for the two other *Anarhichas* species occurring in the northwest Atlantic Ocean that are also Species-At-Risk. Future studies should also assess the quantitative performance of the *A. lupus* eDNA assay. This would necessitate a greater number of samples and a direct comparison between eDNA concentration to trawl captures of *A. lupus* across a study area.

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Author contributions Both authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Marion Chevrinais. The first draft of the manuscript was written by Marion Chevrinais. Geneviève J. Parent commented and improved previous versions of the manuscript. Both authors read and approved the final manuscript.

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Data Availability "The datasets generated during and/or analysed during the current study are available from the corresponding author on request."

Declarations

Competing interests The authors declare no competing interests.

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