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ORIGINAL ARTICLE

# Environmental DNA as a detection and quantitative tool for stream-dwelling salamanders: A comparison with the traditional active search method

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## Abstract

As amphibians are showing significant signs of decline, adequate information and understanding of target species are essential for taking appropriate conservation measures. In recent years, environmental DNA has seen notable growth as a monitoring tool and testing this emergent method with various species has become an important step toward a better understanding of its benefits and limits for studying specific taxa. This study focused on using species-specific qPCR assays developed in our research group to test the eDNA method for three stream-dwelling salamander species of the Plethodontidae family: the Spring salamander (*Gyrinophilus porphyriticus*), the Northern dusky salamander (*Desmognathus fuscus*), and the Northern two-lined salamander (*Eurycea bislineata*). The traditional active search method and the eDNA method were compared for both their ability to detect species as well as to provide a quantitative assessment of populations in 24 headwater streams in Québec, eastern Canada. For all three species, eDNA was detected in every stream where the target species was observed during the active search method. Moreover, eDNA was detected in nine streams where the target species was not identified with the active search. A marginally significant association was found between eDNA concentration and salamander density for *D. fuscus* only. All species showed high variability for eDNA concentration between qPCR technical replicates and between samples of a given stream. The results of this study lead us to conclude that eDNA can be an excellent method for detection of stream-dwelling salamanders. Given the inconsistent quantitative aspect of eDNA with the studied species, the future of these stream-dwelling salamander monitoring most likely lies in the combined use of both eDNA and active search methods. Hence, active search could continue to offer useful small-scale detection and reliable quantitative data while eDNA could be implemented as an efficient and promising tool for large-scale detection.

**KEYWORDS**
amphibian, aquatic biodiversity, conservation, environmental DNA, population monitoring, qPCR, urodela

## 1 | INTRODUCTION

Over the past 20 years, amphibian populations have been experiencing significant signs of decline in a climate of massive anthropological pressure on the natural environment (Alroy, 2015; Houlahan et al., 2000; Stuart et al., 2004). Extensive understanding of a target species' ecology in this class of vertebrates is a critical step toward implementing appropriate conservation and management efforts, and to fully assess the severity of the amphibian species decline. Field monitoring should thus play a central role in the data acquisition process, but remains challenging because of their costly and time-consuming nature (Campbell et al., 2002). Visual or sound monitoring of amphibian species is also challenging since they are known to be often difficult to detect, mostly because of their small size, their cryptic nature or because they vocalize (for anurans) only during short periods of times in a year (Barata et al., 2017). The degree of detection in wildlife monitoring can also be an important issue particularly in situations where the target species are present in very low abundance in the environment (Bailey et al., 2004). The risk of false "absence" data is an additional potential caveat, as this could be consequential for a target species' conservation (Boakes et al., 2016). Multiple solutions exist to circumvent these issues, such as accounting for detection probability and abundance in statistical models (Guillera-Arroita et al., 2014; Mazerolle et al., 2007), as well as developing better monitoring methods and refining the existing ones to increase efficiency and data quality (Burns et al., 2019; Goldberg et al., 2011).

Environmental DNA (eDNA) is an increasingly used method for conducting wildlife monitoring mainly for aquatic macro-organisms (Beng & Corlett, 2020; Carim et al., 2016; Deiner et al., 2021; Goldberg et al., 2011; Tsuji et al., 2019). The eDNA method involves detection of DNA from one or more species in different kinds of substrate, such as soil, sediment, and water (Rees et al., 2014). DNA fragments can be detected from various intracellular or extracellular sources such as feces, gametes, and skin cells that organisms release into the environment (Taberlet et al., 2012; Valentini et al., 2009). The analysis of eDNA can potentially be used to acquire both species-specific detection data (presence/absence) and quantitative data. The detection process can be particularly useful when traditional field monitoring is expensive and logistically complex, or when the organisms under study are present in low abundance (Beng & Corlett, 2020; Goldberg et al., 2016). Using eDNA concentration as a quantitative tool has been examined and discussed by several researchers, who agree that this method shows great potential and should be further tested (Goldberg et al., 2016; Iversen et al., 2015; Yates et al., 2019). Since factors such as transport and degradation could have an important impact on eDNA concentration (Goldberg et al., 2011; Harrison et al., 2019; Laporte et al., 2020), several authors have used a semi-quantitative method by looking at the proportion of positive amplifications performed in quantitative PCR (qPCR) rather than eDNA concentration (e.g., Lacoursiere-Roussel et al., 2016a). In parallel with detection and quantitative aspects, establishing standards for the number of samples and qPCR replicates required for accurate eDNA results will be a central element in future eDNA research, as project managers will need to consider costs and benefits to provide a balanced monitoring strategy (Erickson et al., 2019).

Significant issues associated with amphibians' detection such as high cost of traditional monitoring methods have led several researchers to pioneer the use of eDNA method for species monitoring, in particular for elusive ones (Ficetola et al., 2008; Fukumoto et al., 2015; Olson et al., 2012; Santas et al., 2013). For instance, higher detection of a declining and secretive aquatic salamander, the eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*), was achieved using eDNA compared with a traditional active search method (Spear et al., 2015). The superior detection of a stream salamander with eDNA compared with the traditional field method was also reported for the Idaho giant salamander (*Dicamptodon aterrimus*), especially when the species occurred at low densities (Pilliod et al., 2013). These promising results demonstrate the great potential of eDNA as a monitoring tool for amphibians, thus encouraging further study of this emergent method with species of this taxonomic group.

Among amphibians, stream-dwelling salamanders comprise a well-studied group of species that would greatly benefit from more efficient monitoring methods. Indeed, the cryptic and elusive nature of these species makes them difficult to detect using methods like active search under stream debris such as rocks and wood logs (Pierson et al., 2016; Pilliod et al., 2013; Spear et al., 2015). This is the case for three salamander species of the Plethodontidae family found in eastern Canada: the Spring salamander (*Gyrinophilus porphyriticus*), the Northern dusky salamander (*Desmognathus fuscus*), and the Two-lined salamander (*Eurycea bislineata*) (Figure 1). In addition to the detection difficulties surrounding these species, several characteristics such as slow growth rates, extended aquatic larval stages, and high philopatry make them particularly vulnerable to anthropogenic stresses (Corn & Bury, 1989; Petranka, 1998; Welsh & Ollivier, 1998). Indeed, numerous pressures on their habitat such as elimination of forest cover and increased sedimentation are among the main factors that have led *G. porphyriticus* to be listed as a vulnerable species in the province of Québec (Canada) and a threatened species in Canada (COSEWIC, 2011). Although the active search method can provide useful population monitoring for these three species in small-scale studies, development of large-scale projects is unlikely due to its time-consuming nature. For instance, monitoring an average size stream may involve lifting a high number of rocks and debris, with numbers easily above 1,000 for a 50 m section (Plante et al., unpublished data). Hence, sampling 200 m of a stream can represent a sampling effort of up to eight person/h, which can be a major obstacle considering stream length often exceeds one kilometer (Plante et al., unpublished data). Since species may be present in low abundance down to only a few individuals per stream (COSEWIC, 2011), sub-sampling to save time can lead to false "absence." Moreover, this method has other caveats, including the disturbance of the natural environment when lifting debris and the potential stress inflicted on salamanders when captured.


