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Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*

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Summary

1. Alien invasive species (AIS) are one of the major causes of biodiversity loss and global homogenization. Once an AIS becomes established, costs of control can be extremely high and complete eradication is not always achieved. The ability to detect a species at a low density greatly improves the success of eradication and decreases both the costs of control and the impact on ecosystems.

2. In this study, we compare the sensitivity of traditional field methods, based on auditory and visual encounter surveys, with an environmental DNA (eDNA) survey for the detection of the American bullfrog *Rana catesbeiana* = *Lithobates catesbeianus*, which is invasive in south-western France.

3. We demonstrate that the eDNA method is valuable for species detection and surpasses traditional amphibian survey methods in terms of sensitivity and sampling effort. The bullfrog was detected in 38 sites using the molecular method, compared with seven sites using the diurnal and nocturnal surveys, suggesting that traditional field surveys have strongly underestimated the distribution of the American bullfrog.

4. *Synthesis and applications*. The environmental DNA approach permits the early detection of alien invasive species (AIS), at very low densities and at any life stage, which is particularly important for the detection of rare and/or secretive aquatic species. This method can also be used to confirm the sensitivity of control operations and to better identify the distributions of vulnerable species, making this a very relevant tool for species inventory and management.

Key-words: alien invasive species, DNA barcoding, environmental DNA, inventory, *Lithobates catesbeianus*, species detection

Introduction

Alien invasive species (AIS) constitute one of major causes of biodiversity loss and global homogenization (Vitousek *et al.* 1997; Ficetola, Thuiller & Miaud 2007b; Ehrenfeld 2010; Pyšek & Richardson 2010). They may out-compete native species, act as predators or transmit exotic diseases. For example, in 1991, 68% of freshwater fishes in the continental United States known to have gone extinct since 1890 were negatively affected by introduced non-native fishes (Wilcove & Bean 1994). Once an AIS becomes established, costs of control action can be extremely high, complete eradication cannot always be achieved (Howald *et al.* 2007) and this may negatively affect the environment and compromise the recovery of native species (Myers *et al.* 2000).

During the early stages of AIS introduction, detection of the species is not possible unless its density exceeds a certain threshold (Hulme 2006; Harvey, Qureshi & MacIsaac 2009). The detection threshold depends on the monitoring method used and species detection may only be possible once the

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species is already well-established (Myers *et al.* 2000). The ability to detect an AIS at low densities greatly determines the success of an eradication operation, decreases the costs of control and reduces the impact on ecosystems (Mehta *et al.* 2007). Therefore, there is an urgent need for methods that improve the probability of detection (Harvey, Qureshi & MacIsaac 2009).

Native to Eastern North America, the American bullfrog has been introduced worldwide (Lever 2003). It is considered to be one of the world's 100 worst invasive species (Lowe *et al.* 2000; D'Amore 2012). In Europe, bullfrogs have been introduced in at least 25 countries during the 20th century (Ficetola *et al.* 2007a). Three populations are successfully established in France (Fig. 1), and two of them are subject to control actions consisting of egg mass removal, tadpole trapping and shooting of juveniles and adults (Dejean 2008). In the population established in south-western France (Dordogne), bullfrogs were detected in 35 water bodies in 2006 using traditional survey techniques (calling and visual encounter surveys, Dodd 2010). Control actions started in 2006 and bullfrogs were detected in 19 aquatic sites in 2007 and seven sites in 2008 (Guibert, Dejean & Hippolyte 2010). Control actions seem to reduce the density of bullfrogs, but the relationship between amphibian detection probability and density suggests that small populations are more likely to escape detection (Tanadini & Schmidt 2011). The detection probability of low-density populations can be increased by increasing sampling effort (i.e. increasing the number of visit per site). In this study, we propose to use the newly developed environmental DNA (eDNA) barcoding approach (Hebert *et al.* 2003; Ficetola *et al.* 2008; Valentini, Pompanon & Taberlet 2009), using water samples as the DNA source. This method has been successfully implemented in the detection of invasive (Ficetola *et al.* 2008; Jerde *et al.* 2011) or secretive species (Goldberg *et al.* 2011). We compare and consider the potential errors of both methods (surveys and eDNA).

