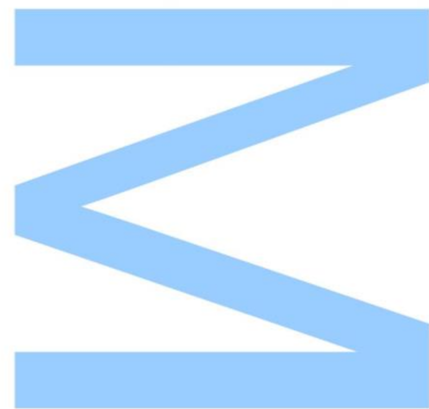
Improving eDNA methodologies to detect amphibians in turbid waters



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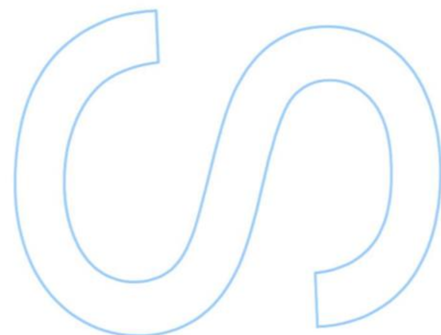

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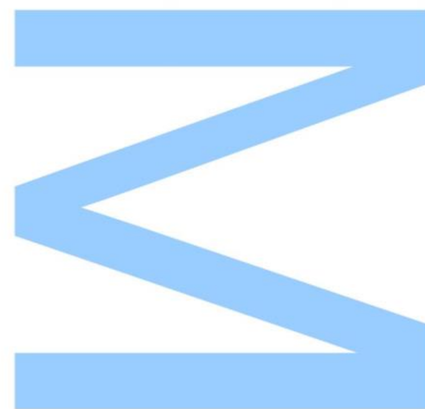

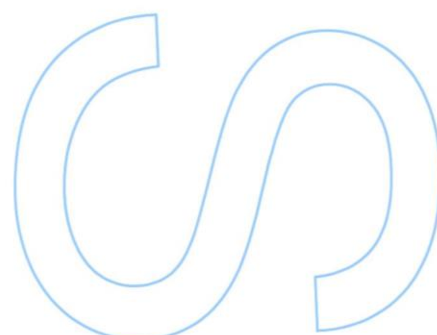

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Resumo

Os anfíbios são alvo de programas de conservação em todo o mundo. Entre todos os grupos de vertebrados, os anfíbios são os mais ameaçados, com cerca de 40% das espécies em vias de extinção. A grande diversidade deste grupo e a sua importância ecológica exigem um conhecimento preciso da sua distribuição, com o intuito de desenvolver ações de conservação que possam retardar o seu declínio. Tal requer um envolvimento ativo entre herpetólogos e investigadores de outras áreas com o objetivo de melhorar as estratégias de monitorização. O DNA ambiental surgiu como uma ferramenta poderosa e não-invasiva para a deteção de espécies, que poderá superar as limitações associadas aos métodos tradicionais.

Apesar da sua crescente aplicação nos últimos anos, a maioria dos estudos de DNA ambiental em sistemas aquáticos tem-se concentrado em águas de baixa turbidez e a eficácia desta técnica em sistemas túrbidos permanece pouco estudada. Independentemente das elevadas quantidades de sedimento, os ambientes aquáticos túrbidos podem albergar uma grande diversidade de espécies de anfíbios. As dificuldades dos programas de monitorização usando DNA ambiental em sistemas túrbidos devem ser melhor compreendidas, e a comparação entre os métodos de amostragem atualmente disponíveis bem como o desenvolvimento de novos métodos precisa de ser abordado. No primeiro capítulo desta tese, três métodos de recolha de água (precipitação, filtração com filtros de disco e filtração com cápsulas) foram avaliados num gradiente de turbidez. A amostragem foi realizada num sistema de metapopulações de anfíbios composto por poças temporárias e pequenos charcos na Reserva Ornitológica do Mindelo, no Norte de Portugal. O desempenho de cada método foi avaliado com base no total de DNA ambiental capturado e nas taxas de deteção de uma única espécie-alvo - *Salamandra salamandra* - com dois métodos (qPCR e sequenciação de alto rendimento). Em comparação com os dois métodos de filtração, a precipitação teve um desempenho inferior tanto em termos de captura de DNA ambiental bem como nas taxas de deteção da espécie-alvo. As cápsulas capturaram uma maior quantidade de DNA ambiental em relação aos filtros de disco, mas a deteção de espécies foi semelhante entre os dois métodos. O preço mais baixo e a possibilidade de detetar espécies em ambientes altamente turvos parecem favorecer o uso de filtros de disco para realizar amostragem em ecossistemas aquáticos turvos, no entanto, mais estudos são necessários no futuro para validar esta conclusão. Embora a sensibilidade do qPCR tenha sido maior do que a de sequenciação de alto rendimento, para DNA

ambiental capturado com filtros de disco, nenhuma diferença entre métodos foi observada de outra forma.