FIGURE 1 (a) The Spring salamander (*Gyrinophilus porphyriticus*), (b) the Northern dusky salamander (*Desmognathus fuscus*), and (c) the Northern two-lined salamander (*Eurycea bislineata*)

Considering the detection constraints for stream-dwelling salamanders and the important conservation issues surrounding them, the analysis of eDNA has the potential to be a promising tool for the monitoring of this group of species. Several studies have already tested this method with similar amphibians, and researchers seem unanimous on the need to further test the approach for both detection and quantitative aspects (Deiner et al., 2021; Goldberg et al., 2016; Spear et al., 2015; Yates et al., 2019). Furthermore, few studies have compared environmental DNA results for similar amphibian species in a single study. Hence, the main purpose of this study was to study three salamander species (*G. porphyriticus*, *D. fuscus*, and *E. bislineata*) to assess the potential benefits and challenges of the eDNA method for its use with these cryptic and elusive amphibians. First, we aimed to compare detection (presence/absence) between eDNA and traditional active search. Secondly, we compared eDNA quantitative data (concentration of eDNA molecules) and semi-quantitative data (qPCR detection) with salamander density estimated from active search. In doing so, we also wanted to assess whether the number of samples and replicates chosen for our protocol were adequate for all three species.

## 2 | METHODS

### 2.1 | Sample collection

Fieldwork occurred from May to September 2019. Although the specific habitat of our three target species differs slightly, they are commonly found in the same headwater streams in eastern Canada, which represents the northern limit of their spatial distribution. Active stream search and eDNA sampling were performed from 24 headwater streams in this region (Figure 2). Since most streams did not have official names, they were associated with a letter from "A" to "X." In every selected stream, both active search and eDNA sampling were conducted within the same 200 m section (one 200 m section per stream). Of the 24 sites, 17 streams were selected based on the known presence of *G. porphyriticus*, which has the smallest distribution of the three species in the study area. Four other streams were in areas where *G. porphyriticus* was observed in the past, but recent monitoring effort could not detect the species. The last three sites were in areas adjacent to known populations of *G. porphyriticus*, but where presence of the species was unknown. Since both *D. fuscus* and *E. bislineata* are more widespread and abundant in the study area, we predicted that they would be detected in most of the 24 streams.

### 2.2 | Active stream search

Active search was done during the day, when the weather conditions allowed good visibility in the water. Consequently, no monitoring was carried out during a rainfall or within 48 h of a heavy rainfall. Periods of drought (more than 10 days without rain) were also avoided so that the water depth would be high enough for water filtration, which was done in the same time period. Active search was done using a standardized protocol developed by the *Ministère des Forêts, de la Faune et des Parcs du Québec* (Bourgault et al., 2017), which consists of lifting debris such as rocks and logs to count salamanders.

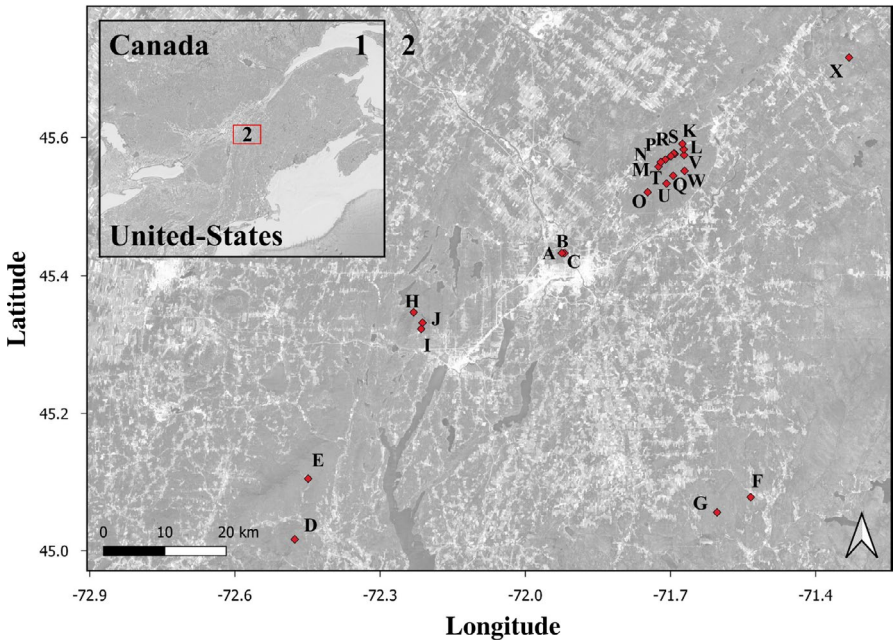


**FIGURE 2** Locations of the sampled streams in the province of Québec, eastern Canada (Map 1). Active search and eDNA sampling were carried out in each of the 24 streams identified with a red diamond (letters "A" to "X," Map 2)

In one section of 200 m per stream, debris more than 6 cm wide located in the stream and on the banks were lifted systematically, if not too heavy. Stream banks were included in the active search, because while juveniles are confined to the water body of the stream due to their external gills, adults often lie under rocks on the banks of the stream. To minimize impacts on the stream structure, only debris buried at less than one third of their volume were lifted. The active search was carried out from downstream to upstream, to avoid recounting a salamander that could potentially escape in the direction of the water flow. Small aquarium dip nets were positioned downstream of the lifted debris to catch individuals that fled with the water flow. Individuals were identified and quickly released at the capture site. For *G. porphyriticus*, juveniles were included in the number of individuals since they can be easily differentiated from other species. Because juveniles of *D. fuscus* and *E. bislineata* cannot easily be differentiated from one another and their small size makes them hard to detect, they were not considered in the count of individuals. A basic stream characterization was done, including stream width (m), corresponding to the natural high-water mark on both sides of the stream, water width (m), and water depth (m) (Table S1). These measurements were repeated 12 times across the 200 m section of the stream, approximatively every 15 m, to get an average value for each stream. Water width and depth measurements were later used with stream estimated slope (%), calculated from elevation data of the upper and lower limit of the 200 m section, to calculate an artificial water flow estimation variable. Stream width over the 200 section was used to determine salamander density.

