

# Droplet digital PCR applied to environmental DNA, a promising method to estimate fish population abundance from humic-rich aquatic ecosystems

Eric Capo<sup>1,2</sup>  | Göran Spong<sup>2,3</sup> | Shuntaro Koizumi<sup>1</sup> | Isolde Puts<sup>1</sup> | Fredrik Olajos<sup>1</sup> | Helena Königsson<sup>2</sup> | Jan Karlsson<sup>1</sup> | Pär Byström<sup>1</sup>

<sup>1</sup>Department of Ecology and Environmental Science, Umeå University, Umeå, Sweden

<sup>2</sup>Molecular Ecology Group, Department of Wildlife, Fish and Environmental Studies, SLU, Umeå, Sweden

<sup>3</sup>Fisheries, Wildlife and Conservation Biology Program, Department of Forestry and Environmental Resources, North Carolina State University, Raleigh, NC, USA

## Correspondence

Eric Capo, Department of Ecology and Environmental Science, Umeå University, Umeå, Sweden.

Email: eric.capo@hotmail.fr

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## Abstract

Measures of environmental DNA (eDNA) concentrations in water samples have the potential to be both a cost-efficient and a nondestructive method to estimate fish population abundance. However, the inherent temporal and spatial variability in abiotic and biotic conditions in aquatic systems have been suggested to be a major obstacle to determine relationships between fish eDNA concentrations and fish population abundance. Moreover, once water samples are collected, methodological biases are common, which introduces additional sources of variation to potential relationships between eDNA concentrations and fish population abundance. Here, we evaluate the performance of applying the droplet digital PCR (ddPCR) method to estimate fish population abundance in experimental enclosures. Using large-scale enclosure ecosystems that contain populations of nine-spined stickleback (*Pungitius pungitius*), we compared the concentrations of fish eDNA (COI mitochondrial region, 134 bp) obtained with the ddPCR method with high precision estimates of fish population abundance (i.e., number of individuals) and biomass. To evaluate the effects of contrasted concentrations of humic substances (potential PCR inhibitors) on the performance of ddPCR assays, we manipulated natural dissolved organic carbon (DOC) concentrations (range 4–11 mg/L) in the enclosures. Additionally, water temperature (+2°C) was manipulated in half of the enclosures. Results showed positive relationships between eDNA concentration and fish abundance and biomass estimates although unexplained variation remained. Still and importantly, fish eDNA estimates from high DOC enclosures were not lowered by potential inhibitory effects with our procedure. Finally, water temperature (although only 2°C difference) was neither detected as a significant factor influencing fish eDNA estimates. Altogether, our work highlights that ddPCR-based eDNA is a promising method for future quantification of fish population abundance in natural systems.

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## KEYWORDS

ddPCR, environmental DNA, fish population estimates, nine-spined sticklebacks, species-specific detection

## 1 | INTRODUCTION

Measures of species-specific environmental DNA (eDNA) could be a cost-efficient and nondestructive method compared to traditional methods to estimate population abundances in aquatic ecosystems (Barnes & Turner, 2016; Coble et al., 2019). Numerous studies have applied molecular methods to natural water samples to estimate the abundance, species composition, and diversity of fish communities (Evans & Lamberti, 2017; Hansen, Bekkevold, Clausen, & Nielsen, 2018; Wilcox, Carim, et al., 2018). However, results are not always straightforward and current knowledge highlights both the potentials and limits of eDNA methods to quantify the abundance of fish populations (e.g., Capo, Spong, Norman, et al., 2019; Levi et al., 2019; Nevers et al., 2018; Wilcox et al., 2016; Wilcox, Young, et al., 2018; Yates, Fraser, & Derry, 2019).

Both abiotic and biotic factors are known to influence eDNA persistence and degradation in the water column (e.g., water retention time, temperature, light, oxygen, pH, salinity, microbial activity; see Hansen et al., 2018 for review). Moreover, once collected, several methodological biases can impact the quality of the eDNA signal from water samples (e.g., sampling volume, sampling representativeness, filtration methods, DNA extraction efficiency, PCR inhibitions, or low detection rate using a quantitative molecular method, Tsuji, Takahara, Doi, Shibata, & Yamanaka, 2019). Among these methodological biases, the presence of high concentrations of terrestrial dissolved organic carbon (DOC) can negatively affect the extraction and amplification of DNA molecules of targeted species, both mechanically (filter clogging) and chemically (PCR inhibitors) (Albers, Jensen, Bælum, & Jacobsen, 2013; Hunter, Ferrante, Meigs-Friend, & Ulmer, 2019; McKee, Spear, & Pierson, 2015). High concentrations of terrestrial DOC also indirectly affect most of the aforementioned environmental conditions (temperature, light, oxygen, pH, microbial activity; Solomon et al., 2015).

