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Investigating the fine scale breeding habitat use of endangered newt species using environmental DNA from water samples

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par

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ABSTRACT

Amphibians are amongst the most threatened species worldwide. To set up efficient conservation plans, a better understanding of their ecology is required. This can be challenging for discreet species such as newts, for which standard visual and acoustic censuses provide largely insufficient detection. Recently, environmental DNA (eDNA) was proposed as an alternative for surveying such species, with improved detection. Nevertheless, to our knowledge, this method was always used in discrete water bodies. Here, we used eDNA metabarcoding approach to determine fine scale breeding habitat use of two endangered newt species (*Lissotriton vulgaris* and *L. helveticus*) in a continuous wet meadow expanse. We characterized the environment of our 50 sampling points by recording 10 environmental variables and measured their impact on the presence probability of newts. *L. vulgaris* was detected up to 340m from their wintering habitats, while it was not predicted to migrate further than hundreds of meters. In contrast, no DNA from *L. helveticus* was detected. None of our environmental variables had a significant influence on the presence probability of *L. vulgaris*. Nonetheless, our results suggest that eDNA is a promising tool to survey inconspicuous species in continuous wetlands habitats.

Key words: metabarcoding, *Lissotriton vulgaris*, *Lissotriton helveticus*, habitat characterization.

RESUME

A l'échelle mondiale, les amphibiens font partie des espèces les plus menacées. Pour mettre en place des plans de conservation efficaces, une meilleure compréhension de l'écologie ces espèces est requise. Cela peut s'avérer compliqué pour les espèces cryptiques comme les tritons, pour qui les recensements visuels et acoustiques standards restent largement insuffisants à leur détection. Récemment, l'ADN environnemental (ADNe) a été proposé comme une alternative à ces méthodes, présentant des taux supérieurs de détections pour de telles espèces. Néanmoins, à notre connaissance, cette méthode a toujours été utilisée dans des plans d'eau discret. Dans cette étude, nous utilisons l'ADNe pour déterminer l'utilisation à petite échelle de l'habitat de deux espèces menacées de tritons (*Lissotriton vulgaris* et *L. helveticus*) pendant la saison de reproduction dans une étendue continue de prairies marécageuses. Nous avons caractérisé l'environnement de 50 points d'échantillonnage en relevant 10 variables environnementales. L'impact de ces variables environnementales sur la probabilité de présence des deux espèces cibles a ensuite été mesuré. *L. vulgaris* a été détecté à plus de 340m de leur habitat d'hivernage, alors qu'il était prédit de ne migrer que d'une centaine de mètre. En revanche, aucun ADN de *L. helveticus* a été retrouvé dans les échantillons d'eau. Aucune des variables environnementales ne semblent affecter la probabilité de présence du *L. vulgaris*. Cependant, nos résultats suggèrent que l'ADNe est un outil prometteur pour recenser des espèces cryptiques dans des zones humides continues.

1 INTRODUCTION

Current biodiversity loss is of major concern because of its well-known human benefits through direct and indirect services (J.S. Singh, 2002; Millennium Ecosystem Assessment, 2003). Biodiversity loss is largely attributed to anthropogenic activities, namely climatic change and habitat loss and degradation (Fahrig, 1997; Pimm & Raven, 2000; Brook et al., 2003). A targeted habitat degradation through human land use and different life history traits characteristics expose species to unequal extinction risks (Mckinney, 1997; Purvis et al., 2000). For instance, amphibian species have shown a rapid population decline over the last 50 years, partly explained by their habitat degradation (Stuart et al., 2004). Indeed, wetlands are amongst the most threatened natural environments and have shown their areas reduced by 87% worldwide during the last three centuries (Davidson, 2014), mainly replaced by human infrastructures (Brinson & Malvárez, 2002). To conserve biodiversity, and thus ecosystems services, protection and restoration of natural areas are needed.

To make efficient conservation management plans, a deep knowledge of population trends and species ecology is essential (Joseph et al., 2006). Thus, rigorous monitoring of endangered species is needed. However, standard survey tools may prove to be inefficient to monitor inconspicuous species. Notably, among amphibian species, newts remain largely undetected using standard survey methods which consist in visual and acoustic censuses as well as trapping (Rödel & Ernst, 2004). Hence, effective survey tools must be developed to increase detectability of such cryptic species.

In this perspective, environmental DNA (eDNA)-based survey methods in ecology and conservation have been developed, originally used by microbiologists (Anderson & Cairney, 2004; Rondon et al., 2000). The eDNA approach defines the extraction of DNA released by individuals –through dead cells, hair, faeces etc.– in environmental samples (soil, water, faeces), thus representing a non-invasive monitoring tool (Taberlet et al., 2012). This method has been recently used to assess recent and ancient biodiversity (Loge et al., 2012; Valentini et al., 2016; Willerslev et al., 2003), to survey endangered (Ficetola et al., 2008; Thomsen et al., 2012) and invasive species (Ficetola et al., 2008; Jerde et al., 2011; Dejean et al., 2012; Smart et al., 2015; Schneider et al., 2016) as well as in diet analyses (Shehzad et al., 2012; De Barba et al., 2014). In that respect, eDNA methods can be used either for single or multiple taxa identifications. The latter is defined as eDNA metabarcoding approach.

Because of its high sensitivity and accurate taxonomic identification, the eDNA approach has been shown to be generally more effective than standard methods in monitoring cryptic

71 species (Biggs et al., 2015; Lopes et al., 2017). Nevertheless, to our knowledge, eDNA-based
72 survey methods have always been used in discrete environments such as distinct water bodies.
73 In this study, we sampled water in two continuous wetland reserves of the Grande Cariçaie
74 (southern shore of Lake Neuchâtel, Switzerland) to investigate the ecology of *Lissotriton*
75 *vulgaris* and *L. helveticus* that are amongst the most threatened species at the swiss scale
76 (Schmidt & Zumbarch, 2005). Although the Grande Cariçaie shelter the largest swiss
77 populations of these latter, a decline in population size was recently observed. The
78 distribution of these species along the Grand Cariçaie is well known since amphibian barriers
79 are regularly placed between wintering (forest) and breeding (wetland) habitats. Nonetheless,
80 their breeding habitat at a smaller scale remains poorly known due to inefficient survey
81 methods. Hence, we aimed at determining the fine scale breeding habitat use of both
82 endangered newt species in continuous wet meadow expanses using eDNA metabarcoding
83 approach. With DNA retrieved in our water sample, we assessed (i) which natural areas and
84 vegetation types are most likely to be suitable for newts during the breeding period; (ii) if
85 recorded environmental variables impact the presence probability of both newt species; (iii)
86 the effectiveness of eDNA-based survey methods compared to the effectiveness of standard
87 survey methods in detecting presence of newts.

88 ## 2 METHODS

89 ### *2.1 Study area*

90 Fieldwork was conducted in the Grand Cariçaie, which includes 660 ha of wet meadows
91 divided into eight reserves distributed along the 40 km of the southern shore of lake Neuchâtel
92 (Switzerland). Two out of the eight reserves were selected − Les Grèves de Cheseaux
93 (Yverdon-Les-Bains, VD) and Les Grèves d'Ostende et de Chevroux (Gletterens, FR),
94 hereafter Yverdon and Gletterens, respectively (figure 1). These reserves were selected since
95 amphibian barriers for prenuptial migration monitoring are present at these locations.
96 Amphibian barriers consist in nets or ducts between amphibians wintering and reproductive
97 habitats. Generally, these barriers are used to protect amphibians during the migration period
98 from road traffic. These barriers also allow to estimate the number of amphibian individuals
99 present in the area, as well as the beginning and the end of the migration period. These
100 barriers are surveyed daily.

101 The two focal newt species are similar in many aspect (body size, morphology, feeding
102 habits) and have shown to exhibit overlapping niches (Griffiths, 1986, 1987). However, *L.*

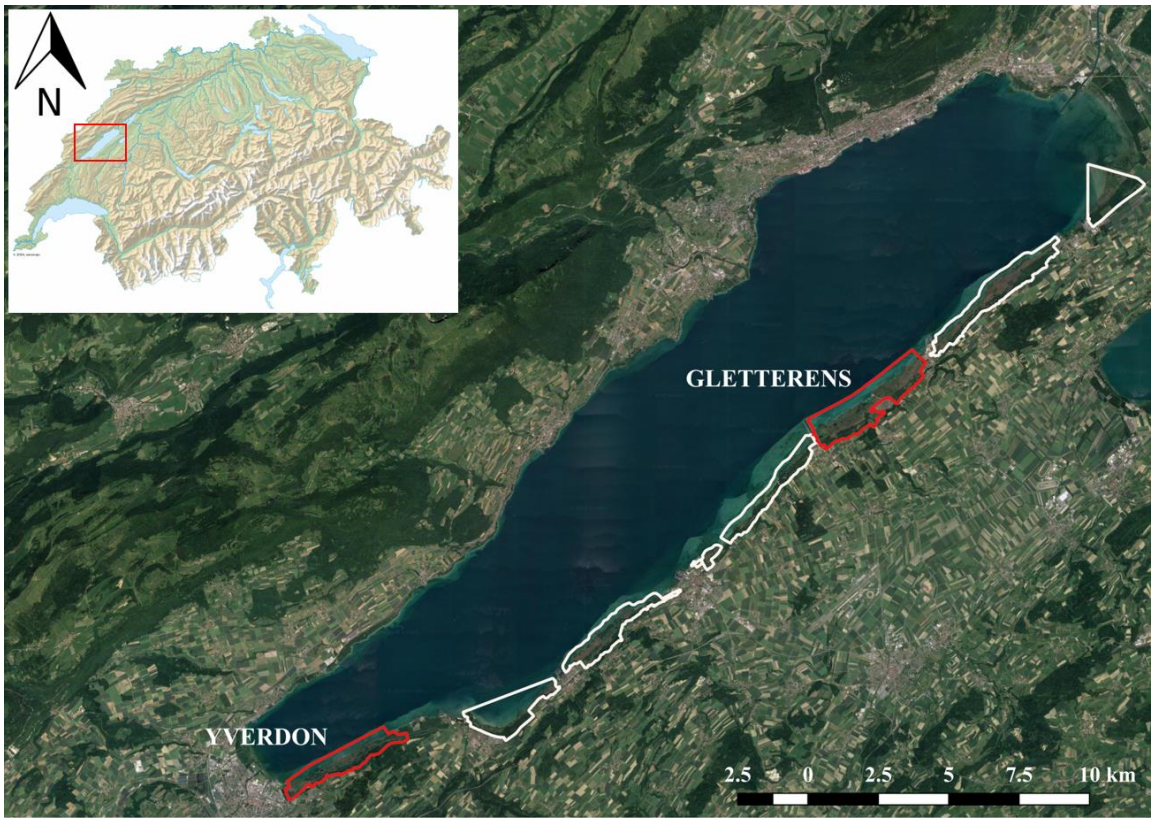

Figure 1: Location of the study areas. *White and red polygons represent the eight reserves constituting the Grande Cariçaie along the southern shore of Lake Neuchâtel. Red reserves correspond to sampled reserves. Map of Switzerland stand in the top left corner. The red square indicates the location of the study area. Background map was extracted from google map using QGIS.*

helveticus is only found in Yverdon reserve whereas *L. vulgaris* is found in the two sampled reserves. Hence, it is of interest to investigate differences in ecology of both species to understand if it might exist a competitive exclusion.

The wet meadow offers the possibility for newts to lay their eggs, since potential predators (i.e. fishes) are rare in this kind of natural environment. Wetlands consist mostly of three vegetations types; sedge meadows dominated by *Carex elata and Cladium mariscus*; reedbeds dominated by *Phragmites australis;* and open water bodies (ponds, ruts) dominated by *Nyphaea alba,* hereafter Magnocaricion, Phragmition and Nymphaion, respectively) (Delarze & Gonseth, 1999).

2.2 Field survey methods

2.2.1 Habitat use

To determine whether a certain vegetation type is preferred by newts for breeding, areas where the latter were the most detected during the migration survey were selected within each of the two reserves. In each reserve, 25 sampling points were randomly assigned amongst the three vegetation types. Each sampling points consisted in a circle of 5 m of diameter. To

ensure spatial independency of sampling point, a minimum distance of 20 m between the center of sampling points was set. Sampling points were assigned using QGIS (version 3.0.1) and a detailed vegetation map provided by the Association de la Grande Cariçaie. Due to field constraints (minimum distance of 20 m between center of sampling points and an unbalanced distribution of the vegetation types), the final number of sampling point per vegetation type was 27 for Magnocaricion, 16 for Nymphaion and 7 for Phragmition.

Ten habitat variables were measured at each sampling point; the vegetation type; the average water and mud depth; average, minimal and maximal water temperature; the percentage of emerged and submerged vegetation; the percentage of emerged land; and the distance to the wintering habitat (nearest forest).