## 2.3 | eDNA sampling

Water was filtered directly into the stream using a portable pump and disposable tips, similarly to the protocol developed by Carim et al., (2016). In each of the 24 streams, eight samples were taken in the stream and one additional sample was used as a negative control using distilled water. For each sample, 2 L of water was filtered through a 1.2 µm Polyethersulfone filter, which was selected since pore size filters below 1.5 µm have been documented to yield the most eDNA (Eichmiller et al., 2016). Starting from the downstream end of the 200 m sections, each sample was taken every 25 m approximately, in places where the bank of the stream was flat and clear enough to lay out the equipment. The tip was placed in a well-mixed area in the middle of the stream. Water temperature was taken four times (once every 50 m) to get an average value (Table S1). Once filtration was completed, each filter was stored in a small sterile bag containing 30 g of silica desiccant. Bags were kept in a dark and cool place before they were stored in a freezer within 2 days of filtration. A minimum period of 48 h was implemented between both methods to minimize the potential impact of active search disturbance on eDNA detection. Sampling with both methods was completed within 7 days for every stream.

## 2.4 | DNA extractions

DNA was extracted from each filter using the QIAshredder and DNeasy Blood and Tissue Kit (Qiagen, Inc.) method from Goldberg et al., (2011). First, each filter was cut in half and both pieces were placed into the same 5 ml tube with 720 µl of ATL Buffer and 80 µl of Proteinase K (Qiagen, Inc.), which was then put away for incubation at 56°C overnight. The next day, both filter and lysate were put into a QIAshredder tube and were centrifuged at 15 871 RCF (G-force). Flow-through lysate was transferred into new 5 ml tubes. Then, 800 µl of AL Buffer was added to all the tubes, which were then vortexed and incubated in a water bath at 70°C for 10 min. After incubation, 800 µl of ethanol 95% was added and mixed by vortexing.

TABLE 1 Species-specific primers for *Gyrinophilus porphyriticus*, *Desmognathus fuscus*, and *Eurycea bislineata* (from Hernandez et al., 2020)

| Common name | Scientific name | Forward primer (5′ -> 3′) | Reverse primer (5′ -> 3′) | TaqMan probe |
|---|---|---|---|---|
| Spring salamander | *Gyrinophilus porphyriticus* | CTTGGATGAATAATTGTTGTATTAACCC | CATGACATGGTTATTTTATTAATATTAGTTGAGG | ACCCTAATTAATTTTTTCATTGTACCTA |
| Northern dusky salamander | *Desmognathus fuscus* | AATATCACAATATCAAACACCATTATTTGTC | GTTAGAAGTATTGTAATTCCTGCTGCTAAA | CCGCTATTTTACTATTATTATCACTACC |
| Northern Two-lined salamander | *Eurycea bislineata* | GTGGTATTAATTTATTTCCCACAATTAACTAC | GATTAGTCATTTTGGTATAAATCCGGAA | TACTCAACTTAACATCAACTAGT |

This mix was transferred into a DNeasy Mini spin column (Qiagen, Inc.) and centrifuged at 15 871 RCF. The spin column was washed by adding 500 µl of AW1 Buffer and was centrifuged at 15 871 RCF. The same column was washed twice more using 500 µl of AW2 Buffer and was centrifuged at 15 871 RCF each time. Finally, 80 µl of nuclease-free water was added and the column was incubated at room temperature for 10 min before being centrifuged at 15 871 RCF. Extracted DNA was then split into two equal parts of 40 µl, and both were frozen at −20°C until amplification. One negative control was added for each site by performing all these steps without a filter to make sure contamination during manipulation could be detected.

## 2.5 | Quantitative PCR

Species-specific primers were used to carry out qPCR (Table 1). Primers were designed and tested by Hernandez and collaborators prior to this study (Hernandez et al., 2020). As mentioned in that study, *D. fuscus* showed cross-contamination from a related species, *Desmognathus ochrophaeus*. Because the known distribution of *D. ochrophaeus* in Québec is very limited and far from our study area (>90 km), we proceeded with these primers. All qPCR reactions were run in MicroAmp Fast Optical 96-Well plates (Applied biosystems, Life Technologies) with the 7,500 Fast Real-Time PCR System (Applied biosystems, Thermo Fisher Scientific). Holding stage was at 50°C for 2 min and then 95°C for 10 min. Cycling stage (50 cycles) was at 95°C for 15 s and 60°C for 1 min. Every eDNA sample, field negative control, and DNA extraction negative control were run in six replicates. Six additional negative controls were also used on every plate to make sure no contamination occurred during the reaction mix preparation. A positive control created from synthetic DNA (gBlocks, Integrated DNA Technologies Inc.) was put on every plate to ensure the reaction mix was well prepared and to control for inhibition. Finally, to quantify the amount of DNA molecules amplified with a standard curve, all plates included five standards in triplicates created from known concentration of synthetic DNA (100 000, 10 000, 1000, 100, and 10 copies). The limit of detection (LOD) of the essay was determined for each species using 10 technical replicates of nine synthetic DNA dilutions (1000, 500, 250, 50, 10, 4, 2, 1, and 0.5 molecule/µl) (Forootan et al., 2017). The LOD was evaluated using a discrete 95% threshold approach (Klymus et al., 2020).

The master mix used for all reactions done in six replicates contained 10 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 1.8 µl of forward primer (10 µM), 1.8 µl of reverse primer (10 µM), and 0.5 µl of specific probe (10 µM). Additionally, the "SPUD" method was used in every reaction to control for inhibition (Nolan et al., 2006). Thus, we added 1.2 µl of SPUD forward primer (10 µM), 1.2 µl of SPUD reverse primer (10 µM), 0.5 µl of specific probe (10 µM), and 1 µl of target DNA. Each of these reactions contained 2 µl of sample DNA (apart from the qPCR negative controls) and 18 µl of master mix. The master mix used for the standards in triplicates contained 10 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 1.8 µl of specific forward primer (10 µM), 1.8 µl

of specific reverse primer (10 μM), 0.5 μl of specific probe (10 μM), and 3.9 μl of RNase-free water.