While many studies used the quantitative PCR method (qPCR) to quantify fish population abundance, the droplet digital PCR (ddPCR) method may be even more suitable for fish eDNA abundance estimation. This method has proved to be powerful for quantifying absolute numbers of DNA sequences, even at very low concentrations such as in aquatic systems with low population abundance (e.g., Capo, Spong, Norman, et al., 2019; Doi, Takahara, et al., 2015; Doi, Uchii, et al., 2015; Hunter et al., 2017). Moreover, the effect of humic substances acting as PCR inhibitors may be reduced by the specific procedure of partitioning a high number of target droplets—because of lowered co-occurrence of humic compounds and DNA molecules in droplets—thereby reducing biases related to PCR inhibition (Hoshino & Inagaki, 2012).

In the present study, we used large-scale experimental enclosure ecosystems containing nine-spined sticklebacks (*Pungitius*

*pungitius*) to test for relationships—under different environmental conditions—between estimates of fish eDNA concentrations and fish population abundance and biomass. More specifically, we designed primers specific at the genus level for *Pungitius* sp. to amplify a 134 bp COI barcode and experimentally tested the performance of a ddPCR-based eDNA approach to quantify fish populations abundance in experimental ponds with contrasted DOC concentrations (from 4 to 11 mg/L).

## 2 | MATERIALS AND METHODS

### 2.1 | Study site and applied treatments

This study was performed in 2018 at the Umeå University Experimental Ecosystem Facility (EXEF, 63°48'34" N, 20°14'33" E). EXEF is a large-scale experimental enclosure system (73 m long, 23 m wide with a depth of 1.6 m) divided into 20 enclosures (11.5 × 6.7 m), separated by water-tight dark-green PVC walls on three sides and a 6.7-m-long natural shoreline. EXEF allows for semi-natural ecosystem studies that span annual and interannual times scales, as the enclosures' ecosystem dynamics and responses include yearly natural ice and snow cover during winter seasons. Each enclosure contains a natural functional ecosystem with a soft bottom benthic habitat, and the food web includes naturally occurring benthic and pelagic primary producers and invertebrate consumers. All enclosures have separate in- and outlets for water and the facility allows for the manipulation of input water characteristics, including warming with heat exchangers for eight of the enclosures to a predetermined level above ambient temperature during the ice-free season.

Warming is performed by circulation water through a filter cube (10 PPI) in each enclosure and a land-based individual heat exchanger and back to the same enclosure. Separate temperature sensors in one of the ambient (i.e., natural season-dependent temperature development) and one heated enclosure continuously control the closed flow system of heated media from an air-source heat pump (to each of the individual heat exchangers). Similar circulation of water is applied to the ambient enclosures but without connected heat exchangers. Here, we used 16 enclosures, of which 8 were subjected to warming ("warm") to a continuous +2°C above ambient ("ambient") temperature development over the summer season 2018 from May to November.

To create a DOC gradient across the 8 ambient and 8 warm enclosures, water from a stickleback-free mid-sized stream 43 km northwest of EXEF (63°57'36" N, 19°25'12" E) with a high DOC concentration (mean ± SD; 18.5 ± 5.0 mg/L over the season) was collected weekly and transported with a trailer to a land-based 40 m<sup>3</sup> tank at EXEF. The DOC water was then continuously fed into the

different enclosures with predetermined input rates, alongside with clear-water additions (mean  $\pm$  SD;  $1.5 \pm 0.3$  mg/L over the season) that came from the municipal groundwater supply. Seasonal means (June to September,  $n = 6$ ) of DOC concentrations ranged from 4.3 to 11.4 mg/L covering a substantial range of DOC concentrations present in lakes in the Northern Hemisphere (Seekell et al., 2015).

On 11 May 2018, 43 adult sticklebacks (total biomass 38.5 g) were introduced into each one of the 16 experimental enclosures. The nine-spined stickleback populations were estimated twice during the ice-free period: on 24 July and 1 October 2018. Population size metrics—the abundance, that is, number of individuals and biomass—were determined by seine-netting 3–6 subsequent times with a specially designed seine net with a size that matched the width and depth of the enclosures. On 24 July, we sampled only 2/3 of each enclosure since the equipment for benthic primary production estimates was positioned at the bottom of each enclosure. We therefore used the average number of individuals of the three first seine net catches as a relative estimate or catch-per-unit-effort of abundance (CPUE) and biomass (BPUE) (see below for methods to estimate biomass of captured individuals). On 1 October, the whole volume of each enclosure was sampled, and population abundances were estimated using the K-pass removal method (Carle & Strub, 1978). At both sampling occasions, all captured fish per seine-haul were stored in white trays, photographed from above, and released back to the enclosure after the final seine-netting effort. Subsamples of captured fish were kept for obtaining length–weight regressions to estimate population biomass. Number and length of fishes were estimated by photo image analysis technique, and relative lengths were transformed into metric units by using a defined reference object as an internal standard. One of the enclosures (#18) was removed from the statistical analyses due to abnormal water conditions and perturbations caused by a nesting muskrat. Seine nettings, methods of sacrifices, and design of all fish sampling strategies in this study comply with the current laws of Sweden and were approved by the local ethics committee of the Swedish National Board for Laboratory Animals in Umeå. (CFN, license no. A20-14 to Pär Byström).