The water and mud depths were calculated by averaging measures taken at the center, at 2.5 m from the center at the four cardinal points and at the four edges of the sampling point (figure S1). Water temperature was measured every hour at each sampling point from May 1st to July 1st, 2018 using thermologgers (1-Wire®/iButton®). Since these thermologgers are not waterproof, they were placed in Falcon tubes sealed with parafilm (hereafter Falcon thermologgers). To investigate the potential bias induced by Falcon tubes, waterproof thermologgers (Onset Hobo®) were also placed at three representative sampling points to get the direct water temperature. For two of the three sampling points, the temperature records between waterproof and Falcon thermologgers did not differ. A larger variation in temperature records was observed for the third sampling point (figure S2). It might be that at this sampling point, thermologgers were not equally exposed to sunlight due to vegetation cover. Thus, this sampling point was assumed to be an outlier and temperature records from the waterproof and Falcon thermologgers were assumed to be generally equal. Hence, for other sampling points, temperature from Falcon thermologgers were taken as such. Then, average, minimal and maximal temperature were computed for each sampling points. Emerged and immerged vegetation cover was sight-estimated in percentage by the same observer to keep consistency in measurements. Distances to the nearest forest were computed using QGIS. All the environmental data was collected from April 21st to 23rd 2018.

2.2.2 Water sample collection

eDNA methods were shown to recover recent presence of focal species in water samples, since DNA became undetectable within two weeks (Dejean et al., 2011; Thomsen et al., 2012). Because DNA is expected to persist longer in sediment than in water (Nielsen et al.,

2007; Barnes & Turner, 2016) and particles from mud are resuspended during environmental data collection, water collection had to be conducted at least two weeks after data collection to determine recent presences of focal species. Thus, the water collection was performed from May 21st to 28th, 2018, corresponding to the breeding season of *L. vulgaris* and *L. helveticus*.

Two liters of water were collected at each sampling point by means of the VigiDNA kit (Spygen). The spoon was attached to a 4m fishing rod to collect water sample away from the sampling point to avoid resuspending particle from mud (figure S3). To avoid cross-contamination, the fishing rod was washed with bottled water between sampling points. Then, the filtration capsules were conserved during two months at room temperature.

2.2.3 Standard newts survey

To compare standard with eDNA survey method, nocturnal sight hunting as well as bottle trapping were performed for some of the sampling points of Yverdon (table S2). To ensure comparable results between traditional and eDNA methods, sight hunting was performed the same day as water collection. Bottle trapping was performed during the same week as the last water sample collection. Both standard survey methods were conducted after water collection for eDNA survey to avoid resuspending particles from mud.

For the sight hunting survey, we stayed on average 20 min per sampling points. Bait traps, consisting in plastic bottles with pig liver inside, were placed at the same sampling points to capture newts. The traps remained in place for 24 hours.

2.3 Laboratory methods

2.3.1 eDNA extraction

DNA was extracted in a room dedicated to low DNA-content samples extraction and pre-PCR setup. DNA extraction protocol was adapted from Pont et al. (2018). The filtration capsules were agitated for 15 minutes on a vortex and another minute by hand to ensure a maximum DNA stood out of the filter. For each capsule, 45 mL of CL1 buffer from the VigiDNA kit were poured in three separate 50 mL falcon tubes and the remaining buffer was kept in the capsule. 33 mL of absolute ethanol and 1.5 mL of sodium acetate were added to each 50 mL Falcon tube and these were incubated at -20°C overnight. Tubes were then centrifuged at 7,800 rpm for 30 min at 6°C. Supernatants were discarded and 720 µL of ATL buffer from the DNeasy Blood & Tissue Extraction kit (Qiagen) were added. Tubes were vortexed, and the supernatant was transferred to a 2 mL Eppendorf containing 20 µL of proteinase K

(Qiagen). Eppendorf tubes were incubated at 56°C for at least 2h. The DNA extraction was performed using the NucleoSpin® Soil (Macherey Nagel) starting from step 6. The three sub-samples were pooled in the extraction column. The elution was performed by adding 100 µL of SE buffer twice.

Eight out of the 50 samples were then tested for inhibitors using qPCR (Biggs et al., 2015). The qPCR mixture contained 1x AmpliTaq^{TM} Gold 360 mix (Applied Biosystem^{TM}); 0.5 µM of tagged forward and reverse BATR01 primers; 2 µM of human-blocking primer; and 10,000-times diluted SyberGreen (ThermoFischer Scientific). These eight samples were diluted 1x, 0.5x or 0.1x and each concentration was replicated three times. Four PCR and four extraction negative controls were included in the qPCR plate. Samples presented no inhibition. Hence, the 50 samples were not diluted for further metabarcoding steps.

2.3.2 Metabarcoding

A fragment of the 12S mitochondrial gene was amplified using BATR01 primers (Valentini et al., 2016). These primers were designed to target amphibian species' 12 S mitochondrial gene sequences. However, sequences from other vertebrate species, such as human, are amplified as well using these primers. For this reason, a human-blocking primer (i.e. a primer that preferentially binds human 12S sequences and prevents its amplification) designed by Valentini et al. (2016) was added to the PCR mix. The PCR mixture was composed of 1x AmpliTaq^{TM} Gold 360 mix (Applied Biosystem^{TM}); 2 µM of human-blocking primers and 0.5 µM of each tagged forward and reverse primers (i.e. primers with eight variable nucleotides added to their 5' end, allowing further sample identification). The final volume was 20 µL including 2 µL of DNA template. Each sample amplification was replicated 12 times in 12 separate PCR plates. Thermocycling conditions were the following: denaturation at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, with a final elongation step of 7 min at 72°C. In each PCR plate, 12 blanks were set in the diagonal as well as seven negative controls and seven positive controls (see Taberlet et al., 2018 p.56 for plate layout). Blanks corresponded to empty wells and allowed to estimate the proportion of tag switches (i.e. false combination of tags used, generating chimeric sequences) occurring during the sequencing process. Positive controls corresponded to DNA from an equimolar assembly of three exotic species (*Pelophylax nigromaculatus, Polypedates maculatus* and *Rana arvalis*) that are not found in the study area and contained comparable DNA concentrations to eDNA samples, estimated using results obtained from the qPCR performed to test sample inhibition.

To ensure that PCRs worked, one out of the seven positive and negative controls per replicate plate were visualized on a 1.5% agarose gel stained by ethidium bromide. The first BATR01 replicate was excluded from further manipulations since no amplification was detected. PCR products from the eleven replicates were subsequently pooled. Amplicons were purified using a MinElute PCR purification kit (Qiagen). Final elution was performed in 15 µL of EB 50%. DNA after purification was quantified using Qubit® 2.0 Fluorometer (Life Technology Corporation) and purification products were visualized on a 1.5% agarose gel stained with ethidium bromide.

Amplicons were size-selected on a 2% agarose gel and purified using MinElute Gel Extraction kit (Qiagen). Library preparation was performed using TruSeq® DNA PCR-Free Library Prep (Illumina) with the following modifications to ensure a maximal yield of DNA, since amplicons are of small size (fragments correspond in average at 110 bp primers included): The "Remove large fragments" phase was skipped, 100 µL of undiluted SPB was added to the 100 µL of end-repaired sample, and the protocol was followed starting from step three of the "Remove small fragments" phase. The final library was quantified by qPCR using KAPA Library Quantification Kit (Roche) and its quality was assessed by a fragment analysis.

Sequencing was carried out at the Genomic Technologies Facility (Lausanne, Switzerland). A 100 pair-end sequencing was performed on an Illumina HiSeq 2500 sequencing system (Illumina). The library was loaded on a single lane.

2.4 Data Analyses

2.4.1 Reference Database

Reference database was constructed by recovering the entire set of DNA sequences from EMBL-European Nucleotide Archive (release 138, standard sequences) and by downloading Taxonomy from NCBI. Those files were converted into an ecoPCR format using *obiconvert* (OBITools software; Boyer et al., 2016). An *in-silico* PCR was performed using ecoPCR (Ficetola et al., 2010) allowing three mismatches per primer with a minimum and a maximum amplicon lengths set at 15 bp and 101 bp, respectively (Bellemain et al., 2010; Valentini et al., 2016).

Since *L. helveticus* was missing in the database, the 12S mitochondrial partial gene was sequenced using Sangers sequencing. *L. helveticus* extracted tissues were amplified using L2519 and H3296 primers targeting a fragment of the 12S mitochondrial gene (Wang et al.,

2017; Supplementary methods and results S1 for PCR details and *L. helveticus* 12S mitochondrial partial gene sequence). Then, the sequence was added manually to the reference database.

2.4.2 Sequences processing

Sequence reads were processed using OBITools software (Boyer et al., 2016). Forward and reverse reads were aligned using *illuminapairedend* with a minimal quality score set at 40 and joined sequences (i.e. unaligned sequences that cannot be used) were discarded using *obigrep*. Sequences were assigned to samples using *ngsfilter*, which identifies tag combination and primers. Subsequently, reads were dereplicated by clustering strictly identical sequences into a unique sequence using *obiuniq.* Singletons were removed, and MOTUs were taxonomically assigned using *ecotag* with the reference database. PCR and sequencing errors were cleaned from the taxonomically attributed sequences using *obiclean* with a minimum ratio between counts of two sequence records set at 0.25. Subsequently, the output was converted to a tab file using *obitab*.

As a final treatment step, this file was processed in R version 3.4.4 (R core Team, 2018). Sequences with a count lower than 10 were removed. Unassigned sequences and sequences with an identity lower than 98% were removed.

To limit false positive occurrence, one must account for contaminant and chimeric sequences (i.e. sequences for which a tag switch occurred) (Schnell et al., 2015). Contaminant sequences induce an overestimation of the number of reads per samples, whereas chimeric sequences are sequences attributed to the wrong sample. In this experiment, negative controls and blanks were set up to estimate and correct sequences stemming from these artifacts, respectively. Since a certain proportion of sequences retrieved in negative controls can be caused by tag switches, it might be too conservative to correct sequences using both blanks and negative controls (PCR and extraction negative controls). Thus, the proportion of sequences retrieved in negatives control and blanks was computed for each of the 11 PCR plate. The proportion of sequences corresponded to the sum of reads retrieved in the 12 blanks or in the 14 negative controls divided by the total amount of reads retrieved in the corresponding PCR plate. Subsequently, the mean proportion and the standard deviation was computed over the 11 PCR plates. The proportion of sequences retrieved in negative controls (0.101 ± 0.018) was higher than the proportion of sequences retrieved in blank (0.081 ± 0.011) (figure S4). Hence, we decided to correct the number of reads per sample using uniquely contaminant sequences (i.e.

sequences of negative controls) assuming it accounts as well for tag switching (i.e. sequences of blanks), since a certain proportion of sequences retrieved in the negative controls can be attributed to chimeric sequences.

To correct sequences from contaminant and chimeric sequences, we decided to remove the mean number of contaminant sequences found in the 14 negative controls by sequence and by PCR replicate to the corresponding samples sequences. This method was assumed to be conservative enough, since through all PCR plates none had more than eleven wells contaminated out of the fourteen wells (figure S5).

To consider a species as present, no consensus threshold is set in the literature (Goldberg et al., 2016; Harper et al., 2018). In the present study we attempted to be conservative to limit occurrence of false-positive and considered a species as present if at least two out of the 11 PCR replicates contained a non-null value of reads after all cleaning steps for a given species (Goldberg et al., 2013; Mahon et al., 2013; Rees et al., 2014; Ficetola et al., 2015).

2.4.3 Statistical analyses

Statistical analyses were conducted in R version 3.4.4 (R core Team, 2018). To analyse the effect of environmental variables on the probability of presence of *L. vulgaris* and *L. helveticus*, generalized linear models (GLMs) with binomial family were performed. The effect of each environmental variable on the newts' presence probability was tested separately. The model contained the newts' presence-absence as response variable and the environmental variables as explanatory variable. The quadratic (polynomials of degree two) effect of the following variables: percentage of emerged land; average water temperature; minimal water temperature; maximal water temperature; and distance to the wintering habitat, were tested as well.

Sampling conditions might differ between both reserves, hence the effect of the reserve location on the newts' presence probability was investigated in each model. Since it was not significant, it was removed from the statistical analyses. To control for multiple testing, *p-values* were adjusted using the Benjamini-Hochberg correction. GLM's assumptions were investigated for each model using DHARMa R package (version 0.2.0, Hartig, 2018).

To discriminate the effect of the three levels of the vegetation type variable (Magnocaricion, Nymphaion and Phragmition), a Tukey test was performed using the function *glht* from the multcomp R package (Hothorn et al. , 2008).

3 RESULTS

The number of raw reads was 182,672,348. After filtering, we obtained a total of 134,637,031 reads among which 53,441,658 were attributed to the Amphibia class corresponding to 39.69% of total reads (figure 2). Three vertebrate classes were identified as well; Actinopterygii; Aves; and Mammalia accounting for 27.19, 2.27 and 28.38% of total reads, respectively. 2.46% of reads corresponded to other taxa regrouping invertebrates, plants and bacteria.