### 2.6 | Data analyses

The 7,500 Software v2.3 (Life Technologies) was used to analyze the qPCR data and to set the detection threshold manually. The threshold was set at the same value for all plates and was placed in the exponential phase of the curves. In each plate, the software used the standard curve and Ct values to calculate the quantity of eDNA material detected (Table S2). Further analyses were done using the R 3.6.1 software (R Core Team, 2019). Considering only streams with eDNA-positive detection, three similar analyses were carried out to compare detection (dependent variable) among the three target species (independent variable). First, the species were compared for the proportion of total positive qPCR amplifications per stream (generally out of 48 amplifications, if no qPCR inhibition occurred). The second analysis aimed at comparing the proportion of positive samples per stream, which corresponded to samples with at least one positive qPCR amplification (generally out of eight samples, if no qPCR inhibition occurred). The third analysis aimed at comparing the proportion of positive qPCR amplifications in a sample (out of six replicates for all stream except for stream "N" where three qPCR replicates were carried out instead of six for *G. porphyriticus* and *D. fuscus* due to a lack of DNA material) on average per stream. For all three analyses, a Shapiro–Wilk's test and a Levene's test were used to assess normality and homogeneity of variance. Since assumptions for a one-way ANOVA were not met for all three analysis, a Kruskal–Wallis test was used. Finally, a Wilcoxon signed-rank test was performed to compare each species for all three analyses.

To examine the relationship between eDNA concentration and salamander density, a hierarchical linear mixed-effects model was used. Negative qPCR amplifications and streams without salamanders were both excluded from the analysis. Both *sampling site* and *sample* were used as random effects in the model. *Sample* (six qPCR replicates per eDNA sample) was nested in *sampling site* (eight samples per site). *Water flow estimation* and *water temperature* were included in the model as additional explanatory variables. The *Water flow estimation* variable was calculated by multiplying water width, water depth, and section length (200 m). This water volume estimation ($m^3$) was then multiplied with the average slope (%) of the stream. Since sampling cells or tissue may cause extreme measurement of eDNA concentration, the model was used with and without outliers, which were associated with the data above 1.5 × IQR (interquartile range) (Klymus et al., 2015; Pilliod et al., 2013). A square root transformation was used to obtain equal variance of errors and normality of errors.

To analyze the variability in the eDNA concentration data, the Relative Standard deviation Error (RSE) was used. Spearman's rank correlation coefficient was applied to assess how within sample RSE was correlated to eDNA concentration, as well as how intersample RSE was correlated to water flow estimation.

To evaluate the relationship between qPCR detection (positive/negative) and salamander density, a similar model to the one described for eDNA concentration was used. A hierarchical logistic mixed-effects model was applied in this case because qPCR detection is a binary variable (either positive or negative). Once again, *sample* (six qPCR replicates per eDNA sample) was nested in *sampling site* (eight samples per site). To reduce scale and convergence problems with the model, *salamander density* was log-transformed and was the only predictor included in this model.

## 3 | RESULTS

### 3.1 | Detection of eDNA (presence/absence)

qPCR inhibition occurred in 19 different samples out of 192, which were discarded for subsequent analyses. The inhibition was observed in the same 19 locations for qPCR of the different species. These samples are from six different streams, which are all small, muddy, and with low water levels (between 1 and 10 cm). The two streams most affected by inhibition have five samples inhibited each, meaning that no stream has all its eight samples inhibited. No detection of the species' DNA occurred in any of the negative control samples (field, eDNA extraction, and qPCR negative control samples). The limit of detection (LOD) for qPCR assays was at 2 DNA copies per reaction for both *G. porphyriticus* and *E. bislineata* and at 4 DNA copies for *D. fuscus*.

For all three species, eDNA was detected in every stream where the target species was observed during the active search (Table 2). *G. porphyriticus'* eDNA was also detected in three streams where no individual was observed during the active search method. Two of these streams ("G" and "M") are located in areas where the species was observed in past years but has been undetected in recent active search monitoring. The third stream ("R") is located in an area adjacent to a known population where dispersal is possible. For *E. bislineata*, eDNA was also detected in two streams ("P" and "T") where no adult was found. However, unidentified larvae (*E. bislineata* or *D. fuscus*) and adults of *D. fuscus* were found at these two sites. Similarly, *D. fuscus*' eDNA was detected at four sites ("F," "L," "U," and "W") where adults were not observed but unidentified larvae and adults of *E. bislineata* were present each time.

Although eDNA was detected in every stream where the target species was observed, the proportion of positive qPCR amplifications, samples, and technical replicates differed between species. Since assumptions for a one-way ANOVA were not all met for the three analyses, a Kruskal–Wallis test was used and showed a significant difference for at least one of the three species for the proportion of total positive amplifications ($p = <0.001$), the proportion of positive samples ($p = <0.001$), as well as the proportion of positive qPCR amplifications in a sample on average per stream ($p = <0.001$). A Wilcoxon signed-rank test was then performed to compare each species for all three analyses. The mean proportion of total positive amplifications

**TABLE 2** For the three target species in each of the 24 streams, number of salamanders observed with active search, number of positive qPCR amplifications, and number of positive samples (samples with at least one positive qPCR amplification)

| | *G. porphyriticus* | | | *D. fuscus* | | | *E. bislineata* | | |
|---|---|---|---|---|---|---|---|---|---|
| Stream | Larvae and adults | Total + qPCR | + Samples | Adults only | Total + qPCR | + Samples | Adults only | Total + qPCR | + Samples |
| A | 0 | 0 / 48 | 0/8 | 35 | 1 / 48 | 1/8 | 49 | 48 / 48 | 8/8 |
| B | 0 | 0 / 18 | 0/3 | 37 | 18 / 18 | 3/3 | 0 | 0 / 18 | 0/3 |
| C | 0 | 0 / 18 | 0/3 | 56 | 18 / 18 | 3/3 | 9 | 5 / 18 | 1/3 |
| D | 40 | 47 / 48 | 8/8 | 21 | 5 / 48 | 2/8 | 3 | 47 / 48 | 8/8 |
| E | 52 | 45 / 48 | 8/8 | 2 | 5 / 48 | 3/8 | 4 | 46 / 48 | 8/8 |
| F | 0 | 0 / 42 | 0/7 | 0 | 5 / 42 | 5/7 | 1 | 42 / 42 | 7/7 |
| G | 0 | 6 / 48 | 4/8 | 1 | 6 / 48 | 7/8 | 8 | 48 / 48 | 8/8 |
| H | 99 | 48 / 48 | 8/8 | 1 | 1 / 48 | 1/8 | 4 | 47 / 48 | 8/8 |
| I | 44 | 47 / 48 | 8/8 | 3 | 3 / 48 | 2/8 | 20 | 48 / 48 | 8/8 |
| J | 46 | 44 / 48 | 8/8 | 0 | 0 / 48 | 0/8 | 5 | 41 / 48 | 8/8 |
| K | 3 | 40 / 42 | 7/7 | 5 | 7 / 42 | 5/7 | 8 | 42 / 42 | 7/7 |
| L | 60 | 48 / 48 | 8/8 | 0 | 9 / 48 | 5/8 | 6 | 48 / 48 | 8/8 |
| M | 0 | 13 / 48 | 5/8 | 12 | 33 / 48 | 6/8 | 3 | 37 / 48 | 7/8 |
| N | 8 | 24 / 24 | 4/4 | 28 | 6 / 24 | 2/4 | 1 | 46 / 48 | 8/8 |
| O | 12 | 45 / 48 | 8/8 | 6 | 14 / 48 | 7/8 | 1 | 48 / 48 | 8/8 |
| P | 19 | 25 / 30 | 5/5 | 7 | 8 / 30 | 3/5 | 0 | 22 / 30 | 5/5 |
| Q | 3 | 9 / 24 | 2/4 | 10 | 6 / 24 | 1/4 | 30 | 24 / 24 | 4/4 |
| R | 0 | 1 / 48 | 1/8 | 14 | 29 / 48 | 8/8 | 3 | 17 / 48 | 7/8 |
| S | 22 | 44 / 48 | 8/8 | 3 | 17 / 48 | 6/8 | 3 | 47 / 48 | 8/8 |
| T | 4 | 48 / 48 | 8/8 | 18 | 4 / 48 | 4/8 | 0 | 42 / 48 | 7/8 |
| U | 35 | 47 / 48 | 8/8 | 0 | 9 / 48 | 3/8 | 14 | 48 / 48 | 8/8 |
| V | 22 | 46 / 48 | 8/8 | 9 | 12 / 48 | 7/8 | 21 | 47 / 48 | 8/8 |
| W | 24 | 43 / 48 | 8/8 | 0 | 1 / 48 | 1/8 | 124 | 48 / 48 | 8/8 |
| X | 14 | 48 / 48 | 8/8 | 6 | 9 / 48 | 6/8 | 1 | 47 / 48 | 8/8 |

