

# Species detection from aquatic eDNA: Assessing the importance of capture methods

Sara Peixoto<sup>1,2</sup>  | Cátia Chaves<sup>1</sup> | Guillermo Velo-Antón<sup>1</sup>  | Pedro Beja<sup>1,3</sup>  | Bastian Egeter<sup>1</sup>

<sup>1</sup>CIBIO/InBIO, Instituto de Ciências Agrárias de Vairão, Vairão, Portugal

<sup>2</sup>Departamento de Biologia da Faculdade de Ciências, Universidade do Porto, Porto, Portugal

<sup>3</sup>CIBIO/InBio, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

## Correspondence

Sara Peixoto, CIBIO/InBIO, Instituto de Ciências Agrárias de Vairão, R. Padre Armando Quintas 7, 4485-661, Vairão, Portugal.

Email: sara.peixoto15@gmail.com

## Present address

Sara Peixoto, Department of Biological and Marine Sciences, University of Hull, Hull, UK

## Funding information

Fundação para a Ciência e a Tecnologia, Grant/Award Number: IF/01425/2014; Horizon 2020 Framework Programme, Grant/Award Number: 668981; BIOPOLIS - Teaming to Upgrade to Excellence in Environmental Biology, Ecosystem Research and AgroBiodiversity, Grant/Award Number: 857251

## Abstract

Environmental DNA (eDNA) is increasingly used for biodiversity monitoring, particularly in aquatic systems. However, each step, from sample collection to bioinformatic analysis, can introduce biases and influence the reliability of results. While much effort has been put into the optimization of laboratory methods, less attention has been devoted to estimate the impacts of eDNA capture methods. To address this issue, water samples were collected at nine small ponds and puddles where up to 10 amphibian species occur, using precipitation, disc filters, and capsules. We focused on targeted detection of an amphibian species, *Salamandra salamandra*, and on the composition of the whole amphibian community. Species detection was performed using a novel qPCR assay for *S. salamandra* and high-throughput sequencing, combined with stringent versus relaxed PCR replication thresholds. Filtration techniques (disc filters and capsules) outperformed precipitation, generating a higher number of detections of *S. salamandra* and higher amounts of captured eDNA, while species detection was identical between disc filters and capsules. There were no significant differences between capture methods regarding amphibian community composition. The variation in detection success associated with capture methods was far higher than that associated with PCR replication, regardless of the detection method used. Our results highlight the importance of choosing a suitable capture method for eDNA studies and suggest that the choice of capture method outweighs the choice of detection method used. To the best of our knowledge, this is the first study to compare high-capacity capsules with common eDNA methods for water samples, such as precipitation and standard disc filters.

## KEYWORDS

Amphibians, capture methods, environmental DNA, PCR replication thresholds, *Salamandra salamandra*

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. *Environmental DNA* published by John Wiley & Sons Ltd

## 1 | INTRODUCTION

Environmental DNA (eDNA) is increasingly used in biodiversity monitoring, both for the targeted detection of particular species (e.g., invasive species or species of conservation concern) and for characterizing the composition of whole biological communities (e.g., Thomsen et al., 2012). This DNA-based monitoring approach can be applied to a range of environments, but most studies in the fields of animal ecology and bioassessment focus on aquatic ecosystems. However, despite the advantages of eDNA to identify aquatic species, its application is still largely under evaluation due to the inherent complexity of the approach. Each step can introduce errors and biases, including sample collection, DNA extraction, DNA amplification, high-throughput sequencing, and bioinformatic pipelines (Zinger et al., 2019). A number of considerations should be taken into account when applying eDNA approaches in aquatic ecosystems and robust experimental designs are needed in order to increase the confidence on the conclusions obtained from eDNA surveys.

While many studies have focused on the optimization of laboratory methods to deal with the challenges associated with biodiversity monitoring using eDNA, comparatively less attention has been given to the evaluation of capture methods. Multiple methods are available for eDNA capture from water bodies, to the extent that few studies share the same methods (Dickie et al., 2018). Environmental DNA capture either by centrifugation (e.g., Caldwell, Raley, & Levine, 2007), precipitation with sodium acetate and ethanol (e.g., Ficetola, Miaud, Pompanon, & Taberlet, 2008), or filtration (e.g., Jerde, Mahon, Chadderton, & Lodge, 2011) has previously been applied in aquatic systems. Centrifugation and precipitation methods are restricted to a low sample volume (usually 15 ml) which can hamper species detection, especially for low-density species (Herder et al., 2014). In contrast, filtration methods allow the capture of eDNA from larger volumes of water with previous studies reporting volumes ranging from 250 ml (Barnes et al., 2014) up to 100 L (Valentini et al., 2016).

The most common filters used in the field are 47 mm disc filters, also called open filters. These are usually associated with small volumes due to their small surface area (c. 17 cm<sup>2</sup>). An alternative, more recent, approach has been the utilization of enclosed filters (hereinafter referred to as capsules) (e.g., Lopes et al., 2017; Valentini et al., 2016). Capsules thus far used by the eDNA community have surface areas ranging from 4.5 cm<sup>2</sup> (Vences et al., 2016; Millex ref SLGV033RS) to 1,300 cm<sup>2</sup> (e.g., Lopes et al., 2017; Valentini et al., 2016; Envirochek HV ref 12099). The larger surface areas allow the filtration of much greater volumes of water, but to the best of our knowledge, a comparison of the performance of high-capacity capsules (defined here as having a surface area of more than 100 cm<sup>2</sup>) to more common eDNA methods is still missing. Vences et al. (2016) did a small test with two high-capacity capsules (1,300 cm<sup>2</sup>; Envirochek HV ref 12099), which the authors noted was not sufficient for statistical comparison. Spens et al. (2017) compared the performance of capsules with ethanol precipitation and disc filters; however, the

surface area of the capsules used was rather small (10 cm<sup>2</sup>; Sterivex ref SVGPL10RC).

Like the capture method, the species detection method applied to a given set of eDNA samples will also play an important role in any eDNA study, as different methods will have different sensitivities. Although PCR-free methods have been used to analyze eDNA samples, the most common approach is to use PCR to facilitate species detection. Currently, two main PCR-based methods are used: quantitative PCR (qPCR) and metabarcoding (PCR followed by high-throughput sequencing, HTS). qPCR is generally utilized as a species-specific assay, while metabarcoding is utilized for the simultaneous detection of multiple species and thus to assess community composition (e.g., Bálint, Nowak, Márton, & Pauls, 2017). The major difference between these two approaches is thus related to the range of organisms the eDNA survey needs to cover and the specificity of the primers used. Metabarcoding studies target a large group of species and often a single primer set is not enough to cover the biodiversity intended, whereas qPCR studies only require one set of primers to detect their target species. Metabarcoding studies are also susceptible to taxon bias, where DNA from some species is amplified more efficiently than others, potentially leading to rare species not being detected for example. The use of multiple markers (nuclear and/or mitochondrial), with different lengths, or the use of multiple primers (both group and species-specific) has previously been suggested to overcome such biases in metabarcoding studies (Harper et al., 2018). Nevertheless, metabarcoding becomes more beneficial in more diverse systems (Thomsen & Willerslev, 2015), allowing the detection of multiple species while being less time-consuming and more cost-efficient than qPCR. A common challenge to both approaches is the existence of errors and incomplete reference databases. Missing sequence information for a particular species will have an impact not only when trying to obtain a species identification but also at an earlier stage when designing the assay. These problems can be minimized using reliable genetic databases such as GenBank, that has been shown to have a very small percentage (<1%) of taxonomic errors and mislabeled sequences (Leray, Knowlton, Ho, Nguyen, & Machida, 2019), despite previous concerns raised about its accuracy (e.g., Harris, 2003). A comparison between qPCR and metabarcoding is thus essential to determine if they provide comparable results. Notwithstanding, it remains unclear which method is the best for species-specific studies, with previous research reporting different outcomes, ranging from a higher performance of qPCR (Lacoursière-Roussel, Dubois, Normandeau, & Bernatchez, 2016) to similar performance between both methods (Murray et al., 2011).

Another source of variation in eDNA research lies on the number of positive samples or replicates needed to consider species presence, to which there are no standard guidelines at the moment (Goldberg et al., 2016). This will have an impact on the final results given that different thresholds will result in different species lists (e.g., Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Allali et al., 2017; Deagle, Thomas, Shaffer, Trites, & Jarman, 2013; Mata et al., 2019). Less stringent thresholds can overestimate the presence of a species (false positives), while strict thresholds might

**TABLE 1** Summary of sampling sites, including the salamander abundance observed in the field and the dimensions of the pond

Site	Sampling date	Latitude	Longitude	Larvae abundance (individuals/m)	Length (m)	Width (m)	Depth (m)	Area (m <sup>2</sup> )
1	13-03-2018	41°23'53.90"N	8°45'05.16"W	0.7	13.5	1.5	0.35	20.3
2	15-03-2018	41°10'37.92"N	8°38'24.24"W	2.0	5.0	2.0	0.40	10.0
3	20-03-2018	41°19'40.44"N	8°44'01.32"W	2.2	33.0	3.5	0.08	115.5
4	20-03-2018	41°19'33.60"N	8°44'03.12"W	1.1	14.0	3.0	0.07	42.0
5	21-03-2018	41°19'41.69"N	8°43'56.85"W	0.5	11.0	8.0	0.20	88.0
6	21-03-2018	41°19'39.07"N	8°43'57.49"W	0.8	6.0	4.0	0.20	24.0
7	22-03-2018	41°19'25.12"N	8°43'58.23"W	3.8	16.0	12.0	0.25	192.0
8	22-03-2018	41°19'29.18"N	8°44'00.02"W	0.8	13.0	2.0	0.05	26.0
9	23-03-2018	41°19'43.44"N	8°44'01.49"W	1.4	11.0	3.0	0.07	33.0

fail to detect it despite its presence in the site (false negatives), with consequences for downstream conservation effort (Thomsen & Willerslev, 2015).

