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ORIGINAL ARTICLE

Proper environmental DNA metabarcoding data transformation reveals temporal stability of fish communities in a dendritic river system

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Abstract

Protecting freshwater biodiversity is considered an ultimate challenge but depends on reliable surveys of species distribution and abundance which eDNA metabarcoding (environmental DNA metabarcoding) may offer. To do so, a better understanding of the sources of temporal variation among species eDNA abundance and of data transformation in eDNA metabarcoding studies is needed. Here, we show that transformation based on relative abundance is critical to suitable analyses of eDNA metabarcoding data and that Hellinger transformation performed slightly better than other methods. Furthermore, we show that site localities significantly explain eDNA metabarcoding variation, while no variation is explained by time of sampling. This indicates that species communities vary more spatially than temporally within a dendritic system composed of small rivers. We then further documented the community structure in the St. Charles River (Québec City, Canada) and six of its tributaries. This revealed the existence of eight species communities explaining 82.1% of eDNA read variation within this river network. Moreover, variation in environmental variables among sites explained 53.0% of eDNA reads, while sampling events and temporal environmental variation explained no eDNA metabarcoding variation. Altogether, this supports the claim that eDNA metabarcoding is a powerful tool to document and monitor fish communities in watersheds composed of small river dendritic systems.

KEYWORDS
data transformation, eDNA, fish communities, metabarcoding, MiFish, ordination analyses

1 | INTRODUCTION

The protection of freshwater biodiversity is considered an ultimate challenge in conservation biology. Prompt and reliable surveys of species distribution and abundance are thus needed for a better management of freshwater biodiversity (Dias et al., 2017; Dudgeon et al., 2006; Vörösmarty et al., 2010; WWF, 2018). Fish distribution and abundance are traditionally evaluated using various methods,

including electrofishing, gill nets, trap nets, seines, and scuba diving, which are generally labor-intensive methods, each having their own biases (Kubečka et al., 2009, 2012; Smith et al., 2015). While similarly affected with its own limitations (Deiner et al., 2021), an increasing number of studies on fish distribution and abundance using the analysis of environmental DNA metabarcoding (eDNA metabarcoding) are revealing the efficiency of this non-invasive and less labor-intensive approach to describe fish community structure and diversity, and as such, showing that eDNA analysis can no longer be ignored for conservation and biomonitoring (Afzali et al., 2020; Berger et al., 2020; Boivin-Delisle et al., 2021; Deiner et al., 2021; Handley et al., 2019; Hänfling et al., 2016; Valentini et al., 2016). However, understanding the effect of data transformation is needed to avoid erroneous conclusions from a dataset, for example, by correctly estimating the distances among sites in ordination analyses (Legendre & Gallagher, 2001; Legendre & Legendre, 2012; ter Braak and Smilauer, 1988). Moreover, despite the growing number of studies describing fish communities using eDNA metabarcoding, further investigations on spatiotemporal and environmental effects are needed to reach the full potential of this promising method (but see Handley et al., 2019).

eDNA is the residual DNA collected in environmental samples (e.g., water, soil or air) released by organisms via their epidermis, feces, mucus, hair, gametes, and other sources (Levy-Booth et al., 2007; Rees et al., 2014). In aquatic ecosystems, several studies confirmed the positive relationships between eDNA concentration and abundance and/or biomass measured by quantitative PCR (species-specific DNA primers) as well as by eDNA metabarcoding (using multispecies DNA primers), both in the wild and in controlled conditions (Afzali et al., 2020; Boivin-Delisle et al., 2021; Doi et al., 2017; Evans et al., 2016; Hänfling et al., 2016; Kelly et al., 2014; Klobucar et al., 2017; Lacoursiere-Roussel et al., 2016; Maruyama et al., 2018; Pont et al., 2018; Shaw et al., 2016; Takahara et al., 2012; Thomsen et al., 2012, 2016; Wilcox et al., 2016; Yates et al., 2020). Moreover, it has been shown that relationship between fish community structure and environment can be detected using eDNA metabarcoding and that sample replicates show high reproducibility (Afzali et al., 2020; Berger et al., 2020; Boivin-Delisle et al., 2021; Civade et al., 2016). However, despite recent studies investigating the effect of seasonality on eDNA (de Souza et al., 2016; Goldberg et al., 2011; Handley et al., 2019; Sevellec et al., 2020; Sigsgaard et al., 2017; Stoeckle et al., 2017; Thalinger et al., 2021; Wacker et al., 2019), sources of temporal variation in abundance in species eDNA need further investigation to improve the reliability of long-term surveys of species community in aquatic ecosystems.

In numerical ecology, Hellinger transformation is considered a gold standard to study species abundance in ordinate analyses (Legendre & Gallagher, 2001; Legendre & Legendre, 2012). This transformation is a square root on relative abundance that results in a Hellinger distance after being projected by principal component or redundancy analyses. The Hellinger transformation also corrects for the double 0 problem when assessing similarities among sampling sites (Legendre & Gallagher, 2001; Legendre & Legendre, 2012). Species are known to have unimodal distributions along environmental conditions and are absent from sites that differ too much from their privileged environmental conditions (ter Braak & Prentice, 1988). Even though sequence and species counts share many similarities, they also differ in magnitude. Whereas species counts per sample rarely exceeds hundreds of specimens, sequence counts in eDNA metabarcoding studies can be several orders of magnitude higher. Moreover, normalization of raw counts using relative abundances is necessary to control for different total numbers of reads among sites, as well as other biases (Sard et al., 2019; Ushio et al., 2018). To our knowledge, relative abundance, relative abundance on Bray–Curtis dissimilarity, and Hellinger transformation are the three most used data transformations for eDNA metabarcoding, but no comparison of their effect on model performance has been performed yet.

Here, we first test for the effects of eDNA metabarcoding data transformation in inferring the spatiotemporal variation of fish community structure in a small dendritic river system, the St. Charles River basin near Quebec City, Canada. This river basin has an area of 550 km^2 and shows important environmental and physicochemical variation among its tributaries and is the most densely populated river basin in Québec, with an average of 600 inhabitants per km^2 (Statistics Canada, 2010a, 2010b, 2010c). This river basin constitutes the main potable water source of Quebec City and drains into the St. Lawrence River, which drags 25% of the world freshwater reserves. More specifically, Hellinger transformation, relative abundance, Bray–Curtis dissimilarity, and Bray–Curtis dissimilarity on eDNA sequence relative abundance were compared based on model residuals. The relative importance of temporal and sampling site location effects, as well as model residuals, was examined on these five versions of data transformation for two different datasets with variation partitioning analyses (datasets: inter-annual and inter-seasonal sampling). Then, we studied the effects of environmental variation and re-sampling on the inter-seasonal dataset to evaluate the proportion of eDNA variation explained by (i) re-sampling, (ii) environmental variation, and (iii) environmental variation linked to seasonal changes. We then documented the overall variation in fish community structure within the St. Charles River basin using K-means and redundancy analyses. Finally, we investigated the effect of fluvial distance on fish eDNA sequence correlation among sampling sites within the St. Charles River.

2 | MATERIALS AND METHODS

2.1 | eDNA sampling

Prior to sampling, and for each sample, a sealed kit comprising a piston syringe with a glass fiber 0.7-µm filter (Whatman GF/F), a plastic bottle (1 L), a pair of plastic gloves, and forceps was sterilized with UV light for 1 h and was prepared for a total of 28 sampling sites X temporal replicates/site = 56 sampling kits). All kits were prepared 48 h before the respective sampling events. Water

sampling (1 L) was performed as described by Leduc et al. (2019) and Boivin-Delisle et al. (2021) for piston syringe sampling, which is a commonly used filtration method that is simple to use while also being efficient to minimize contamination in the field. In September 2016 and September 2017, 10 sites were sampled on the main arm of the St. Charles River (Figure 1; Table 1). These 10 sites will hereafter be called the "inter-annual" dataset. Then, another 17 sites from eight tributaries of the St. Charles River (i.e., des Hurons, Noire, Hibou, Trois-Petits-Lacs, Nelson, Savard, Valet, and Jaune rivers) and one in the upstream section of the St. Charles River were sampled in July 2017 and September 2017 (Figure 1; Table 1). These 18 sites will hereafter be called the "inter-seasonal" dataset. Field negative controls (n = 7) were performed during each sampling events. All filters were individually stored at −20°C until DNA extraction.