Fig. 1. Distribution of introduced bullfrog populations in France. 1: Colonized area in Gironde (introduction in 1968), 2: colonized region in Dordogne (introduction in 1990). This study was performed in this region (see Fig. 2). 3: colonized region in Loir et Cher (introduction suspected in the 2000). The comparisons of costs between traditional and environmental DNA (eDNA) surveys were performed in this area (see Discussion). (Map kindly provided by J. Lescure and J.-C. de Massary).

Materials and methods

STUDY AREA

The study was conducted within the Natural Regional Park of Perigord-Limousin, in the south-west of France (Fig. 2). About 80 aquatic sites were identified in the 5×5 km study area. The ponds are natural field depressions and old (*c.* 17th century) fishery ponds. The fishery activity ceased at the end of the 19th century. The Dronne river and its two tributaries (Fig. 2) are the only running water in this area.

BULLFROG DETECTION ESTIMATE

The presence of the American bullfrog was first detected in this region in the early 90s. To estimate detection probability of bullfrogs with traditional field survey methods, a study was conducted in eight ponds where bullfrog presence had been confirmed for several years (Dejean 2008). These ponds were sampled on four occasions (8, 20, 27 and 29 June 2006, methods described below). The weather conditions (temperature and wind) during these survey days were optimal for bullfrog activity in south-western France (Ficetola *et al.* 2007a).

Detection probability and site occupancy were modelled according to MacKenzie *et al.* (2006). We tested two models (detection probabilities were either constant or varied among the four sampling occasions) for each bullfrog category (juveniles, males and females) with the software PRESENCE 3.1 (MacKenzie *et al.* 2006). Evaluation of the relative performance of the models was based on Akaike Information Criterion (AIC) (Burnham & Anderson 2002), and models with lower AIC are considered better candidates than those with higher AIC.

ERADICATION PROGRAM

The staff of the PNR decided to implement an eradication program in 2005. Forty-nine ponds were selected (most of unselected sites within the study area were small ponds with temporary water, Dejean 2008), including the eight ponds described above, that were surveyed to determine bullfrog detection probability. The eradication methods included egg mass removal, tadpole trapping and shooting of juveniles and adults (Dejean 2008). In late spring and summer 2006, 601 bullfrogs were detected and shot in 32 ponds, of these 515 were juveniles, 74 were males and 12 were females. In 2007, 412 individuals were shot in 19 ponds, including 339 juveniles, 59 males and 14 females. In 2008, 334 individuals were shot in seven ponds, including 313 juveniles, 14 males and seven females (Guibert, Dejean & Hippolyte 2010).

TRADITIONAL FIELD SURVEYS

Surveys were performed in 2006 (eight ponds, see above for methods) and 2008 (49 ponds, from 16 to 20 June) with the help of skilled herpetologists: (i) During the day, a visual encounter survey was performed on the borders of each pond (up to 1 h depending on the pond size): the shoreline was followed and investigated using binoculars, to detect adults and juveniles. The

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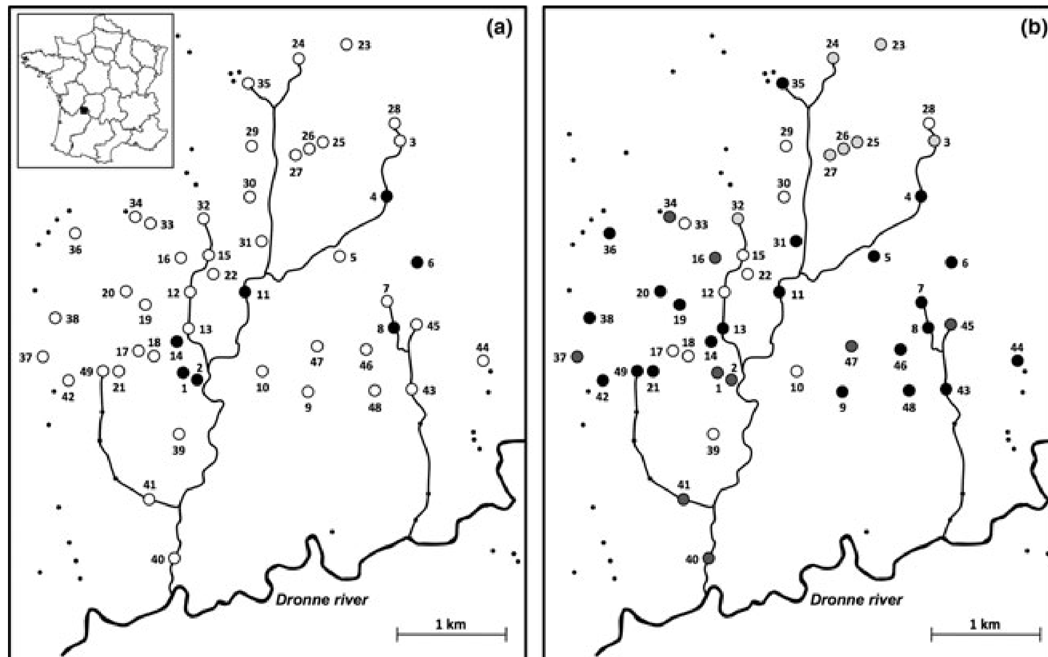