*Note:* Total positive (+) qPCR denominators represent the number of reactions carried out (generally eight samples of six technical replicates for a total of 48). Below 48 reactions correspond to streams with occurrence of inhibition, except for the stream "N" where three qPCR replicates were done instead of six due to a lack of DNA material for *Gyrinophilus porphyriticus* and *Desmognathus fuscus*. Positive samples denominators below eight correspond to streams with occurrence of inhibition.

**TABLE 3** Comparing the three species considering only streams with eDNA-positive detection: 1. The mean proportion of total positive qPCR amplifications per stream (generally out of 48), 2. The mean proportion of positive samples per stream (generally out of eight samples), and 3. The mean proportion of positive qPCR amplifications in a sample (generally out of six replicates)

| | *Gyrinophilus porphyriticus* | | *Desmognathus fuscus* | | *Eurycea bislineata* | |
|---|---|---|---|---|---|---|
| | *Mean* | *Standard deviation* | *Mean* | *Standard deviation* | *Mean* | *Standard deviation* |
| 1. Proportion of total positive amplifications (%) | 80.4 | 32.0 | 27.6 | 28.2 | 86.7 | 27.1 |
| 2. Proportion of positive samples (%) | 88.8 | 24.6 | 57.2 | 29.2 | 95.5 | 14.2 |
| 3. Proportion of positive replicates in each sample (%) | 84.4 | 25.4 | 42.6 | 28.9 | 93.6 | 13.5 |

*Note:* A standard deviation measure is presented for each mean.

for *D. fuscus*, which was 27.6%, was significantly lower than for *E. bislineata* ($p$ = <0.001) and for *G. porphyriticus* ($p$ = <0.001), while there was no significant difference between *E. bislineata* and *G. porphyriticus* ($p$ = 0.141) (Table 3, Figure 3a). Similarly, the mean proportion of positive samples for *D. fuscus* was 57.2%, which was significantly lower than for *E. bislineata* ($p$ = <0.001)

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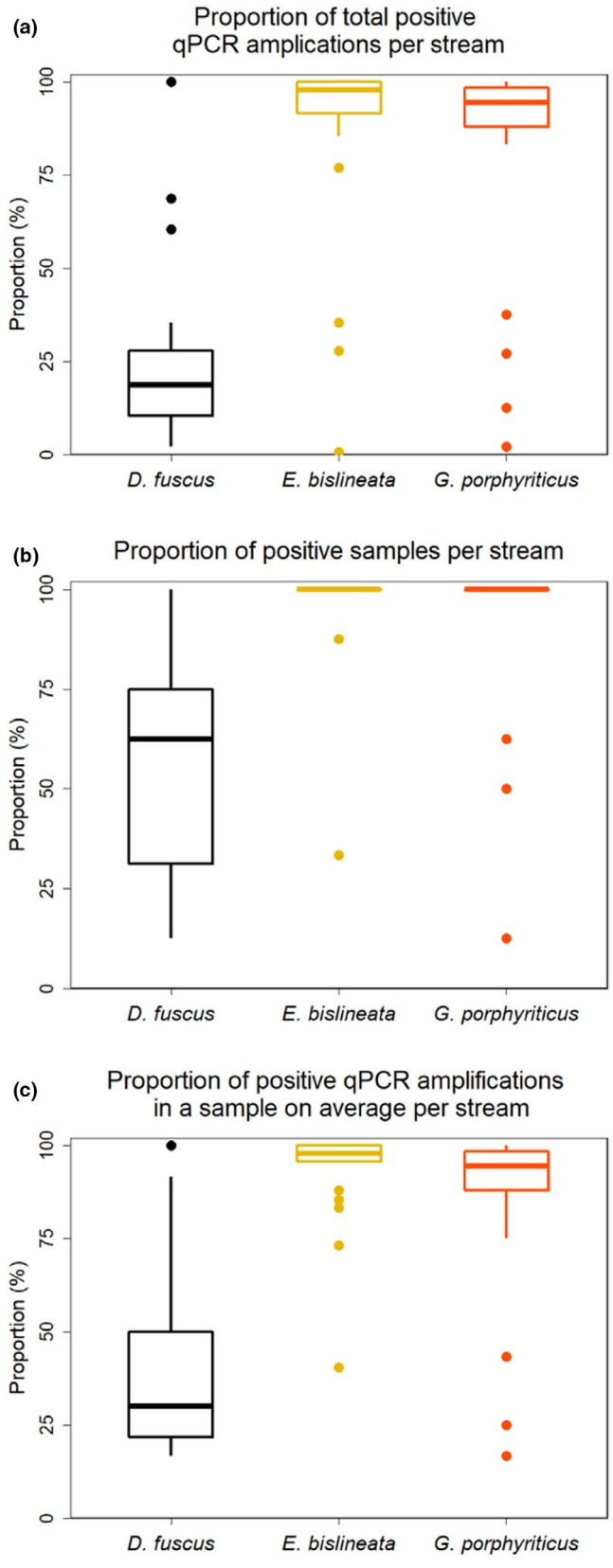