Ten amphibian taxa were recovered; five are part of the fauna of the Grande Cariçaie (*Hyla arborea, Bufo bufo, L. vulgaris, Rana temporaria* and *Pelophylax ridibundus*); three species belong to the PCR positive control (*R. arvalis, Pelophylax sp.* and *Pseudacris sp.*); and two exotic amphibian species (*Xenopus tropicalis* and *Rhinella sp.*). Regarding *L. vulgaris*, 6,141,079 reads were obtained representing 11.49% of the total number of Amphibia reads (figure 2). No *L. helveticus* sequence was recovered (supplementary methods S2).

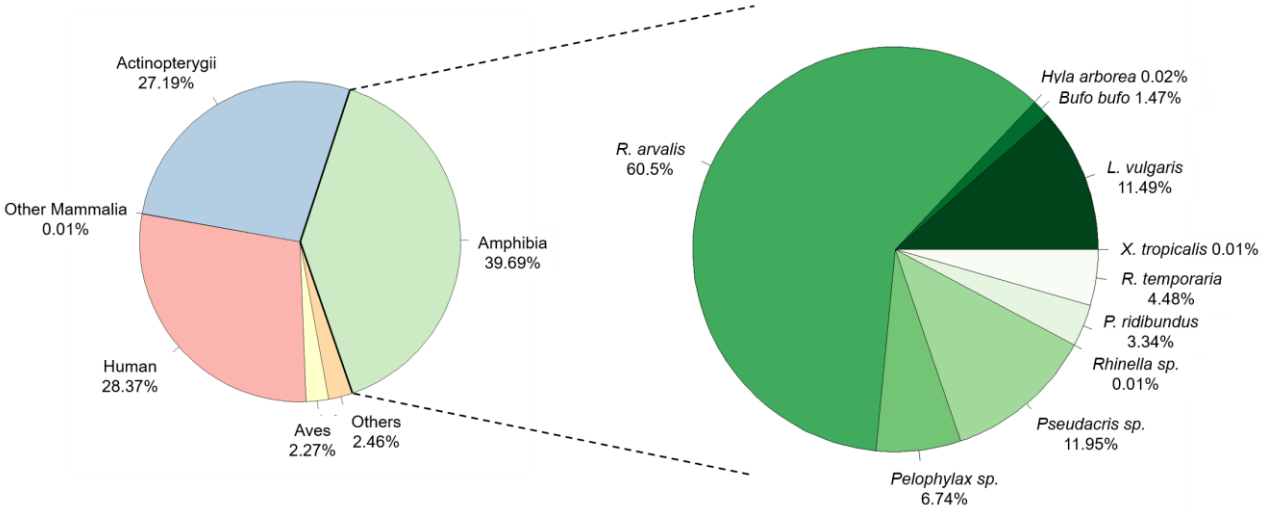

Figure 2: Pie charts representing the percentage of reads per Class (left chart) among total number of reads; or per amphibian species (right chart) among total number of Amphibia reads. *Total number of reads after the filtering was 134,637,031. The total number of Amphibia reads was 53,441,658.*

During the prenuptial migration (i.e. migration from wintering to breeding habitat) monitoring, the number of *L. vulgaris* individuals recorded was 495 in Yverdon and 74 in Gletterens. The number of *L. helveticus* individuals in Yverdon was 112.

Using eDNA approach, *L. vulgaris* was detected in 11 out of the 50 sampling points, distributed in both reserves (Yverdon n=6, Gletterens n=5; figure 3).

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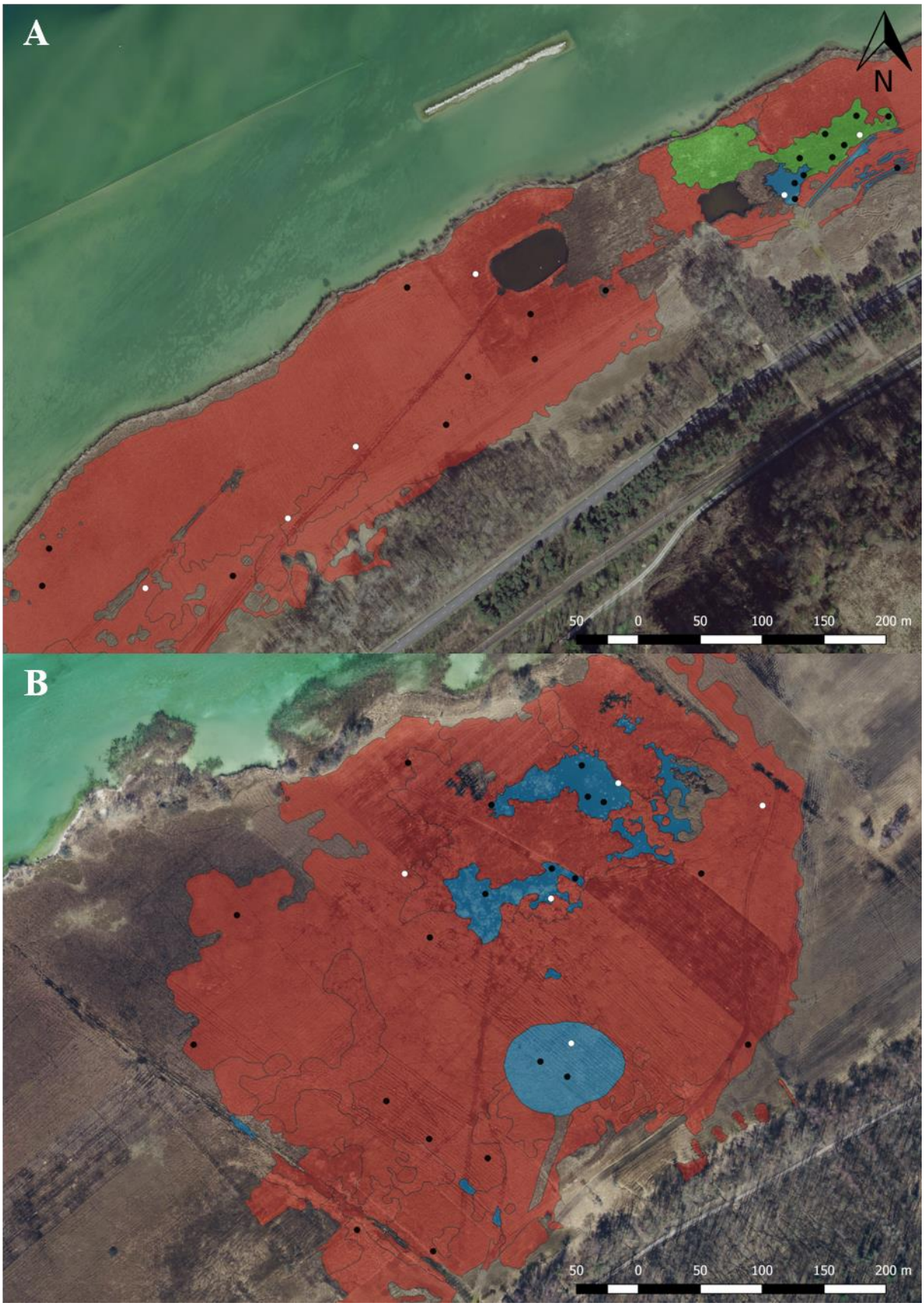

Figure 3: Sampling points according to L. vulgaris' presences and absences in both reserves. *Red, dark blue and green polygons represent respectively, the Magnocaricion, the Nymphaion and the Phragmition. black points represent sampling points where L. vulgaris was absent (n = 39) and white points represent sampling points where L. vulgaris was present (n = 11).* ***A.*** *Yverdon reserve (L. vulgaris' presence n = 6),* ***B.*** *Gletterens reserve (L. vulgaris' presence n = 5). The size of the points is at scale and of 5m diameter. Background picture obtained from the Swiss Federal Office of Topography: www.swisstopo.admin.ch.*

We were interested in exploring whether one of the three vegetation types -Magnocaricion, Nymphaion and Phragmition- was preferred by newts for their breeding. The proportion of sampling point where *L. vulgaris* was present did not differ amongst the three vegetation types (*p-value>0.05*; figure 4, table S1).

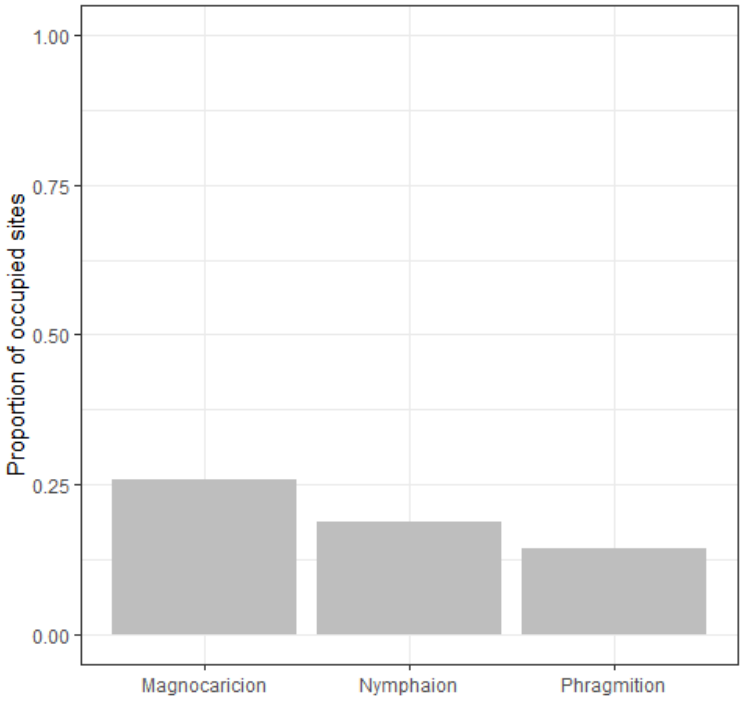

Figure 4 : Proportion of occupied site by L. vulgaris per vegetation types. *Total number of sampling point per vegetation type was: Magnocaricion n = 27, Nymphaion n = 16 and Phragmition n = 7. Number of occupied site per vegetation type was: Magnocaricion n = 7, Nymphaion n = 3, Phragmition n = 1.*

The effect of ten environmental variables on the presence probability of the focal species was investigated. None of the environmental variables affect significantly the *L. vulgaris'* presence probability (table S1).

To be able to compare standard and eDNA methods, presence data on four other amphibian species (*B. bufo*, *H. arborea*, *R. temporaria* and *P. ridibundus*) based on DNA retrieved in water samples was also analysed. No *L. vulgaris* was detected using standard methods. Globally, 34 presences, all species combined, were detected using eDNA method, whereas bottle trapping and sight hunting detected respectively six and five presences (figure 5, table S2). Seven presences found using eDNA approach were confirmed using either one or both standard methods. Two false negatives were detected using eDNA method. Indeed, we detected tadpoles of *P. ridibundus* at two separate sampling points using sight hunting and bottle trapping methods, without recovering DNA of this species at these points. No species presence was detected using uniquely the two standard survey methods.

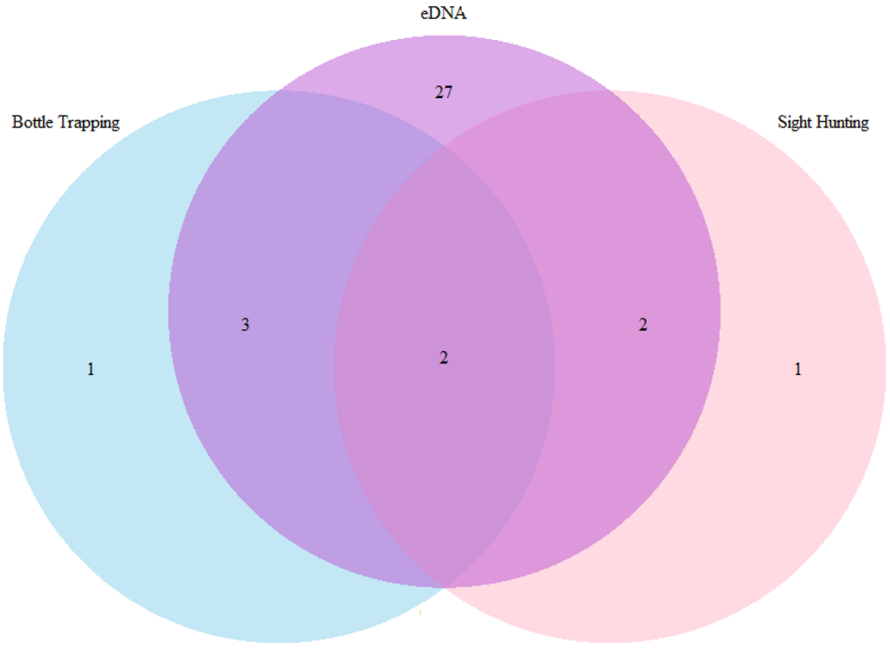

Figure 5: Venn Diagram representing the number of presences of L. vulgaris, B. bufo, H. arborea, R. temporaria and P. ridibundus identified with each survey methods. *eDNA survey allowed to detect 34 presences of the five amphibian species, whereas sight hunting and bottle trapping allowed to detect five and six presences respectively. eDNA and bottle trapping shared three detection of individuals, eDNA and sight hunting shared two detection of individuals. Two individual presences were identified using the three methods.*

4 DISCUSSION

It is of prime interest to develop efficient survey tools to increase understanding of endangered species ecology, as well as population trends to implement effective conservation management plans (Joseph et al., 2006). Recently, eDNA approaches were proposed as a promising tool to monitor cryptic species, showing increased detection compared to standard survey methods (Biggs et al., 2015; Lopes et al., 2017). So far, eDNA methods have been used in discrete natural environment, such as distinct water bodies, to study species ecology. However, such approaches have never been used, to our knowledge, to investigate the fine scale habitat use of amphibian species in a continuous wet meadow expanse. Here, we used eDNA metabarcoding approach to determine the fine scale breeding habitat use of two endangered newt species -*L. vulgaris* and *L. helveticus*- in a continuous wet meadow expanse.