Water chemistry and light conditions were sampled 6 times during the 2018 season, while water temperature in each enclosure was continuously recorded with temperature loggers. In this study, we used DOC, pH, light extinction coefficient ( $K_d$ ), and warming as potential factors influencing eDNA estimates of fish abundance and biomass. For DOC measurements, an integrated water sample cross surface to 1 m depth was filtered through a 0.45- $\mu$ m filter (Sarstedt Filtrapur S 0.45). DOC samples were acidified with 100  $\mu$ l HCl 1.2 M/10 ml sample and stored in a refrigerator. DOC measurements were performed through high-temperature combustion in a Formacs

HT-I analyzer (Skalar). pH was measured on the same water sample using a pH meter (Mettler Toledo, Seven easy). For  $K_d$  estimates, PAR (photosynthetically active radiation) was measured at the surface, 0.2, 0.5, 1, and 1.5 m depth using a Li-250A radiometer equipped with a spherical quantum sensor Li-193SA (Li-Cor). The  $K_d$  was calculated as the slope of the linear regression of the natural logarithm of PAR versus depth (Karlsson et al., 2009).

## 2.2 | Sampling and filtration of water samples for eDNA analysis

Water samples were collected from the 15 experimental enclosures at four time points: 9 May (Sample Time 1—ST1, i.e., 2 days prior to fish introduction), 28 May (ST2), 23 July (ST3), and the 27 September 2018 (ST4). For each enclosure, 1 L of surface water was collected in sterile Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd) from the center of the enclosure. Sampling controls consisted of two 1 L MilliQ water opened bottles during sampling at each sampling date. Water samples (from enclosures and sampling controls) were first prefiltered through a 50- $\mu$ m mesh and then filtered sequentially through 3- $\mu$ m pore size, 47-mm-diameter polycarbonate filters (Isopore™), and 0.2- $\mu$ m Supor® 200 47-mm diameter polyether-sulfone filters (PALL Corporation) with a peristaltic pump. Full 1 L water samples were filtered through 50- $\mu$ m and 3- $\mu$ m filters, but for some samples, 0.2- $\mu$ m filters were clogged before 1 L. The resulting filtered water volume is reported in Table S2 for each sample. All filters were stored in 2-ml microcentrifuge tubes with 900  $\mu$ l of a buffer TES (50 mM Tris-HCl, 40 mM EDTA, 0.75 M sucrose) at 22/20°C until further analyses. Sampling and filtration equipment were sterilized with 5% bleach and rinsed with MilliQ water before and between each sampling and filtration, respectively.

## 2.3 | Design of species-specific primers for nine-spined stickleback

DNA was extracted from approximately 500 mg of tissue samples, preserved in 95% ethanol, from 5 individual nine-spined stickleback (*Pungitius pungitius*) using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol (Table S1).

PCR amplifications were then performed using primer sets targeting a portion of the COI mitochondrial gene (see sequences of COI\_fw and COI\_rv and amplicon length in Table 1). Each PCR was performed in a total volume of 25  $\mu$ l including 12.5  $\mu$ l of 2\*Qiagen Multiplex PCR Master Mix, 7  $\mu$ l of ultrapure water, 1.5  $\mu$ l of each

**TABLE 1** Nucleotide sequences of primers used in the present study

Primers F/R	Forward primer 5'–3'	Reverse primer 5'–3'	Amplicon length (bp)
COI_fw/COI_rv	TCAACCAACCACAAAGACATTGGCAC	AGTGTTCACAGTGTGTAGGC	1,330
COI_Pf/COI_Pr	ACCGCAACCTAAACACCACCTT	ATTCCGAAGCCTGGAAGGATGAGGA	134

primer (300 nM), and 4  $\mu$ l of 25 ng/ $\mu$ l DNA extract. The applied touchdown PCR protocol includes an initial denaturation at 95°C for 3 min followed by 8 cycles of 30 s at 94°C, 90 s of annealing 62°C (lowered by 0.5°C compared to each previous cycle), 120 s at 72°C and 25 cycles of 30 s at 94°C, 90 s at 58°C and 120 s at 72°C. The amplicons were then subjected to a final 5-min extension at 72°C.

Sanger sequencing was applied to PCR amplicons using a 3730 DNA Analyzer (Applied Biosystems). Forward and reverse reads were then cleaned and merged using the software BioEdit (Hall, 1999) and MEGA 7.0.26 (Kumar, Stecher, & Tamura, 2016). Consensus sequences are provided in Table S1. DNA sequences from *Pungitius* species were downloaded from GenBank (date: 12/07/2018) combining this genus name with the search terms "COI." The online DNA sequences were aligned with obtained sequences. The software Primer3Plus (Untergasser et al., 2007) was used to design primers (Table 1) fitting the following criteria: amplicon length around 50–150 bp, primers length around 15–30 bp, the total number of Gs and Cs in the last five nucleotides at the 3' end of the primer should not exceed two (GC clamp), GC content between 30% and 80%. The primer melting temperatures ( $T_m$ ) were at 62 and 64°C for forward and reverse primers, respectively.