A eficiência de DNA ambiental em sistemas aquáticos pode ser afetada por vários fatores bióticos e abióticos. Entender os processos que limitam a deteção de DNA ambiental é crucial para programas de monitorização precisos baseados nesta técnica. A contabilização desses fatores através da otimização de protocolos de amostragem fornecerá melhores estimativas da distribuição das espécies e aumentará a confiança em pogramas de monitorização através de DNA ambiental. No segundo capítulo desta tese, a probabilidade de deteção de espécies de anfíbios foi avaliada de acordo com o volume de água filtrada e várias variáveis ambientais (área e profundidade do charco, claridade da água, condutividade, pH e temperatura). A amostragem foi realizada num parque natural na planície costeira do sudoeste de Portugal. Foi possível observar uma influência significativa na deteção de espécies para a maioria das variáveis, apesar de não seguir um padrão consistente entre espécies. De uma forma geral, o aumento da profundidade do charco e da condutividade da água diminuíram a deteção das espécies, enquanto que o aumento da área do charco e do pH aumentou a deteção das espécies. A única variável que influenciou a deteção em mais de uma espécie foi a claridade da água, exibindo padrões opostos.

De uma forma geral, este estudo traz novos conhecimentos em relação a estudos de DNA ambiental em ecossistemas aquáticos. O trabalho desenvolvido neste estudo fornece comparações precisas entre três métodos de amostragem, oferecendo esclarecimentos importantes sobre o melhor método de amostragem para monitorização da biodiversidade em ambientes aquáticos turvos, bem como os efeitos de variáveis ambientais na deteção de DNA ambiental de anfíbios em lagoas temporárias do Mediterrâneo. Este trabalho também ajudou a identificar novas áreas de pesquisa e a destacar aspetos importantes que têm sido negligenciados por estudos anteriores, levando potencialmente a conclusões erradas. Esta informação irá proporcionará no futuro o desenvolvimento de protocolos de amostragem otimizados que possam aumentar a eficiência e a confiança de programas de monitorização em habitats aquáticos com base no DNA ambiental e ajudar a combater de uma forma eficaz o declínio global de anfíbios.

Palavras-chave: águas turvas, avaliação da biodiversidade, cápsulas, conservação de anfíbios, DNA ambiental, filtros de disco, lagoas temporárias do Mediterrâneo, modelos de ocupação, precipitação, *Salamandra salamandra*

Abstract

Amphibians are a focus of conservation programs worldwide. Among all vertebrate groups, amphibians are the most threatened, with about 40% of species facing extinction. The large diversity of this group and their ecological importance requires accurate knowledge of species distributions in order to develop conservation and management actions that can mitigate amphibian decline. This requires an active engagement between amphibian specialists and researchers from other areas in order to improve survey and monitoring strategies. Environmental DNA has emerged as a powerful, non-invasive tool for species detection, which will potentially overcome the limitations associated with traditional biodiversity surveys.

Despite its increasing application in recent years, most eDNA surveys in aquatic systems have focused on low-turbidity waters and the efficacy of eDNA methods in turbid systems remains understudied. Regardless of the high sediment loads, turbid aquatic environments can harbour a large diversity of amphibian species. The challenges of biodiversity assessment using eDNA in turbid environments should be better understood, and the comparison of currently available sampling methods as well as the development of new methods needs to be addressed. In the first chapter of this thesis, three capture methods (precipitation, filtration with disc filters and filtration with capsules) were evaluated across a turbidity gradient. Sampling was conducted in an amphibian meta-population system composed of temporary puddles and small ponds at Mindelo Ornithological Reserve, northern Portugal. The performance of each method was evaluated based on both total captured eDNA and detection rates of a single target species - *Salamandra salamandra* – with two approaches (qPCR and high-throughput sequencing). Compared to the two filtration methods, precipitation underperformed both in terms of total eDNA captured and species detection rates. Capsules captured a higher quantity of total eDNA than disc filters, but species detection was similar among the two methods. The lower price and the possibility to detect species in highly turbid environments seem to favour the use of disc filters for sampling in aquatic turbid ecosystems, however, more research is needed in the future to validate this conclusion. Although the sensitivity of the qPCR assay was higher than that of high-throughput sequencing, for eDNA captured using disc filters, no difference between assay sensitives was noted otherwise.

The efficiency of eDNA methods in aquatic systems can be affected by several biotic and abiotic factors. Understanding the processes limiting eDNA detection is crucial in order for accurate eDNA-based surveys. Accounting for those factors by optimizing

sampling designs will provide better estimates of species distribution and increase the reliability of eDNA biodiversity monitoring. In the second chapter of this thesis, the probability of detection of amphibian species was assessed according to the volume of water filtered and several environmental variables (pond area and depth, water clarity, conductivity, pH and temperature). Sampling was conducted in a natural park on the coastal plain of southwest Portugal. A significant influence on species detection was observed for most of the variables measured, but not in a consistent pattern among species. Generally, increasing pond depth and water conductivity decreased species detection, while increasing pond area and pH increased species detection. The only variable influencing detection in more than one species was water clarity, exhibiting opposite patterns.