2.2 | eDNA extraction, PCR amplification and sequencing

DNA extraction of 2016 samples was performed using phenol–chloroform (Lacoursière-Roussel et al., 2018), while DNA extraction of 2017 samples was performed using a QIAshredder and DNeasy Blood and Tissue kit (Qiagen) according to Goldberg et al. (2011). To eliminate any possible contamination in the laboratory, extractions were performed under a UV hood with bleached and UV-treated instruments. For each extraction batch, a negative extraction control was performed. The MiFish primers were used to target a hypervariable region of the 12S rRNA gene (174 bp) (Miya et al., 2015) and proved to be efficient to reliably document freshwater and marine fish communities from Québec (Afzali et al., 2020; Berger et al., 2020; Boivin-Delisle et al., 2021; Garcia-Machado

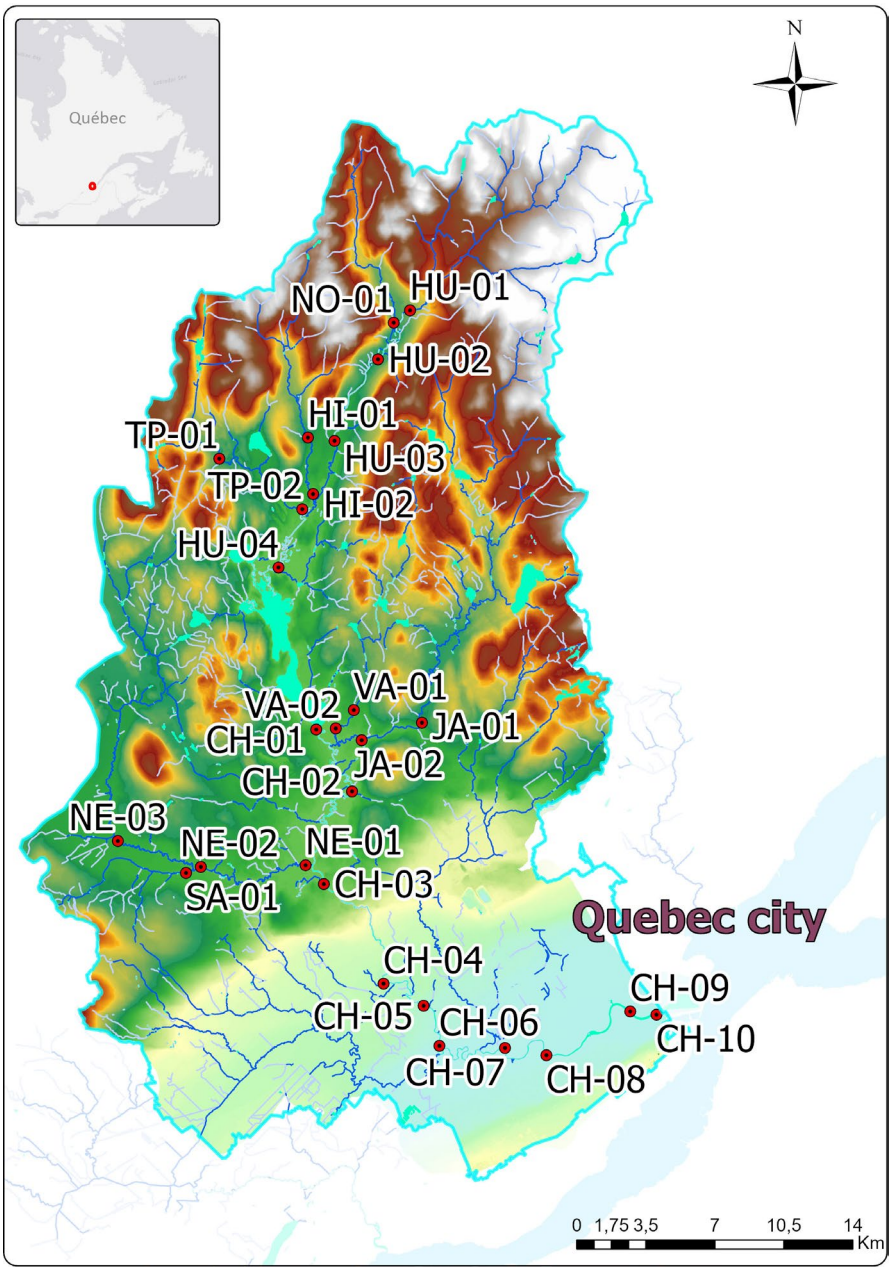

FIGURE 1 Map of the sampling area showing the 27 localities sampled in the St. Charles River and its tributaries. Darker colors represent area with higher altitude

TABLE 1 Sampling sites distributed among nine tributaries with their geographic coordinates and sampling time (year and month) and used in different dataset analyzed in this study

Site	River	Latitude and longitude	Year	Month	Dataset
CH-01	Saint-Charles	46.9106°, −71.3717°	2016–2017	July–September	IA – IS – EN – DC
CH-02	Saint-Charles	46.8912°, −71.3557°	2016–2017	September	IA – DC
CH-03	Saint-Charles	46.8625°, −71.3691°	2016–2017	September	IA – DC
CH-04	Saint-Charles	46.8311°, −71.3425°	2016–2017	September	IA – DC
CH-05	Saint-Charles	46.8241°, −71.3244°	2016–2017	September	IA – DC
CH-06	Saint-Charles	46.8115°, −71.3174°	2016–2017	September	IA – DC
CH-07	Saint-Charles	46.8106°, −71.2876°	2016–2017	September	IA – DC
CH-08	Saint-Charles	46.8082°, −71.2687°	2016–2017	September	IA – DC
CH-09	Saint-Charles	46.8215°, −71.2303°	2016–2017	September	IA – DC
CH-10	Saint-Charles	46.8204°, −71.2184°	2016–2017	September	IA – DC
HI-01	Hibou	47.0016°, −71.3737°	2017	July–September	IS
HI-02	Hibou	46.9840°, −71.3717°	2017	July–September	IS – EN
HU-01	des Hurons	47.0407°, −71.3264°	2017	July–September	IS – EN
HU-02	des Hurons	47.0256°, −71.3413°	2017	July–September	IS – EN
HU-03	des Hurons	47.0004°, −71.3616°	2017	July–September	IS – EN
HU-04	des Hurons	46.9613°, −71.3879°	2017	July–September	IS – EN
JA-01	Jaune	46.9123°, −71.3235°	2017	July–September	IS
JA-02	Jaune	46.9071°, −71.3510°	2017	July–September	IS – EN
NE-01	Nelson	46.8684°, −71.3773°	2017	July–September	IS – EN
NE-02	Nelson	46.8693°, −71.4251°	2017	July–September	IS – EN
NE-03	Nelson	46.8767°, −71.4627°	2017	July–September	IS – EN
NO-01	Noire	47.0369°, −71.3339°	2017	July–September	IS – EN
SA-01	Savard	46.8664°, −71.3819°	2017	July–September	IS – EN
TP-01	des Trois-Petits lacs	46.9953°, −71.4142°	2017	July–September	IS – EN
TP-02	des Trois-Petits lacs	46.9793°, −71.3767°	2017	July–September	IS – EN
VA-01	du Valet	46.9356°, −71.3496°	2017	July–September	IS
VA-02	du Valet	46.9109°, −71.3628°	2017	July–September	IS – EN

Abbreviations: DC, distance effect on correlation between sites; EN, environmental effects; IA, inter-annual; IS, inter-seasonal.