**FIGURE 3** Comparing the three species considering only streams with eDNA-positive detection: (a) The proportion (%) of total positive qPCR amplifications per stream (generally out of 48), (b) The proportion of positive samples per stream (generally out of eight samples), and (c) The proportion of positive qPCR amplifications in a sample (generally out of six replicates) on average per stream

and for *G. porphyriticus* ($p$ = <0.001), while there was no significant difference between *E. bislineata* and *G. porphyriticus* ($p$ = 0.706) (Table 3, Figure 3b). Lastly, the mean proportion of positive qPCR amplifications in a sample was 42.6% for *D. fuscus*, which was significantly lower than for *E. bislineata* ($p$ = <0.001) and for *G. porphyriticus* ($p$ = 0.002), while there was again no significant (albeit near significance) difference between *E. bislineata* and *G. porphyriticus* ($p$ = 0.071) (Table 3, Figure 3c).

## 3.2 | Quantitative and semi-quantitative aspects

A hierarchical linear mixed-effects model used to examine the relationship between eDNA concentration and *G. porphyriticus* density showed no significant association between eDNA concentration and salamander density ($t$ = 1.183, $p$ = 0.258), water flow estimation ($t$ = 1.379, $p$ = 0.192), or water temperature ($t$ = 0.997, $p$ = 0.338) while considering outliers. Results were similar when outliers above 1.5x interquartile range were removed, with no significant association between eDNA concentration and salamander density ($t$ = 1.343, $p$ = 0.202), water flow estimation ($t$ = 1.427, $p$ = 0.179), or water temperature ($t$ = 0.710, $p$ = 0.492). Results for *E. bislineata* also showed an absence of significant association between eDNA and salamander density ($t$ = 1.238, $p$ = 0.233), water flow estimation ($t$ = −0.465, $p$ = 0.648) and water temperature ($t$ = −0.959, $p$ = 0.352) with outliers. Removing outliers did not improve the correlations. In the case of *D. fuscus*, a significant association was found between eDNA concentration and salamander density ($t$ = 3.600, $p$ value = 0.003), but not for water flow estimation ($t$ = 0.365, $p$ = 0.720) and water temperature ($t$ = 0.014, $p$ = 0.989). When outliers were removed, the relationship between eDNA concentration and salamander density remained significant ($t$ = 2.072, $p$ = 0.050). A hierarchical logistic mixed-effects model used to determine the relationship between qPCR detection (positive/negative) and salamander density showed no significant effect for *G. porphyriticus* ($z$ = 0.649, $p$ = 0.516) and *E. bislineata* ($z$ = 0.041, $p$ = 0.967). As for *D. fuscus*, a significant effect was detected by the model ($z$ = 3.138, $p$ = 0.002). The estimated increase in qPCR detection per one unit of salamander density (one salamander per 10 $m^2$) generated by the model was 0.201%, thus showing very low influence of the species density on the qPCR positive amplifications.

The mean eDNA concentration for *G. porphyriticus*, *D. fuscus*, and *E. bislineata* was 158.5 molecules/L, 299.2 molecules/L, and 386.1 molecules/L, respectively. While *D. fuscus* eDNA concentration averaged 36.4 molecules/L across 21 streams, the mean concentration of the two streams with the highest salamander density (stream "B" and "C") had much higher concentrations, with 2790,0 molecules/L and 232.1 molecules/L, respectively (Figure 4).