Overall, this study brings new insights into eDNA research in aquatic environments. The work developed in this study provides accurate comparisons between three sampling methods, offering important elucidations regarding the best method for biodiversity assessment in turbid aquatic environments as well as the effects of environmental variables on amphibian eDNA detection in Mediterranean temporary ponds. This work has also helped to identify new research areas and highlight important aspects that have been overlooked by previous studies, potentially leading to erroneous conclusions. This will provide the development of optimized sampling designs in the future that can increase the efficiency and reliability of eDNA biodiversity monitoring in aquatic habitats and help combat global amphibian declines effectively.

Keywords: amphibian conservation, biodiversity assessment, capsules, environmental DNA, disc filters, Mediterranean temporary ponds, *Salamandra salamandra*, site occupancy models, precipitation, turbid waters

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List of abbreviations

AIC – Akaike information criterion

anova – analysis of variance

BHQ – Black Hole Quencher

BLAST – Basic Local Alignment Search Tool

BOLD – Barcode of Life Data System

bp – base pairs

COI – cytochrome c oxidase I

diH_2O – deionized water

DNA – deoxyribonucleic acid

eDNA – environmental DNA

EDTA - ethylenediamine tetraacetic acid

FAM – 6-carboxyfluorescein

glmer – generalized linear mixed-effects model

HCl – hydrochloric acid

HTS – high-throughput sequencing

lmer – linear mixed-effects model

mS - milli Siemens

NCBI – National Center for Biotechnology Information

PCR – polymerase chain reaction

qPCR – quantitative polymerase chain reaction

rpm – revolutions per minute

rRNA – ribosomal ribonucleic acid

SE – standard error

UV – ultraviolet

$\bar{X}$ - mean

Chapter 1. General introduction

1.1 Global amphibian decline

The world is currently experiencing a biodiversity crisis, mainly resulting from anthropogenic disturbances, with major impacts on the planet's sustainability (Ceballos et al., 2015; Barnosky et al., 2011). Conservation efforts to save biodiversity depend on continuous biological monitoring to obtain accurate information on species distribution and protect global and regional hotspots of biodiversity. As faunal populations worldwide continue to decline, the need for rapid and cost-efficient methods for biodiversity assessment is becoming increasingly important.

Among all vertebrate groups, amphibians are currently considered the most threatened worldwide (Wake & Vredenburg, 2008), with an estimated 40% of species in danger of extinction (Bishop et al., 2012). Despite their large distribution range, inhabiting every continent in the world except Antarctica, amphibians are particularly sensitive to environmental changes. Group-specific traits, such as low mobility, water dependence and highly permeable skin, which easily absorbs substances from the environment, are important factors determining their high sensitivity (Bishop et al., 2012).

Several potential causes have been described to explain the observed decline. Habitat loss and fragmentation (Cushman, 2006), overexploitation, invasive species, infectious diseases (Daszak et al., 1999), chemical contamination (Blaustein et al., 2003) and climate change are considered the most relevant (Bishop et al., 2012; Vredenburg & Wake, 2007; Stuart et al., 2004). The mechanisms behind these causes are often complex and can act together, aggravating species declines (Vredenburg & Wake, 2007).

Combating amphibian decline has proven to be one of the greatest conservation challenges of the century, but of utmost importance (Bishop et al., 2012). Amphibians are important members of ecosystems and their decline is likely to affect entire communities. They are present in numerous habitats and act as both prey and predators, being considered key components of food webs (Vredenburg & Wake, 2007). In addition, they are considered important bioindicators (Bishop et al., 2012), being used to infer the quality of the surrounding environment (Welsh & Ollivier, 1998).

The large diversity of amphibians and their ecological importance requires a continuous and growing effort by researchers all over the world in order to increase the knowledge about this vertebrate class. Only then will it be possible to apply efficient conservation measures, that can counter amphibian decline.

1.1.1 Iberian species

Iberian herpetofauna is composed of 39 amphibian species, spread over 7 families and with 13 Iberian endemisms: *Calotriton arnoldi, Lissotriton boscai*, *Chioglossa lusitanica*, *Triturus pygmaeus, Salamandra longirostris, Rana iberica*, *Alytes cisternasii*, *Alytes dickhilleni*, *Alytes muletensis*, *Discoglossus jeanneae*, *Discoglossus galganoi*, *Pelodytes atlanticus* and *Pelodytes ibericus*.

Iberian amphibians can be found in a variety of habitats, such as coastal dunes, deciduous forests, urban, rural, agricultural and mountainous areas. The temporary ponds associated with dune areas harbour important populations of some species, such as the western spadefoot (*Pelobates cultripes*), the sharp-ribbed salamander (*Pleurodeles waltl*) or the natterjack toad (*Epidalea calamita*) that reproduce in these sites (Ferreira & Beja, 2013; Maravalhas & Soares, 2018).