et al., 2021; Miya et al., 2020). For each sample, a one-step dual-indexed approach using 91pb MiFish primers including Illumina Nextera adaptor and index was performed. PCRs were conducted in a total volume of 25 µl including 12.5 µl of Master Mix (Qiagen multiplex PCR kit 1000), 2 µl of each primer (10 µM), 5.5 µl of diH_2O, and 3 µl of eDNA sample. The PCR program was run under the following conditions: 15 min at 95°C, 35 cycles of amplification (30 s at 94°C, 90 s at 65°C, 60 s at 72°C), and a final elongation step of 10 min at 72°C. For each eDNA and field negative control sample, five PCR replicates were performed and pooled after amplification, and a PCR negative control on gel was also added for each index combination to assess the occurrence (or not) of laboratory contamination. No mock community were specifically designed here, but MiFish primers are reliable to represent proportion of biomass from a subgroup of species present in our study area (Boivin-Delisle et al., 2021). In addition, while positive controls are crucial when aiming to detect a particular species, this is less of a concern in community studies (Berger et al., 2020); thus, no PCR positive control was added. PCR products were visualized on a 1.5% agarose gel. Before sequencing, no amplification of the PCR negative controls was observed. PCR products were purified using a paramagnetic bead-based post-PCR clean up kit (Axygen). After elution in 35 µl of water, DNA concentration of each sample was determined using the TECAN Spark 10 M 223 Reader and the Ultra High Sensitivity dsDNA Quantitation kit (AccuClear). Samples were randomly pooled in equal concentration and repurified to equalize sequencing depths across sampling sites. The concentration and fragment size distribution of the library were analyzed on an Agilent 2100 Bioanalyzer. All samples were sequenced together in a same run at the Plateforme d'Analyses Génomiques (PAG) of the Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Québec (http://www.ibis.ulaval.ca/). Sequencing was performed using Illumina MiSeq (Illumina) and the MiSeq Reagent Kit V3 with paired-end 300 bp reads (Illumina).

2.3 | Bioinformatics analyses and data cleaning

Raw forward and reverse reads were trimmed, merged, and classified using the Barque v1.7.0, an eDNA metabarcoding pipeline (Mathon et al., 2021; www.github.com/enormandeau/barque). More precisely, forward and reverse sequences were trimmed and filtered using Trimmomatic v0.30 with the following parameters: (TrimmomaticPE, -phred33, LEADING:20, TRAILING:20, SLIDINGWINDOW:20:20, MINLEN:200) (Bolger et al., 2014). Read pairs were merged with FLASH v1.2.11 (Fast Length Adjustment of Short reads) with the following options: (-t 1 -z -O -m 30 -M 280) (Magoč & Salzberg, 2011). Reads containing both primer sequences and reads with lengths inside the expected range were kept. Chimeric sequences were removed using VSEARCH v 2.15.12 (uchime denovo command and the default parameters) (Rognes et al., 2016). Finally, a supplemented 12S database consisting of the mitofish sequences and others sequenced by our team was used to annotate the most likely species of the sequences with a threshold of 97% similarity. This database is distributed as part of Barque. To remain conservative, species for which sequences were detected less than 20 times among samples were removed from the analysis as they could be caused by sequencing artifacts (Brown et al., 2015) or sample misidentification (Schnell et al., 2015). Among cases of non-unique taxonomic assignment, if only one of such species could potentially be present in St. Charles River basin (MDDELCC, 2016; Richard, 2010) (e.g., others totally out of their range of distribution), the sequences were added to this species. Finally, sequences attributed to non-fish species and fish species not occurring neither the St. Charles River nor in the St. Lawrence River in the Quebec City vicinity (Bernatchez & Giroux, 2012; MDDELCC, 2016; Richard, 2010) were discarded, except for potential invasive species (Table S1).

2.4 | Comparison of data transformations for multivariate analyses

All statistical analyses were performed using the package *vegan*, *ade4*, *scales and segmented* of the R software version 3.6 (Dray & Dufour, 2007; Oksanen et al., 2019; R Core Team, 2018; Vito & Muggeo, 2008; Wickham, 2018). To compare the effect of different data transformations to minimize model residuals, we produced five versions of both inter-annual and inter-seasonal datasets (i.e., raw data, Hellinger transformation, relative abundance, Bray–Curtis dissimilarity, and Bray–Curtis dissimilarity on relative abundance). Note that here, raw data and Bray–Curtis dissimilarity datasets are analyzed to evaluate the potential biases that may arise when data are not normalized in eDNA metabarcoding studies (see Paliy & Shankar, 2016 for further precisions). Hellinger transformation and relative abundance were computed with the function *decostand* and Bray–Curtis dissimilarity with the functions *vegdist*. The function *pcoa* was used with versions that include a dissimilarity matrix, a step needed to perform a distance-based redundancy analyses on distance or dissimilarity matrices. The five different data transformations were then compared on their capacities to explain variation in fish eDNA sequence abundance among sampling sites and time periods based on their model residuals using adjusted coefficient of determination (Legendre & Legendre, 2012).

2.5 | Relative importance of spatial and temporal variation on fish eDNA abundance and distribution

For each version of both inter-annual and inter-seasonal datasets, we performed variation partitioning using the function *varpart* after conducting redundancy analyses, where an eDNA metabarcoding matrix surrogating fish abundance variation was explained by the variables "site" (10 sites for inter-annual and 18 sites for inter-seasonal datasets) and "time" (September 2016 and 2017 for the inter-annual and July and September 2017 for the inter-seasonal datasets), both as categorical factor in the model (Table 1). Variation partitioning is a method using coefficient of determination to fraction the variation of a response matrix into four fractions (Borcard et al., 1992). Two of these fractions are the part of the variation exclusively explained by one of the two explanatory variables, one is the proportion shared by the two explanatory variables and the last one is the part non-explained by the model. The explained proportion was compared to document the relative importance of variation in species community among sampling sites and time periods, while the proportion unexplained by the model was used to rank the power of transformation data as mentioned above.

2.6 | Effect of environmental variables, seasonal variations, and re-sampling

Since residuals resulting from Hellinger transformation were among the lowest while also offering the advantage of being producing data that is usable with principal component and redundancy analyses (see Section 3), subsequent analyses were performed with this transformation only. A subset of 15 sites out of the 18 comprised in the inter-seasonal dataset (for which data from environmental surveys were available) was used to study the effect of environment on fish community structure (Table 1). A principal component analysis with center-reduction was performed on 14 environmental variables (i.e., ammoniacal nitrogen (µg N/L), total nitrogen (mg N/L), chlorophyll *a* (µg/L), fecal coliforms (UFC/100 ml), conductivity (µS/cm), fDOM (QSU),chloride ions (mg/L), nitrites–nitrates (mg N/L), dissolved oxygen (%), total phosphorus (µg P/L), pH, total dissolved soluble (g/L), water temperature (°C), and turbidity (NTU)), all extracted from a database developed by AGIRO, CRAD (Centre de Recherche en Aménagement et Développement de l'Université Laval), Watershed Monitoring©, and the environmental department of Quebec city (Behmel, 2018) for the month of July and September 2017 (Table S2). PC scores were then used as an explanatory matrix surrogating environmental conditions among sampling sites and time periods, which also control for variance inflation factor problem

since they are orthogonal (Legendre & Legendre, 2012). A backward selection using the function *ordistep* was used to keep the PC axes that explained a significant proportion ($p < 0.01$) of the eDNA matrix. Then, a variation partitioning was produced to estimate the relative proportion of eDNA variance explained by environment, by re-sampling as well as by changes in environmental conditions between re-sampling (i.e., shared by both variables). Since no variation was explained by re-sampling, neither shared by both variables (see Section 3), a redundancy analysis was then performed to associate fish community structure with the heterogeneity in environmental conditions.

2.7 | Identification of species communities

Since all tests revealed that the variable "time" did not significantly explain a proportion of variance in fish eDNA sequence (see Section 3), we used the 27 sites sampled in September 2017 to identify species that most significantly explained spatial variation in fish communities (Table 1). The *kmeans* function was used with 1000 random sets, to perform a K-means analyses (Hartigan–Wong algorithm) to identify groups of sites sharing similar species community (Legendre & Legendre, 2012). The number of groups was decided based on the within group sum of squares method (Legendre & Legendre, 2012). We then produced a redundancy analysis with the variable group as an explanatory variable to identify species being most strongly associated with these groups (two-tailed normal distribution on RDA axes species loadings: $p < 0.1$; see Forester et al., 2018 for more details). To visualize fish community variation throughout the study area, a schematic representation of the regions and a heatmap (function *heatmap*) were then produced.