Fig. 2. Distribution of bullfrog with traditional surveys (a) filled circles represent the ponds where the American bullfrog was detected, and open circles represent the pond where the species was not detected. Distribution of bullfrog with environmental DNA (eDNA) survey (b) filled light grey circles represent the ponds where the American bullfrog was detected in one of the three water samples, filled dark grey circles represent the ponds where it was detected in two of the three water samples, filled black circles represent the ponds where it was detected in all the water samples and open circles represent the pond where the species was not detected. Dots represent the ponds that were not surveyed. This study area refers to the colonized region 2 in Fig. 1.

water near the shoreline was also searched for egg masses and tadpoles. (ii) From about 10:00 PM (sunset) to 02:00 AM, the ponds were visited again, and a calling survey was performed. The ponds were approached quietly to a distance of about 50 m from the pond edge. Upon reaching the survey position, the surveyor waited for 5 min before carrying out the auditory survey. Each survey was carried out for a maximum of 15 min and was concluded as soon as a bullfrog call was detected.

eDNA SURVEY

The sampling strategy used for bullfrog detection with eDNA followed the protocol as described by Ficetola *et al.* (2008). Sampling occurred in the same time period as the traditional survey (16–20 June 2008), and three 15-mL water samples were collected from different sections of each pond where the species was most likely present (i.e. mainly in areas rich in aquatic vegetation). For each pond, the samples were collected the same day. Immediately after collection, a solution composed of 1·5 mL of sodium acetate 3 M and 33 mL absolute ethanol was added to the water samples and the tubes were then stored at −20 °C until DNA extraction. A total of 147 water samples were collected from 49 ponds. All samples for the eDNA survey were composed by clear water.

DNA extraction and amplification methods were adapted from Ficetola *et al.* (2008). The mixture was centrifuged at 9400 g for 1 h at 6 °C to recover DNA and/or cellular remains. The DNA from the resulting pellet was extracted using QIAmp Blood and Tissue Extraction Kit (GmbH; Qiagen, Hilden, Germany), following manufacturer's instructions. DNA extraction was performed in a room dedicated for degraded DNA samples. Control extractions were systematically performed to monitor possible contaminations. Bullfrog DNA was amplified with specific primers (Ficetola *et al.* 2008). DNA amplifications were carried out in a final volume of 25 μL, using 3 μL of DNA extract as template. Three PCR replicates were performed per DNA sample. The amplification mixture contained 1 U of Ampli*Taq* Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 10 mM Tris-HCl, 50 mm KCl, 2 mM of MgCl2, 0·2 mM of each dNTPs, 0·2 μm of each primer and 0·005 mg of bovine serum albumin (BSA; Roche Diagnostics, Basel, Switzerland). After 10 min at 95 °C (*Taq* activation), the PCR cycles were performed as follows: 55 cycles of 30 s at 95 °C, 30 s at 61 °C. PCR products were visualized using electrophoresis on 2% agarose gel. Negative (UHQ water) as well as positive samples (DNA extracted from American bullfrog tissue) were systematically added during the PCR step.