Similar to amphibian species worldwide, Iberian amphibians have experienced population declines. Invasive species (Cruz et al., 2008; Rebelo et al., 2010), infectious diseases (Rosa et al., 2013; Rosa et al., 2017) and chemical contamination (Marques et al., 2008) are dominant causes for the observed decline.

1.2 Traditional amphibian survey methods

Due to the large range of habitats occupied by amphibians, several techniques are available for amphibian surveys. The goal of the study, the habitat of the target species and the resources available for the project are important factors that will influence the choice of the sampling method to use. Common traditional methods are: visual and audio surveys, quadrat sampling, straight-line drift fences with pitfall traps and quantitative sampling of larvae, through dipnetting for example. Moreover, since amphibian densities change within habitats, sampling specific patches of a pond – patch sampling – or along a transect that crosses all habitats – transect sampling – is also suggested (Heyer et al., 1994).

Nevertheless, traditional methods are usually time consuming and might fail to detect some species. Visual and audio surveys are likely to miss species that are not visible or audible at the time of the survey, respectively. Moreover, species identification based on morphological traits and species-specific calls can be challenging, requiring training and expertise. Likewise, pitfall traps are not suitable to capture species with climbing/jumping abilities, such as *Hyla sp.* or *Pelophylax sp.*, that can easily escape the traps (Heyer et al., 1994).

Amphibian preservation requires an active engagement between amphibian conservationists and researchers from other areas in order to improve conservation strategies (Bishop et al., 2012; Vredenburg & Wake, 2007). In a number of cases, recent molecular techniques have proved to be more efficient than traditional field surveys for amphibian species detection (Dejean et al., 2012; Smart et al., 2015).

1.3 eDNA: definition and applications

Environmental DNA (eDNA) is described as DNA that can be extracted from environmental samples such as soil (fig. 1A), water (fig. 1B) or even air, without the need to manipulate any organism. The first eDNA study dates back to 1987, where Ogram and colleagues extracted DNA from sediment samples to detect microorganisms (Ogram et al., 1987). A few years later, one of the first studies on macro-organisms arises where DNA was extracted from cigarette butts, with potential for forensic applications (Hochmeister et al., 1991). However, only at the beginning of the 2000s, with the development of high-throughput sequencing (HTS) technologies, this field became more accessible and attracted the interest of researchers, being applied across many biological fields (e.g. Willerslev et al., 2003).

Fig. 1 Examples of eDNA sources: soil **(A)** and water **(B)**.

As they move around in the environment, animals leave traces of their DNA via faeces, urine, hairs, carcasses or skin cells, that can later be collected and analysed (Herder et al., 2014). An environmental sample can contain information from several different organisms, whose DNA can be either cellular or extracellular (Herder et al., 2014). This type of sample can be obtained either from ancient or modern ecosystems (Bohmann et al., 2014; Thomsen & Willerslev, 2015).

DNA-based detection methods rely on the use of suitable genetic markers capable of identifying the target species and ideally excluding non-target ones (Darling & Mahon, 2011), in order to maximise the usefulness of the generated sequence data. The use of appropriate barcodes will strongly influence the output of an eDNA study (Coissac et al., 2012). Among the properties of an ideal barcode, high taxonomic coverage and high

resolution are crucial. A high taxonomic coverage allows the application to a wide number of taxa, whereas a high resolution provides a successful distinction between species (Ficetola et al., 2010). Likewise, the length of eDNA barcodes should be carefully taken into consideration. After being released into the environment, DNA starts to degrade due to multiple factors and the selection of short DNA fragments will increase the chances of recovering DNA from an environmental sample (Valentini et al., 2009). High quality barcodes thus require a short but variable enough DNA sequence, flanked by highly conserved regions, which still remains a challenge (Ficetola et al., 2010; Coissac et al., 2012).

Although some authors do not consider it a suitable marker (Deagle et al., 2014), the standard DNA barcode currently used for most animal groups is a 658 bp fragment of the mitochondrial COI gene (Valentini et al., 2009). The high copy number per cell of mitochondrial genes makes them suitable targets for eDNA studies, as they are more likely to be captured than single-copy nuclear DNA (Thomsen & Willerslev, 2015). Other mitochondrial markers such as cytochrome *b*, 12S and 16S have also been suggested as equally suitable for eDNA studies (Ficetola et al., 2010; Deagle et al., 2014).

The identification of the species source of DNA can be performed using either a single-species or a multiple-species approach, the latter being particularly useful in biodiversity surveys (Thomsen & Willerslev, 2015). A multiple-species approach has the power to identify multiple species within a single sample, increasing the amount of information we can retrieve, as well as the complexity of the study. It requires the use of HTS technologies (fig. 2) and can be applied to different biological areas, such as diet analysis studies (Shehzad et al., 2012), reconstruction of past ecosystems (Jorgensen et al., 2012) and assessment of community composition (Andersen et al., 2012).