2.8 | Fish eDNA abundance spatial correlations along the St. Charles River

We used the 10 sites of the inter-annual dataset in 2017 to study correlation along sites of the St. Charles River (Table 1). The "segmented" function of the software R was used to produce a segmented linear regression explaining the eDNA sequences spatial correlation among sampling sites (Pearson's *r*) with their fluvial distance (km) measured with Google earth Pro v7.3.3.7786 software. A breaking point was estimated to find the average distance at which a switch in fish community can be observed in the river.

3 | RESULTS

3.1 | Dataset summary and quality

Following sequencing and data filtering using the Barque pipeline, a total of 9,680,837 sequences were obtained (average of 172,872 reads per sample SD ± 75,368). More precisely, 2,287,286 sequences were obtained for the 10 sites of the inter-annual dataset (average per filter 114,361 SD ± 72,999) and 7,393,611 sequences for the 18 sites of the inter-seasonal dataset (average per filter 205,378 SD ± 54,684). This could be explained by the age of the sample or by the phenol–chloroform DNA extraction since the 2016 inter-annual samples had significant lower sequence number in comparison with the three other groups (inter-annual 2016: average per site 54,393 SD ± 44,392; inter-annual 2017: 174,330 SD ± 35,881; inter-seasonal July: 213,070 SD ± 45,296; and inter-seasonal September 197,686 SD ± 63,083; $F = 23.74$, $p < 0.001$). Yet, sequence coverage of all samples falls within the range routinely observed in similar studies. All negative controls showed an average of 1224 SD ± 1950 sequences (0.7% SD ± 1.1%), indicating that this small percentage of contamination should have negligible impact on results (Barnes et al., 2014). After removing non-fish species, fish species not occurring in the study area and fish species with sequences count inferior to 20 (see Table S1 for details), we identified a total of 49 species belonging to 39 genera. In comparison, 36 fish species have been physically observed in the St. Charles River basin, among which we detected 30 (MDDELCC, 2016; Richard, 2010; see Table S1 for details). The sequences of the six species that were physically observed but not found with eDNA metabarcoding were present in our reference database. No difference in overall species richness among stations was observed between sampling years (inter-annual dataset; paired *t*-test: $t = 0.61$, df = 9, $p = 0.56$), neither between sampling months (inter-seasonal dataset; paired *t*-tested: $t = 0.12$, df = 17, $p = 0.90$), indicating that species richness is temporally stable within site. As mentioned in the materials and methods section, if a sequence was linked to more than one species, the sequence was associated with the species that was physically observed in the St. Charles River basin (MDDELCC, 2016; Richard, 2010). An exception to this was for *Percina caprodes* and *P. copelandi* species. These two species showed a total of 8087 and 120 sequences, respectively, but 17,120 sequences were associated with both species without being able to be assigned to either. Because the reference dataset needs additional specimens to efficiently tell apart these sequences and because *P. copelandi* is a relatively rare species that was never observed in this river basin and in the Québec City region, those sequenced were all considered to represent *P. caprodes*, which is a common species in the system. Such modification should not affect the conclusion of the present study considering the low number of sequences linked to *P. copelandi* ($n = 120$). The other exception was for two lamprey species *Ichthyomyzon fossor* and *I. unicuspis* that represented 39 sequences in total and that we label under the name *Ichthyomyzon* spp. considering that both species have been reported previously in the St. Lawrence River near Québec City (Scott & Crossman, 1976). Finally, we observed high correlations between temporal re-sampling based on species raw number of sequences (average correlation was 0.82 SD ± 0.10 among the 10 sites of the inter-annual dataset and 0.95 SD ± 0.08 for the 18 sites of the inter-seasonal dataset), testifying of a high and reliable re-sampling and temporal stability of fish community in the St. Charles River basin.

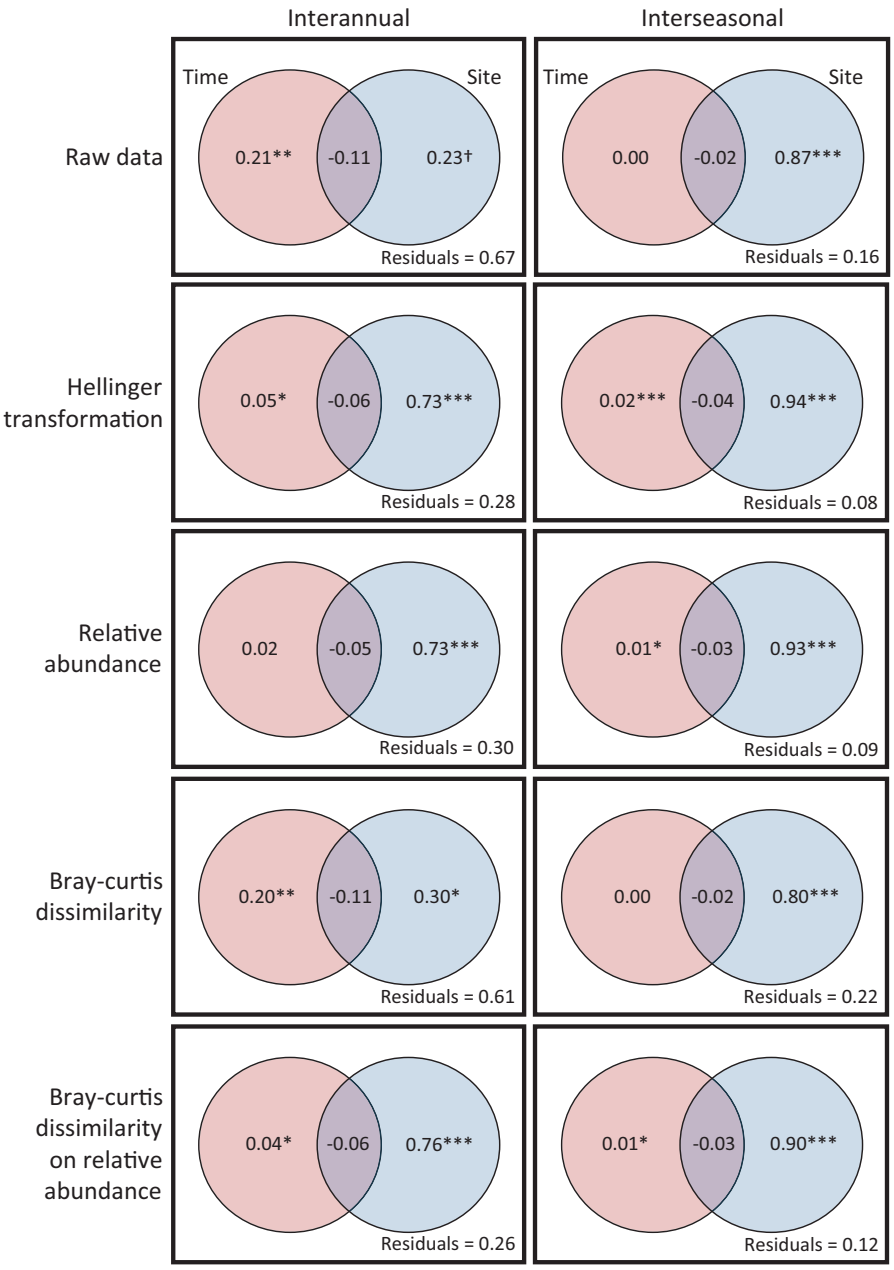

FIGURE 2 Venn diagrams representing partition variations based on redundancy analyses showing model residuals and relative explained variation by time and site locations alone and the intersection of both variables for two datasets of eDNA metabarcoding (columns: inter-annual and inter-seasonal) after five data transformations (rows: raw data, Hellinger transformation, relative abundance, Bray–Curtis dissimilarity, and Bray–Curtis dissimilarity on relative abundance)

3.2 | Effects of data transformation, time period, and sampling location on fish eDNA abundance and distribution