Results

TRADITIONAL FIELD SURVEYS

The models with constant detection probability provided a better explanation of observed survey data collected in 2006 for each bullfrog category (Table 1): the detection probability was 0·80 ± 0·073 in juveniles, 0·73 ± 0·080 in males and 0·27 ± 0·081 in females, respectively.

The presence of bullfrogs was detected in seven of the 49 ponds surveyed in 2008 (occurrence = 0·143). Tadpoles were observed during diurnal visual encounter surveys in three ponds while males were detected by nocturnal calling surveys in four other ponds (Fig. 1a and Table 2).

Table 1. Model selection for bullfrog detection probability inference using traditional visual methods (encounter and calling surveys) for amphibian detection

	Model	AIC	Model likelihood	Nb parameters
Juveniles	*P* constant	34·02	1·00	2
	P variable	34·99	0·62	5
Males	*P* constant	38·79	1·00	2
	P variable	43·25	0·11	5
Females	*P* constant	38·79	1·00	2
	P variable	38·81	0·99	5

Aic, Akaike Information Criterion; Nb, number.

eDNA SURVEY

Bullfrog eDNA was successfully amplified in water samples collected in 38 out of 49 ponds. Positive bullfrog DNA signal was obtained for all three replicates in 22 ponds, for two of the three replicates in nine ponds and for one of the three replicates in seven ponds. Three PCR replicates were performed for each DNA extract (i.e. a total of nine PCRs for each pond). The overall average bullfrog DNA amplification success was 0·53 ± 0·03 (min: 1/3; max: 3/3, $N = 38$). The eDNA survey produced a bullfrog occurrence of 0·775 (Fig. 1b and Table 2).

COMPARING TRADITIONAL AND eDNA SURVEYS

Positive results were obtained for the eDNA survey from all seven ponds where bullfrogs were detected using traditional survey methods (Table 2). The amplification success in these ponds was 0·62 ± 0·06 (min: 3/9; max: 8/9, $N = 7$). It is not possible to infer relation between eDNA amplification success and bullfrog density because the surveys were not designed to estimate this parameter.

Excluding these seven ponds as well as the 11 ponds where bullfrogs were not detected (using both traditional and eDNA surveys), the amplification success was 0·52 ± 0·03 (min: 1/9; max: 9/9) in the remaining 31 ponds.

No bullfrog presence was detected in pond number #41 from using the traditional survey method, whereas two of three water samples and five of nine PCRs were positives (Table 2). Similarly, four ponds (#13, 19, 20 and 21) showed amplification of bullfrog DNA in all water sample replicates (seven of nine positive PCRs) while no bullfrog presence was detected at these ponds using traditional survey methods.

Overall, the eDNA method indicated bullfrog occurrence in 38 out of 49 ponds (0·775) which is more than five times higher than that found in traditional surveys (seven out of 49; 0·143).

Table 2. Detection of bullfrog using traditional and environmental DNA (eDNA) surveys (June 16–20, 2008)

Traditional surveys			eDNA surveys		
Pond	Visual encounter detection	Calling detection	Detection	Water samples positives	Positive PCRs
1	+		+	2/3	6/9
2		+	+	2/3	6/9
3			+	1/3	1/9
4		+	+	3/3	3/9
5			+	3/3	8/9
6	+		+	3/3	9/9
7			+	3/3	6/9
8		+	+	3/3	3/9
9			+	3/3	6/9
10				0/3	0/9
11	+		+	3/3	4/9
12				0/3	0/9
13			+	3/3	7/9
14		+	+	3/3	8/9
15				0/3	0/9
16			+	2/3	3/9
17				0/3	0/9
18				0/3	0/9
19			+	3/3	7/9
20			+	3/3	7/9
21			+	3/3	7/9
22				0/3	0/9
23			+	1/3	2/9
24			+	1/3	2/9
25			+	1/3	1/9
26			+	1/3	1/9
27			+	1/3	2/9
28				0/3	0/9
29				0/3	0/9
30				0/3	0/9
31			+	3/3	9/9
32			+	1/3	1/9
33				0/3	0/9
34			+	2/3	2/9
35			+	3/3	6/9
36			+	3/3	4/9
37			+	2/3	6/9
38			+	3/3	8/9
39				0/3	0/9
40			+	2/3	3/9
41			+	2/3	5/9
42			+	3/3	4/9
43			+	3/3	4/9
44			+	3/3	6/9
45			+	2/3	3/9
46			+	3/3	6/9
47			+	2/3	2/9
48			+	3/3	8/9
49			+	3/3	9/9