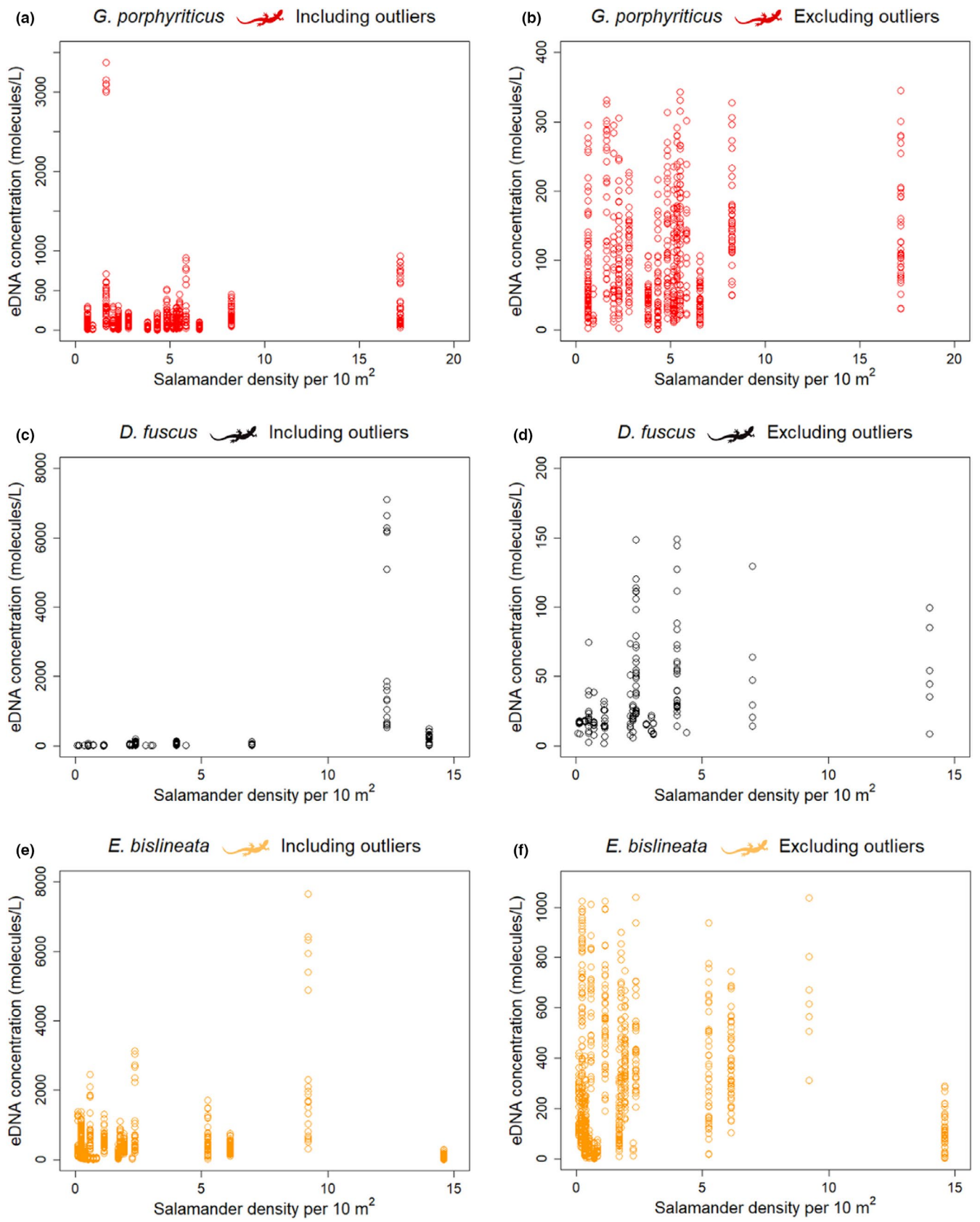


FIGURE 4 The relationship between eDNA concentration (molecules/L) and salamander density (salamanders per 10 $m^2$) for the three species, excluding negative qPCR amplifications and streams without salamanders. Each dot represents one qPCR amplification and data are represented with (a, c, e) and without (b, d, f) outliers (1.5 × interquartile range)

### 3.3 | Variance among replicates and samples

High variance among replicates and samples within sites was clearly visible when examining the distribution of the data set (Figure 4). Two elements of this variability were distinguished: the within samples relative standard variation error (RSE) (between the six qPCR technical replicates of a given sample), and the intersample RSE (between the eight samples from a given stream) (Table 4). The RSE within samples was 22.3% for *G. porphyriticus*, 18.6% for *E. bislineata*, and 63.9% for *D. fuscus*. The Spearman's rank correlation between RSE within samples and eDNA concentration was −0.39 for *G. porphyriticus* ($p = 0.091$), −0.63 for *E. bislineata* ($p = 0.002$), and −0.54 for *D. fuscus* ($p = 0.008$). As for the variability between samples, RSE was 32.8% for *G. porphyriticus*, 25.7% for *E. bislineata*, and 52.7% for *D. fuscus*. Spearman's rank correlation between intersample RSE and water flow estimation was very weak and non-significant, with −0.10 for *G. porphyriticus* ($p = 0.681$), −0.24 for *E. bislineata* ($p = 0.269$), and −0.06 for *D. fuscus* ($p = 0.774$).

## 4 | DISCUSSION

### 4.1 | Detection of eDNA (presence/absence)

In this study, we successfully demonstrated the potential of the eDNA method for the detection of elusive amphibians by targeting three stream-dwelling salamander species. Positive detection with the eDNA method not only occurred in 100% of streams where individuals were observed with the active search method, but also in nine streams where the target species was not detected with active search. Such detection results from three different species in 24 sites are clear evidence that the eDNA method can provide valuable presence/absence data and superior detection in comparison with the traditional method, which is consistent with several studies investigating the performance of eDNA methods with amphibians in lotic systems (Bjordahl et al., 2020; Pierson et al., 2016; Pilliod et al., 2013; Spear et al., 2015). Since none of the field and laboratory negative control samples showed a positive amplification, we are confident that contamination is unlikely to have occurred. Hence, the detection of eDNA in sites where the target species was not detected with the active search could be explained in two other ways. First, the detected eDNA could be produced by one or more individuals located upstream of the 200 m section. eDNA transport is known to occur over 200 m in lotic systems, which makes detection further downstream likely (Harrison et al., 2019; Pilliod et al., 2013; Wood et al., 2019). Secondly, the detected eDNA could be produced from one or more individuals located within the 200 m which were undetected during the active search method. The stream active search is a method with imperfect detection, and an unknown number of individuals could very well contribute to the local eDNA pool. This is particularly the case for *D. fuscus* and *E. bislineata* since juveniles of these species were not considered in the count of individuals because of the difficulties related to their detection and identification.

Our results show that the eDNA method could offer a more effective detection than the active search in some cases. A prominent example of its effectiveness is clearly shown within one individual stream (stream "G"; Figure 2), where *G. porphyriticus* was observed in past years, but no pictures and GPS locations were taken. Considering the federal and provincial status of this species in Québec, land managers and local conservationists wanted to confirm its presence with certainty. Thus, 550 m of the stream was thoroughly monitored using the active search method, and around 4200 rocks were lifted over almost 12 person/h (Plante et al., unpublished data). Although this represents a high sampling effort for a single stream, the species still went undetected. However, our eDNA data obtained with much less effort confirmed the presence of the species. This example shows the enhanced potential of this method for the detection of cryptic species.

The number of samples and qPCR replicates used in eDNA studies vary notably, and selecting the adequate sample size and number of replicates is a key issue for future eDNA research and use in monitoring (Erickson et al., 2019). As it is the case with all monitoring methods, project managers will need to consider costs and benefits according to the study's objectives to provide an appropriate monitoring strategy. For example, adequate number of samples and qPCR replicates might not be the same for all species. Such a situation occurred in our study, as the proportion of detection among total amplifications, samples, and technical replicates was significantly lower for *D. fuscus*. This important discrepancy clearly indicates that it can be a misstep to presume eDNA results will not differ across ecologically similar species.

Given that the number of positive eDNA amplification within a stream was in some cases significantly low in this study, the occurrence of inhibition should be considered seriously. This is especially the case with small headwater streams, where water depth is frequently low. In these conditions, we observed a few times

**TABLE 4** The average relative standard deviation error (RSE) for streams with eDNA detection was calculated within samples (for qPCR technical replicates) and between samples (eight samples per stream)

| | *Gyrinophilus porphyriticus* | *Desmognathus fuscus* | *Eurycea bislineata* |
|---|---|---|---|
| Technical replicates RSE | 22.3 | 63.9 | 18.7 |
| $r_s$ with eDNA concentration | −0.39 | −0.54 | −0.63 |
| $r_s$ *p* value | 0.091 | 0.008 | 0.002 |
| Samples RSE | 32.8 | 52.7 | 25.7 |
| $r_s$ with water flow estimation | −0.10 | −0.06 | −0.24 |
| $r_s$ *p* value | 0.681 | 0.774 | 0.269 |

*Note:* Spearman's rank correlation coefficient ($r_s$) was used to estimate the correlation between within samples RSE and eDNA concentration, as well as between samples RSE and water flow estimation.

that filtration collected several substances that were most likely the cause of qPCR inhibition. These small streams can even dry up when the rains are not frequent enough, thus preventing monitoring from being carried out. Since such small streams represent the typical habitat for *D. fuscus*, filtration work should be planned carefully when monitoring this species. While a feasible solution for this caveat could be to avoid long periods without rain, one must consider that this represents a noteworthy limitation to the eDNA method with stream-dwelling salamanders and ecologically related species.