Variation partitioning showed that Hellinger transformation, relative abundance, and Bray–Curtis dissimilarity on relative abundance performed the best according to their coefficient of determination (adj. R^2) (Figure 2). The Hellinger transformation showed the second lowest and the lowest model residuals for the inter-seasonal (0.28) and inter-annual (0.08) datasets, respectively. Bray–Curtis dissimilarity on relative abundance ranked first and third for the inter-annual (0.26) and inter-seasonal (0.12), while relative abundance performed third and second for the inter-annual (0.30) and inter-seasonal (0.09) datasets. In comparison, raw data and Bray–Curtis dissimilarity on raw data performed poorly (residuals were, respectively, 0.67 and 0.61 for inter-annual and 0.16 and 0.22 for the inter-seasonal datasets) (Figure 2). In all versions of both datasets, the variable "site" always explained a higher proportion of eDNA variation compared to "time." Inversely, the variable "time" always explained little eDNA variation despite a higher effect in the inter-annual dataset for the raw data and Bray–Curtis dissimilarity versions, which can be explained by the lack of normalization and a significant lower number of raw eDNA sequences in 2016 (Figure 2). Except for these two latter cases, the variable "site" explained at least 14.6 times more the eDNA variation than the variable "time" (Figure 2). This indicates that species communities vary much more spatially than temporally, even at relatively small spatial scale. Finally, the shared fractions between the two explanatory variables showed negative values

(Figure 2). This occurs when two variables together explain the response matrix better than the sum of the individual effects of these variables (Legendre & Legendre, 2012), which implies that the variable "time" without any control from the variable "site" explained no proportion of fish eDNA abundance variation.

3.3 | Environmental effects on fish eDNA abundance and distribution based on Hellinger transformation

Since the Hellinger transformation shows among the lowest residuals while also offering the advantage of producing data that can be used with principal component and redundancy analyses, subsequent analyses were performed with this transformation only. Indeed, principal coordinates and distance-based redundancy analyses can be performed with a distance matrix, but at the cost of losing information on species loading. Variation partitioning among the 15 sites of the inter-seasonal dataset kept for the environmental analysis revealed that the variable "time" did not explain any eDNA variation, but environmental variables did (Figure 3). Indeed, five PC axes (PC-1, PC-2, PC-3, PC-4, and PC-8) significantly ($p < 0.001$) explained 53.0% of the variation in species eDNA abundance (Figure 3). eDNA abundance and distribution of the species *Ameiurus nebulous*, *Esox lucius*, *Lepomis gibbosus*, *Notemigonus crysoleucas*, and *Perca flavencens* were associated with environment with higher chlorophyll *a* and temperature (Figure 3). Inversely, *Salvelinus fontinalis* and *Rhinichthys cataractae* eDNA were more abundant in environment with lower temperature and chlorophyll *a* (Figure 3). Finally, *Gasterosteus aculeatus* eDNA abundance was correlated with higher specific conductivity, total nitrogen, and total phosphorus (Figure 3).

3.4 | Fish community structure in the St. Charles River basin

For all 27 sites that were sampled in September 2017, species richness per site ranged between 13 (NO-01 and SA-01) and 36 (CH-10) (average 22.19 SD ± 5.90) (Figure 4). Note that the second site with the highest species richness was CH-09 ($n = 35$), which is the second closest to the St. Lawrence River after the site CH-10 (Figures 1 and 4). K-means analysis suggested the occurrence of eight spatially structured fish communities among the 27 sites that were clearly differentiated in the heatmap (Figure 4). To simplify visualization, we regrouped species with less than 20,000 sequences in the dataset and labeled them as "Others." This grouping revealed a likely influence of the St. Lawrence River to explain the higher species richness in the two most downstream sampling sites CH-09 and CH-10 sites (Figure 4). For example, the Round goby (*Neogobius melanostomus*) which is an invasive fish species highly abundant in the St. Lawrence River and that has never reported in the St. Charles River basin was detected only in CH-09 ($n = 1682$) and CH-10 ($n = 2872$) sites. We then identified species associated with these eight fish communities with a redundancy analysis (RDA). The eighth communities explained 82.1% ($p < 0.001$) of eDNA abundance variation among the 27 sites, and a total of 16 species were identified as species differentiating communities among seven significant RDA axes ($p < 0.05$) (Figure 5). Note here that for conservative purpose, we changed the threshold of the two-tailed distribution ($p < 0.05$ instead of $p < 0.1$) for the RDA axes 5, 6, and 7 because all species loadings were under 0.4. The first two RDA axes already separated six of the eight fish communities (Figure 5a). The two remaining groups (TP-01 -02 and HU-04 vs. NE-01 -02 -03 and SA-01) were differentiated by the RDA axes 3 and 4 (Figure 5b). Using heatmap and species identified on the seven RDA axes, we produced a schematic map of the system

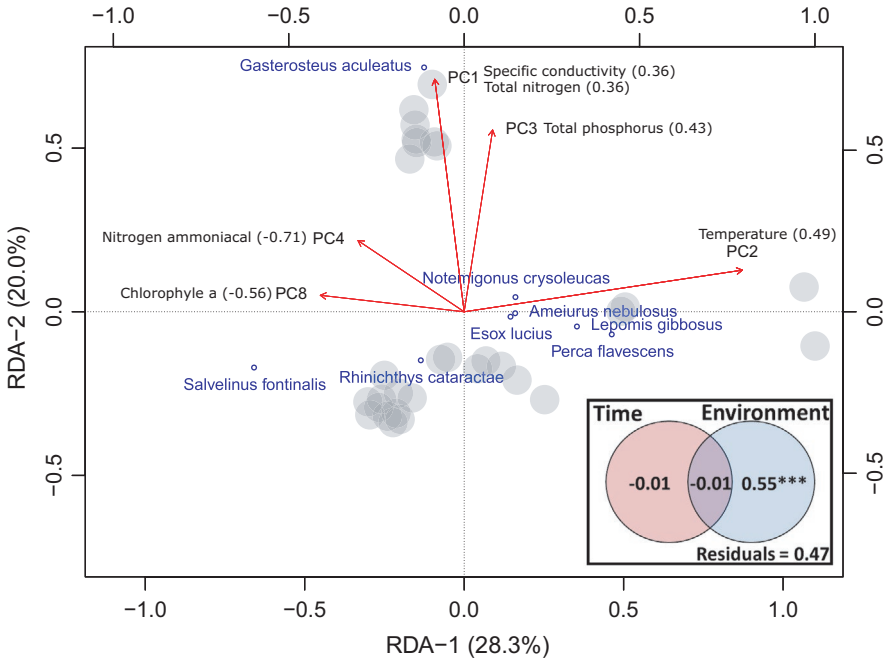

FIGURE 3 Redundancy analysis plot showing four PC axes representing environmental variation (red arrows, higher loadings for each PC axis are shown) explaining sampling variation in sequence reads (gray spot). Species loadings significantly differentiating samples are presented in blue. A partition variation at the right bottom shows that the global model explains 53.0% of sampling variation but that sampling month and the intersection between sampling month and environmental variation explain no sampling variation, leaving environmental variation among site as significant variables. *** $p < 0.001$