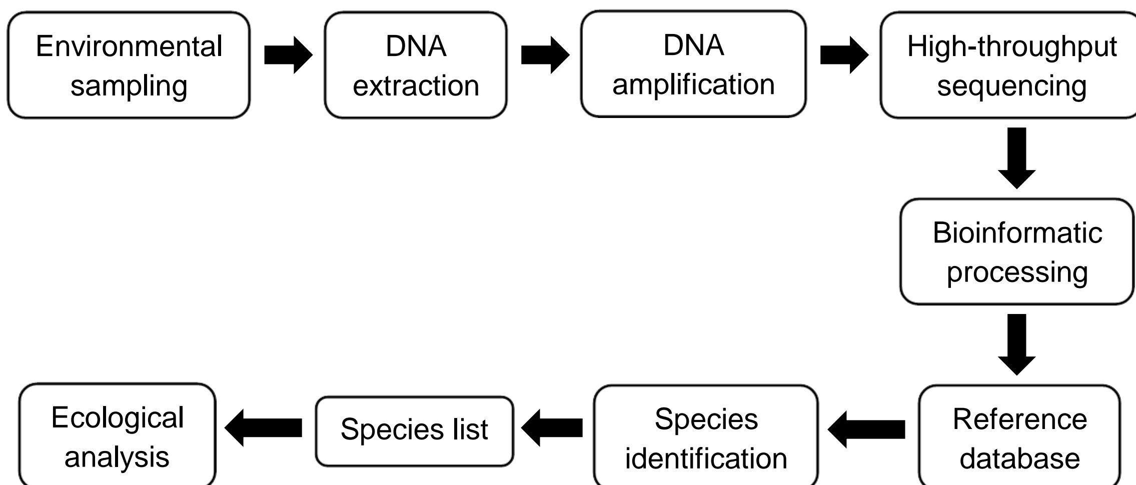


Fig. 2 An example of workflow using a multiple-species approach. Adapted from Herder et al. (2014).

Surveys and inventories provide essential data for monitoring species and are considered important tools towards biodiversity conservation. Considering the current threats to amphibians it is essential to perform this type of study using efficient and powerful tools such as those offered by eDNA methods. Aquatic habitats are particularly difficult to sample accurately using traditional surveys, making the use of eDNA promising in these ecosystems.

1.4 Traditional vs eDNA methods

1.4.1 Advantages of eDNA methods

Researchers worldwide have progressively applied eDNA methods for biodiversity assessments. This technique has been increasingly used in the last years, offering some advantages over conventional approaches. One of the most perceptible and important advantages of utilising eDNA is the fact that non-invasive samples can be used, since it does not require the capture of organisms in order to detect their presence (Herder et al., 2014). This prevents animal stress often caused by traditional approaches, minimizes the disturbance to habitats and reduces the transfer of diseases (Bohmann et al., 2014; Thomsen & Willerslev, 2015). Additionally, in many cases, no special authorization is needed for the collection of samples, in contrast to the many permits usually required when working with protected species through traditional approaches (Herder et al., 2014).

Moreover, eDNA methods are often considered cheaper and less time consuming when compared to traditional methods. This can vary according to the target species, but it is often true for secretive and rare species (Hunter et al., 2015), and in high-diversity regions (Bálint et al., 2018). Previous studies with the great crested newts (Biggs et al., 2014) and the invasive American bullfrogs (Dejean et al., 2012) demonstrated that eDNA monitoring can be more cost-efficient. Nevertheless, in some circumstances, traditional methods may be more suitable than eDNA methods. According to Spikmans et al. (2008), the spined loach (*Cobitis taenia*) and the bitterling (*Rhodeus amarus*) are two species that are easy to catch, with minor effort, thus the authors conclude that traditional methods are a more appropriate approach. Ultimately, the most appropriate survey method will vary according to the species and research goals.

Likewise, eDNA studies on amphibians showed higher detection probability when compared to traditional approaches (Smart et al., 2015; Valentini et al., 2016; Dejean et al., 2012), even at very low population densities (Ficetola et al., 2008). This can provide an early detection of alien/invasive species (Jerde et al., 2011; Dejean et al., 2012), increasing the chances of successful eradication, thus decreasing the economic costs

and impacts on the ecosystem (Herder et al., 2014). Nevertheless, the detection probability of a species will depend on different ecological features (see section 3.1), therefore the interpretation of eDNA results is not always straightforward and caution needs to be exercised.

Finally, performing biodiversity surveys based on eDNA techniques can provide increased taxonomic resolution. Species identification based on morphological traits can be difficult for many taxa, requiring long training and experience. The identification to species level can be challenging, especially for species with cryptic life styles (Thomsen & Willerslev, 2015) or when working with juvenile forms, since most identification keys focus on adult life stages (Darling & Mahon, 2011; Herder et al., 2014). The use of DNA-based identification can often distinguish species where traditional methods fail. Notwithstanding, it is critical to note that this can only be achieved if the genetic markers used are appropriate, capable of distinguishing closely related species, and the assays are suitably validated (Herder et al., 2014).