Discussion

The precise understanding of species distribution is a key requirement for conservation management, especially when the focal species is invasive (Magurran 2003; Harvey, Qureshi & MacIsaac 2009). The ability to detect a species at a low density greatly influences management decisions (Mehta *et al.* 2007), making the development of methods to improve detection probabilities a high priority. In this study, we take advantage of a control action to manage the invasive American bullfrog in the Natural Regional Park of Périgord-Limousin to compare traditional field surveys and eDNA surveys. The control actions performed from 2006 onwards were considered as effective because 35 water bodies were detected as colonized in

2006, 19 in 2007 and seven in 2008 (Guibert, Dejean & Hippolyte 2010). However, if the observed trend in occurrence is correlated with a population size decrease, there is a high risk of overestimating the success of this action because of the well-known relationship between detection and density in amphibians (e.g. Tanadini & Schmidt 2011).

The eDNA methods constitute a promising tool in ecology (Ficetola *et al.* 2008); however, it is important to assess the efficiency by comparing different survey methods to determine their relative reliabilities.

BULLFROG DETECTION COMPARED WITH TRADITIONAL SURVEY

The reliability of amphibian surveys, as any other species detection method, can be compromised by the possibility of false positives (Type I errors, species is detected where it is not present) and/or false negatives (Type II errors, species is not detected where it is present). Bullfrog detection was based on calls, tadpoles and spawns determination. This species is easy to identify among the other native amphibians of the studied area, and we consider that the possibility of false positives is closed to null.

On the other hand, a bullfrog not detected where it is present is a classical issue in amphibian population monitoring (see Dodd 2010 for a review). Amphibian occurrence or abundance strongly depends on species detectability, which vary with many factors; these include the date and time of day, meteorological conditions, population size and observer experience (Crouch & Paton 2002; Genet & Sargent 2003; Schmidt & Pellet 2010; Tanadini & Schmidt 2011).

Calling surveys are one of the most popular methods (e.g. Pellet & Schmidt 2005; de Solla *et al.* 2005; Weir *et al.* 2005), because male vocalizations to attract females are easily identifiable, making their detection and identification relatively simple when weather conditions are favourable (e.g. Pellet & Schmidt 2005). In the Périgord-Limousin region, bullfrog detectability (combining calling and visual encounter surveys) was similar and rather high for juveniles and males (0·80 ± 0·073 and 0·73 ± 0·080, respectively) while it decreased to 0·27 ± 0·081 for females. The reproductive biology and behavioural differences between life stages can explain this result (e.g. no calling activity and territory defence in females, Ryan 1980). Pellet & Schmidt 2005 showed that for the common toad (*Bufo bufo*), a pond-breeding amphibian, at least six visits (15 min each) were necessary to infer the presence of the species with 95% confidence. If the decrease in bullfrog density from 2006 to 2008 leads to a reduced detection probability, this phenomenon can be counterbalanced by an increased sampling effort (i.e. the number of visit per site). The large difference in bullfrog occurrence estimates in 2008 between the two methods (traditional vs. eDNA) suggested that the bullfrog distribution was underestimated using traditional field surveys. Following this result, the staff of the PNR Périgord-Limousin was highly motivated to perform a new field survey (more than five visits per site) on the 38 ponds identified as bullfrog positive with eDNA. Bullfrogs were detected in the previous seven ponds, and in 11 'new' water bodies. The estimated occurrence of bullfrogs thus reached 0·47 with this strong sampling effort, in a set of ponds that the eDNA method identified as bullfrog positive. Goldberg *et al.* (2011) demonstrated the sensitivity of the eDNA method in a case study involving Asian carps in North America, by comparing it with classical electrofishing. At the lowest carp density, only eDNA was able to detect carp presence. One carp was detected in one pool after 93 person-days of electrofishing effort that was motivated and targeted by the discovery of carp eDNA.