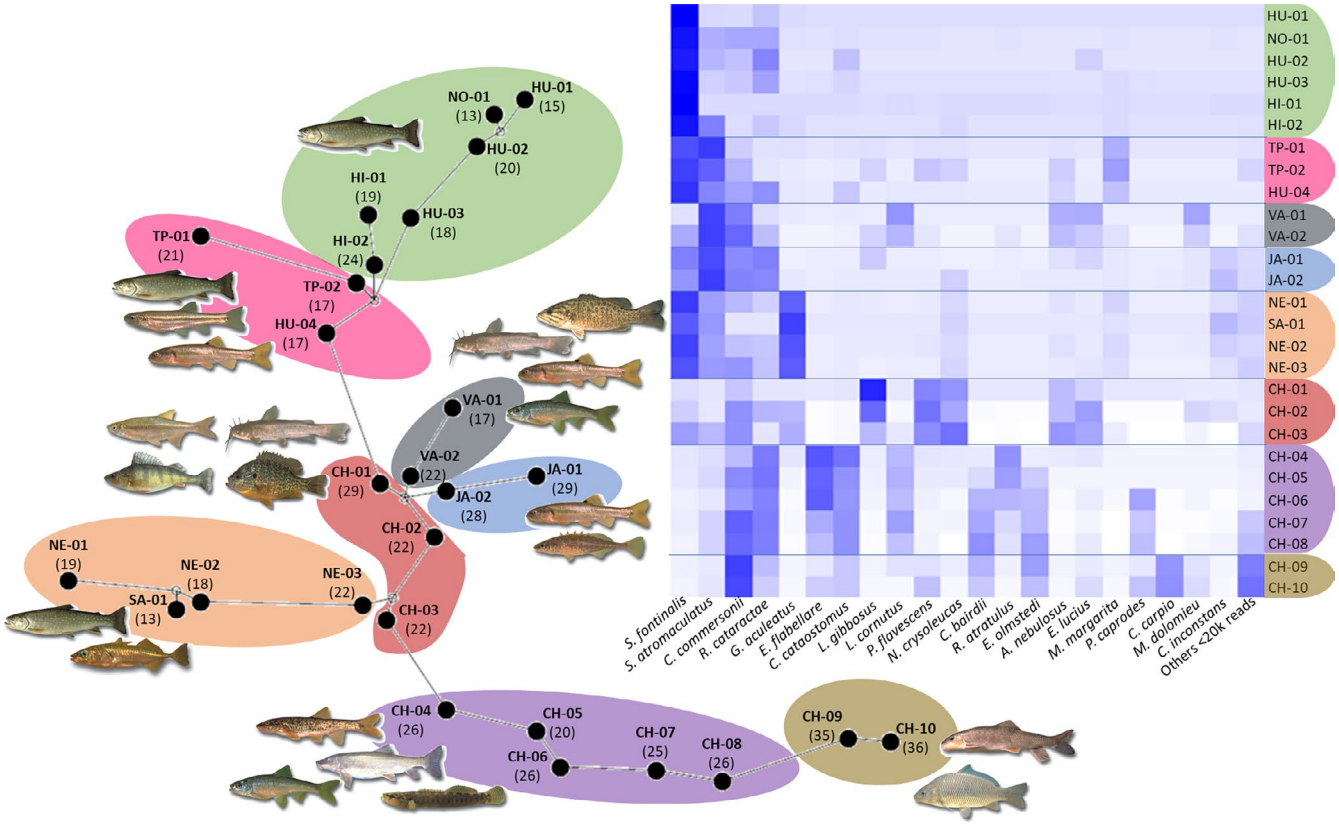

FIGURE 4 Left: A schematic representation of sampling area showing the eight fish communities obtained from k-means analyses. Species present in each community have been obtained from redundancy loading statistical test (see Figure 5 and text for precision). Right: Heatmap of species relative abundance for each sampling sites in September 2017. Absence of species is represented by the color white and darker the blue is, more the relative abundance of the species is. Species with less than 20,000 reads among all sites have been regroups and labeled as "Others <20k reads" for visibility purpose

to visualize the eight species communities of the St. Charles River basin (Figures 4 and 5).

3.5 | Effect of fluvial distance on fish communities along the St. Charles River

The segmented linear regression showed that fluvial distance significantly explained the correlation in eDNA fish sequences among sites in the St. Charles River (Figure 6) ($p < 0.001$, adj. $R^2 = 0.71$). A breaking point was observed at 16.14 km, representing the average distance for a switch in species communities along the river (Figure 6). However, considering the three fish communities found by the K-means analysis in the St. Charles River (upstream: CH-01 -02 -03; center: CH-04 -05 -06 -07 -08 and downstream: CH-09 -10) we found two distinct spatial gradients where upstream sites were always poorly correlated with sites from the two other groups (Figure 6). Furthermore, correlation between CH-03 and CH-04 sites (separating the upstream and central fish communities) was 0.10 and only 5.55 km separated these sites, indicating that a switch in species communities could be observed within 6 km with eDNA metabarcoding in the St. Charles River (Figure 6). Interestingly, CH-01 -02 -03 are in a section of the river where a sudden increase in altitude can be observed (Figure 1).

4 | DISCUSSION

In this study, we (i) compared the performance of different dataset transformations, (ii) examined the inter-annual and inter-seasonal effects on eDNA abundance and distribution, (iii) tested for the effects of environmental variables and fluvial distance on fish eDNA abundance, and (iv) detected eight fish communities spatially distributed among the different tributaries of the St. Charles River basin. Briefly, our results point out that using a data transformation based on relative abundance is crucial to study fish community variation with eDNA metabarcoding. Furthermore, we show that re-sampling and temporal effects (season or annual) had a very weak effect on eDNA relative abundance and distribution, which implies that eDNA varies much more spatially than temporally (either between years or seasons) in this river basin for the tested period. Moreover, our results showed that the community structure detected was associated with habitat heterogeneity, with environmental variables explaining 53% of eDNA relative abundance variation. Finally, we observed an

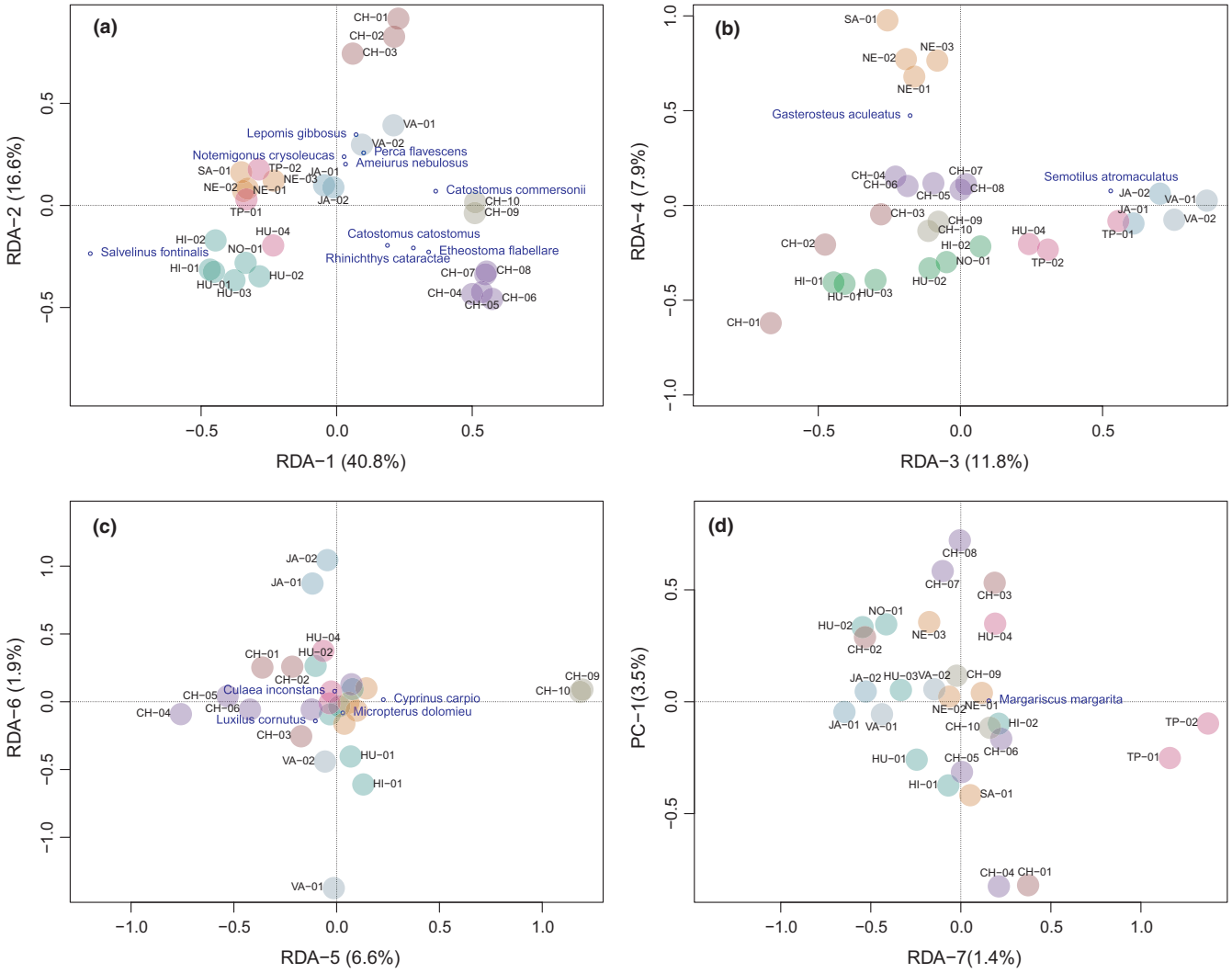

FIGURE 5 Redundancy analysis plots representing each of the seven significant RDA axes (a: axes 1 and 2; b: axes 3–4; c: axes 5–6; and d: axes 7 with the first unconstrained axis) where eight communities were obtained by k-means analysis explaining 82.1% of sequence read variation among sampling sites. Species with significant loadings differentiating sites are indicated in blue