## 4.2 | Quantitative and semi-quantitative aspects

We found no relationship between eDNA concentration and salamander density for both *G. porphyriticus* and *E. bislineata*, and a marginally significant effect obtained for *D. fuscus*. In a similar manner, no relationship was found between qPCR detection and salamander density for *G. porphyriticus* and *E. bislineata* and only a small effect was found for *D. fuscus*. As other studies have shown, linking eDNA concentration with species abundance in natural environment varies substantially among species and study context (Goldberg et al., 2016; Spear et al., 2015; Yates et al., 2019). In the case of the three stream-dwelling salamanders under study, multiple factors could explain our results. The eDNA detected in the 200 m section might be greatly influenced by the individuals located upstream of the section (Harrison et al., 2019; Jane et al., 2015), and the abundance detected with active search in the sampled section might not necessarily reflect the abundance upstream of the section.

Stream-dwelling salamander habitat also poses question for the quantitative aspect of eDNA, as adults of some species can hide under rocks either in the water or on the bank of the stream. Hence, individuals located in the water could potentially generate more eDNA in the water than the individuals on the bank, where eDNA could remain mostly trapped in the substrate (Harrison et al., 2019). Low densities of individuals for some species or populations, combined with individual variations in behavior (individuals in the water or on the banks), mean that a few individuals may have a large impact on the amount of eDNA detected in the water. Another problem with *D. fuscus* and *E. bislineata* was that juveniles were not included due to the identification and detection difficulties. Since juveniles were sometimes seen in large abundance during active search ($n > 100$) and considering they are restricted to the water body, their contribution to eDNA production could be substantial. Lastly, the abundance was calculated with the results of the active search, which does not necessarily detect all salamanders. This imperfect detection might have introduced additional variation to the observed relationship between abundance and eDNA. Although we are confident in the quality of the traditional monitoring method, an ideal monitoring method would include a probability of detection to account for imperfect detection (Mazerolle et al., 2007), but the high cost of active search, both in time and effort, made it impossible to perform it multiple times to evaluate the probability of detection.

The relative standard variation error (RSE) within and between eDNA samples was very high, as above 20% is considered high heterogeneity (McCune & Grace, 2002). Such variation in eDNA concentration has been found in other studies (Lacoursiere-Roussel et al., 2016b; Pilliod et al., 2013). The Spearman correlation found between RSE within samples and eDNA concentration might partly explain this high variation. Because eDNA concentration found was often low, high variance might have blurred any relationship with salamander density. While we suspected that water flow could have an impact on the homogeneity of eDNA particles in the water column (Harrison et al., 2019), the absence of correlation between water flow and RSE between samples does not support this assumption. Overall, considering the high RSE within and between samples, using six qPCR replicates and eight samples per stream might be too few to perform quantitative work with these species. While using more qPCR replicates and samples seems like a simple solution, the increased cost related to it might be an important limitation in most field monitoring.

## 4.3 | The case of *D. fuscus*

Our eDNA data for *D. fuscus* offer an interesting lead worth exploring. Although ecologically very similar to the other two species of this study and with an average density per 10 $m^2$ slightly higher than *E. bislineata*, *D. fuscus* showed a much lower eDNA detection. The higher limit of detection (LOD) in qPCR essays found for *D. fuscus* might partially explain this difference, but it does not explain why eDNA concentration for this species was significantly lower than the two other species in almost every stream. An interesting difference between the three target species is the duration of the aquatic larval stage, which is around four years for *G. porphyriticus*, two to three years for *E. bislineata*, and only eight to twelve months for *D. fuscus* (Bruce, 2005). Since a shorter larval stage could mean a higher proportion of individuals on the banks of the stream, this difference could possibly explain why the detection of *D. fuscus* was much lower than the other two species.

Another plausible explanation may lie in the species' habitat use (in the water or on the banks), which may vary depending on stream size. Of the 23 streams where *D. fuscus* was detected with eDNA, only the two smallest streams (stream "B" and "C") had adults that were found in majority under rocks in the water, compared to the other 21 larger streams where adults were found almost exclusively on stream banks. While eDNA detection for this species was rather low in these 21 larger streams, with an average of 20.1% of positive amplifications, we observed a 100% detection rate in the two smaller streams. eDNA concentration also showed important distinction, with an average of 36.4 molecules/L for the 21 larger streams and 2790.0 molecules/L and 232.1 molecules/L for the two smaller streams ("B" and "C," respectively). This large difference can possibly be explained by the fact that salamanders in the water might contribute to a much greater extent to eDNA concentration in the water than individuals located on the bank of the stream.

Moreover, stream-dwelling salamanders are known to disperse along a moisture gradient that reduces the overlap of ecological niches between species (Grover, 2000; Smith & Pough, 1994). In addition to being the most aquatic species of this study, *G. porphyriticus* is larger in size and known to be an important predator and a good competitor in headwater streams (Bruce, 1972; Gustafson, 1994). Consequently, the presence of *G. porphyriticus* could partly explain why *D. fuscus* was often detected on the banks of the stream rather than in the water. Detection of *D. fuscus* in streams with absence of *G. porphyriticus* could thus potentially lead to a higher eDNA detection, but we cannot adequately tackle this assumption with our data since *G. porphyriticus* is present in most sampled streams. While *E. bislineata* could undergo similar competition from *G. porphyriticus*, the longer duration of the larval stage compared with *D. fuscus* could explain why the detection of eDNA remains excellent for this species.

## 5 | CONCLUSION

In conclusion, this study unequivocally showed the high potential of environmental DNA for the detection of stream-dwelling salamander species, thus representing an additional tool for improved monitoring and conservation of such elusive species. Although our three target species were ecologically quite similar, major detection differences clearly showed why eDNA should preferably be tested for each target species. We encourage future research to take into consideration ecological knowledge of the target species when designing eDNA projects, as tackling this poorly understood and understudied aspect of eDNA might set this method one step closer to a better and more efficient monitoring tool. Given the apparently limited potential of the quantitative aspect of eDNA with the studied species, the future of these stream-dwelling salamander monitoring most likely lies in the combined use of both eDNA and active search methods. Hence, active search could continue to provide useful small-scale detection and reliable quantitative data while eDNA could be implemented as an efficient and promising tool for large-scale detection.

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### CONFLICT OF INTEREST

We have no conflict of interest to declare.

### AUTHOR CONTRIBUTIONS

FP led experiment design, data acquisition, data analysis, and manuscript writing. PB, YD, and LB provided critical feedback and helped shape the research, analysis, and manuscript. PB also contributed to data acquisition.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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## SUPPORTING INFORMATION

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

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