DETECTION PROBABILITY WITH eDNA SURVEY

The reliability of the genetic method can be compromised by the possibility of false positives and/or negatives (Type I and II errors, see above), that is, non-specificity of the primers used for DNA amplification, contamination or protracted DNA persistence after the death of the organism, poor sampling or poor protocol efficiency (Darling & Mahon 2011). The protocol used had been shown to be reliable in a previous study (Ficetola *et al.* 2008): before the eDNA analysis, primer reliability, robustness and specificity were tested, first *in silico* [using ECOPCR software (Taberlet *et al.* 2007)] and then on high-quality DNA (extracted from tissues samples), and PCR conditions were optimized (Ficetola *et al.* 2008). Because of the rarity of DNA in the water samples, the analysis was performed with similar precautions as those used for ancient DNA studies to reduce contamination and poor-quality DNA results (Taberlet *et al.* 1996; Cooper & Poinar 2000). This means that DNA was extracted in a dedicated room for rare DNA, mock samples without DNA and positive samples were analysed in parallel, the number of PCR cycles was increased, the analysis was performed on several field samples and three PCR replicates per sample were performed. Based on three samples per pond, the amplification success was 0·37 ± 0·1 in ponds where bullfrogs were present at low densities and 0·79 ± 0·08 in ponds where bullfrogs were present at high densities (Ficetola *et al.* 2008). In the present study, this amplification rate was 0·53 ± 0·03. It is too early to infer quantitative (i.e. abundance or density) information from eDNA survey results, but the amplification rate could be useful to that end in the future.

Several ponds within the study area cannot be considered to be discrete units, because they are connected by small streams (Fig. 1). It is thus possible that DNA moves from one site to another, leading to false positives (bullfrog eDNA detected in bullfrog free pond). However, in our study, eDNA revealed the presence of bullfrog in ponds 32 and 13 (about 1 km apart, Fig. 1), and not in ponds 12 and 15 located between them on the same tributary. Note that there is no water flow in the stream at this time of year, which limits eDNA dispersal.

Finally, bullfrogs can move from one pond to another, leaving eDNA in an unoccupied pond at the time of sampling, which will lead in turn to false positives. The mean distance between adjacent ponds in the sampling area is lower than adult bullfrog potential dispersal (Ingram & Raney 1943; Willis, Moyle & Baskett 1956). However, adult and juvenile

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dispersal inferred with radiotracking and pit-fall trap methods in south-western France (Berroneau, Detaint & Coïc 2007) showed that both life stages stayed in aquatic sites during the June study period. In addition, the persistence of eDNA in water has been tested (Dejean *et al.* 2011) and bullfrog eDNA was detected only for a maximum of 2 weeks after the removal of the source animal. It is thus highly probable the bullfrog was present or had been present at maximum 2 weeks prior to the time the water sampling was performed, which further limits the possibility of type I errors.

The use of eDNA as a survey tool in ecology is in a developmental phase. The reliable detection of aquatic vertebrates was confirmed in wetlands (Ficetola *et al.* 2008), in a large river and canal system (study of the risk of invasion of the Laurentian Great Lakes region by Asian Carp, Jerde *et al.* 2011) and streams (inventory of secretive Rocky Mountain tailed frogs, *Ascaphus montanus*, and Idaho giant salamanders, *Dicamptodon aterrimus*, in the north-western region of the United States, Goldberg *et al.* 2011). These studies show higher detectability for rare and/or secretive aquatic species, at all life stages and at low densities. In pond-breeding amphibians (this study), detection with traditional methods is very sensitive to meteorological conditions and often limited in the season (short stay in water of breeding adults). Water sampling for eDNA can be performed whatever the weather conditions and for a longer period of time because more cryptic individuals (e.g. tadpoles) stay in the water. In another introduced bullfrog population (32 colonized ponds, Loir et Cher, Central France, Fig. 1), a preliminary estimate of traditional and eDNA survey costs showed that the eDNA method was 2·5 times cheaper and 2·5 times less time consuming than the traditional survey (based on a 2-person fieldwork time and complete molecular analysis) (Michelin, Heckly & Rigaux 2011).

The sensitivity of eDNA across taxa and environments remains to be determined. The method is efficient for vertebrate inventory in freshwater environments, with rare and/or secretive species, at low densities and at several life stages. This method is particularly promising for other taxonomic groups (e.g. microorganisms, plants) and other environments (e.g. soils).

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