important switch in species communities between sites with less than 6 km of fluvial distance (e.g., between sampling sites CH-03 and CH-04), implying a relatively weak effect of eDNA transportation on downstream sites, at least in this river section. Overall, this study adds support to the view that eDNA metabarcoding can provide useful information on fish community structure and represents an efficient means to perform long-term monitoring in lotic systems. Below, we discuss the necessity for proper data transformation in addition to the consideration of relative importance of temporal, environmental and spatial variation toward the goal of producing prompt and reliable biomonitoring of fish communities.

4.1 | Data transformation of fish community structure

Our results show that raw data or a transformation not based on relative abundance are suboptimal and potentially erroneous for explaining eDNA variation in eDNA abundance and distribution. Indeed, residuals based on adjusted coefficient of determination were higher for these versions of dataset. Moreover, technical problems occurring from sampling to sequencing (i.e., field filtration, DNA extraction, and sequencing runs) can also erroneously explain variation in eDNA quantities in a dataset. Here, we observed a significantly lower number of sequences per site from the 2016 campaign in comparison to sampling campaigns done in 2017 (same sampling effort for both years), which was not expected since overall fish biomass and abundance would be unlikely to increase by a 3-fold in one year in a river at the same period of the year (54,393 vs. 174,330 average sequences per sites). Two non-exclusive hypotheses could explain this result. First, DNA extraction with phenol–chloroform introduced PCR inhibitors that could reduce the final amount of DNA to be sequenced (Hu et al., 2015). Second, long-term DNA storage at −80°C and −164°C (or dried) is recommended to conserve DNA for years and decades, respectively (Oxford Gene Technology, 2011). Here, we stored DNA at −20°C, which could explain the lower

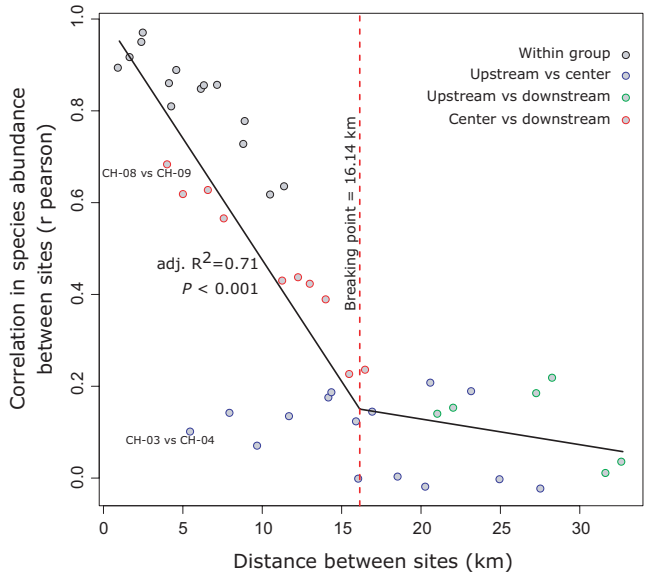

FIGURE 6 Segmented linear regression plot showing a breaking point between fluvial distance (km) and correlation in eDNA metabarcoding species abundance between pairwise sites

number of sequences in 2016. Other confounding variables could not be ruled out since this observation is based on a single group. Nevertheless, we observed that using transformation based on relative abundance can correct, at least partially, such problems, since no effect of time was observed on the relative eDNA abundance and distribution of different species between 2016 and 2017. This suggests that the DNA degradation rate was overall similar among species in the different temporal samples. Finally, Legendre and Gallagher (2001) showed that in ecological studies, Hellinger transformation was the most appropriate data transformation to study species counts in ordination analyses. Since Hellinger transformation performs slightly better and because the transformed data could directly be used in PCA and RDA analyses, as opposed to a distance or dissimilarity matrix, we propose to privilege this data transformation for eDNA community structure analyses. Admittedly, the analyses of additional datasets comprising different sample sizes from different ecosystems and types of community will be needed to assess the generality of our observations.

4.2 | Temporal and re-sampling effects on eDNA relative abundance and variation

No seasonal effect on eDNA variation was observed despite the fact environmental difference recorded between the 2 months sampled in 2017. This observation differs from other studies in which the effect of seasonality on eDNA variation has been documented. For example, de Souza et al. (2016) observed that detection probability of the amphibian Alabama waterdog (*Necturus alabamensis*) had a negative correlation with warm season, while a positive correlation was observed for the flattened musk turtle (*Sternotherus depressus*). Goldberg et al. (2011) showed that the probability of detection among seasons was related to species-specific changes in population density between the Idaho giant salamander (*Dicamptodon aterrimus*) and the Rocky mountain tailed frog (*Ascaphus montanus*). Wacker et al. (2019) showed a 20-fold increase in eDNA concentration during summer reproduction for the freshwater pearl mussel (*Margaritefera margaritefera*) and Thalinger et al. (2021) observed that longitudinal and lateral fish eDNA distribution is impacted by changes in seasonal water discharge. Change in eDNA abundance and species richness has also previously been reported in fish communities showing the effect of seasonality in marine and estuarine environments (Sigsgaard et al., 2017; Stoeckle et al., 2017), as well as in Artic metazoan communities (Sevellec et al., 2020). Finally, Handley et al. (2019) showed that lake thermal stratification in summer changes spatial eDNA distribution for several fish species. Here, no annual and seasonal effects were observed on eDNA variation and we did not observe any change in species richness in both inter-seasonal and inter-annual datasets within sites. Overall, our results suggest that the detected fish species remained associated with the same habitats during the seasonal period we covered, such that community structure remained the same. This was reinforced by high correlations between temporal re-sampling based on species raw number of sequences for both inter-annual (average r of Pearson = 0.95 SD ± 0.08) and inter-seasonal (r = 0.82 SD ± 0.10) datasets. Of course, this does not rule out the possibility that temporal effect would have been detected between other seasonal periods, for instance, when comparing summer vs. winter period or comparing reproductive vs. non-reproductive period, as observed in other systems (Handley et al., 2019; Sigsgaard et al., 2017; Stoeckle et al., 2017). In any case, the pronounced temporal stability (both inter-seasonally and inter-annually) in spatial variation of eDNA abundance for different species we observed provides further evidence that eDNA metabarcoding is reliable to identify fish communities within a dendritic river system such as the St. Charles River basin.