Using eDNA metabarcoding approach, we were able to identify presences of *L. vulgaris* among the sampling points, as well as of four other amphibian species belonging to the fauna of the Grande Cariçaie -*B. bufo, H. arborea, R. temporaria* and *P. ridibundus*. On the other hand, *L. helveticus'* DNA was not recovered in water samples. The absence of *L. helveticus* was expected in the Gletterens reserve, however, presences in the sampled area of Yverdon

were expected. During the prenuptial migration monitoring, we captured 112 *L. helveticus* individuals and 495 *L. vulgaris* individuals along the sampled area of Yverdon. Lower numbers of *L. helveticus* than *L. vulgaris* individuals could explain the non-detection of *L. helveticus'* DNA in water samples. Nonetheless, *L. vulgaris'* DNA was detected in five sampling points of Gletterens, although a low number of individuals (74) were captured during the prenuptial migration monitoring. Hence, low number of individuals does not seem to impact the detection of species using eDNA approach. The *L. helveticus* DNA absence in water samples can be explained either by a failure in its detection using eDNA methods, or by its absence in the sampled areas. Our results support this second hypothesis, since all other species expected to be present in the sampled area were found using eDNA approach. The only other species that might have been present in the sampled environment was *Ichthyosaura alpestris.* However, during the prenuptial migration monitoring only four and one males were respectively censused in Yverdon and Gletterens. Therefore, its density is too low to be detected in the sampled area.

Differences between the ecology of both focal newt species are unclear. Indeed, *L. vulgaris* and *L. heleveticus* are similar in many aspects (body size, morphology and behavior) and have been shown to expose overlapping niches with similar feeding habits (Griffiths, 1986, 1987). However, our results might suggest that both species do not share the same microenvironment during the breeding season in the wet meadows of the Grande Cariçaie. *L. helveticus* might occupy either different vegetation types than the ones sampled in this study, or individuals might remain at the edges of the sampled area. In fact, *L. helveticus* was shown to exhibit short prenuptial migration distances, migrating about 150 m from wintering to breeding habitats (Diego-Rasilla & Luengo, 2007).

Similar to *L. helveticus*, *L. vulgaris* has short prenuptial migration distances. Kovar et al. (2009) showed that most *L. vulgaris* individuals migrate not more than 280 m before reaching breeding habitats, with some individuals migrating 500 m. Once in the water body, they are thought not moving much, especially breeding individuals (Bell, 1977). Newts most likely do not overwinter at the edges of the forests. For instance, amphibian ducts along the Yverdon reserve are located upstream the forest border. During the migration monitoring, newts are captured indicating that newts most likely overwinter in remote habitat from wetlands. Hence, *L. vulgaris* individuals might already migrate hundreds of meters before reaching the edges of the Grande Cariçaie wet meadows. Furthermore, *L. vulgaris* were found to be opportunist in respect to their breeding sites choice (Cirovic et al., 2008). It was thus expected to recover

presences of this species at sampling points located at short distances from wintering habitat. The analysis of the effect of distance to the wintering habitat on the *L. vulgaris'* presence probability revealed that some individuals are found up to 340 m in the wet meadow expanse from wintering habitats (figure S6). This result might suggest that *L. vulgaris* individuals might move more in their breeding habitat than expected.

We were interested in determining which of the three vegetation types is the most suitable to shelter newts during the breeding season. Our results suggested that none of them is preferred by *L. vulgaris* (figure 4). Furthermore, none environmental variable recorded to characterize the newts breeding habitat seems to affect the *L. vulgaris* probability of presence, suggesting an opportunistic behavior concerning the breeding habitat choice as described by Cirovic et al. (2008). Nonetheless, results suggested that the average and minimal temperature might affected the *L. vulgaris* presence probability, with an average water temperature optimum around 20°C, although these results showed not significance (figure S7 and S8, table S1).

The impact of environmental variables on the presence probability of the four other amphibian species (*B. bufo, H. arborea, R. temporaria* and *P. ridibundus*) were investigated as well to contrast results obtained for newt species. None species seems to present preferences for one of the three sampled vegetation types (figure S9). However, the unbalanced number of sampling points in each vegetation type might induce a bias. Furthermore, similar to *L. vulgaris*, none environmental variables affected significantly the presence probability of either species. Nevertheless, results suggest trends in presence probability of species in response to recorded environmental variables. *R. temporaria* presence probability seems to be higher at low distances from wintering habitat (figure S6, table S1) and *B. bufo* presence probability seems reaching an optimum in temperate environments (figure S7 and S8, table S1). It might be that 50 sampling points are not enough to obtain sufficent presence-absence data to build an explanatory model with adequate statistical power. Moreover, many other environmental parameters, such as precipitations, might vary among years. Sattler et al., (2005) argued that the environmental conditions during the study period could lead to unrepresentative and ungeneralizable results. Hence, higher number of sampling points as well as repeated sampling through time must be performed to get reliable data to investigate species habitat use. However, increasing the sample size and replicate sampling events through time may lead to large increase in costs. This is a significant limitation in the perspective of implementing eDNA methods as a tool for monitoring the habitat use of endangered species.

In this study, we assessed in a qualitative manner the efficiency of standard survey methods, consisting in sight hunting and bottle trapping, compared to the efficiency of eDNA approach. eDNA method was shown to be more effective than standard methods in amphibian species detection (figure 3), especially for *L. vulgaris* and *H. arborea* that were not detected using standard methods (table S2). With eDNA two false negatives were obtained that might be explained by a morphological misidentification of the tadpoles. Indeed, standard survey methods were performed by amateurs and tadpoles of *P. ridibundus* and *R. temporaria* can easily be confused. Nevertheless, the highest detection performance using eDNA approach has been confirmed by several studies (Biggs et al., 2015; Lopes et al., 2017). The higher species detectability observed with eDNA approach, compared to standard method, could be attributed to false-positive. Indeed, false-positive occurrence when using eDNA approaches cannot be completely excluded. However, in this study, we attempted to reduce the occurrence of these false-positive through accounting for contaminations and by considering a species as present if at least two PCR replicates contained positive amount of sequences for a given species. This method is considered as a conservative method (Ficetola et al., 2015).

Despite a high effectiveness of eDNA approaches, many factors influencing DNA detection remain poorly known. For instance, to determine the scale at which environmental variables must be measured to describe the species habitat, DNA diffusion and persistence potential must be considered. Previous studies have investigated persistence of DNA in water in laboratory or mesocosm conditions (Thomsen et al., 2012), but DNA diffusion potential in natural environments remains unknown. Each environment differs in nucleases composition, UV exposition, pH, salinity or temperature, that individually affect degradation of DNA (Nielsen et al., 2007) leading to differential DNA detection among natural environments. Natural environments, such as the Grande Cariçaie, composed of different vegetation types and of a high proportion of emerged lands, might particularly differ in terms of persistence and DNA diffusion potentials. In the present study, we attempted to investigate the DNA diffusion in the continuous wet meadows by releasing free DNA of 21 exotic species at different distances from the center of our sampling points (Supplementary methods S3). No DNA from these exotic species was retrieved in water samples. It might be that either the filter used to collect DNA from our water samples does not bind free DNA, or DNA was instantly degraded in the environment. Though, free DNA persistence in marine or freshwater environment is estimated from hours up to few days (Nielsen et al., 2007). Thus, it might be that free DNA from the exotic species used in this study was not degraded until the water was

collected and it might be that the filter used did not capture free DNA. If so, sampling methods as described in Schneider et al. (2016) must be considered to recover free DNA. On the other hand, it may be that the DNA concentration released in this experiment was too low to be detected. Further studies aiming at determining the habitat use of species in continuous environments using eDNA approach must implement preliminary assessments of DNA diffusion to accurately interpret results.

Sequences from three other vertebrate classes were recovered, namely birds, mammals and ray-finned fishes. Thus, primers that were used in the present study for eDNA metabarcoding analysis are not fully specific in targeting amphibian group. The non-specificity is mainly due to the small length of the amplicon. Nevertheless, among the four different vertebrate classes, amphibians obtained the highest number of reads. Among mammalian species, humans get the largest proportion of reads. Contaminations from human sequences are probably inevitable, since even though precautions were taken in the lab to reduce them, such as the addition of a blocking primer, human sequences are still detected in large amount. Higher concentration of human-blocking primer could have been used, however, these primers might inhibit the DNA replication. Among amphibian sequences, the largest proportion of reads were attributed to the positive control. Hence, we recommend using lower concentration of DNA for positive controls to be able to recover more reads of the focal species. Here, we attempted to get comparable DNA concentration between positive control and water samples. However, the quality of DNA extracted from environmental sample might be lower than the quality of DNA extracted from tissues inducing a potential PCR bias (i.e. primers might bind preferentially sequences of high quality). Surprisingly, two exotic amphibian species -*X. tropicalis* and *Rhinella sp.*- were identified as well in our water samples. These contaminations might have appeared during lab manipulations. However, DNA from amphibian species has never been brought in the pre-PCR lab where manipulations were conducted. Thus, these contaminant sequences might probably stem from errors during PCR or sequencing leading to a wrong taxonomical attribution of the original sequence. For instance, *Rhinella sp.* is a subgenus of *Bufo* s.l.. It might be that errors during PCRs and sequencing process occurred leading to a mutated sequence of *B. bufo* that matched preferentially *Rhinella sp.* sequences. To account for these contaminations, one must search for the maximal number of reads among the contaminant sequences among all samples, blank, negative and positive controls included. This maximal number of reads should be subsequently subtracted to all other sequences retrieved in all samples of each PCR replicate.

Our study showed that eDNA metabarcoding is a powerful tool to monitor biodiversity, since DNA from five out of the six species expected to be found in sampled environment was retrieved. The absence of *L. helveticus* in water samples suggests, rather than failure in DNA detection using eDNA approach, that this latter and *L. vulgaris* do not exhibit overlapping niches in the wet meadows of the Grande Cariçaie. We showed that *L. vulgaris* breeding individuals might have greater movements in their breeding habitat than expected. These results suggest that eDNA is a promising and powerful tool to study species ecology even at a small scale among a continuous wet meadow expanse. To obtain reliable data on species habitat use using eDNA methods, we recommend, however, to investigate the diffusion of DNA in the environment, and to repeat sampling events through years. Globally, our results showed that eDNA approach has the potential to investigate species habitat, leading to a better understanding of their ecology. Increased knowledge of species ecology will allow to plan efficient conservation policy to protect endangered species by conserving and restoring threatened environments.