The species specificity of primers was verified in silico using the software Primer-BLAST with default settings (Ye et al., 2012). Results show that primers match at 100% only for DNA sequences from *Pungitius* sp. The specificity of primers to amplify the desired target was verified applying the cloning-sequencing approach to the DNA extracts obtained from the water from experimental enclosures: PCRs were performed in a total volume of 10  $\mu$ l following the protocol described above. The PCR protocol is the same as the one described above. PCR amplicons were cloned using the CloneJet PCR cloning kit (Thermo Scientific), followed by purification and Sanger sequencing (Eurofins). Sequencing results confirmed the specificity of the primer set, the DNA sequences obtained from the environmental samples being identical to those obtained from fish tissues.

## 2.4 | DNA extractions from water samples

The DNA extraction was performed from the 0.2- $\mu$ m filters using a custom DNA extraction protocol. Filters, previously stored at -20°C in TES buffer, were thawed and placed for rotation (1 g, 56°C for 24 hr). The liquid was transferred into new tubes with the addition of 50  $\mu$ l SDS and 10  $\mu$ l proteinase K and incubated at 37°C for 1 hr followed by 55°C for 20 min. After centrifuging (3 min, 9,500 g), the supernatant was transferred to new tubes, to which one equivalent volume of sodium acetate (3 M, pH 5.2) and GenElute (TM-LPA, 1  $\mu$ l) were added. Following 5 min at room temperature, cold isopropanol was added and centrifuged (10 min, 5,600 g). The supernatant was removed, and the pellet was cleaned by adding ethanol (200  $\mu$ l, 80%) and centrifuged (10 min, 5,600 g). After removing the supernatant, the remaining ethanol was evaporated with a 20-min Speedvac step. Finally, the pellet was eluted in 25  $\mu$ l of TE solution, incubated 1 hr

at 37°C, and stored at -20°C. DNA extraction controls ( $n = 7$ ) were performed alongside with samples. The concentration (ng/ $\mu$ l) of bulk eDNA was estimated with two methods: (a) nanodrop method based on the UV absorbance measurement at 260 nm (UV spectroscopy) with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific) and (b) qubit method that uses a fluorescent dye bound to double-stranded DNA (fluorescence spectroscopy) with a Synergy HTX Multi-mode reader (Biotek) with an HS dsDNA Assay kit. The comparison between the estimated DNA concentrations obtained from both methods was used to estimate the presence of contaminants (putative PCR inhibitors) in DNA extracts, the nanodrop method being prone to overestimation of DNA quantity when other compounds, such as phenols, are co-extracted with DNA (Thermo Fisher Scientific, 2010).

## 2.5 | Droplet digital assays

We performed ddPCR assays using the primers previously designed (i.e., primers COI\_Pf and COI\_Pr, Table 1). Each ddPCR mixture contained 2  $\mu$ l of 10-fold dilution DNA extract, 200 nM of primers, 10  $\mu$ l of 1 $\times$  Bio-Rad Evagreen Supermix (Bio-Rad) with ultrapure sterilized water up to a total volume of 22  $\mu$ l. From this reaction mix, 20  $\mu$ l was mixed with Bio-Rad droplet generator oil and partitioned in up to 20,000 droplets using the Bio-Rad QX-200 droplet generator (Bio-Rad). PCRs were performed in sealed 96-well plates with the following conditions: 5 min at 95°C, 40 cycles of denaturation for 30 s at 95°C, and extension for 64 s at 62°C, followed by 5 min at 4°C, 5 min at 95°C and held at 4°C. After PCR amplification, plates were transferred to the Bio-Rad QX-200 droplet reader (Bio-Rad). PCR optimizations were previously performed to select suitable primer concentrations and extension temperature for the PCR amplification. The ddPCRs were run in triplicates for a total number of 73 DNA extracts (58 from enclosure's water samples, 8 from sampling controls, 7 from DNA extraction controls) alongside with ddPCR controls. We failed to extract DNA from two filters (sample id: ST2-P6 & ST4-P2).

## 2.6 | Data analysis

The Bio-Rad's QuantaSoft software version 1.7.4.0917 was used to quantify the number of copies of target DNA by  $\mu$ l of DNA extract. Droplets out of the fluorescence range of 8,000 to 14,000 were considered as outliers and thus discarded. The ddPCRs with less than 8,000 droplets accepted were discarded from the analysis. False positives in ddPCR assays were represented by one or two droplets detected in the fluorescence range of positive controls in a part of control samples (Table S2). However, such patterns were never detected in any of the triplicate ddPCR assays and were therefore considered as random noise and discarded with the following procedure: (a) ddPCR assays with <3 droplets were considered as negative, (b) only samples for which subsequent positive droplets were found in at least two of the three replicates were considered as positive