4.3 | Identification of fish communities

A total of eight spatially distributed fish communities were detected in the St. Charles River basin, explaining 82.1% of fish eDNA sequence variation. The salmonid *S. fontinalis* (Brook charr) is highly present in three of these groups located at higher altitudes and more remote from urban development and dominates these communities except for the Nelson tributary, where the habitat is shared with a population of three-spined stickleback (*G. aculeatus*). The five other groups are associated with species resistant to more eutrophic environments, such as centrarchids, ictalurids, percids, and cyprinids families. These eight groups are clearly differentiated except for the two groups closest to the St. Charles R. mouth, where gradients can be observed for several species. Indeed, some species show a reduction in eDNA sequences through CH-04 to CH-10 sites (*R. cataractae*: Longnose dace, *Etheostoma flabellare*: Fantail darter, *Catastomus cataostomus*: Longnose sucker, *R. atratulus*: Blacknose dace), while

others show an increase (*C. commersonii*: White sucker, *E. olmstedi*: Tesselated darter, and several species within the group "others"). The fish eDNA diversity at the mouth of the St. Charles River appears to be influenced by species living in the St. Lawrence River which can punctually enter the St. Charles River or whose eDNA can be introduced by the freshwater tides. Further surveys would be needed to differentiate the occurrence of permanent, punctual species and exogenous eDNA in this lower stretch of the river. This will be particularly important for the round goby (*N. melanostomus*), an invasive species that we have detected for the first time in the St. Charles River. Indeed, we observed round goby sequences in sites CH-09 and CH-10 (1682 and 2872 sequences, respectively). Indeed, it would be crucial to know whether the detection of round goby eDNA represents a new invader within the St. Charles R. or a mere exogenous eDNA source from the St. Lawrence River toward the goal of producing a fast management response to preserve St. Charles River basin ecological integrity.

4.4 | Environmental effects on fish communities with eDNA metabarcoding

It should be noted here that abiotic factors such as temperature, pH, UV, and O_2 have previously been associated with degradation of eDNA plumes in both freshwater and marine ecosystems (Afzali et al., 2020; Boivin-Delisle et al., 2021; Buxton et al., 2017; Klymus et al., 2015; Laramie et al., 2015; Robson et al., 2016; Seymour et al., 2018; Stewart, 2019), which could partly hamper a reliable description of community structure (Dickie et al., 2018; Goldberg et al., 2016; Ruppert et al., 2019; Stewart, 2019). Nevertheless, if abiotic effects on degradation are relatively small in comparison with fish abundance and distribution on eDNA sequences counts, repeatable results describing fish communities should be observed through time if nothing else change. Here, we observed neither effect of seasonality, nor of environmental changes between seasons, supporting the idea that differential eDNA degradation by environmental heterogeneity is not a major factor explaining eDNA variation in our dataset. Thus, we are confident that our description of fish community structure in this system is reliable. This is also supported by the fact that association between fish species and environmental conditions (explaining 53% of the measured variation) makes sense from an ecological standpoint. For example, sites showing a community dominated by Brook charr (*S. fontinalis*) are associated with colder temperature (PC-2), lower chlorophyll *a* (PC-8), total phosphorus (PC-3), total nitrogen (PC-1), and nitrogen ammoniacal (PC-4). Brook charr is a salmonid well known to be sensitive to warm water (Smith & Ridgway, 2019), eutrophication (i.e., water enrichment by nitrogen and/or phosphorus nutriments leading to higher concentration in chlorophyll *a*; IMAP, 2017), and ammonia (Tudorache et al., 2010). Similarly, Longnose dace (*R. cataractae*) is less abundant in sites with higher total nitrogen (PC-1), total phosphorus (PC-3), and chlorophyll *a* (PC-8), which have been associated with an alteration of their physiology (Jeffries et al., 2008). Inversely, Northern pike (*E. lucius*), Brown bullhead (*Ameiurus nebulosus*), Golden shiner (*N. crysoleucas*), Yellow perch (*P. flavenscens*), and Pumpkinseed (*L. gibbosus*) sequence reads were associated with higher temperature (PC-2), chlorophyll *a* (PC-8), and ammoniacal nitrogen (PC-4). All those species are known to show preference and/or tolerance to higher temperatures and eutrophication level (Bernatchez & Giroux, 2012; Scott & Crossman, 1976). Finally, the three-spined stickleback (*G. aculeatus*) read abundance is associated with higher specific conductivity and total nitrogen (PC-1) and total phosphorus (PC-3). Interestingly, it was observed that anthropogenic saltwater inlet, which increase specific conductivity, might benefit the euryhaline three-spined stickleback by reducing interspecific competition and parasite infection pressure (Lugert et al., 2017). Considering that no three-spined stickleback have been captured in the Nelson tributary in 1999 (Richards, 2010), this raises the hypothesis that surrounded recent urban densification may have led to a more suitable environment for the species via the increase of road de-icing salts runoff (Statistics Canada, 2010a, 2010b, 2010c). Overall, our study, as previously shown by Berger et al. (2020), supports the view that eDNA metarbacoding can be used to detect ecological relationships affecting fish species abundance and community structure.

4.5 | Upstream effect of eDNA transport in describing fish communities

In addition to degradation, eDNA transportation in lotic environments may complicate inference of spatial distribution of species based on their eDNA (Dickie et al., 2018; Goldberg et al., 2016; Ruppert et al., 2019; Stewart, 2019; Wilcox et al., 2016). For instance, Deiner and Altermatt (2014) and Pont et al. (2018), respectively, reported that eDNA could be detected a long distance from its source, namely up to 12.3 km for the invertebrate *Daphnia longispina* and up to 60 km for the European whitefish (*Coregonus lavaretus*). These observations support the concept that eDNA is a conveyor belts of biodiversity; that is, eDNA samples in a river mouth can provide biodiversity information of the entire basin (Deiner et al., 2016). However, association between eDNA quantities and downstream distance from the eDNA source shows varying results from good correlations (Nukazawa et al., 2018; Tillotson et al., 2018) to no significant decreasing concentrations with distance up to 9 km (Sansom & Sassoubre, 2017; Wacker et al., 2019). Recently, Laporte et al. (2020) showed that hydrodynamic modeling eDNA dispersion is a better indicator than downstream distance to explain eDNA concentration and dispersion from a given source. Moreover, Berger et al. (2020) associated fish read abundance with environmental variables within a 5-km transect despite the high flow rate of the St. Lawrence River. Here, we observed pronounced changes in fish communities after 5.55 km (e.g., between sampling site CH-03 and CH-04), supporting the idea that fish community structure can be inferred in a dendritic river system by eDNA metabarcoding. Together, this suggests that eDNA metabarcoding can be both (i) a conveyor belt of biodiversity information and yet (ii) a powerful tool to describe community structure in

a dendritic river system. In fact, a relatively weak upstream effect should poorly affect the overall signal of fish community because of the low proportion of exogenous eDNA, which is supported by the present study and by Berger et al. (2020). In parallel, our study does not deny the fact that eDNA is a conveyor belt of biodiversity. Indeed, when combining the sites CH-09 and CH-10, only eight of the 49 species were absent (i.e., *Anguilla rostrata*, *Carpiodes cyprinus*, *Chromosomus eos*, *C. neogaeus*, *Couesius plumbeus*, *Pimephales promelas*, *Semotilus corporalis*, and *Salmo salar*). Moreover, apart from *C. eos* and *C. neogaeus*, these eight species were detected with relatively few sequences reads in the entire dataset which support the view that (i) those species are rare in the system and (ii) eDNA is, at minimum, a good conveyor belt of fish biodiversity in this system (84% of fish species detection). Nevertheless, *C. eos* (1798 sequences in nine samples) and *C. neogaeus* (433 sequences in three samples) are mainly present in the Nelson tributary, the closest one to St. Charles River mouth. These species should have been detected in sites CH-09 and CH-10 considering that species with lower sequences count present in more upstream and remote sites were detected. Thus, specific physicochemical conditions (e.g., UV exposition, temperature, salinity, primary productivity) and hydrodynamic features (e.g., slope, sinuosity, and turbidity) of the river should be further studied for a better understanding of the potential limits of the biodiversity information that sampling a river mouth can provide and on the conditions that could affect the upstream effect on an in situ fish community.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

ML and LB designed the study, ML performed the statistical analyses and wrote the first draft, ML, ER-N, and VC contributed to the fieldwork, CH and BB carried out the laboratory work, EN performed the bioinformatics analyses, and SB and CC provided the environmental database and produced the map (Figure 1). All authors participated to the final writing of the paper.

DATA AVAILABILITY STATEMENT

All raw data are available at NCBI under the accession number: PRJNA729765.

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SUPPORTING INFORMATION

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