1.4.2. Disadvantages of eDNA methods

No method is flawless and eDNA techniques still have some limitations to overcome. One of the major disadvantages of this method is the difficulty in quantifying species density from an environmental sample. In controlled environments, several authors already observed a correlation between species density and eDNA concentration (Takahara et al., 2012; Thomsen et al., 2012). However, under natural conditions there are numerous factors that can influence the amount of eDNA available (see section 3.1). Consequently, it can be extremely difficult to link eDNA concentration from an environmental sample to the species biomass. In fact, previous studies conducted under natural conditions obtained different results, ranging from weak positive correlations (Jane et al., 2015) to no correlation at all (Spear et al., 2015). Currently, eDNA can only provide some indications of species densities. Different approaches such as the use of sequence reads abundance (Elbrecht & Leese, 2015), frequency of occurrence estimates, i.e., the proportion of samples where the organism is detected (Shehzad et al., 2012) or the use of correction factors (Thomas et al., 2014; Thomas et al., 2015) have been applied for quantifying species abundance and shed some light on future research.

Another challenge with eDNA studies is related to the misidentification of species due to inappropriate databases. Public databases such as GenBank often contain many errors and mislabelled species. The use of well-curated databases, such as BOLD (http://www.boldsystems.org/), where the information is previously verified could be a

solution (Herder et al., 2014). Moreover, for many taxonomic groups substantial databases do not yet exist and are not likely to for many years, currently limiting the applicability of eDNA research to groups that are already well-studied. According to Coissac et al. (2012), the construction of specific databases for each study can overcome this limitation.

An additional constraint of eDNA surveys is associated to the amount of information that can be collected. This method can only detect species presence or absence, not being able to distinguish live and dead organisms or to obtain information regarding the life stages, demographic structure or body condition of the target species. Additionally, as most markers target mitochondrial DNA, the differentiation between hybrids or individuals of the same species is often difficult (Thomsen & Willerslev, 2015; Herder et al., 2014; Goldberg et al., 2016). This type of information can usually be obtained through traditional methods and can be useful depending on the purpose of the study.

Lastly, the probability of occurrence of false positive results, mainly due to contamination from external sources, increases. This source of error has consequences for downstream application in biodiversity conservation or eradication of invasive species, causing an overestimation of species occurrence (Thomsen & Willerslev, 2015) and therefore wasting resources for the application of unnecessary measures.

eDNA is a promising tool for biodiversity assessment. This non-invasive sampling method has been increasingly used in the last years, overcoming some difficulties associated with traditional sampling techniques. Instead of replacing them, eDNA methods should be implemented together with traditional monitoring techniques, in order to maximize the information obtained from biodiversity surveys (Thomsen & Willerslev, 2015; Herder et al., 2014).

1.5 Methods for eDNA capture and species detection in aquatic systems

1.5.1 Sample collection

Despite the applicability of eDNA techniques to a range of environments, such as soil (Andersen et al., 2012; Bienert et al., 2012), air (Leung et al., 2014; Yooseph et al., 2013), or snow (Dalén et al., 2007), the majority of studies in the field of animal ecology and bioassessment focus on aquatic ecosystems.

The goal of sample collection is to obtain DNA in sufficiently high amounts and preserving this DNA until it can be processed in the laboratory. Nevertheless, the low-

quality and low-quantity characteristics of eDNA samples makes them very easy to contaminate (Goldberg et al., 2016). To minimize the risk of contamination, fieldworkers should be aware of all possible sources of contamination prior to sample collection. The equipment used should be properly decontaminated after each usage, or discarded, if possible (Bohmann et al., 2014). Field negative controls should also be collected: either samples taken from locations where the target species is absent (Bohmann et al., 2014) or by filtering ultra-pure or distilled water along the equipment before each sample (Rees et al., 2014). In addition, fieldworkers should use gloves every time and avoid entering the water body (Herder et al., 2014).

Multiple methods are available for eDNA capture from water bodies, to the extent that very few studies share the same methods (Dickie et al., 2018). Concentration of DNA either by centrifugation (e.g. Caldwell et al., 2007), precipitation with sodium acetate and ethanol (e.g. Ficetola et al., 2008), or filtration (e.g. Jerde et al., 2011), have previously been applied to capture DNA from aquatic systems. Likewise, the use of resin beads, particles with an anionic exchange area that attract negatively-charged molecules, have recently been used as well (Williams et al., 2017), although not successfully.

Using centrifugation and precipitation methods all eDNA present in the water sample is captured (Herder et al., 2014), maximizing the probability of detection. However, the low sample volume (usually 15 ml) associated with these methods can hamper species detection, especially for low density and low mobility species (Herder et al., 2014). Notwithstanding, the collection of multiple samples at different sampling points within the study area can overcome this problem (Ficetola et al., 2015).