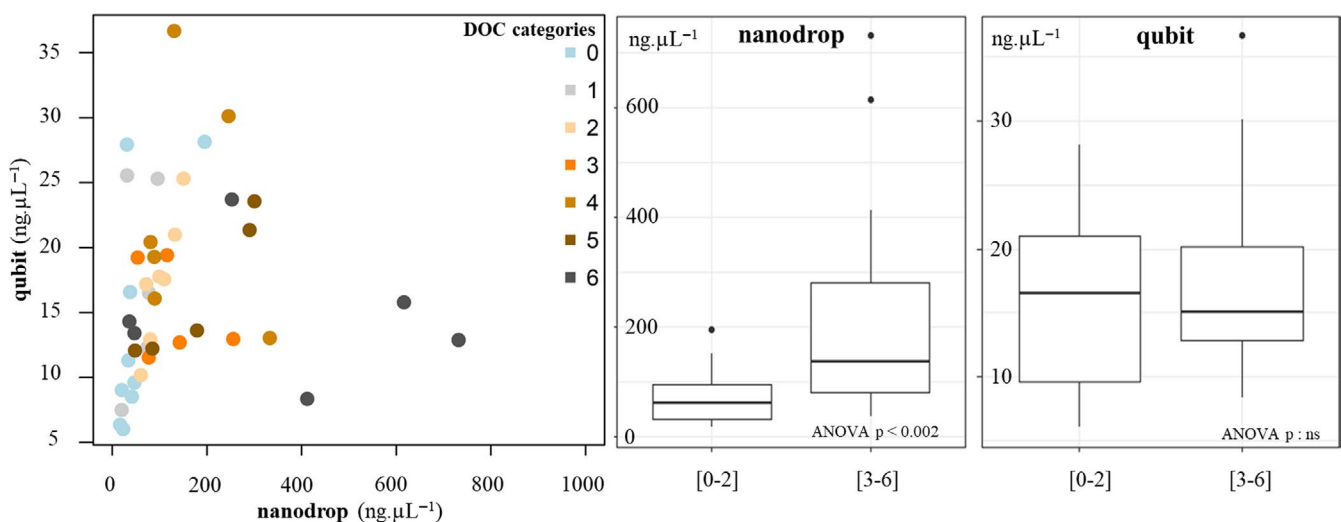
and (c) for remaining samples, a mean number of positive droplets was calculated. All controls (sampling, DNA extraction, and ddPCR controls) were found to be negative. When a high variability in the number of positive droplets were found within triplicates, additional ddPCR assays were performed, and mean values were calculated from all performed ddPCR replicates to reduce the effects of variability on the estimation of the number of positive droplets (Table S2). Then, for each sample, a mean DNA copy number per DNA extract was calculated by dividing the mean number of positive droplets by the volume used in ddPCR (1.8  $\mu\text{L}$ ) and multiply by the dilution factor (10) and the total volume of the DNA extract (25  $\mu\text{L}$ ) resulting in an estimate of stickleback eDNA concentrations per sample. Estimated stickleback eDNA concentrations in water samples were thus calculated as follows: the stickleback eDNA concentrations were divided by the amounts of water filtered (in ml) and multiplied by a factor of 1,000. Those values were called stickleback eDNA concentrations in the manuscript and were used for all statistical analysis. Bivariate correlations were calculated between stickleback eDNA concentrations and abundance and biomass estimates as well as DOC and water temperature using Pearson correlation coefficients. One-way ANOVAs were applied to study the relationships between DOC categories of each enclosure and the measures of extracted DNA estimated both with nanodrop and qubit methods. To identify the factors that may influence stickleback eDNA concentrations, we applied generalized linear models (GLM) with the functions *glm* (lme4 R package, Bates, Mächler, Bolker, & Walker, 2015). A total of four models were performed: two for ST3 (1 for abundance estimates, 1 for biomass estimates) and two for ST4 (1 for abundance estimates, 1 for biomass estimates). The GLM modeling was performed with the Poisson distribution family, and numeric predictor variables (abundance/biomass estimates, DOC, and water temperature) centered and scaled to have a mean of 0 and a standard deviation of 1.

The two other variables—the light extinction coefficient ( $K_d$ ) and pH values—were excluded to avoid overfitted models. The collinearity of all environmental variables was assessed using Spearman's correlation coefficient and variance inflation factors (vif function from R package car; Fox & Weisberg, 2011). None of the variables were considered as collinear (i.e., with Spearman  $r > 0.3$  and VIF  $> 3$ ). For the four models, significance was tested using a likelihood ratio test with the *drop1* R function.

### 3 | RESULTS

DNA was successfully extracted from the water samples from the 15 enclosures except for two samples (ST2-P6 and ST4-P2). The total eDNA concentrations ranged from 17 to 732  $\text{ng}/\mu\text{L}$  and from 6 to 38  $\text{ng}/\mu\text{L}$  with nanodrop and qubit methods, respectively (Table S2). The predetermined input rates of DOC water to each enclosure were used as explanatory factors in statistical analysis, after being categorized into DOC categories from control enclosures (category 0;  $4.5 \pm 1.4$   $\text{mg}/\text{L}$ , mean  $\pm$  SD) to enclosures with the highest input of DOC-rich water (category 6:  $10.7 \pm 1.1$   $\text{mg}/\text{L}$ , mean  $\pm$  SD) (Table S2). DNA extracts from the high DOC enclosures (categories 3–6) displayed significantly higher total DNA concentrations than the DNA extracts from low DOC enclosures (categories 0–2) when using the nanodrop method (Figure 1). However, no difference was found in the total eDNA concentrations measured using the qubit method comparing samples from the low and high DOC enclosures (Figure 1).