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Table S1 : Statistical analyses. *Shown are logistic binomial regressions for the effect of the percentage of submerged and emerged vegetation cover; percentage of emerged land; water and mud depth; average, minimal and maximal water temperature; distance to the forest (wintering habitat) on the five amphibian species' presence probability. Also shown, Tukey test comparing the effect of the three vegetation types (Magno, Nympha and Phrag, respectively Magnocaricion, Nymphaion and Phragmition). "P BH" correspond to p-values corrected using Benjamini-Hochberg correction.*

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	L. vulgaris				***B. bufo***				***H. arborea***				***R. temporaria***				***P. ridibundus***			
Source	d.f	Z	P	P BH	d.f	Z	P	P BH	d.f	Z	P	P BH	d.f	Z	P	P BH	d.f	Z	P	P BH
Magno – Nympha	49	-0.54	0.85	0.97	49	1.26	0.41	0.55	49	-1.25	0.42	0.82	49	-0.95	0.57	0.91	49	-0.79	0.70	0.89
Magno – Phrag	49	-0.64	0.79	0.97	49	-1.24	0.42	0.55	49	-0.80	0.70	0.87	49	0.01	1	1	49	-1.52	0.28	0.84
Phrag – Nympha	49	-0.26	0.96	0.97	49	-1.98	0.11	0.33	49	0.12	0.99	0.99	49	0.01	1	1	49	0.87	0.66	0.89
Submerged vegetation	49	1.28	0.20	0.84	49	1.55	0.12	0.33	49	-0.40	0.70	0.87	49	-0.40	0.69	0.91	49	0.19	0.85	0.84
(Submerged vegetation)^2	*Na*	*Na*	*Na*	*Na*	49	-1.38	0.17	0.33	49	1.09	0.28	0.69	49	1.20	0.30	0.91	*Na*	*Na*	*Na*	*Na*
Emerged vegetation	49	-0.27	0.78	0.97	49	-0.54	0.59	0.69	49	0.36	0.72	0.87	49	0.27	0.79	0.91	49	1.13	0.26	0.84
(Emerged vegetation)^2	*Na*	*Na*	*Na*	*Na*	49	-2.27	**0.02**	0.21	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*
Emerged Land	49	0.04	0.97	0.97	49	-0.12	0.90	0.90	49	0.21	0.21	0.69	49	0.74	0.46	0.91	49	1.25	0.21	0.84
(Emerged Land)^2	49	-0.47	0.64	0.97	49	0.50	0.62	0.69	49	-2.13	**0.03**	0.49	49	-0.26	0.79	0.91	*Na*	*Na*	*Na*	*Na*
Water depth	49	0.72	0.47	0.97	49	0.43	0.66	0.70	49	-1.28	0.20	0.69	49	-0.76	0.45	0.91	49	-1.49	0.14	0.84
(Water depth)^2	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	49	1.24	0.21	0.91	*Na*	*Na*	*Na*	*Na*
Mud depth	49	-0.61	0.54	0.97	49	0.78	0.43	0.55	49	-0.11	0.91	0.98	49	0.65	0.51	0.91	49	-0.56	0.57	0.89
Average water T°C	49	1.61	0.11	0.72	49	2.60	**0.009**	0.17	49	-0.31	0.76	0.87	49	0.31	0.75	0.91	49	0.38	0.70	0.89
(Average water T°C)^2	49	-1.53	0.13	0.72	49	-1.23	0.22	0.36	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*
Min water T°C	49	1.67	0.09	0.72	49	1.94	0.05	0.31	49	-1.14	0.25	0.69	49	0.54	0.59	0.91	49	0.78	0.44	0.89
(Min water T°C)^2	49	-1.04	0.30	0.97	49	-1.42	0.15	0.33	*Na*	*Na*	*Na*	*Na*	49	0.64	0.52	0.91	*Na*	*Na*	*Na*	*Na*
Max water T°C	49	-0.42	0.67	0.97	49	1.51	0.13	0.33	49	-1.34	0.18	0.87	49	-1.26	0.21	0.91	49	0.13	0.90	0.89
(Max water T°C)^2	49	0.09	0.92	0.97	49	1.62	0.10	0.33	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*
Distance to forest	49	-0.07	0.93	0.97	49	1.26	0.21	0.36	49	-0.44	0.65	0.87	49	-1.98	**0.05**	0.76	49	0.32	0.74	0.89
(Distance to forest)^2	49	-0.66	0.51	0.97	*Na*	*Na*	*Na*	*Na*	49	0.77	0.44	0.82	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*	*Na*

Table S2: Detection of the five amphibian species using the three survey methods. *Is shown the presences (1) and absences (0) of five amphibian species (R. temporaria, L. vulgaris, B. bufo, H. arborea and P. ridibundus) using three survey methods (Bottle trapping, sight hunting and eDNA). The survey methods comparison was performed in the Yverdon reserve. In this reserve, 25 sampling points were survey using eDNA, 19 sampling points were using Sight hunting and 13 sampling points were survey using Bottle trapping.*

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	Bottle trapping					**Sight hunting**					**eDNA**				
Sampling points	*R. temporaria*	*L. vulgaris*	*B. bufo*	*H. arborea*	*P. ridibundus*	*R. temporaria*	*L. vulgaris*	*B. bufo*	*H. arborea*	*P. ridibundus*	*R. temporaria*	*L. vulgaris*	*B. bufo*	*H. arborea*	*P. ridibundus*
100	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1
102	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1
104	NA	NA	NA	NA	NA	0	0	0	0	0	0	0	0	0	1
108	NA	NA	NA	NA	NA	0	0	0	0	0	1	0	0	1	0
109	0	0	1	0	0	0	0	0	0	0	1	0	1	1	1
111	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
118	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0
123	NA	NA	NA	NA	NA	0	0	0	0	0	1	1	1	1	1
124	NA	NA	NA	NA	NA	0	0	0	0	0	1	0	0	0	1
125	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1
127	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1
131	1	0	0	0	0	1	0	0	0	1	1	0	0	0	1
133	NA	NA	NA	NA	NA	0	0	0	0	0	1	0	1	0	1
140	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
141	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1
142	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
144	NA	NA	NA	NA	NA	0	0	0	0	0	1	0	0	0	0
146	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
148	1	0	0	0	0	1	0	0	0	0	1	0	0	0	1

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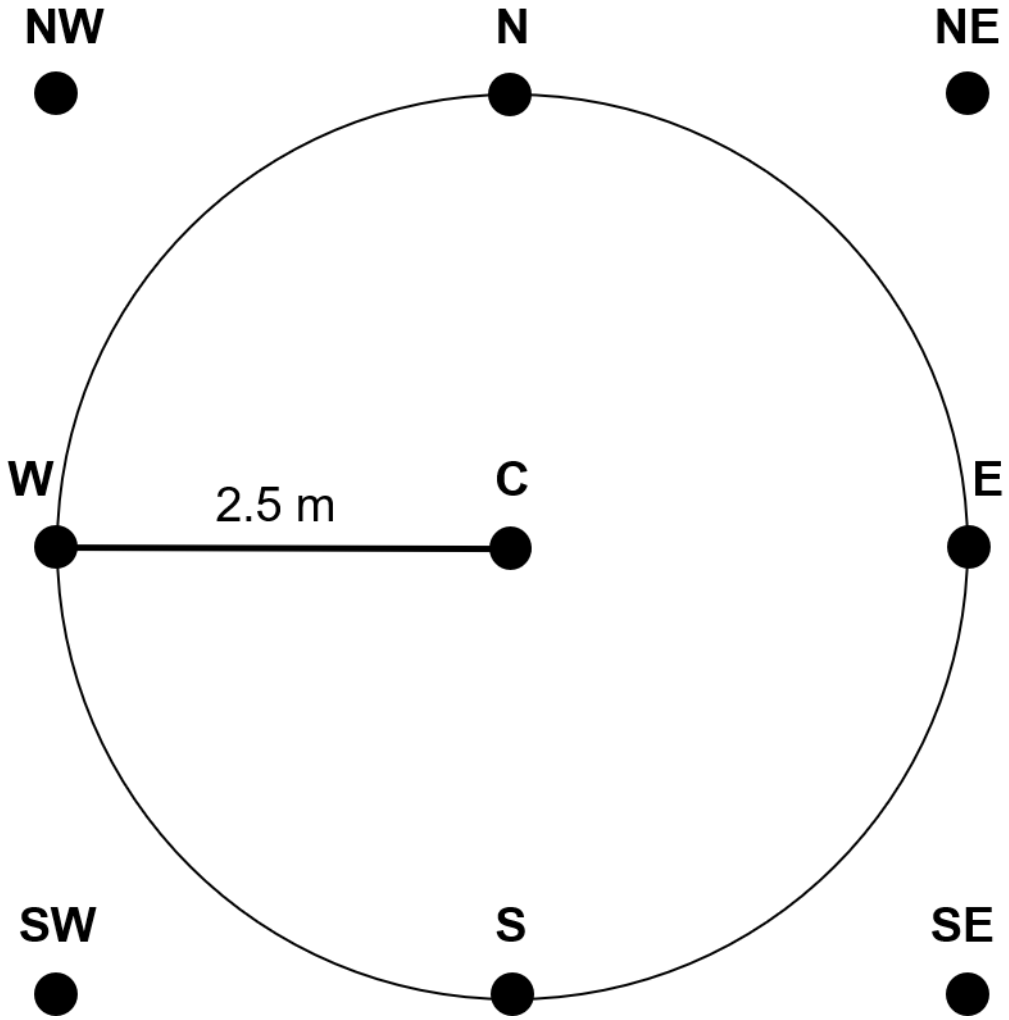

Figure S1 : Scheme of where the water and the mud depth measurements were taken at each sampling point. *Measurements were taken at 2.5 m from the center. C = Center; W = West; NW = Northwest; N = North; NE = Northeast; E = East; SE = Southeast; S = South; SW = Southwest. The circle represents the sampling point with a diameter of 5 m.*

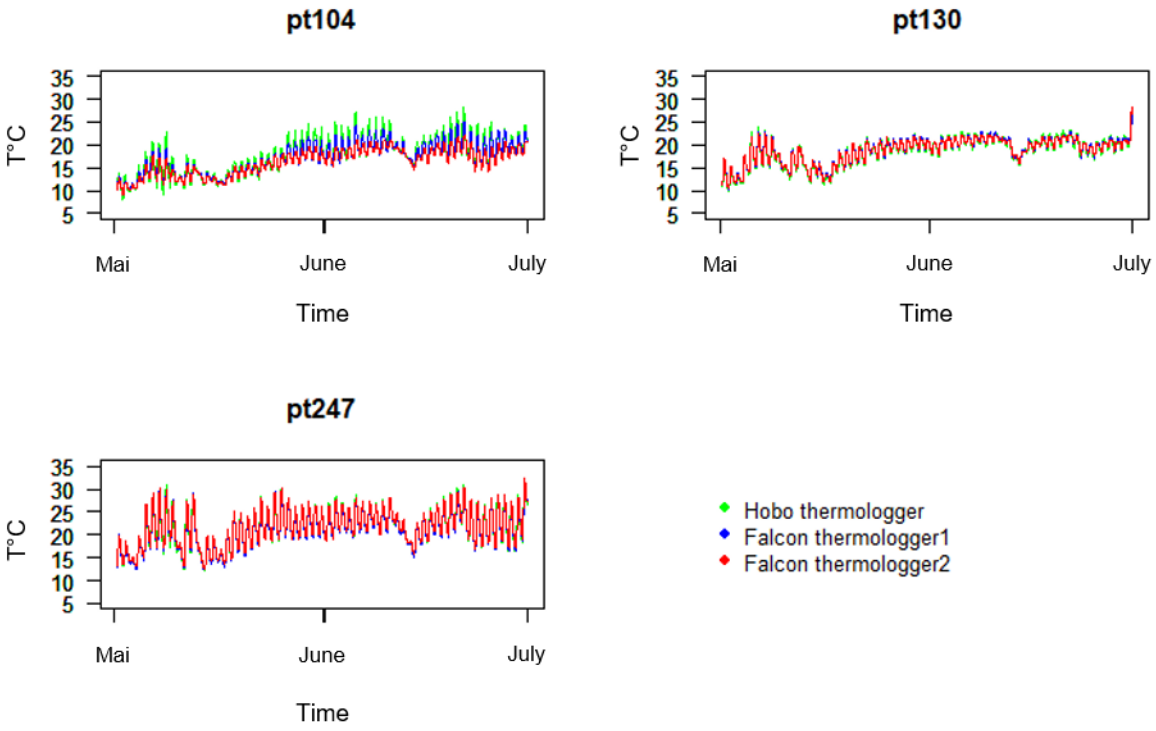

Figure S2 : Comparison of temperature records between the waterproof thermologger and the two thermologgers contained in Falcon tubes at three sampling points. *Temperature was recorded from May 1st to July 1st. Pt104 is located in the Magnocaricion in the Yverdon reserve. Pt130 is located in the Phragmition in the Yverdon reserve. Pt247 is located in the Nymphaion in the Gletterens reserve.*

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Figure S3 : Sampling material. *Spoon from the kit VigiDNA (Spygen) attached to the four-meter fishing rod by means of two electric grippers and a belt. The water body presented on the right picture does not reflect environmental conditions of the Grande Cariçaie wetlands. Pictures were taken in Fontanezier (Switzerland, VD).*