In the present study, we focused on amphibians, which are currently considered the most threatened group of vertebrates worldwide (Wake & Vredenburg, 2008), with an estimated 40% of species in danger of extinction (Bishop et al., 2012). Given their declines, the need for powerful and cost-effective methods for amphibian surveys is becoming increasingly important. The use of molecular eDNA techniques has been shown to be more efficient than traditional field surveys for amphibian detection in several cases (Dejean et al., 2012; Smart, Tingley, Weeks, Van Rooyen, & McCarthy, 2015; Valentini et al., 2016). However, amphibians often inhabit turbid environments (Lobos, Cattán, Estades, & Jaksic, 2013; Schmutzer, Gray, Burton, & Miller, 2008), such as agricultural ponds (Ferreira & Beja, 2013; Knutson et al., 2004) or shallow lakes (Jackson & Moquin, 2011), where sampling of eDNA is challenging due to reduced performance of filtration methods associated with high-sediment loads clogging filters (Hinlo, Gleeson, Lintermans, & Furlan, 2017). The efficiency of eDNA studies in turbid waters remains poorly known, and few studies have addressed the difficulties of biodiversity assessment in these environments (Egeter et al., 2018). Due to their large surface area, capsules allow the filtration of large volumes (Valentini et al., 2016) and could help overcome the clogging problem.

While much effort has been put into the optimization of eDNA laboratory methods, less attention has been devoted to estimate the impacts of capture methods. To address this issue and better understand what influences species detection in aquatic systems, water samples were collected from turbid environments using three eDNA capture methods (precipitation, disc filters, and capsules) and their efficiency was compared in terms of volume filtered, eDNA recovered, and species detection. To cover the usual range of applications in eDNA monitoring, the study considered both the targeted detection of a ubiquitous species (the fire salamander, *Salamandra salamandra* Linnaeus, 1758) and the characterization of the overall amphibian community composition, using two species detection

methods (qPCR and HTS) and two PCR replication thresholds (stringent and relaxed).

## 2 | MATERIAL AND METHODS

### 2.1 | Target species and pond selection

The study was conducted at the Ornithological Reserve of Mindelo and two nearby localities at Porto, Portugal, where the target species has previously been studied (e.g., Alarcón-Ríos, Nicieza, Kaliontzopoulou, Buckley, & Velo-Antón, 2020). The species selected for targeted detection, the fire salamander (*Salamandra salamandra*), is a urodele species widespread across Europe. The populations occurring in our study area are larviparous. Pregnant female salamanders deliver up to 90 larvae into water bodies (i.e., ponds, puddles, and streams) during the reproductive periods (Autumn and Spring), where the larvae stay until they complete metamorphosis (Velo-Antón, Santos, Sanmartín-Villar, Cordero-Rivera, & Buckley, 2015). This species was chosen due to its abundance in the study area and the relative ease with which it is detected using traditional pond sampling methods. The study system includes small ponds and temporary puddles of similar dimensions, providing suitable biological replicates, as the salamander population densities at the time of sampling were relatively homogeneous across sampling points (Table 1). Diurnal surveys were conducted in a range of ponds and puddles throughout the Porto region in late March 2018, and a total of nine ponds/puddles were sampled (Table 1).

At each sampling site, the physical characteristics of ponds (length, width, and depth) were measured. Larvae were detected using visual surveys, and larvae abundance was recorded using a transect sampling approach (Heyer, Donnelly, McDiarmid, Hayek, & Foster, 1994) (i.e., number of individuals per meter; Table 1). Several other amphibian species were detected and recorded during fieldwork, but abundance was only measured for fire salamanders, as this was the focal species for the comparison of capture and detection methods. Turbidity was measured using a Secchi disc housed in a

turbidity tube (Anderson & Davic, 2004; Myre & Shaw, 2006). The level on the turbidity tube at which the Secchi disc was no longer visible was recorded.

## 2.2 | Water sampling

Water collection was performed over a 10-day period using three capture methods: precipitation, disc filters, and capsules. For each site, all sampling was completed within a single sampling event (1–2 h). Precipitation samples were taken by collecting 15 ml surface water in a sterile 50-ml falcon tube. Immediately after collection, 1.5 ml of sodium acetate 3 M and 33.5 ml of absolute ethanol were added to the 15 ml water aliquots (Ficetola et al., 2008). Disc filters, with a surface area of approximately 17.4 cm<sup>2</sup>, were used together with a 500-ml filtering cup (Nalgene™ Polysulfone Filter Holder with Funnel, Thermo Scientific). The capsules used were Waterra FHT-45 (Waterra USA Inc.) disposable groundwater filters, with a surface area of 600 cm<sup>2</sup>. Both disc filters and capsules had a polyethersulfone hydrophilic membrane and a pore size of 0.45 μm. For capsules and disc filters, water was pumped using a peristaltic pump (Solinst 410, Solinst Canada Ltd.), powered by a car battery. The water was filtered until the filter membrane clogged. The volume filtered with each method was recorded (Table S1), and the filters were stored in sterile bags. At each pond, surface water was collected at two different sampling points, at opposing ends of the pond. At each sampling point, all three methods were employed, giving a total of 18 samples for each method. All the samples were stored at ambient temperature (average of 10°C (IPMA, 2018)) inside a cooler box and transported to the laboratory, within four hours of collection. No storage buffer was added to the filters to minimize contamination levels that can arise from handling liquids in non-sterile environments. Preservation on ice during fieldwork was not considered necessary as temperatures were low, and sampling sites were close to the research facilities. Once in the laboratory, samples were stored at –20°C until DNA extraction as this storage method has shown to improve DNA yields for long-term storage compared to refrigerating (4°C) or storing samples at room temperature (20°C) (Hinlo et al., 2017). Equipment was sterilized between ponds with a 10% dilution of household bleach for at least 30 min and later rinsed with distilled water to remove any bleach residues. Two negative controls were collected at each pond. For the first, 15 ml of distilled water brought from the laboratory was added to a 50-ml falcon tube, along with 1.5 ml of sodium acetate 3 M and 33.5 ml of absolute ethanol. For the second, to ensure that all tubing and other reusable filtering apparatus was clean, 100 ml of distilled water was pumped through a filtering unit with a disc filter.

## 2.3 | DNA extraction and quantification

All DNA extractions were performed in a low-copy DNA laboratory (at CIBIO, Portugal) equipped with UV radiation. Strict protocols

were followed to prevent contamination, including disposable laboratory wear, UV sterilization of all equipment before entering the laboratory and having workbenches and all the equipment needed for extraction cleaned with a 60% dilution of household bleach between extraction batches. Handling and cutting of the filters was performed on disposable aluminum sheets, changed between each filter, using forceps and scissors, which were cleaned with ethanol and flame-sterilized between samples. Additionally, a negative control was included in each batch of extractions ( $n = 6$  batches), containing an average of 12 samples per batch.

Capsules were filled with 100 ml of resuspension buffer (50 mM Tris, 10 mM EDTA), both ends were covered with parafilm, and they were agitated manually for five minutes (e.g., Civade et al., 2016; Lopes et al., 2017). To concentrate the material in the buffer to a volume suitable for downstream extraction, the buffer was then poured into a sterile container and filtered through a sterile 0.45 μm 47 mm disc filter. Disc filters were cut into small pieces and placed into a 15-ml falcon tube with 2 ml n-lauroylsarcosine based buffer (Maudet, Luikart, Dubray, Von Hardenberg, & Taberlet, 2004). DNA extraction of precipitated samples followed the protocol by Ficetola et al. (2008), with minor modifications, where samples were centrifuged at 3184 g for 45 min, at 10°C. The supernatant was discarded, and 2 ml of n-lauroylsarcosine based buffer was added to the tubes. After addition of n-lauroylsarcosine based buffer, the samples from all three capture methods were kept at 54°C for 30 min. From here, the E.Z.N.A.® Tissue DNA kit (Omega Bio-Tek) was used following manufacturer's instructions, but using 300 μl BL Buffer, 300 μl ethanol, and 50 μl Elution Buffer. In the end, the volume of each sample was measured with a micropipette (Eppendorf Research) to more precisely calculate the total mass of DNA (ng) captured, as the volume of the final elution can vary somewhat between extractions.

For capsules, the extraction controls consisted of adding resuspension buffer to a clean unused capsule. Extraction controls for disc filters consisted of a sterile 2 ml Eppendorf with only n-lauroylsarcosine based buffer and no filter, while for precipitation samples, controls consisted of 50-ml falcon tubes with distilled water.

Double-stranded DNA was quantified by fluorometry (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes), following the manufacturer's instructions. Readings were performed three times, and an average was obtained for each sample.