No stickleback eDNA was found in samples collected before fish introduction at the first sampling time (ST1) while stickleback eDNA was detected at ST2 (19 days after fish introduction) in some but not all enclosures (Table S2). At ST3, positive correlations were found between the estimates of stickleback eDNA concentrations



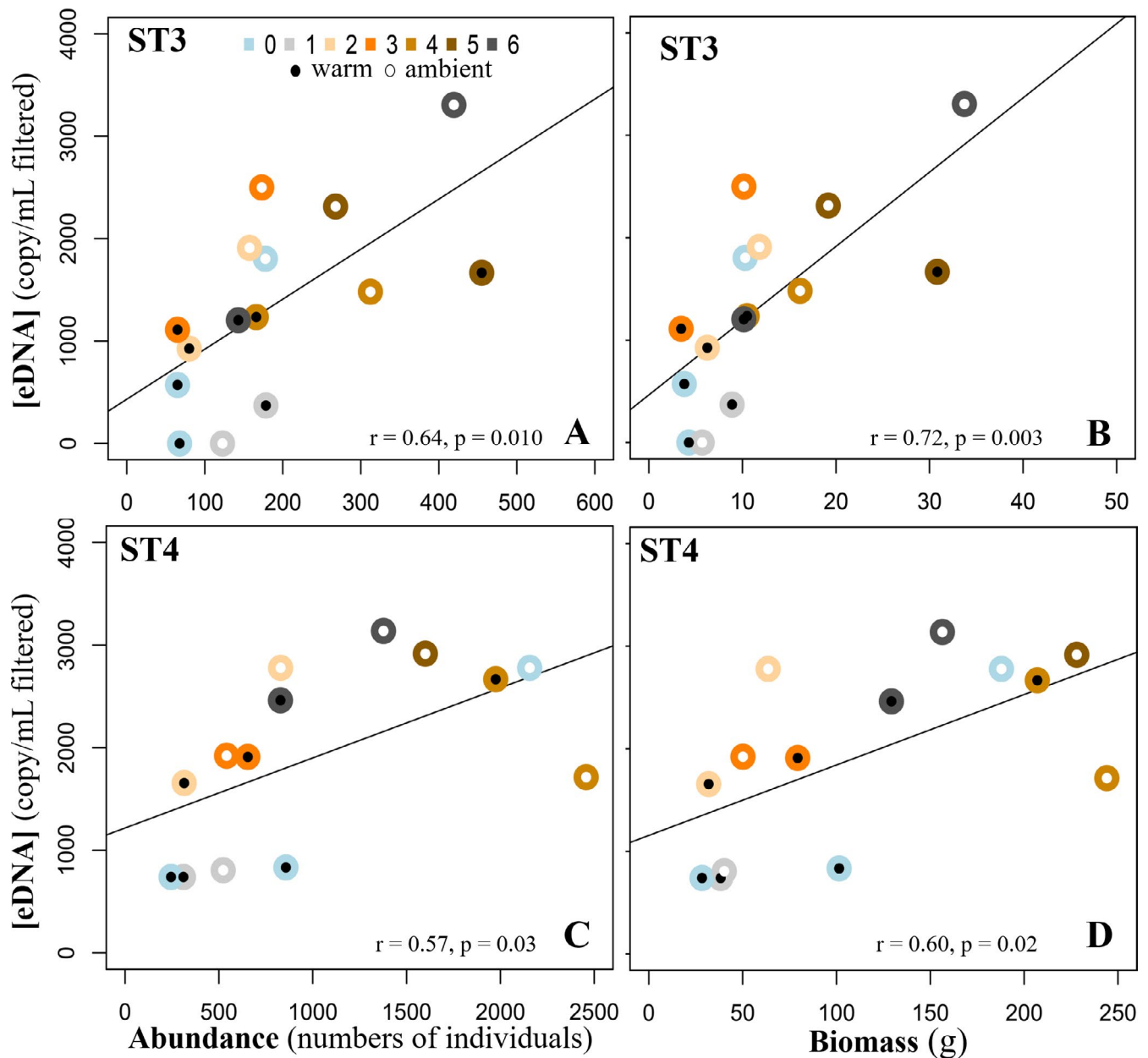
**FIGURE 1** The relationships between the DNA concentrations measured with the nanodrop and qubit methods. Each dot corresponds to a DNA extract colored considering the DOC categories defined for each enclosure. The boxplots highlight, for both methods independently, the range of DNA concentrations measured for DNA extracts from low DOC enclosures [0–2] and high DOC enclosures [3–6]. The results of a one-way ANOVA are displayed in the plots. Only DNA extract collected after the stickleback populations have established were used for these plots (i.e., from ST2, ST3 & ST4)

and abundance (i.e., number of individuals;  $r = 0.64$ ,  $p = .010$ ) and biomass ( $r = 0.72$ ,  $p = .003$ ) (Figure 2). Similarly, at ST4, positive correlations were found between stickleback eDNA concentrations and abundance ( $r = 0.57$ ,  $p = .03$ ) and biomass ( $r = 0.60$ ,  $p = .02$ ) (Figure 2).