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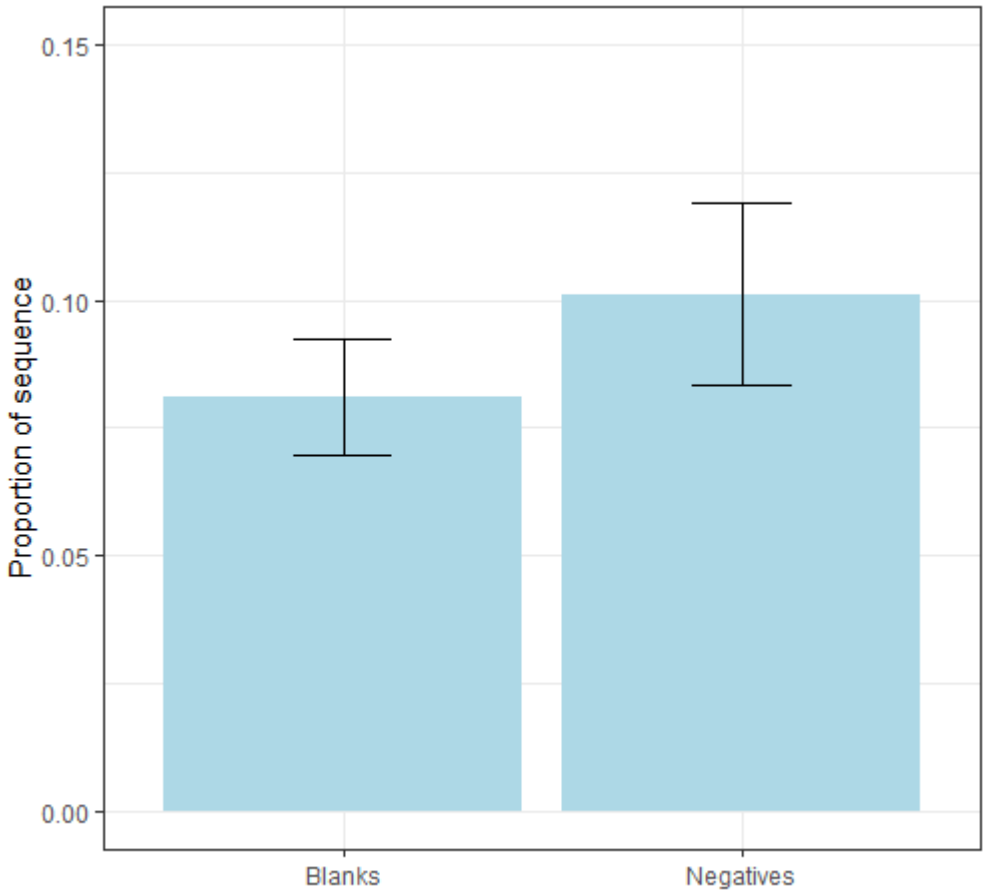

Figure S4: Proportion of sequence retrieved in blanks and negative PCR and extraction controls over the 11 retained PCR plates. *Proportion were calculated for each PCR plate and then the average and standard deviation were computed over the 11 replicates. Blue bars represent the mean proportion of sequences and error bars correspond to the standard deviation. The mean proportion of sequences retrieved in blanks is of 0.081 ± 0.011 and retrieved in negative controls is of 0.101 ± 0.018.*

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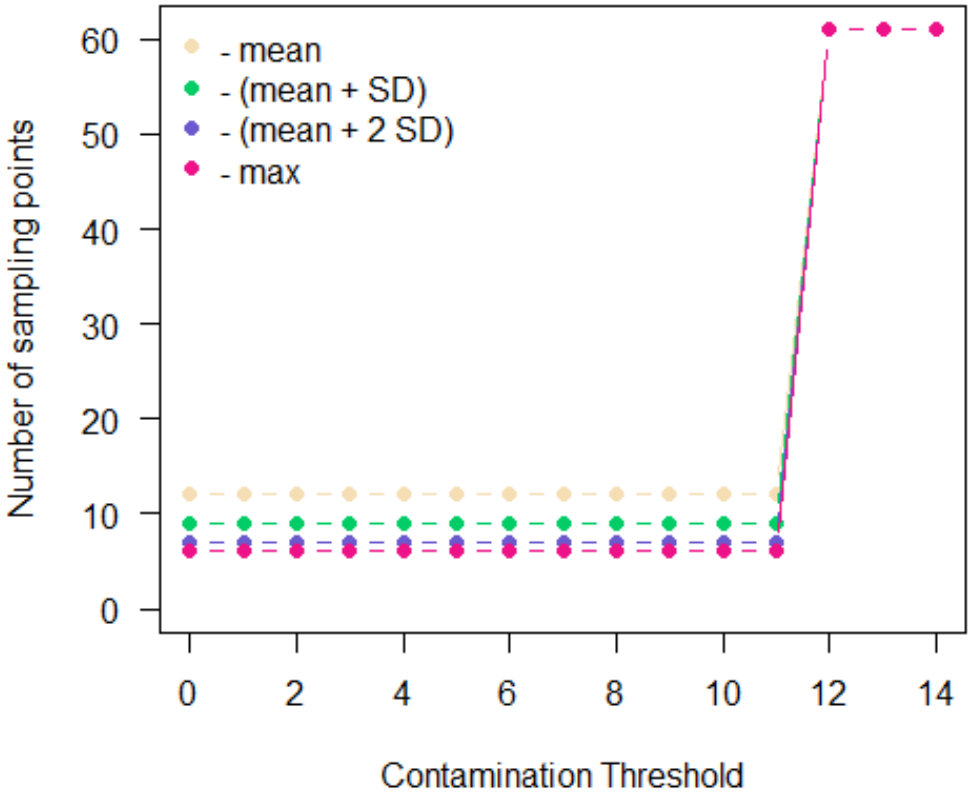

Figure S5: Comparing sequences correction methods. *To correct sequences from contaminant and chimeric sequences, we decided to test four different correction methods consisting in subtracting (i) the mean; (ii) the mean plus the standard deviation; (iii) the mean plus two standard deviation; and (iv) the maximum number of reads contained in the 14 negative controls per PCR plate (seven PCR and seven extraction negative controls). On the x-axis is represented the contamination threshold consisting in the number of non-null negative control from which (i), (ii), (iii) and (iv) are calculated. On the y-axis is represented the number of sampling points at which L. vulgaris is present.*

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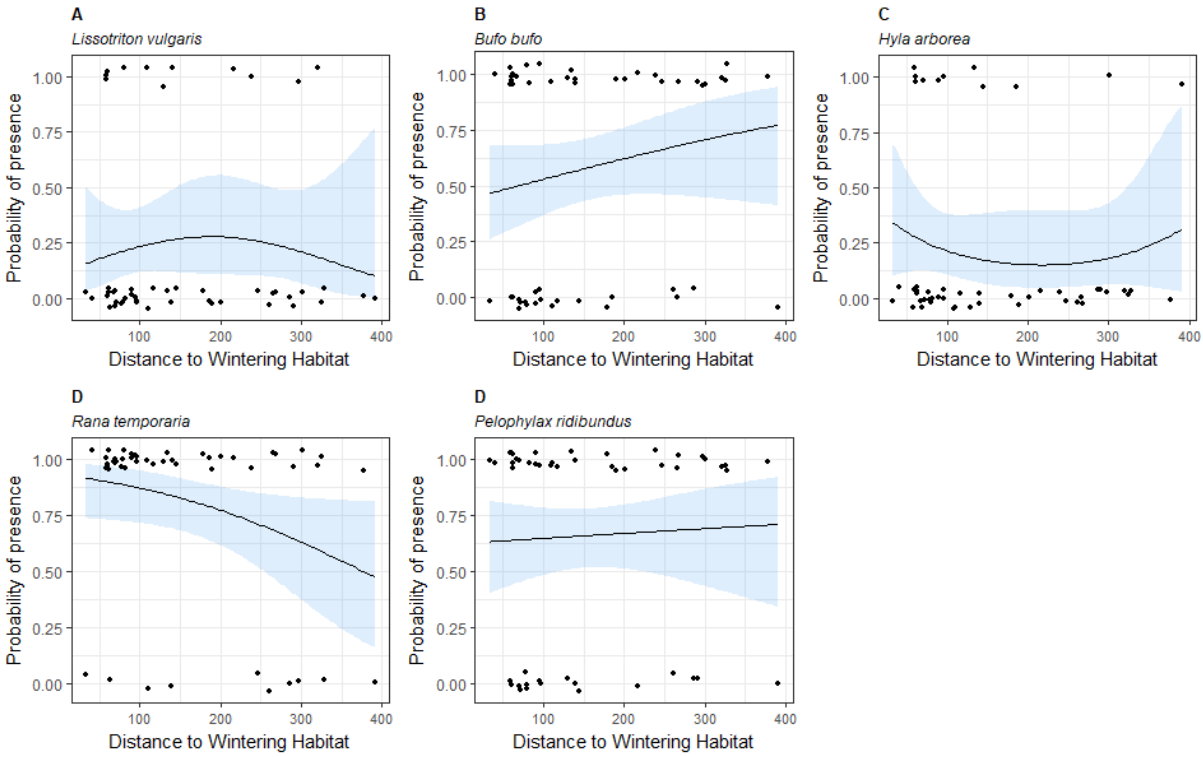

Figure S6 : Effect of distance to wintering habitats on the presence probability of five amphibian species. A. *L. vulgaris;* ***B.*** *B. bufo;* ***C.*** *H. arborea;* ***D.*** *R. temporaria;* ***E.*** *P. ridibundus. The dark line represents the distribution of presence probability predicted by the logistic binomial regression. The 95% interval confidence is represented in blue.*

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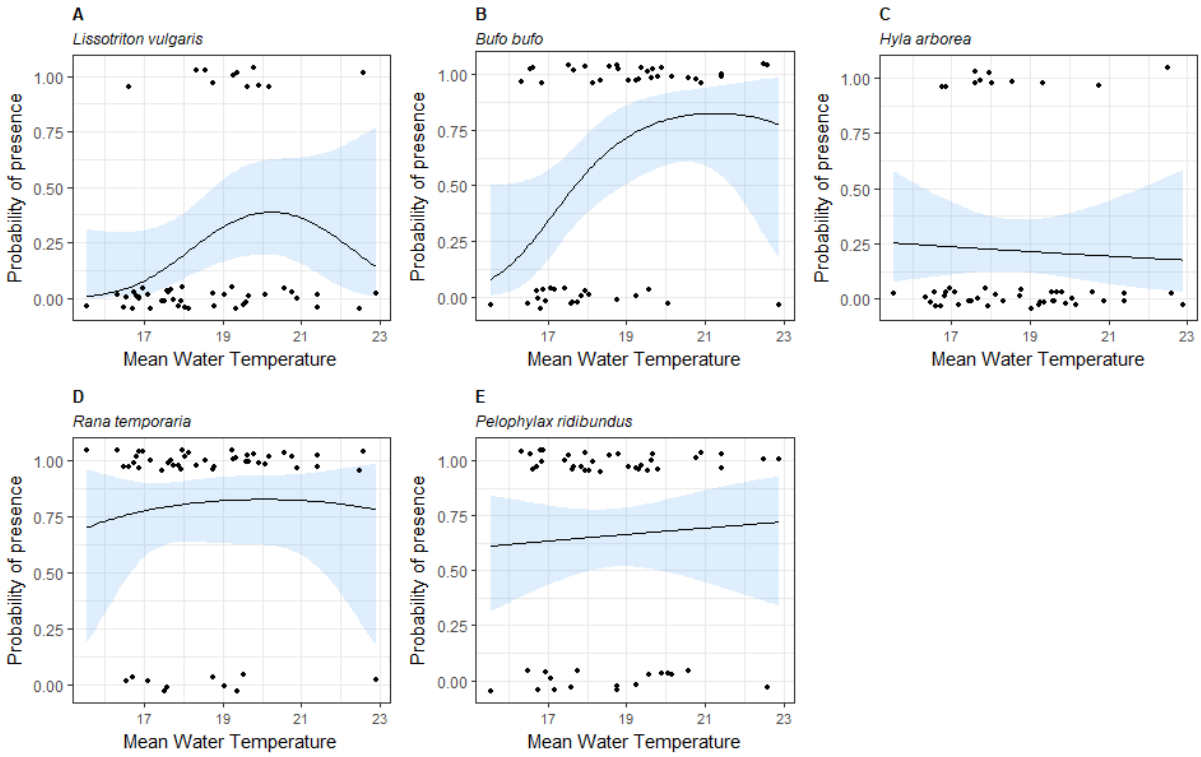

Figure S7 : Effect of the average water temperature on the presence probability of five amphibians species. A. *L. vulgaris;* ***B.*** *B. bufo;* ***C.*** *H. arborea;* ***D.*** *R. temporaria;* ***E.*** *P. ridibundus. The dark line represents the distribution of presence probability predicted by the logistic binomial regression. The 95% interval confidence is represented in blue.*