## 2.4 | qPCR

Species-specific primers targeting a small fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene of *S. salamandra* were designed using AlleleID 7 software and available COI sequences from all Portuguese amphibian species, downloaded from the National Center for Biotechnology Information's (NCBI) Nucleotide database. COI was chosen as there were available sequences in GenBank for all species present in northern Portugal, and the high variability of this gene makes the development of single-species assays feasible. Additionally, COI has been selected

as the barcode marker of preference for amphibians (Murphy et al., 2012). Two candidate primer sets were tested in vitro on DNA extracted from *S. salamandra* tissue, as well as DNA at similar concentrations from all nontarget amphibian species known to occur in the study area (*Alytes obstetricans*, *Discoglossus galganoi*, *Pelodytes atlanticus*, *Pelobates cultripes*, *Epidalea calamita*, *Bufo spinosus*, *Lissotriton boscai*, *Lissotriton helveticus*, *Triturus marmoratus*; see Appendix S1). One primer set was found to be more specific and thus was chosen for further optimization: primer forward (Peixoto\_Sal\_2019-F 5'-CACCTTATTCGTATGATCTGTC-3'), primer reverse (Peixoto\_Sal\_2019-R 5'-GTAGTGTTTAGGTTTCGATCTG -3') and probe (Peixoto\_Sal\_2019-PR 5'-/6-FAM/ACCGCAATCCTACTCC TCCTATCTCT/BHQ\_1/-3'). To ensure specificity, the primer selected was tested against further nontarget samples, including one DNA sample from four other vertebrate classes (Reptilia, Actinopterygii, Aves and Mammalia—including human), as well as DNA from a mixed sample of invertebrate species. In addition, eDNA samples collected from ponds where the target species does not occur were also included as negative controls. Finally, to determine optimum PCR conditions, a gradient PCR was conducted (see Appendix S1).

Final qPCR conditions were performed in a total volume of 10  $\mu$ l, including 5  $\mu$ l of TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.6  $\mu$ l (10  $\mu$ M) of each primer, 0.17  $\mu$ l (10  $\mu$ M) of probe, 2.63  $\mu$ L of H<sub>2</sub>O (HPLC grade water), and 1  $\mu$ l of extracted DNA. PCR cycles were as follows: 10 min of denaturation at 95°C, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 60°C for 60 s. Fluorescence was measured at the extension step, after binding of the species-specific probe to the target DNA and cleavage of the fluorophore (5' 6-FAM) by the exonuclease activity of DNA polymerase, generating a positive signal.

To evaluate assay sensitivity and generate a standard curve, a PCR product produced by the Peixoto-Sal-2019 primer pair was gel-extracted and cleaned with the QIAquick<sup>®</sup> gel extraction kit (Qiagen), following the manufacturer's instructions. The purified DNA was quantified on Qubit™ following the manufacturer's instructions, the number of copies was calculated using the software DNA CALCULATOR (Sint, Raso, & Traugott, 2012), and six 10:1 serial dilutions ranging from 3.00E + 06 to 3.00E + 01 copies/ $\mu$ l were generated.

Standards were performed in triplicate and eDNA samples and negative controls in duplicate. A total of three plates were run, giving a total of six negative qPCR controls. Additionally, strict protocols were followed to prevent contamination, such as the use of separate rooms for pre- and post-PCR work, as well as the cleaning of workbenches and pipettes with bleach and ethanol before and after each use.

qPCR reactions were considered positive if a sample's fluorescence intersected the threshold line, and negative otherwise. The threshold was defined as 10 times the fluorescence value of the average standard deviation of baseline cycles (e.g., Barletta, Edelman, & Constantine, 2004; Sails, Fox, Bolton, Wareing, &

Greenway, 2003). To ensure that positive results corresponded to amplification of *S. salamandra*, 25 of the positive eDNA amplicons were randomly selected and sequenced by Sanger sequencing, and resultant sequences were BLASTed (Zhang, Schwartz, Wagner, & Miller, 2000) against the NCBI Nucleotide database to validate species identification.

## 2.5 | High-throughput sequencing

### 2.5.1 | Library preparation

Amplicons for the Illumina MiSeq platform were generated by means of a two-step PCR following the protocol detailed in Egeter et al. (2018), with minor modifications. This protocol uses a primer designed to amplify vertebrates (12SV5.1\_F ACTGGGATTAGATACCCC and 12SV5.1\_R TAGAACAGG CTCCTCTAG; Riaz et al., 2011), targeting the mitochondrial 12S rRNA gene. We first confirmed by PCR that this assay amplified DNA from tissue samples of all amphibians known to occur in the study area (see Appendix S2). PCR reactions for eDNA samples were performed in duplicate and the optimized first PCR conditions were as follows: an initial step of denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 47°C for 30 s, and 72°C for 30 s, followed by a final extension step of 72°C for 10 min. Each sample replicate was performed in separate plates, and a third plate with DNA of two amphibian species not occurring in the area (*Oreolalax omeimontis* and *Glyphoglossus molossus*) was also included to control for tag jumping. A PCR negative control was included in each plate, for a total of three. The resulting PCR product was diluted 1:4 with HPLC grade water and used as input for the second PCR, where sample-specific indexes and Illumina adaptors were incorporated. Different indexes were used for the forward and reverse primer, and a different combination was generated for each sample. To validate the increase in amplicon size, indicating that indexes and adaptors had been incorporated, two random samples per plate were visualized on a 2% agarose gel stained with GelRed (Biotium).

Indexed PCR products were cleaned with 0.9x AMPure XP beads (Beckman Coulter) following the manufacturer's instructions. Cleaned PCR products were quantified by spectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific), normalized to 15 nM using HPLC grade water, and pooled together by combining 5  $\mu$ l of each sample. The quality of the final pool was assessed with Qubit™ (Robin, Ludlow, LaRanger, Wright, & Shay, 2016) following the manufacturer's instructions. Additionally, to validate fragment sizes, amplicons were analyzed in a 2200 TapeStation (Agilent Technologies). The pool was then diluted to 4 nM with Tris 10 mM pH 8.5 and 0.1% Tween, and the concentration was measured once more with Qubit™, in triplicate. The final 10 pM denatured library was mixed with 20% PhiX control, and amplicon libraries were sequenced on a MiSeq Illumina System platform housed at Instituto

Gulbenkian de Ciência, Lisbon, Portugal, using a 500-cycle Illumina MiSeq V2 Kit (Illumina). During library preparation, the same protocols described for qPCR were applied to prevent contamination.

## 2.6 | Bioinformatic pipeline

Reads produced on the MiSeq platform (Illumina) were demultiplexed according to the sample-specific indexes using BASESPACE (basespace.illumina.com). Sequence data were processed using the MBC pipelines package (Galhardo et al. in prep.; commands used are provided in Appendix S3). Within the package, paired-end reads were aligned using flash2 (Magoč & Salzberg, 2011) and primers were removed with cutadapt (Martin, 2011). Sequences were dereplicated, singletons were removed, and sequences outside the expected amplicon lengths (70–130 bp, excluding primers) were removed using vsearch (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). The majority of commands used the default settings. Exceptions to this were (a) as we did not expect overlaps above 100 bp in paired-end reads, we specified `--max-overlap = 100` using flash2 and (b) to filter low-quality reads we applied a maximum expected error of 1 using the vsearch `--fastq_filter` command. The exact sequence variants (ESVs) were mapped against a 12S amphibian database containing all species potentially occurring in the study area (Table S2), using the MEGABLAST algorithm (Zhang et al., 2000), and 100 results per query were kept. Hits with <70% query cover or >0.001 *e*-value were discarded. To discard lower-identity matches, when better matches were found, hits that had a percentage identity that was not within 1% of the top hit were discarded (similar to Piñol, Mir, Gomez-Polo, & Agustí, 2015). ESVs were placed in taxonomic bins using the metabin program in metabinkit (v0.1.0; <https://doi.org/10.5281/zenodo.3855032>; Egeter B. et al., unpublished; see Appendix S3). The procedure used by metabin was, for each taxonomic level, to consider only hits that fall within the sequence identity thresholds (species = 98%, genus = 95%, family = 92%, higher-than-family = 80%). Then for each query, find the lowest common ancestor (LCA) of the considered hits. If the LCA is at species-level, that is the final bin assigned to the ESV. Failing a species-level assignment, binning at genus level is attempted. Failing a genus-level assignment, binning at family level is attempted and failing a family-level assignment, order and above are assigned. We assessed appropriate binning thresholds for the primer set used and the target taxa and confirmed that the binning thresholds applied in the current study would not result in incorrect taxonomic assignments (Appendix S4). To remove potential tag jumping between samples, detections that were <0.1% of the total read count for the respective taxon were removed, as were detections that were <0.1% of the total read count for the respective sample (for further information on tag jumping controls see Appendix S5). To further remove any potentially spurious results, all detection <100 reads were discarded. Only species-level detections were used for amphibian community analyses.