The efficiency of capturing eDNA with filtration methods is highly dependent on the pore size of the filter. eDNA fragments are highly degraded and usually around 150 bp (Valentini et al., 2009). Large pore sizes may not capture all eDNA present in the water (Herder et al., 2014), whereas small pore sizes can easily clog with suspended sediment and organic material (Piaggio et al., 2014), hampering the capture of DNA. The trade-off between the volume of water filtered and the ability to capture large amounts of DNA needs to be considered when selecting the pore size of the filter.

Several studies have already compared the efficiency of different pore sizes on eDNA recovery. For example, Turner et al. (2014) suggested a pore size of 0.2 μm as the optimal to capture macrobial aqueous eDNA, Majaneva et al. (2018) proposed a 0.45 μm pore size as the best indicator of metazoan community composition, while Li et al. (2018) stated that 0.8 μm is the optimal membrane size to assess fish community. The

conclusions obtained are not consistent across studies and the optimal pore size seems to change according to the target species and purpose of the study.

Similar to pore size, a comparison between the efficiency of different filter membranes has already been performed. Once more, depending on the study, a wide range of filter membranes has been suggested for filtration: cellulose nitrate (e.g. Spear et al., 2015), polyethersulfone (e.g. Renshaw et al., 2015), glass fibre (e.g. Jerde et al., 2011), nylon (e.g. Bálint et al., 2017), polycarbonate (e.g. Takahara et al., 2012) and cellulose acetate (e.g. Takahara et al., 2013).

Filtration methods allow the concentration of eDNA from larger volumes of water when compared to centrifugation or precipitation methods. Previous studies reported volumes ranging from 250 ml (e.g. 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 (fig. 3A), generally used in combination with cups or filter funnels (fig. 3B). These filters are usually associated with small volumes (less than 2 L typically) due to their small surface area. Alternatively, enclosed filters (hereinafter called capsules) (fig. 3C), a recent method in eDNA studies (Valentini et al., 2016; Lopes et al., 2017), generally have a higher surface area allowing the filtration of much higher volumes of water (more than 10 L typically).

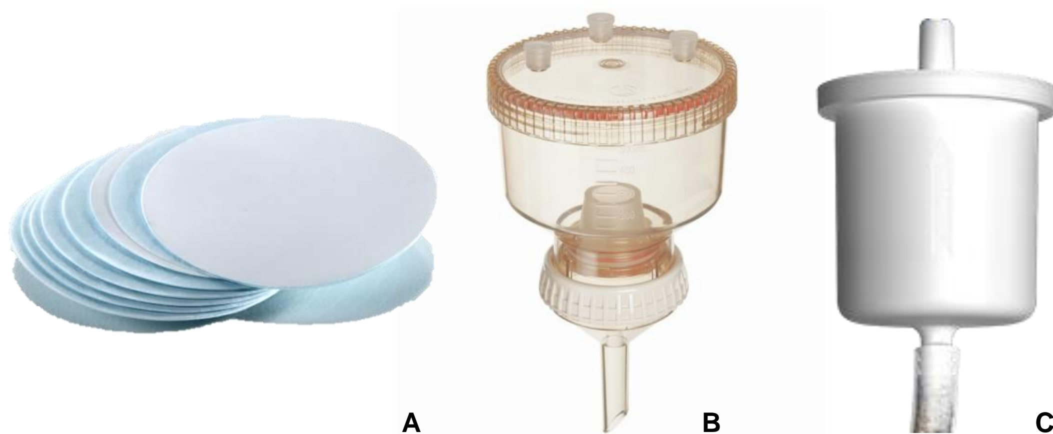


Fig. 3 Types of filters used for water collection in eDNA studies: open filters **(A)** used together with filter funnels **(B)**, and enclosed filters **(C)**.

Nonetheless, in turbid waters, the performance of filtration methods may decrease. Due to the high-sediment loads typical of these ecosystems, filters become easily clogged and filtration time may increase significantly (Hinlo et al., 2017). A previous study demonstrated that filter clogging in turbid waters limited filtering capacity to as low as 200 ml (Robson et al., 2016). The use of large-pore filters (Robson et al., 2016) or multiple filters (Hinlo et al., 2017) may help overcome these problems.

Despite the possibility of processing larger volumes than precipitation, filtration methods are more expensive and time consuming. Additionally, by filtering higher water volumes, inhibitors could be highly concentrated in the final sample (Herder et al., 2014), which may constrain the subsequent steps in the laboratory.

1.5.2 DNA extraction

After collection in the field, environmental samples are subjected to DNA extraction, where DNA is isolated and purified for downstream analyses. To minimize the risk of contamination, DNA extraction should be performed in isolated rooms, equipped with positive air pressure and UV radiation. This room should be physically separated from rooms where high-quality DNA is extracted or where PCR products are handled. Additionally, all lab surfaces should be thoroughly cleaned with bleach or other DNA decontaminating agent, and a negative control should be included with every batch of samples (Herder et al., 2014; Goldberg et al., 2016; Rees et al., 2014).