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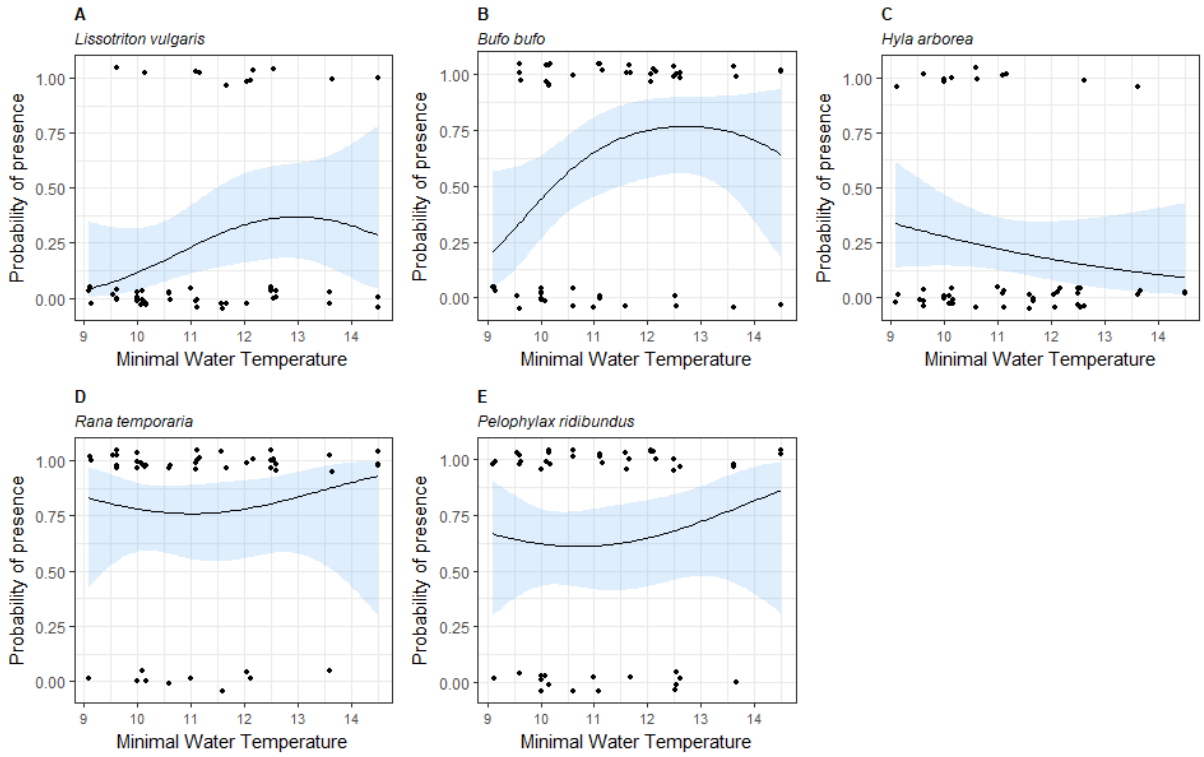

Figure S8 : Effect of the minimal water temperature on the presence probability of five amphibians species. A. *L. vulgaris;* ***B.*** *B. bufo;* ***C.*** *H. arborea;* ***D.*** *R. temporaria;* ***E.*** *P. ridibundus. The dark line represents the distribution of presence probability predicted by the logistic binomial regression. The 95% interval confidence is represented in blue*

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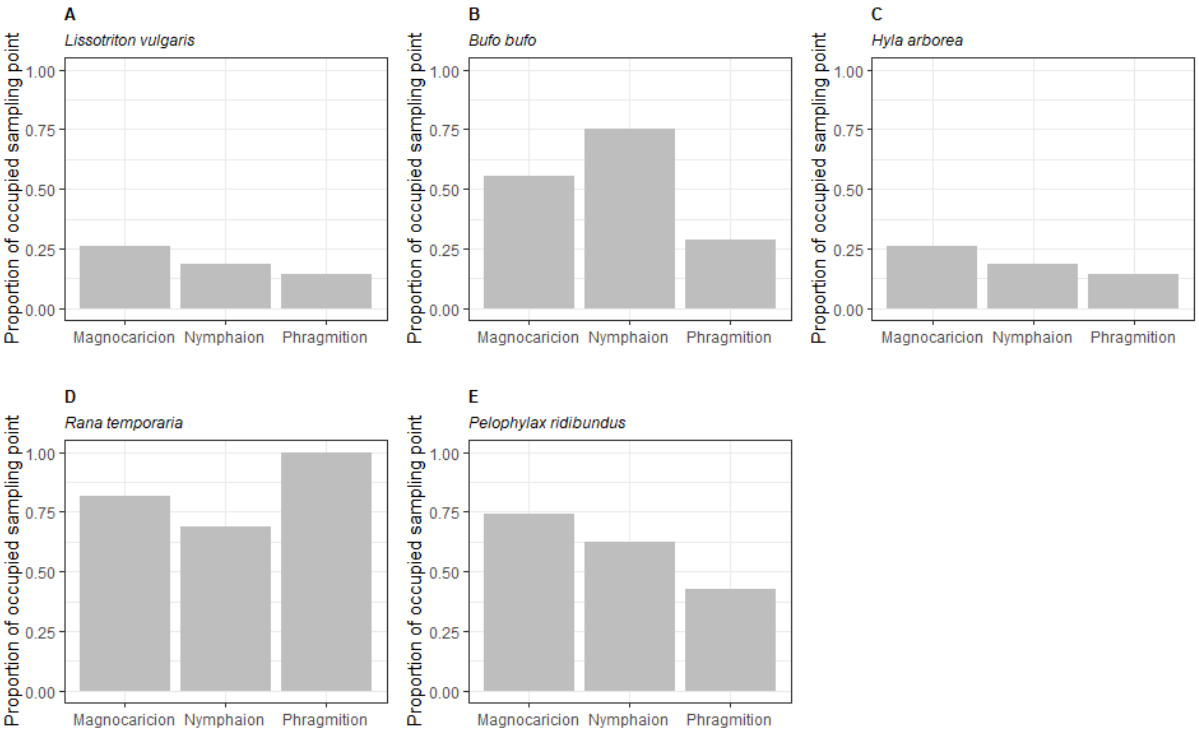

Figure S9 : Proportion of occupied sampling points by five amphibian species per vegetation type (Magnocaricion, Nymphaion and Phragmition). *Total number of sampling point per vegetation type was: Magnocaricion n = 27, Nymphaion n = 16 and Phragmition n = 7.* ***A.*** *L. vulgaris; number of occupied site per vegetation type was: Magnocaricion n = 7, Nymphaion n = 3, Phragmition n = 1.* ***B.*** *B. bufo; number of occupied site per vegetation type was: Magnocaricion n = 15, Nymphaion n = 12, Phragmition n = 2.* ***C.*** *H. arborea; number of occupied site per vegetation type was: Magnocaricion n = 8, Nymphaion n = 2, Phragmition n = 1.* ***D.*** *R. temporaria; number of occupied site per vegetation type was: Magnocaricion n = 22, Nymphaion n = 11, Phragmition n = 7.* ***E.*** *P.ridibundus; number of occupied site per vegetation type was: Magnocaricion n = 20, Nymphaion n = 10, Phragmition n = 3.*

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Supplementary methods and results S1: *Lissotriton helveticus* 12S partial gene sequenced using Sanger sequencing.

Since interest portion of the 12 S mitochondrial gene of *L. helveticus* was missing in EMBL, it was sequenced using Sanger sequencing. Previously, a PCR was performed on extracted *L. helveticus* tissues. The PCR mixture contained 1 U of AmpliTaq Gold polymerase, 1x PCR gold buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.5 µM of forward and reverse primers, 0.2 mg/mL of bovine serum albumin and 2 µL of template DNA, resulting in a final volume of 25 µL. Thermocycling conditions were as follows; denaturation and activation of the polymerase at 95 °C for 10 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, followed by a final elongation at 72 °C for 7 min.

To ensure that amplicons contains the targeted amplicon from amplification with BATR01, a nested PCR was performed. Same PCR mixture was used and the thermocycling conditions were 10 min at 95°C for DNA denaturation, followed by 10 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, followed by a final elongation of 7 min at 72°C.

Amplicon amplified with primers L2519 and H3296 was then sequenced using Sanger sequencing.

12S partial sequence of *L. helveticus* amplified with L2519 and H3296 primers (Wang et al., 2017)	5'- GAGTACTACGAGCAACAGCTTAAAACTCAAAGGACTTGGCGGTGCCCTATACCCAC CTAGAGGAGCCTGTTCTTTAATCGATAACCCCCGATAAACCTCACCATTTATTGCCA ATACAGCCTATATACCACCGTCCAGCCCACCCTTTAAAGGCTAAACAGTAGGCACA ACTACAAACATAAAAACGTCAGGTCAAGGTGTAGCAAATAAAATGGGAAGAAATG GGCTACATTTTCTAACCTAGAAAACACGGAAAAGTTTATGAAATTAAACTTTGAAGG AGGATTTAGCAGTAAAAAGAAAAAAGAGTGTTCTTTTTAACCCGGCAATGGAGCGC GCACACACCGCCCGTCACCCTCTTCAAATACCACAATATAATAGATAAACACAGTA ATAAAAGAAGAAGAGGCAAGTCGTAACATGGTAAGCTTACCGGAAGGTGAGCTTGG AACATCAGTTTATAGCTTAACTAAAGCATCCTGCTTACACCAGGAAAACGCTCGTTA AACTCGAGTTAGATTGAGTTTTACTCCTAGCCAAAACAAACACAACCCCAACTAGCT AAACTAAACCATTTAATCAAACAGTATAGGCGATAGAAAATTTTTATGAGCAATAG AAAAGTACTGCAAAGGAAAGGTGAAATAAAAATGAAATAAATGGCAAAACAATAA AAAGAAAAGATTAAGCCTTGTACCTTTTGCATMATGGGGTCTAGCAA -3'
12S partial sequence of *L. helveticus* amplified with BATR01 primers	5'- CTTCAAATACCACAATATAATAGATAAACACAGTAATAAAAGAAGAAGAGG -3'

Supplementary methods and results S2: Investigating the absence of *Lissotriton helveticus* DNA in water samples.

The absence of *L. helveticus* was expected in the sampled area of Gletterens, however, it was expected to recover DNA of this species in water samples from Yverdon.

Thus, we tested to map sequences from water samples to the sequenced 12S mitochondrial partial gene of *L. helveticus* using *bwa* and *samtools*. 36 sequences were found to match the *L. helveticus* 12S partial gene (hereafter matching sequences). To investigate phylogenetical distances among the 36 matching sequences and the *L. helveticus* 12S partial gene, a tree was constructed using MEGA (figure S10). The 12S mitochondrial partial gene of *L. helveticus* was found to be an outgroup of matching sequences. The 36 matching sequences are shown to be grouped with the *L. vulgaris* 12S partial gene.

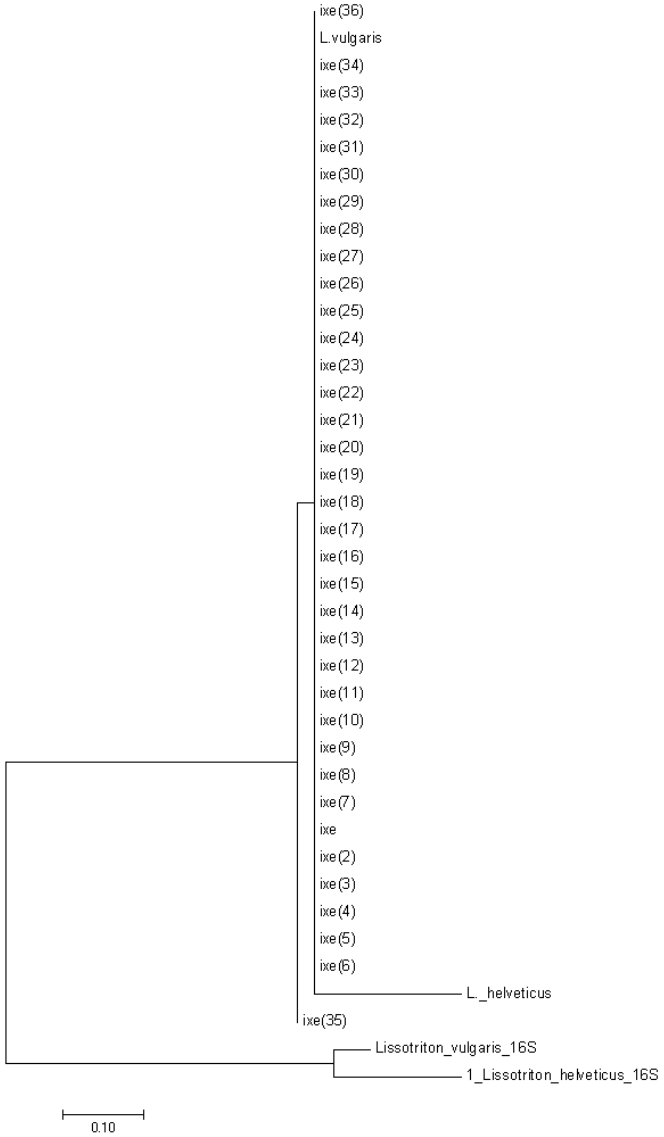

Figure S10 : Phylogenetical distances between 12S and 16S partial gene of L. helveticus and L. vulgaris as well as the 36 matching sequences recovered using samtools. *Ixe(1-36) correspond to matching sequences. L_helveticus corresponds to the 12s partial sequence of this species. L_vulgaris corresponds to the 12S partial gene sequence of this species.*

Supplementary methods S3: Investigation of DNA diffusion: design and protocol

To get reliable information relative to the habitat use of endangered species using eDNA approaches, one must know at which scale environmental variables must be measured. Hence, the DNA diffusion in natural environment must be investigated since DNA is not static in the environment. Here, we used DNA from several exotic species as a diffusion marker. Solutions of this DNA were released at different distances from the center of the sampling point. To get realistic assessment, the amount of DNA released by an individual in a natural area was first estimated and DNA solutions with the same amount of DNA were then prepared to be released in the environment.