## 2.7 | Statistical data analysis

For both qPCR and HTS data, two thresholds were used for salamander detection, when *S. salamandra* was detected in at least one (relaxed) or both (stringent) PCR replicates of the respective sample. Statistical analyses were performed with R (R Development Core Team, 2008) using linear modeling approaches implemented in the lme4 (v. 1.1-17) package (Bates, Mächler, Bolker, & Walker, 2015) and further assessed using the car (v. 3.0-0) package ANOVA function (Fox & Weisberg, 2011) and emmeans (v. 1.2.3) package emmeans function (Lenth, 2018). Linear mixed-effects models were used to assess the effects of capture method on the volume of water filtered and the mass of eDNA captured, as well as the effects of turbidity and salamander abundance on qPCR copies per liter of water filtered. Generalized linear mixed-effects models were used to assess the effects of capture methods on the detection of *S. salamandra* (with detection as a binary response; logit link and binomial errors) and on total amphibian species richness (as a count response; exponential link and Poisson errors). To account for nonindependence of samples within sites, site was included in all models as a random factor affecting the intercept. For continuous response variables, normality (tested with Shapiro–Wilk test) and homoscedasticity (verified with fitted values versus residuals plots) were assessed for each model and, if necessary, variables were transformed to common logarithm to meet assumptions. To ensure that variation in salamander abundance, pond area or pond depth was not confounding results, they were initially included as predictors in all models that had salamander detection and amphibian detection (only pond area and depth) as a response variable. As these factors did not contribute significantly to any models, they were subsequently excluded from final models. To compare the contributions of capture and laboratory methods to variation in *S. salamandra* detection, PCR replicate was nested within capture method and the mean sum of squares was used to estimate the contribution of each component (Mata et al., 2019). Although other amphibian species were incidentally detected by the HTS approach, their abundances were not measured as they were not the target study species. However, as all three capture methods were conducted at each sampling point, it was possible to assess the effect of capture method on the amphibian community composition. Moreover, overall pond effects associated with variation in nontarget species abundances were controlled by specifying sites as random factors in the final mixed models. To assess the effect of capture method on the amphibian community composition, presence/absence data (binary) were used to construct a distance matrix of Jaccard dissimilarities between eDNA samples using the vegdist function from the vegan (v. 2.5-6) package (Oksanen et al., 2019). The matrix was used as the response variable for a permutational multivariate analysis of variance using distance matrices (PERMANOVA) model with capture method as the main factor, implemented using vegan's adonis function (10,000 permutations, with Site as strata to account for nonindependence between samples from the same Site).

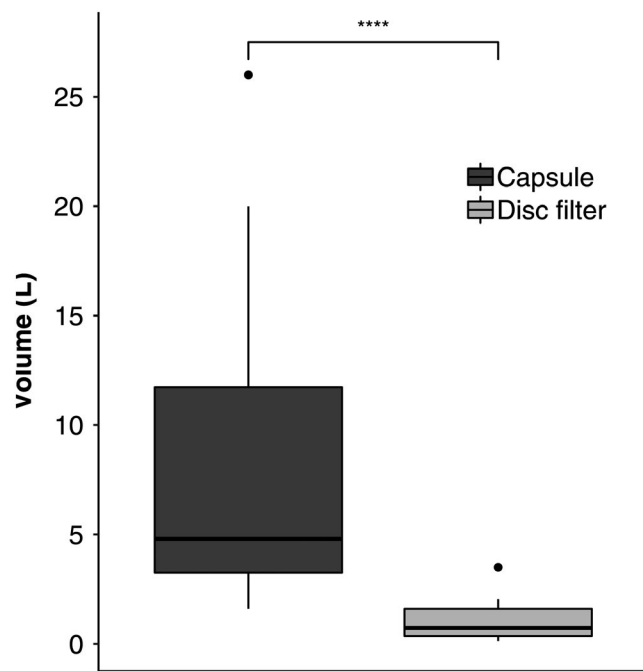
### 3 | RESULTS

A total of 54 water samples, 18 field negatives, six extraction negatives, and three PCR negatives were processed. From all controls, one field negative, from site 3, contained reads assigned to *D. galganoi* ( $n = 1,137$ ), and so detections of this taxon were removed from all samples from this site. Approximately, 2.2 million reads were obtained for the sample set after demultiplexing, of which c. 355,000 were assigned to amphibian species and passed all bioinformatic filters. Information on volume of water filtered and DNA amount is provided in the Supplementary Material (Table S1), as well as the final taxa table with the number of reads assigned to each species (Table S3) and a summary of the read counts at each bioinformatic step (Figure S2).

#### 3.1 | Water sampling and eDNA capture

The volume of water filtered was significantly different ( $p < .0001$ ) between capsules ( $\bar{x} = 7.89$  L,  $SE = 6.79$ ) and disc filters ( $\bar{x} = 1.10$  L,  $SE = 1.03$ ; Figure 1). The volume processed for precipitation was always 0.015 L. Turbidity was negatively correlated with the volume of water filtered for disc filters ( $R^2 = .64$ ;  $p < .0001$ ) but not for capsules ( $R^2 = .35$ ;  $p > .05$ ).

There was a significant difference ( $p < .0001$ ; Figure 2) in the quantity of eDNA captured between capture methods, with capsules capturing more eDNA ( $\bar{x} = 366.61$  ng,  $SE = 261.66$ ) than disc



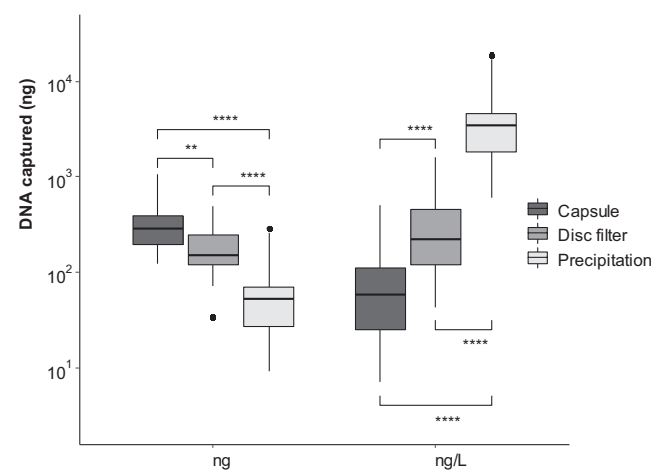
**FIGURE 1** Volume of water filtered using either capsules or disc filters. The three hinges starting from below correspond to the 25th percentile, median, and 75th percentile, respectively, extending to the smallest and highest value recorded. Significance values are represented by stars: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  and \*\*\*\* $p < .0001$

filters ( $\bar{x} = 194.29$  ng,  $SE = 113.74$ ), which in turn captured more than precipitation ( $\bar{x} = 69.91$  ng,  $SE = 74.22$ ). Taking into account the volumes of water processed, there was also a significant difference between capture methods ( $p < .0001$ ; Figure 2) but with the opposite trend, with precipitation capturing more eDNA per L ( $\bar{x} = 4,660.91$  ng/L,  $SE = 4,947.93$ ) than disc filters ( $\bar{x} = 413.54$  ng/L,  $SE = 475.28$ ), which in turn captured more eDNA per L than capsules ( $\bar{x} = 112.32$  ng/L,  $SE = 144.59$ ).

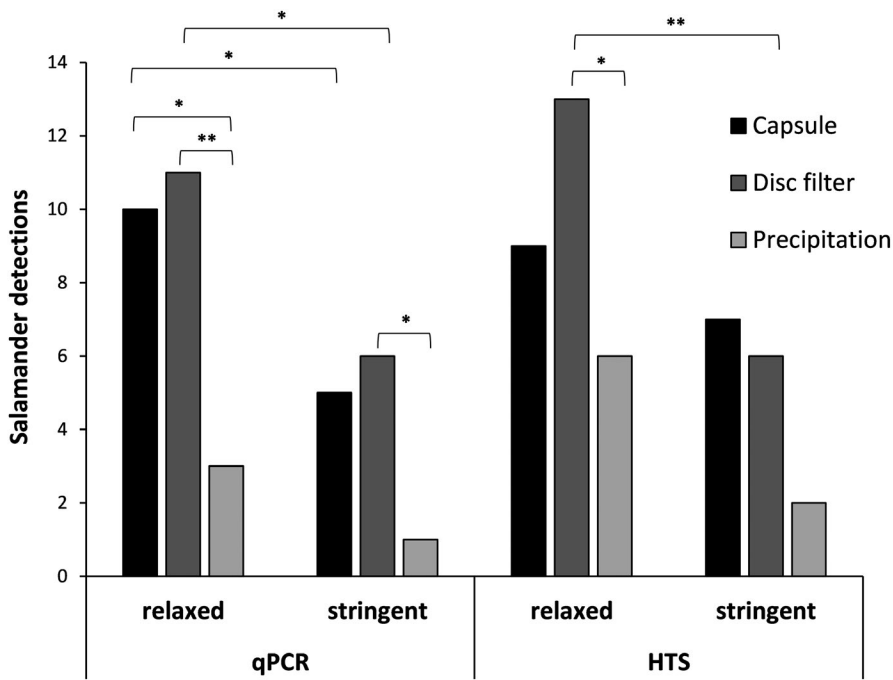
#### 3.2 | *Salamandra salamandra* detection

The final qPCR assays exhibited 92%–100% efficiency,  $R^2$  between .993–.998 and slope between  $-3.33$  and  $-3.53$  (Figure S1). Information on Cq-values and copies/ $\mu$ l for each sample is provided in the Supplementary Material (Table S4). All 25 randomly selected qPCR positives sequenced by Sanger sequencing were identified as *S. salamandra* (97%–100% matches, with no other Portuguese amphibian species occurring in the first 100 hits provided by BLAST), confirming the specificity of the primers developed. Using the HTS approach, of the c. 355,000 reads in the final taxa table, almost 105,000 were assigned to *S. salamandra*.