According to the likelihood ratio test applied to our 4 models, abundance and biomass estimates, DOC concentrations, and water temperatures were all identified as factors influencing stickleback eDNA concentrations at ST3 and ST4 (Table 2). Bivariate correlations showed significant correlations at ST3 between eDNA concentrations and DOC concentrations ( $r = 0.61$ ,  $p = .01$ ) and water temperatures ( $r = -0.56$ ,  $p = .03$ ) but no correlation was found for these parameters at ST4 ( $p$ -values  $> .1$ ).

## 4 | DISCUSSION

Our work, aiming to test the use of a ddPCR-based eDNA method to quantify fish population abundances, showed positive and significant correlations between nine-spined stickleback population estimates (abundance and biomass) and stickleback eDNA concentrations. Such findings are in line with many recent studies (Klobucar, Rodgers, & Budy, 2017; Klymus, Richter, Chapman, & Paukert, 2015; Lacoursière-Roussel, Côté, Leclerc, Bernatchez, & Cadotte, 2016; Nevers et al., 2018; Takahara et al., 2012; Wilcox et al., 2016) highlighting that eDNA may be a promising tool for estimating fish population abundance and biomass in aquatic systems.



**FIGURE 2** Relationships between stickleback eDNA concentrations (in copy number per mL of water filtered) and estimates of (a) CPUE (number of individuals) for ST3, (b) BPUE (biomass) for ST3, (c) fish abundance (number of individuals) for ST4, or (d) fish biomass for ST4 obtained in each enclosure. The outer color of each dot corresponds to the DOC categories defined for each enclosure. The inner color of each dot discriminates warm and ambient enclosures

**TABLE 2** Results of the GLM models of the effects of environmental parameters on stickleback eDNA concentrations in water samples

Time	Parameters	eDNA concentrations		Parameters	eDNA concentrations	
		$\chi^2$	p-value		$\chi^2$	p-value
ST3	Fish abundance	510.2	<2.2e-16	Fish biomass	840.0	<2.2e-16
	DOC (mg/L)	183.4	<2.2e-16	DOC (mg/L)	11.8	6.1e-4
	Temp (°C)	1,222.6	<2.2e-16	Temp (°C)	1,287.1	<2.2e-16
ST4	Fish abundance	458.2	<2.2e-16	Fish biomass	625.3	<2.2e-16
	DOC (mg/L)	192.5	<2.2e-16	DOC (mg/L)	45.0	2.0e-11
	Temp (°C)	193.9	<2.2e-16	Temp (°C)	260.3	<2.2e-16

Note: Models were performed using the Poisson distribution family, enclosures modeled as a random effect and numeric predictor variables (fish abundance and biomass, DOC concentrations, water temperatures) centered and scaled to have a mean of 0 and a standard deviation of 1 as fixed variables. Model testing was conducted using likelihood ratio tests.

We performed our analysis from water samples collected in experimental enclosures for which DOC concentrations and water temperature were controlled. As illustrated by the meta-analysis from Yates et al. (2019), correlations between fish eDNA concentration and fish population abundance can be stronger for studies performed in controlled conditions when compared to those from natural systems. We therefore discuss below the precision in our estimates—for both eDNA concentrations and stickleback population abundance—compared to similar applications in natural systems. First, our eDNA estimates are likely more homogeneous than most estimates of fish eDNA concentrations from natural and substantially larger lakes due to the small size of the enclosures. Second, the spatial variability in eDNA concentrations should be lower in our enclosures than in natural systems due to lower spatial and structural complexity of the lake ecosystem per se (e.g., Capo, Spong, Norman, et al., 2019; Lawson Handley et al., 2019). Third, commonly various types of catch-per-effort estimates come with considerable variation and uncertainty (e.g., Capo, Spong, Norman, et al., 2019 and references therein). However, as we sampled more than 2/3 of the area of each enclosure via seine net at ST3, we believe that the uncertainties in our CPUE estimate are relatively small. Fourth, at ST4, we obtained actual population abundance estimates by sampling the whole enclosure volume at least three times and by applying the K-pass removal method (Carle & Strub, 1978). However, even under our controlled conditions and small spatial scale, our estimated population abundances with the eDNA method came out in some cases with large variation (Table S2), although excluding those enclosures (#1, #6, #14) from the analysis did not substantially improve statistical relationships (results not shown). Finally, the biomass of sticklebacks in the October sampling (ST4) ranged between 4 and 42 kg/ha (Table S2) which is in the range of species-specific abundance estimates in temperate and northern lakes of freshwater species valuable for fisheries, management, and for ecosystem function, for example, perch (*Perca fluviatilis*); 1.8–41.1 kg/ha (Sumari, 1971); 19–40 kg/ha (Rask & Arvola, 1985), 160–1100 individuals/ha (Persson, Andersson, Wahlstrom, & Eklov, 1996), Arctic char (*Salvelinus alpinus*); 780 individuals, respectively, 7.9 kg/ha (Finstad, Jansen, & Langeland, 2001), 80–460 individuals, respectively, 10–32 kg/ha (Ask 2010), 460–680 individuals/ha