DNA solutions corresponded to 12S mitochondrial gene amplified with primers from Wang et al. (2017) (Primer names: L2519 and H3296) of 21 exotic amphibian species. The aim of having chosen exotic amphibian species as diffusion marker is that they are amplified with BATR01 primers (Valentini et al., 2016), used for further metabarcoding amplification, and their sequences can be discriminate from other amphibian species since they are not present in the study area. To choose amphibian species, an *in-silico* PCR was performed using ecoPCR to ensure these species are amplified with both pair of primers (L2519 and H3296, and BATR01) and contained SNPs. Primers amplifying larger fragments of 12S genes than BATR01 primers were chosen in case of end degradation of the DNA molecules in the environment.

To obtain realistic amount of DNA released normally by individuals in their environment, the quantity of DNA contained in the solutions was calculated based on Thomsen et al., (2012). Thomsen et al. quantified the amount of *Triturus cristatus* cytochrome B molecules present in 15 mL of water. It was assumed that *T. cristatus* and *L. vulgaris* as well as *L. helveticus* release similar amount of DNA in the environment. A simple quantification of total extracted DNA is not sufficient to approximate the amount of DNA to be released, since Thomsen et al. (2012) had not quantified total amount of DNA released by an individual but the amount of part of the mitochondrial DNA. Thus, quantity had to be calculated to be adjusted to 12S gene marker used in this study. Calculations were as follow:

From Thomsen et al., (2012), an individual of *Triturus cristatus* releases 71.15 molecules of DNA after 44 days in 15 mL. In total the number of molecules is:

$$q_{tot} = q_{init}\frac{V_{tot}}{V_{init}} = 71.15 \times \frac{80'000}{15} = 379'466.67\ molecules$$

Where q_{tot} and q_{init} represent respectively the total and the initial number of DNA molecules and V_{tot} and V_{init} represent respectively the total and the initial volume. This number of molecules corresponds in mole at:

$$\frac{Molecule\ number}{N_A} = \frac{379'466.67}{6.02\ \times\ 10^{-23}} = 6.303 \times 10^{-19}\ moles$$

Where N_A correspond to the Avogadro number. The molecular weight of nucleotides is:

Nucleotide	Molecular weight	Average
A	331.2122 [g/mol]	326.9596 [g/mol]
T	322.2085 [g/mol]	
G	347.2212 [g/mol]	
C	307.1971 [g/mol]	

Hence, the molecular weight of a DNA molecule is: $81 \times 326.9596 = 26'483.732\ [\frac{g}{mol}]$.

Mass of a DNA molecule:

$$mass = mole \times molecular\ weight = 6.303 \times 10^{-19} \times 26'483.732 = 1.67 \times 10^{-14} g$$

Thus, there is $1.67 \times 10^{-5}\ ng$ of DNA in 80 L of water.

Since the solution that will be released in the natural environment will be of 1 mL $0.2 \times 10^{-9}\ ng$ of DNA have to be taken per amplified exotic species.

To prepare DNA solutions of exotic amphibians, a PCR was performed using L2519 and H3296 primers. The PCR mixture contained 1 U of AmpliTaq Gold polymerase, 1x PCR gold buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.5 µM of forward and reverse primers, 0.2 mg/mL of bovine serum albumin and 2 µL of template DNA, resulting in a final volume of 25 µL. Thermocycling conditions were as follows; denaturation and activation of the polymerase at 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, followed by a final elongation at 72 °C for 7 min. To ensure the further amplification with BATR01 primers for metabarcoding amplification, a nested PCR was performed. Same PCR mixture was done and the thermocycling conditions were 10 min at 95°C for DNA denaturation, followed by 10 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, followed by a final elongation of 7 min at 72°C. Then, the 12S amplicon amplified with L2519 and H3296 primers from exotic species was quantified using QBit (table S3). Subsequently,

amplified DNA was diluted to correspond to the estimated quantity of DNA released by an individual in the environment (0.2×10^{-12} [ng/uL]).

DNA solutions were released at different distances from the center of the sampling point (distances of 0, 0.5, 1, 2, 4 and 8 meters) at the four cardinal points (figure S11). The order in which the DNA solutions were released was randomized using R (version 3.4.4). DNA solutions were released one, three or five days before water samples were collected at the sampling point (table S4 and S5 for release order). Thus, DNA diffusion over time and degradation in such natural environments is estimated. For each condition, five replicates were made and chosen at random among the sampling points of the habitat use determination experiment (point 2.2.1 of Method; released order table S4 A and B).

Table S3 : Concentration of amplified DNA of the 21 Exotics species used as diffusion marker.

	Concentration [ng/uL]	**First dilution**	**[uL]**	**Final quantity [ng]**	**Letter**
Pelobates fuscus	25.3	10^6	6.120	0.2×10^{-9}	A
Pelodytes punctatus	31.5	10^6	4.915	0.2×10^{-9}	B
Pelodytes ibericus	29.2	10^6	5.303	0.2×10^{-9}	C
Bombina bombina	36.1	10^6	4.289	0.2×10^{-9}	D
Rana iberica	47.2	10^6	3.280	0.2×10^{-9}	E
P. vespertines	33.4	10^6	4.636	0.2×10^{-9}	F
Pelobates syriacus	18.1	10^6	8.555	0.2×10^{-9}	G
Hyla intermedia	28.8	10^6	5.376	0.2×10^{-9}	H
Rana yvapaiensis	43.4	10^6	3.568	0.2×10^{-9}	I
Rana berlandieri	39.9	10^6	3.881	0.2×10^{-9}	J
Rana kukunoris	21.4	10^6	7.235	0.2×10^{-9}	K
Rana sphenocephala	33.9	10^6	4.567	0.2×10^{-9}	L
Rana chiricahuensis	40.1	10^6	3.861	0.2×10^{-9}	M
Rana latasei	36.4	10^6	4.254	0.2×10^{-9}	N
Rana tarahumarae	28.4	10^6	5.452	0.2×10^{-9}	O
Rana macrocnemis	37.8	10^6	4.096	0.2×10^{-9}	P
Rana montezumae	46.7	10^6	3.316	0.2×10^{-9}	Q
Rana dybowskii	25.5	10^6	6.072	0.2×10^{-9}	R
Rana saharicus	25.3	10^6	6.120	0.2×10^{-9}	S
Rana italica	32.1	10^6	4.824	0.2×10^{-9}	T
Rana sylvatica	39.9	10^6	3.881	0.2×10^{-9}	U
Rana chensinensis	33.9	10^6	4.567	0.2×10^{-9}	V

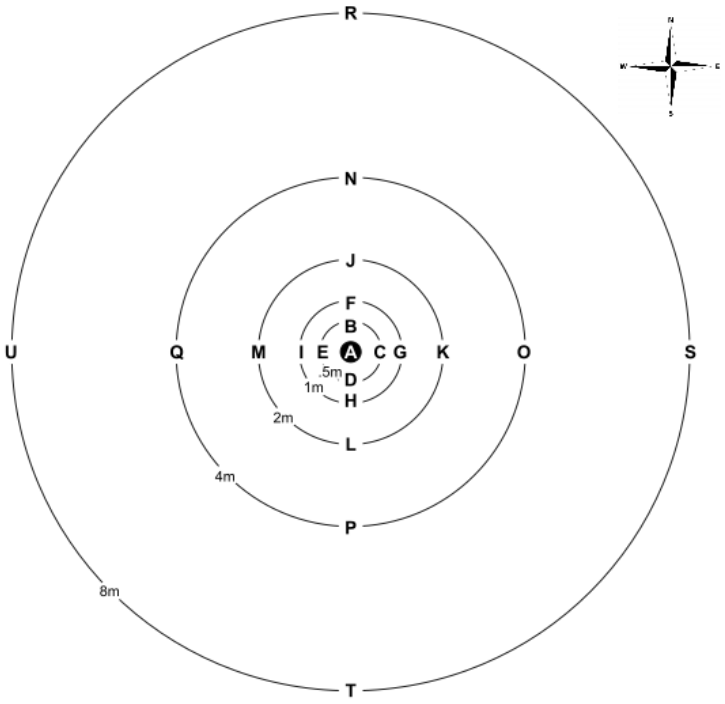

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Figure S 11 : Scheme of where DNA of the 21 exotic species was released from the center of the sampling point. *Letters A to U, represent exotic species DNA solution released at 0, 0.5, 1, 2, 4 and 8m.*

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Table S4: Sampling point and order at which DNA from 21 exotic species were released. *The DNA was released 1, 3 or 5 days before water was collected.* ***A.*** *Yverdon reserve;* ***B.*** *Gletterens*

A- YVERDON

Treatment	Point	Hour	Distance	Center	North	East	South	West
Day 1	125	9:30	0	D	-	-	-	-
			0.5	-	E	I	R	M
			1	-	J	Q	L	K
			2	-	T	C	H	G
			4	-	S	A	F	O
			8	-	P	N	U	B
	118	10:10	0	I	-	-	-	-
			0.5	-	H	U	S	A
			1	-	Q	J	D	M
			2	-	R	N	K	B
			4	-	L	P	F	C
			8	-	O	G	E	T
	145	18:00	0	A	-	-	-	-
			0.5	-	H	R	O	T
			1	-	Q	M	P	J
			2	-	I	B	E	D
			4	-	C	F	N	L
			8	-	S	U	V	G
Day 3	147	9:00	0	N	-	-	-	-
			0.5	-	B	M	D	F
			1	-	K	I	O	L
			2	-	T	H	G	J
			4	-	R	C	U	P
			8	-	S	Q	E	A
	123	9:50	0	O	-	-	-	-
			0.5	-	R	D	L	J
			1	-	N	G	B	S
			2	-	M	Q	C	H
			4	-	U	P	I	T
			8	-	A	K	F	E
Day 5	133	8:15	0	I	-	-	-	-
			0.5	-	S	H	R	D
			1	-	A	J	P	G
			2	-	N	Q	K	E
			4	-	O	C	U	B
			8	-	T	M	F	L
	111	9:00	0	K	-	-	-	-
			0.5	-	V	U	F	M
			1	-	G	Q	L	B
			2	-	R	H	T	P
			4	-	D	A	I	C
			8	-	E	N	J	S

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B- GLETTERENS

Treatment	Point	Hour	Distance	Center	North	East	South	West
Day 1	211	18:00	0	D	-	-	-	-
			0.5	-	R	H	A	I
			1	-	L	N	T	K
			2	-	C	O	Q	P
			4	-	E	S	F	G
			8	-	J	B	M	U
	242	18:43	0	E	-	-	-	-
			0.5	-	S	A	K	U
			1	-	I	J	M	P
			2	-	F	G	R	H
			4	-	C	O	N	L
			8	-	T	Q	D	B
Day 3	249	11:50	0	C	-	-	-	-
			0.5	-	K	S	U	O
			1	-	N	H	I	M
			2	-	F	G	A	Q
			4	-	D	T	J	L
			8	-	B	R	E	P
	223	13:10	0	K	-	-	-	-
			0.5	-	E	L	R	H
			1	-	P	I	N	J
			2	-	M	O	S	Q
			4	-	F	U	C	D
			8	-	G	B	T	A
	261	14:10	0	C	-	-	-	-
			0.5	-	V	S	T	Q
			1	-	P	H	K	F
			2	-	D	A	U	M
			4	-	E	O	L	J
			8	-	G	R	B	I
Day 5	200	19:29	0	M	-	-	-	-
			0.5	-	A	U	N	L
			1	-	S	D	B	T
			2	-	R	I	H	O
			4	-	P	J	E	C
			8	-	K	Q	G	F
	207	20:00	0	D	-	-	-	-
			0.5	-	T	G	E	N
			1	-	R	P	H	M
			2	-	K	O	S	U
			4	-	F	J	I	C
			8	-	A	Q	B	L
	233	20:35	0	E	-	-	-	-
			0.5	-	G	N	U	Q
			1	-	J	C	R	B
			2	-	F	H	V	I
			4	-	P	S	A	M
			8	-	O	D	L	K

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