The HTS approach generally resulted in more salamander detections than the qPCR assay, but not significantly so (Figure 3). Precipitation consistently provided the lowest numbers of *S. salamandra* detections, while capsules and disc filters showed similar results (Figure 3). PCR replicability was similar using either the qPCR or the HTS methods, as indicated by the similar values for the mean sum of squares for the nested fixed effect of Method:PCR\_replicate (Table 2). The variation in detection success associated with the choice of capture method was over 10 times higher than that associated with PCR replication, regardless of the detection method used (Table 2). As expected, applying the more stringent PCR replication



**FIGURE 2** Amount of eDNA captured for each capture method: (a) Total mass and (b) mass per L of water sampled. The three hinges starting from below correspond to the 25th percentile, median, and 75th percentile, respectively, extending to the smallest and highest value recorded. Significance values are represented by stars: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  and \*\*\*\* $p < .0001$



**FIGURE 3** Number of samples in which *S. salamandra* was detected by each capture method and with each detection method and PCR replication threshold ( $n = 18$  in all cases). Significance values are represented by stars: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  and \*\*\*\* $p < .0001$

**TABLE 2** Variation estimated from a linear mixed-effects model in *S. salamandra* detection success associated with capture method and PCR replicate using either the qPCR or HTS approaches. PCR replicate was nested within capture method

Nested fixed effects	Degrees of freedom	Sum of squares	Mean sum of squares	$p$ -value
qPCR				
Method	2	2.72	1.35	<.01
Method:PCR_replicate	3	0.16	0.05	>.05
Residuals	102	21.11	0.21	
HTS				
Method	2	1.80	0.90	<.05
Method:PCR_replicate	3	0.25	0.08	>.05
Residuals	102	23.83	0.23	

threshold consistently resulted in fewer *S. salamandra* detections, although not always to a statistically significant degree (Figure 3).

For capsules, the number of reads assigned to *S. salamandra* was positively correlated with the volume filtered: for each increase of 1 L filtered, reads increases 358 ( $R^2 = .47$ ,  $p < .01$ ); while for disc filters no such trend was evident. Additionally, we did not observe a significant relationship between turbidity and salamander abundance with the total qPCR copies obtained per liter of water filtered.

### 3.3 | Amphibian community composition

Overall, there was a total of 49 detections (the sum of the number of species detected in all field samples) using the stringent replication

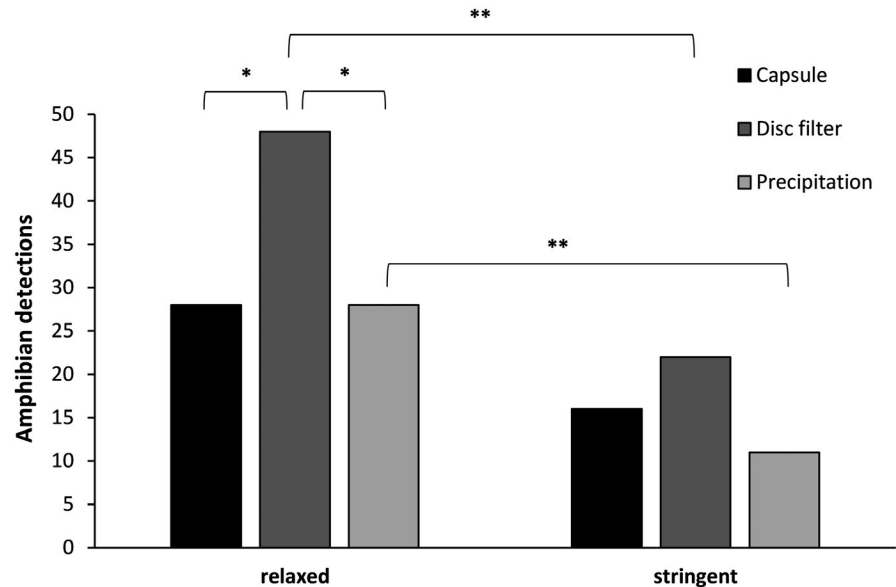
threshold and a total of 104 detections using the relaxed replication threshold. Using the relaxed replication threshold, disc filters resulted in significantly higher amphibian detections than the other two capture methods ( $p < .05$ ; Figure 4). However, there was no significant difference between capture methods when using the stringent replication threshold, although disc filters still resulted in highest detection levels (Figure 4; Table 3). Using the relaxed replication threshold, the total number of amphibian detections was significantly higher than for the stringent replication threshold for both disc filters ( $p < .01$ ) and precipitation ( $p < .01$ ) (Figure 4), but not for capsules. There were no significant differences in amphibian community composition estimated by the three capture methods ( $MS = 0.35$ ,  $F_{Model} = 1.37$ ,  $R^2 = .04$ ,  $p = .33$ ; Figure 5). This was true regardless of modeling the results of single or combined PCR replicates (data only shown for combined).

## 4 | DISCUSSION

In the present study, filtration techniques outperformed precipitation, generating a higher number of detections of *S. salamandra* and captured eDNA, while species detection was identical between disc filters and capsules. However, amphibian community characterization (i.e., species richness and composition) was not significantly affected by the choice of capture method. Overall, *S. salamandra* detection was similar with both qPCR and HTS. Relaxed PCR replication threshold consistently generated higher detection levels than the stringent replication threshold, although differences were not always statistically significant. It is unlikely that these key results were affected by methodological biases or artifacts, namely eventual problems associated with temporal or spatial variations in sampling conditions. For instance, while seasonal variation in eDNA



**FIGURE 4** Number of amphibian detections (the sum of the number of species detected in all field samples), using each capture method and with each PCR replication threshold ( $n = 104$  and  $49$  for the relaxed and stringent PCR replication thresholds, respectively). Significance values are represented by stars:  $*p < .05$ ,  $**p < .01$ ,  $***p < .001$  and  $****p < .0001$



**TABLE 3** Percentage (%) of ponds ( $n = 9$ )/field samples ( $n = 18$  for each capture method) in which each species was detected by each capture method and with each PCR replication threshold. Species are sorted alphabetically

	Capsule		Disc filter		Precipitation	
	Relaxed	Stringent	Relaxed	Stringent	Relaxed	Stringent
<i>Alytes obstetricans</i>	0/0	0/0	11/06	0/0	0/0	0/0
<i>Bufo spinosus</i> <sup>a</sup>	0/0	0/0	0/0	0/0	0/0	0/0
<i>Discoglossus galganoi</i>	33/22	11/11	44/39	33/28	22/22	22/22
<i>Epidalea calamita</i>	22/11	11/06	56/39	33/22	44/39	11/06
<i>Lissotriton boscai</i>	11/11	11/11	11/11	11/11	11/06	11/06
<i>Lissotriton helveticus</i>	44/28	11/11	78/50	22/17	33/28	11/06
<i>Pelobates cultripes</i>	0/0	0/0	0/0	0/0	11/06	0/0
<i>Pelodytes atlanticus</i>	22/17	22/11	33/28	11/06	11/11	11/11
<i>Salamandra salamandra</i>	56/50	56/39	89/72	44/33	44/33	22/11
<i>Triturus marmoratus</i>	22/17	0/0	33/22	11/06	22/11	0/06

<sup>a</sup>*Bufo spinosus* was initially detected but it did not pass the bioinformatic filters applied.

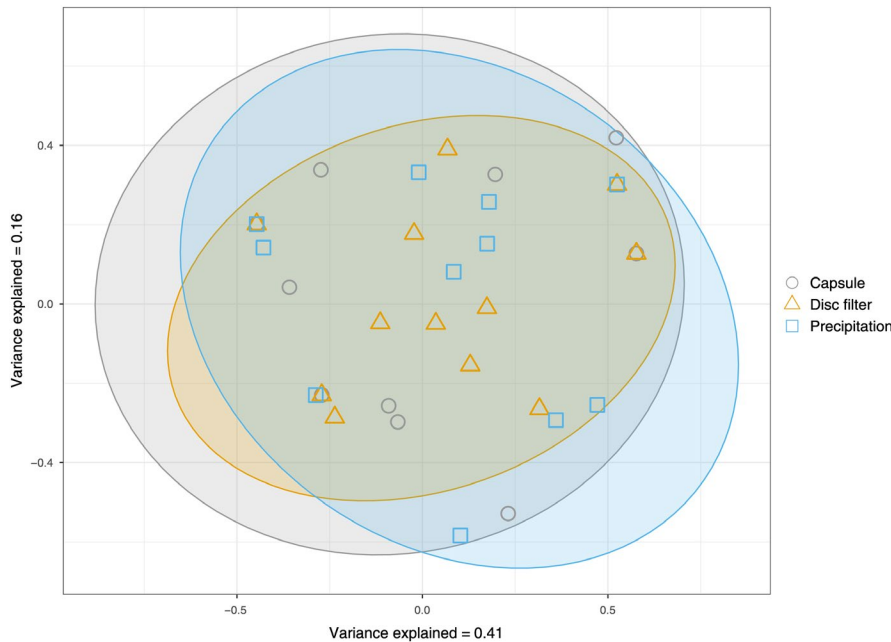
concentration has previously been observed in freshwater environments (de Souza, Godwin, Renshaw, & Larson, 2016), this is unlikely to have affected our results because our study was performed over a 10-day sampling period. Also, it is unlikely that spatial variations affected the results given that the three capture methods were employed at each sampling point on the same sampling occasion, and differences among ponds in environmental conditions and eDNA concentrations were controlled statistically through our mixed-model approach.