(Byström, Andersson, Persson, & De Roos, 2004), 30–170 individuals/ha (Klobucar et al., 2017), brown trout (*Salmo trutta*); 50–200 individuals/ha (Borgström, 1992), Northern pike (*Esox lucius*); 7 kg/ha (Rask & Arvola, 1985), 23–47 individuals/ha (Persson et al., 1996), 3.3–60 individuals/ha (Pierce & Tomcko, 2003). While some of the above density estimates are only based on individuals (by ha), the corresponding biomass estimates most likely do not differ substantially from the range of fish biomass estimates of fish in our study (based on information in these studies on the individual length and/or weight measures). Hence, despite the smaller spatial scale of our experimental system, our results are likely relevant to natural lake systems and provide support for the use of estimate of fish eDNA concentrations in water samples as proxies of fish population abundance. Nevertheless, despite the significant relationships between eDNA concentration and fish abundance and biomass estimates, substantial unexplained variation remained, which also points to the fact that further development of sampling strategies and methods may be needed to obtain higher precision of eDNA-based fish population abundance estimates.

In the present study, we further aimed to evaluate whether fish eDNA concentrations obtained by the ddPCR method are not biased due to the presence of high amounts of organic matter, as relatively high concentrations of DOC can be found in lakes worldwide (Seekell et al., 2015). Many eDNA-based studies have used end-point PCR (presence/absence of the targeted DNA sequences) and qPCR to successfully detect and quantify DNA from fishes in freshwaters (Collins, Armstrong, Holyoake, & Keeling, 2013; Eichmiller, Best, & Sorensen, 2016; Eichmiller, Miller, & Sorensen, 2016; Jerde et al., 2013; Jerde, Mahon, Chadderton, & Lodge, 2011; Lacoursière-Roussel, Côté, et al., 2016; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016; Mahon et al., 2013; Minamoto, Yamanaka, Takahara, Honjo, & Kawabata, 2012; Takahara, Minamoto, & Doi, 2013). However, end-point PCR and qPCR are prone to PCR inhibitions, especially for DNA extracts obtained from systems with a relatively high amount of humic substances such as high DOC ponds and humic-rich lakes (Albers et al., 2013; McKee et al., 2015). By contrast, the ddPCR method can overcome the effect of inhibitors during PCR amplification because of the partitioning of PCR

mixture in thousands of droplets (Hoshino & Inagaki, 2012). In our experimental enclosures, we controlled DOC concentrations to mimic the natural variability in organic matter concentrations in lakes (Seekell et al., 2015) with values up to 11 mg/L DOC. The overestimation of total DNA concentrations measured from water samples with the nanodrop method highlighted the presence of compounds co-extracted with DNA such as humic substances (phenols) that can act as inhibitors during PCR amplifications (Thermo Fisher Scientific, 2010). Still, taken fish abundance estimates into account, the estimated stickleback eDNA concentrations were not lower in high DOC enclosures compared to low DOC enclosures, suggesting that our ddPCR-based procedure method is suitable to avoid biased estimates of fish eDNA concentrations due to potential PCR inhibition, as previously suggested (Capo, Spong, Königsson, & Byström, 2019; Doi, Uchii, et al., 2015; Hoshino & Inagaki, 2012).

Among other abiotic factors, water temperature is known to affect the degradation of DNA molecules (Barnes & Turner, 2016; Eichmiller, Miller, et al., 2016; Jo, Murakami, Yamamoto, Masuda, & Minamoto, 2019; Strickler, Fremier, & Goldberg, 2015). In our study, we did detect a negative correlation between stickleback eDNA estimates and water temperature—eDNA estimates being lower in enclosures with +2°C warmer temperature—but only for one sampling time (ST3). However, such relationship may be hampered by the fact that fish abundance/biomass is influenced by differences in water temperature, having thus an indirect effect of stickleback eDNA estimates. Moreover, Strickler et al. (2015) found an increase in DNA degradation rates with increased temperature when studied a much larger range in water temperature (from 5 to 35°C).

## 5 | CONCLUSIONS

This study aimed to evaluate the potential effects of natural variability in environmental conditions—here, the presence of humic substances in the water and increased temperatures—on estimates of fish eDNA concentrations retrieved from water samples. Despite the co-extraction of compounds with DNA from high DOC enclosures, as shown by overestimated DNA concentrations by the nanodrop method, significant correlations were found between abundance/biomass estimates and stickleback eDNA concentrations. This highlights the strength of the applied procedure to quantify fish populations in water bodies with various levels of humic substances. Hence, we suggest that the application of a droplet digital PCR-based protocol provide promising results for the development of eDNA methods to estimate fish population abundances in water bodies.

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None declared.

## CONFLICT OF INTEREST

None declared.

## AUTHORS' CONTRIBUTIONS

PB, GS, EC, and HK conceived the ideas and designed methodology; EC, SK, IP, and FO collected and analyzed the data; EC and PB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

## DATA AVAILABILITY STATEMENT

The raw data are available on figshare (<https://doi.org/10.6084/m9.figshare.11493777.v1>).

## ORCID

Eric Capo  <https://orcid.org/0000-0001-9143-7061>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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