#### 4.1 | Filtration and precipitation capture methods

Filtration and precipitation are currently the two main approaches to capture eDNA in aquatic ecosystems (Herder et al., 2014; Hinlo et al., 2017; Li, Handley, Read, & Hänfling, 2018). Filtration is more common with disc filters, while capsules have only recently been

applied in eDNA studies (e.g., Civade et al., 2016; Lopes et al., 2017). In the present study, the choice of capture method influenced eDNA recovery and species detection, with filtration methods capturing more eDNA and detecting the target species in a higher number of samples than precipitation. Previous studies in aquatic environments have reported similar results, where precipitation resulted in lower detection rates than filtration (Eichmiller, Miller, & Sorensen, 2016; Hinlo et al., 2017; Piggott, 2016; Spens et al., 2017). The higher amounts of eDNA captured and species detection observed for filtration methods were likely associated with their higher sample volumes (Raemy & Ursenbacher, 2018).

Previous research has demonstrated that filter attributes such as pore size and membrane material can influence eDNA recoveries and detection rates (e.g., Deiner et al., 2018; Djurhuus et al., 2017; Jeunen et al., 2019). The present study did not include a comparison of pore sizes or membrane types, but as both disc filters and capsules had a polyethersulfone hydrophilic membrane and a pore size



**FIGURE 5** Principal coordinate analysis plot of amphibian community dissimilarity of eDNA samples in the present study, using Jaccard distances, based on presence/absence data. Ellipses are drawn with a confidence level of 0.9.

of 0.45  $\mu\text{m}$ , it allowed for a direct comparison of the two filtration methods used. Within these, both the volume of water filtered and the amount of eDNA captured were significantly higher with capsules than disc filters. In a similar study, Spens et al. (2017) reported higher eDNA concentrations and lower Cq-values for low-capacity capsules when compared to disc filters. The higher performance of capsules in our study was likely due to their larger surface area (almost 35 times the surface area of disc filters). This feature can also help explain why turbidity was negatively correlated with the volume of water filtered for disc filters but not for capsules, which do not clog as easily. Previous studies have demonstrated the capacity of filtering large volumes with this type of filters in natural systems, such as 20 L (Vences et al., 2016), 45 L (Civade et al., 2016) and even 100 L (Valentini et al., 2016). As more water is sampled, the chances of eDNA fragments being captured increases, thus explaining the higher amounts of eDNA recovered for capsules. Nevertheless, *S. salamandra* detection and amphibian community composition was similar between both filtration methods and did not reflect the higher performance of capsules regarding volume and eDNA recovered. Even though disc filters provided a higher number of amphibian detection events, amphibian community composition was identical for both filtration methods. The most likely explanation is that, given the relatively small pond sizes utilized in this study, amphibian eDNA was sufficiently abundant to be detected despite filtering lower volumes with disc filters. Further studies comparing disc filters and capsules across other taxonomic groups and study systems would provide a better understanding of the efficiency of each filtration method.

Filtering higher water volumes may not always be advantageous as it might increase the concentration of inhibitors in the sample (Herder et al., 2014), usually abundant in turbid waters, constraining downstream laboratory procedures. However, we did not observe any obvious PCR inhibition in the capsule-derived eDNA samples.

The percentage of PCRs that successfully amplified was identical between capsules and precipitation samples, and both were higher than for disc filters. This indicated that inhibition was not occurring to a greater degree in capsule-derived PCRs, compared to the other capture methods. Also, we did not normalize DNA concentrations prior to the first PCRs. It might be expected that, by capturing more eDNA, the concentration of nonamphibian eDNA increases in the extracted DNA elution, which could lead to a higher proportion of nonamphibian eDNA being amplified. However, the percentage of nonamphibian reads was lower for capsules (median = 36%) than for disc filters (median = 50%), so this does not help to explain the results. Overall, capsules may be more appropriate for running waters or larger water bodies, where eDNA is more diluted (Herder et al., 2014) and filtering larger volumes can increase species detection (Lopes et al., 2017), whereas disc filters might be more suitable for smaller stagnant water bodies, where eDNA is less diluted (Herder et al., 2014). Disc filters may also prove to be more cost-effective in many situations, particularly when funds are limited, as the capsules used in our study cost c. €25 at the time of writing, while disc filters cost less than €1. One benefit of using capsules over disc filters is that they require less handling in the field, which may decrease the risk of contamination. A further alternative not tested in this study is the use of Sterivex capsules (e.g., Raemy & Ursenbacher, 2018; Sigsgaard et al., 2017). Even though their surface area is smaller than the ones compared here, they have shown to outperform disc filters (Spens et al., 2017) and are usually cheaper than high-capacity capsules.

Previous protocols using capsules have also used 5 min agitation, but followed by a centrifugation at 15,000 g of 50-ml tubes (e.g., Civade et al., 2016; Lopes et al., 2017). As not all laboratories have centrifuge equipment for these larger volumes at high speeds, we concentrated the material captured in each capsule by filtering it through a standard 47 mm disc filter. While some eDNA may have

been lost at this step, far more was still captured using this method than by filtering in the field directly through a standard disc filter.

## 4.2 | qPCR versus. HTS

Species detection with eDNA methods can be accomplished with either a single species or a multi-species approach. Single-species detection is generally used for endangered (e.g., Piggott, 2016) or invasive species (e.g., Hunter et al., 2015), where the knowledge on species distribution gained from eDNA analyses can aid the development of appropriate management measures. In this study, salamander detection was similar with both qPCR and HTS. Few studies so far have compared the efficiency between both detection methods for a target species. Murray et al. (2011) demonstrated that qPCR and HTS approaches displayed very similar results when attempting to detect four prey species from penguin scats. In contrast, a more recent eDNA study using water samples has demonstrated a higher sensitivity of qPCR for the detection of a turtle species when compared to HTS (Lacoursière-Roussel et al., 2016). According to the authors, the differences between the performance of detection methods might be explained by the amplicon size of the primers used for each method (Lacoursière-Roussel et al., 2016; Murray et al., 2011). The fragment sizes used in the present study were very similar, differing by only 7 bp (qPCR target fragment: 112 bp; HTS target fragment: 105 bp), which could help explain the similar results observed between both methods. However, different primers and target regions were used for the two detection methods (COI gene for qPCR and 12S rRNA gene for HTS), which is likely to affect detection success. Therefore, the contributions of the effect of the target regions cannot be assessed in the current study. We did not attempt to design new metabarcoding primers for the COI region (used for the qPCR assay) as the 12SV5.1 primer pair was effective on the tissue samples and has been used successfully for vertebrates in a number of previous studies (Kelly, Port, Yamahara, & Crowder, 2014; Port et al., 2016; Rodgers et al., 2017). Previous studies have also successfully applied qPCR methods for detecting *S. salamandra* (Preißler, Watzal, Vences, & Steinartz, 2018). While HTS is often more advantageous and cost-efficient to detect multiple species (Thomsen & Willerslev, 2015), single-species detection with qPCR is generally cheaper (Harper et al., 2018) and less time-consuming. Additionally, HTS approaches add a level of complexity to data analyses due to the bioinformatic filtering steps required to remove sequence reads that might originate from sequencing errors or contamination (Thomsen & Willerslev, 2015).

## 4.3 | PCR replication threshold

An additional source of variation in eDNA research lies on the thresholds applied to the data. The use of strict filtering to reduce false positives likely reduces detection rates and thus inflates false negatives. The opposite is true for relaxed thresholds, reducing false

negatives but generally at the expenses of increasing false positives and overestimating the presence of a species. The results of the present study indicate that there is much higher variability introduced from the choice of capture method than from PCR replication threshold. There were, however, differences in detection events between the thresholds used, particularly for disc filters, which suggests a higher stochasticity in PCR replicates for this capture method (Figure 4). PCRs from capsules were more replicable, displaying overall less disparity between stringent (species detected in both replicates) and relaxed (species detected in either replicate) PCR replication thresholds for HTS, most likely due to having a greater starting mass of eDNA. Similar to our results, a recent study using water samples and both qPCR and HTS approaches for the detection of the great crested newt has demonstrated that stringent thresholds reduce detection levels of a target species (Harper et al., 2018). To enhance the reliability of a study, a balance between false positives and negatives is required, highlighting the importance of careful consideration of the most suitable threshold to apply when inferring species presence-absence, especially for endangered or protected species. According to Ficetola et al. (2015), site occupancy models can be a useful tool to estimate error rates regarding species detection and determine taxon-specific thresholds by adjusting the minimum number of replicates required to consider the presence of a species, therefore increasing confidence in the results.

## 5 | CONCLUSIONS

To the best of our knowledge, this is the first study to compare high-capacity capsules with common eDNA methods, such as precipitation and filtration with standard disc filters, highlighting the importance of choosing a suitable capture method for eDNA studies. The results indicate that the use of either disc filters or high-capacity capsules outperforms precipitation, but no major differences were found between filtration methods. Based on species detections, we cannot recommend the use of capsules over standard filters. However, as capsules filter more water and capture more eDNA, their application may be beneficial in other field situations, such as detecting low abundance species in larger or fast-flowing water bodies. The results suggest that, if eDNA assays are well-designed, the choice of capture method outweighs the choice of laboratory detection method used. However, PCR replication thresholds applied also affect the reliability of results. Identifying the best capture method is essential for accurate biodiversity surveys using eDNA techniques, and further research with larger sample sizes and a multi-taxon approach would provide a better understanding of the efficiency of each capture method, particularly relevant for capsules and disc filters.

## ACKNOWLEDGEMENTS

We thank Filipa Martins, Joana Veríssimo, Joana Pinto and Sandra Afonso that kindly provided lab advice, and Pamela Puppo for help with the fieldwork. We thank Mafalda Galhardo for guidance with implementing the MBC pipelines. This project has received funding

from the European Union's Horizon 2020 research and innovation programme under grant agreement No 668981. G.V.A. is supported by FCT – Foundation for Science and Technology (IF/01425/2014). This work has been supported by the European project BIOPOLIS - Teaming to Upgrade to Excellence in Environmental Biology, Ecosystem Research and AgroBiodiversity, Grant Agreement Number 857251.

## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

S.P., G.V.A., B.E. and P.B. designed the study, S.P. collected all samples with help from G.V.A. and B.E. and S.P. performed all laboratory work with help from C.C. to prepare the sequencing run. Data analysis were conducted by S.P., with help from B.E., and the manuscript was written by S.P. with inputs from all authors.

## DATA AVAILABILITY STATEMENT

All demultiplexed fastq files generated by the Illumina Miseq sequencer, and all final fasta sequences for the sanger-sequenced amphibian tissue samples, are available on ENA, Project Accession PRJEB35424, along with all relevant sample metadata. The final taxa table used for statistical analysis is available on BioStudies (Accession No. S-BSST308) as is a summary of the number of reads at each bioinformatic step (see also Figure S2).

## ORCID

Sara Peixoto  <https://orcid.org/0000-0003-1558-1613>

Guillermo Velo-Antón  <https://orcid.org/0000-0002-9483-5695>

Pedro Beja  <https://orcid.org/0000-0001-8164-0760>

## REFERENCES

- Alarcón-Ríos, L., Niecieza, A. G., Kaliontzopoulou, A., Buckley, D., & Velo-Antón, G. (2020). Evolutionary history and not heterochronic modifications associated with viviparity drive head shape differentiation in a reproductive polymorphic species, *Salamandra salamandra*. *Evolutionary Biology*, 47(1), 43–55. <https://doi.org/10.1007/s11692-019-09489-3>
- Alberdi, A., Aizpuru, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, 9(1), 134–147. <https://doi.org/10.1111/2041-210X.12849>
- Allali, I., Arnold, J. W., Roach, J., Cadenas, M. B., Butz, N., Hassan, H. M., ... Azcarate-Peril, M. A. (2017). A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. *BMC Microbiology*, 17(1), 194. <https://doi.org/10.1186/s12866-017-1101-8>
- Anderson, P., & Davic, R. D. (2004). Use of transparency tubes for rapid assessment of total suspended solids and turbidity in streams. *Lakes and Reservoir Management*, 20(2), 110–120. <https://doi.org/10.1080/07438140409354355>
- Bálint, M., Nowak, C., Márton, O., & Pauls, S. U. (2017). Twenty-five species of frogs in a liter of water: eDNA survey for exploring tropical frog diversity. *BioRxiv*, 176065.
- Barletta, J. M., Edelman, D. C., & Constantine, N. T. (2004). Lowering the detection limits of HIV-1 viral load using real-time immuno-PCR for HIV-1 p24 antigen. *American Journal of Clinical Pathology*, 122(1), 20–27. <https://doi.org/10.1309/529T2WDNEB6X8VUN>
- Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., & Lodge, D. M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science and Technology*, 48(3), 1819–1827. <https://doi.org/10.1021/es404734p>
- Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1–48.
- Bishop, P. J., Angulo, A., Lewis, J. P., Moore, R. D., Rabb, G. B., & Moreno, G. J. (2012). The amphibian extinction crisis – What will it take to put the action into the Amphibian Conservation Action Plan? *SAPIENS. Surveys and Perspectives Integrating Environment and Society*, 5(2), 97–111.
- Caldwell, J. M., Raley, M. E., & Levine, J. F. (2007). Mitochondrial multiplex real-time PCR as a source tracking method in fecal-contaminated effluents. *Environmental Science & Technology*, 41(9), 3277–3283. <https://doi.org/10.1021/es062912s>
- Civade, R., Dejean, T., Valentini, A., Roset, N., Raymond, J.-C., Bonin, A., ... Pont, D. (2016). Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in a natural freshwater system. *PLoS One*, 11(6), e0157366. <https://doi.org/10.1371/journal.pone.0157366>
- de Souza, L. S., Godwin, J. C., Renshaw, M. A., & Larson, E. (2016). Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS One*, 11(10), e0165273. <https://doi.org/10.1371/journal.pone.0165273>
- Deagle, B. E., Thomas, A. C., Shaffer, A. K., Trites, A. W., & Jarman, S. N. (2013). Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: Which counts count? *Molecular Ecology Resources*, 13(4), 620–633. <https://doi.org/10.1111/1755-0998.12103>
- Deiner, K., Lopez, J., Bourne, S., Holman, L., Seymour, M., Grey, E. K., ... Lodge, D. M. (2018). Optimising the detection of marine taxonomic richness using environmental DNA metabarcoding: The effects of filter material, pore size and extraction method. *Metabarcoding and Metagenomics*, 2, e28963. <https://doi.org/10.3897/mbmg.2.28963>
- Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., & Miaud, C. (2012). Improved detection of an alien invasive species through environmental DNA barcoding: The example of the American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology*, 49(4), 953–959.
- Dickie, I. A., Boyer, S., Buckley, H. L., Duncan, R. P., Gardner, P. P., Hogg, I. D., ... Weaver, L. (2018). Towards robust and repeatable sampling methods in eDNA-based studies. *Molecular Ecology Resources*, 18(5), 940–952. <https://doi.org/10.1111/1755-0998.12907>
- Djurhuus, A., Port, J., Closek, C. J., Yamahara, K. M., Romero-Maraccini, O., Walz, K. R., ... Chavez, F. P. (2017). Evaluation of filtration and DNA extraction methods for environmental DNA biodiversity assessments across multiple trophic levels. *Frontiers in Marine Science*, 4, 314. <https://doi.org/10.3389/fmars.2017.00314>
- Egeter, B., Peixoto, S., Brito, J. C., Jarman, S., Puppo, P., & Velo-Antón, G. (2018). Challenges for assessing vertebrate diversity in turbid Saharan water-bodies using environmental DNA. *Genome*, 61(11), 807–814. <https://doi.org/10.1139/gen-2018-0071>
- Eichmiller, J. J., Miller, L. M., & Sorensen, P. W. (2016). Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. *Molecular Ecology Resources*, 16(1), 56–68. <https://doi.org/10.1111/1755-0998.12421>
- Ferreira, M., & Beja, P. (2013). Mediterranean amphibians and the loss of temporary ponds: Are there alternative breeding habitats? *Biological Conservation*, 165, 179–186. <https://doi.org/10.1016/j.biocon.2013.05.029>
- Ficetola, G. F. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, 4(4), 423–425. <https://doi.org/10.1098/rsbl.2008.0118>

- Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguët-Covex, C., De Barba, M., ... Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, 15(3), 543–556. <https://doi.org/10.1111/1755-0998.12338>
- Fox, J., & Weisberg, S. (2011). *An R companion to applied regression* (2nd ed.). Thousand Oaks CA: Sage.
- Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., ... Gilbert, M. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, 7(11), 1299–1307.
- Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., ... Hänfling, B. (2018). Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution*, 8(12), 6330–6341.
- Harris, D. J. (2003). Can you bank on GenBank? *Trends in Ecology and Evolution*, 18(7), 317–319. [https://doi.org/10.1016/S0169-5347\(03\)00150-2](https://doi.org/10.1016/S0169-5347(03)00150-2)
- Herder, J., Valentini, A., Bellemain, E., Dejean, T., van Delft, J., Thomsen, P. F., & Taberlet, P. (2014). *Environmental DNA - a review of the possible applications for the detection of (invasive) species*. Stichting RAVON, Nijmegen. Report 2013–104.
- Heyer, W. R., Donnelly, M. A., McDiarmid, R. W., Hayek, L.-A.-C., & Foster, M. S. (1994). *Measuring and monitoring biological diversity: Standard methods for amphibians*. Washington, DC: Smithsonian Institution.
- Hinlo, R., Gleeson, D., Lintermans, M., & Furlan, E. (2017). Methods to maximise recovery of environmental DNA from water samples. *PLoS One*, 12(6), e0179251. <https://doi.org/10.1371/journal.pone.0179251>
- Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C. T., ... Hart, K. M. (2015). Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PLoS One*, 10(4), e0121655. <https://doi.org/10.1371/journal.pone.0121655>
- IPMA (2018). *Boletim climatológico mensal - Março de 2018 de Portugal Continental*. ISSN 2183–1076.
- Jackson, L. J., & Moquin, P. A. (2011). Turbidity of shallow prairie lakes. *Lakeline*, 31, 36–40.
- Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, 4(2), 150–157. <https://doi.org/10.1111/j.1755-263X.2010.00158.x>
- Jeunen, G.-J., Knapp, M., Spencer, H. G., Taylor, H. R., Lamare, M. D., Stat, M., ... Gemmill, N. J. (2019). Species-level biodiversity assessment using marine environmental DNA metabarcoding requires protocol optimization and standardization. *Ecology and Evolution*, 9(3), 1323–1335. <https://doi.org/10.1002/ece3.4843>
- Kelly, R. P., Port, J. A., Yamahara, K. M., & Crowder, L. B. (2014). Using environmental DNA to census marine fishes in a large mesocosm. *PLoS One*, 9(1), e86175. <https://doi.org/10.1371/journal.pone.0086175>
- Knutson, M. G., Richardson, W. B., Reineke, D. M., Gray, B. R., Parmelee, J. R., & Weick, S. E. (2004). Agricultural ponds support amphibian populations. *Ecological Applications*, 14(3), 669–684. <https://doi.org/10.1890/02-5305>
- Lacoursière-Roussel, A., Dubois, Y., Normandeau, E., & Bernatchez, L. (2016). Improving herpetological surveys in eastern North America using the environmental DNA method. *Genome*, 59(11), 991–1007. <https://doi.org/10.1139/gen-2015-0218>
- Lenth, R. (2018). *emmeans: Estimated marginal means, aka least-squares means*. R package version 1.2.3. Retrieved from <https://CRAN.R-project.org/package=emmeans>
- Leray, M., Knowlton, N., Ho, S.-L., Nguyen, B. N., & Machida, R. J. (2019). GenBank is a reliable resource for 21st century biodiversity research. *Proceedings of the National Academy of Sciences of the United States of America*, 116(45), 22651–22656. <https://doi.org/10.1073/pnas.1911174116>
- Li, J., Handley, L. J. L., Read, D. S., & Hänfling, B. (2018). The effect of filtration method on the efficiency of environmental DNA capture and quantification via metabarcoding. *Molecular Ecology Resources*, 18(5), 1102–1114. <https://doi.org/10.1111/1755-0998.12899>
- Lobos, G., Cattán, P., Estades, C., & Jaksic, F. M. (2013). Invasive African clawed frog *Xenopus laevis* in southern South America: Key factors and predictions. *Studies on Neotropical Fauna and Environment*, 48(1), 1–12.
- Lopes, C. M., Sasso, T., Valentini, A., Dejean, T., Martins, M., Zamudio, K. R., & Haddad, C. F. B. (2017). eDNA metabarcoding: A promising method for anuran surveys in highly diverse tropical forests. *Molecular Ecology Resources*, 17(5), 904–914. <https://doi.org/10.1111/1755-0998.12643>
- Magoč, T., & Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27(21), 2957–2963. <https://doi.org/10.1093/bioinformatics/btr507>
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *Embnet journal*, 17(1), 10–12. <https://doi.org/10.14806/ej.17.1.200>
- Mata, V. A., Rebelo, H., Amorim, F., Beja, P., Mccracken, G. F., & Jarman, S. (2019). How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis. *Molecular Ecology*, 28(2), 165–175. <https://doi.org/10.1111/mec.14779>
- Maudet, C., Luikart, G., Dubray, D., Von Hardenberg, A., & Taberlet, P. (2004). Low genotyping error rates in wild ungulate faeces sampled in winter. *Molecular Ecology Notes*, 4(4), 772–775. <https://doi.org/10.1111/j.1471-8286.2004.00787.x>
- Murphy, R. W., Crawford, A. J., Bauer, A. M., Che, J., Donnellan, S. C., Fritz, U., ... Zhang, Y.-P. (2012). Cold Code: The global initiative to DNA barcode amphibians and nonavian reptiles. *Molecular Ecology Resources*, 13(2), 161–167. <https://doi.org/10.1111/1755-0998.12050>
- Murray, D. C., Bunce, M., Cannell, B. L., Oliver, R., Houston, J., White, N. E., ... Haile, J. (2011). DNA-based faecal dietary analysis: A comparison of qPCR and high throughput sequencing approaches. *PLoS One*, 6(10), e25776. <https://doi.org/10.1371/journal.pone.0025776>
- Myre, E., & Shaw, R. (2006). *The turbidity tube: Simple and accurate measurement of turbidity in the field*. Houghton, Michigan: Michigan Technological University.
- Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlenn, D., Wagner, H. (2019). *vegan: Community Ecology Package*.
- Piggott, M. P. (2016). Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. *Ecology and Evolution*, 6(9), 2739–2750. <https://doi.org/10.1002/ece3.2083>
- Piñol, J., Mir, G., Gomez-Polo, P., & Agustí, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, 15(4), 819–830. <https://doi.org/10.1111/1755-0998.12355>
- Port, J. A., O'Donnell, J. L., Romero-Maraccini, O. C., Leary, P. R., Litvin, S. Y., Nickols, K. J., ... Kelly, R. P. (2016). Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. *Molecular Ecology*, 25(2), 527–541. <https://doi.org/10.1111/mec.13481>
- Preißler, K., Watzal, A. D., Vences, M., & Steinfartz, S. (2018). Detection of elusive fire salamander larvae (*Salamandra salamandra*) in streams via environmental DNA. *Amphibia-Reptilia*, 40(1), 50–64.
- R Development Core Team (2008). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <http://www.R-project.org/>
- Raemy, M., & Ursenbacher, S. (2018). Detection of the European pond turtle (*Emys orbicularis*) by environmental DNA: Is eDNA adequate for reptiles? *Amphibia Reptilia*, 39(2), 135–143.

- Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). EcoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*, *39*(21), e145. <https://doi.org/10.1093/nar/gkr732>
- Robin, J. D., Ludlow, A. T., LaRanger, R., Wright, W. E., & Shay, J. W. (2016). Comparison of DNA quantification methods for next generation sequencing. *Scientific Reports*, *6*, 24067. <https://doi.org/10.1038/srep24067>
- Rodgers, T. W., Xu, C. C. Y., Giacalone, J., Kapheim, K. M., Saltonstall, K., Vargas, M., ... Jansen, P. A. (2017). Carrion fly-derived DNA metabarcoding is an effective tool for mammal surveys: Evidence from a known tropical mammal community. *Molecular Ecology Resources*, *17*(6), e133–e145. <https://doi.org/10.1111/1755-0998.12701>
- Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ Preprints*, *4*, e2409v1. <https://doi.org/10.7717/peerj.2584>
- Sails, A. D., Fox, A. J., Bolton, F. J., Wareing, D. R. A., & Greenway, D. L. A. (2003). A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. *Applied and Environmental Microbiology*, *69*(3), 1383–1390. <https://doi.org/10.1128/AEM.69.3.1383-1390.2003>
- Schmutzer, A. C., Gray, M. J., Burton, E. C., & Miller, D. L. (2008). Impacts of cattle on amphibian larvae and the aquatic environment. *Freshwater Biology*, *53*(12), 2613–2625. <https://doi.org/10.1111/j.1365-2427.2008.02072.x>
- Sigsgaard, E. E., Nielsen, I. B., Carl, H., Krag, M. A., Knudsen, S. W., Xing, Y., ... Thomsen, P. F. (2017). Seawater environmental DNA reflects seasonality of a coastal fish community. *Marine Biology*, *164*(6), 128. <https://doi.org/10.1007/s00227-017-3147-4>
- Sint, D., Raso, L., & Traugott, M. (2012). Advances in multiplex PCR: Balancing primer efficiencies and improving detection success. *Methods in Ecology and Evolution*, *3*(5), 898–905. <https://doi.org/10.1111/j.2041-210X.2012.00215.x>
- Smart, A. S., Tingley, R., Weeks, A. R., Van Rooyen, A. R., & McCarthy, M. A. (2015). Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications*, *25*(7), 1944–1952. <https://doi.org/10.1890/14-1751.1>
- Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S. T., ... Hellström, M. (2017). Comparison of capture and storage methods for aqueous microbial eDNA using an optimized extraction protocol: Advantage of enclosed filter. *Methods in Ecology and Evolution*, *8*(5), 635–645.
- Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., ... Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. <https://doi.org/10.1111/j.1365-294X.2011.05418.x>
- Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18. <https://doi.org/10.1016/j.biocon.2014.11.019>
- Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., ... Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, *25*(4), 929–942. <https://doi.org/10.1111/mec.13428>
- Velo-Antón, G., Santos, X., Sanmartín-Villar, I., Cordero-Rivera, A., & Buckley, D. (2015). Intraspecific variation in clutch size and maternal investment in pueriparous and larviparous *Salamandra salamandra* females. *Evolutionary Ecology*, *29*(1), 185–204. <https://doi.org/10.1007/s10682-014-9720-0>
- Vences, M., Lyra, M. L., Perl, R. G. B., Bletz, M. C., Stanković, D., Lopes, C. M., ... Steinfartz, S. (2016). Freshwater vertebrate metabarcoding on Illumina platforms using double-indexed primers of the mitochondrial 16S rRNA gene. *Conservation Genetics Resources*, *8*(3), 323–327. <https://doi.org/10.1007/s12686-016-0550-y>
- Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 11466–11473. <https://doi.org/10.1073/pnas.0801921105>
- Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology*, *7*(1–2), 203–214. <https://doi.org/10.1089/10665270050081478>
- Zinger, L., Bonin, A., Alsos, I. G., Bálint, M., Bik, H., Boyer, F., ... Taberlet, P. (2019). DNA metabarcoding – Need for robust experimental designs to draw sound ecological conclusions. *Molecular Ecology*, *28*(8), 1857–1862. <https://doi.org/10.1111/mec.15060>

## SUPPORTING INFORMATION

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

**How to cite this article:** Peixoto S, Chaves C, Velo-Antón G, Beja P, Egeter B. Species detection from aquatic eDNA: Assessing the importance of capture methods. *Environmental DNA*. 2021;3:435–448. <https://doi.org/10.1002/edn3.130>