Inefficient extraction protocols, along with the co-extraction of PCR inhibitors might constrain downstream amplification reactions, possibly leading to false negative results (Goldberg et al., 2015). A post-extraction step to remove these substances has been shown to decrease PCR inhibition and increase species detection. McKee et al. (2015) demonstrated spin-column purification as an effective method to decrease qPCR inhibition. The authors further suggest the addition of bovine serum albumin (BSA) to PCR reactions and using alternative DNA polymerase or other PCR reagents for reducing inhibition in environmental samples.

In a similar study, Williams et al. (2017) used a post-extraction inhibitor removal kit to successfully increase the probability of eDNA detection of the target species. However, in some samples, DNA was lost during the treatment and not detected afterwards. The trade-off between removing inhibitors and the risk of losing DNA in a sample that has already low concentrations of target DNA molecules needs to be considered within each study.

1.5.3 Species detection

Although PCR-free methods have been used to analyse eDNA samples, by far the most common approach is to use PCR to facilitate species detection. Polymerase chain reactions (PCR) exponentially amplify a few copies of a specific DNA fragment, generating millions of copies. Similar to sample collection and DNA extraction, negative controls should be included to monitor for possible contamination (Rees et al., 2014; Herder et al., 2014; Goldberg et al., 2016).

In general, three PCR-based methods are used: conventional PCR (PCR followed by gel electrophoresis (fig. 4A) or Sanger sequencing), quantitative PCR (qPCR) and metabarcoding (PCR followed by HTS). Conventional PCR is cheaper than qPCR, but usually less sensitive (Herder et al., 2014). qPCR can be performed with two different techniques: either using non-specific fluorochromes that bind to all double stranded DNA in a sample (SYBR ® Green) or using hybridization probes that bind specifically to the target species (e.g. TaqMan ®), releasing fluorescence upon amplification (fig. 4B). Both methods allow the quantification of target DNA in the sample, but probe-based qPCR is considered to be more specific (Goldberg et al., 2016).

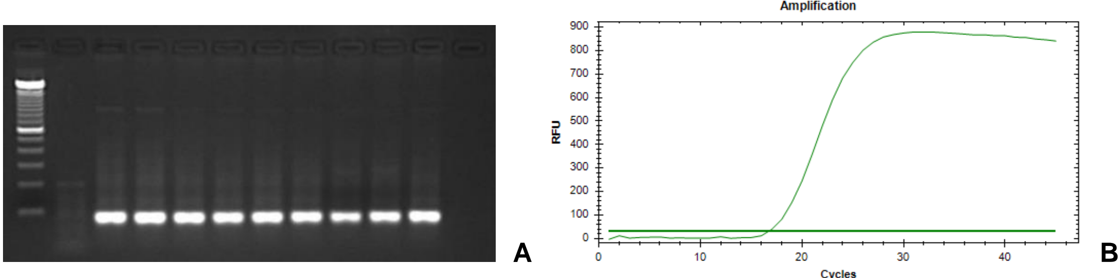


Fig. 4 Species detection with conventional PCR **(A)** and quantitative PCR **(B)**.

HTS techniques allow the simultaneous detection of multiple species within a sample, useful for assessing community composition (e.g. Bálint et al., 2017). It can be considered more expensive when compared to other methods. Nevertheless, the costs of sequencing have rapidly decreased over time and continue to do so (Sboner et al., 2011), thus encouraging the use of this powerful tool.

To decrease false negative results, PCR replicates are usually performed. Even though there is currently no criterion for the number of replicates necessary (Goldberg et al., 2016), as the optimal replication level strongly differs among studies (Ficetola et al., 2015), most researches use between three and ten (Rees et al., 2014). Similarly, the use of site occupancy models can help overcome the uncertainty associated with false negatives. These models account for imperfect species detection, using the information on species presence/absence to calculate detection probabilities (Mackenzie et al., 2002), thus making them a useful tool for eDNA-based surveys.

1.6 Objectives

1.6.1 Comparison of eDNA capture and species detection methods in turbid waters (chapter 2)

Despite its increasing application in recent years, most eDNA biodiversity surveys in aquatic systems have focused on low turbidity waters. Nevertheless, the efficiency of this technique across different ecosystems, turbid waters in particular, needs to be further

explored. In spite of the high levels of suspended sediment, turbid waters usually contain a large diversity of taxa. However, the assessment of biodiversity in turbid environments poses a unique set of challenges. This chapter aims to compare different eDNA capture methods across a gradient of turbidity and evaluate their performance in terms of a) eDNA capture and b) species detection, using both qPCR and HTS techniques.

1.6.2 Factors influencing species detection in Mediterranean ponds (chapter 3)

eDNA detectability is not consistent across sites nor species and is highly influenced by a range of ecological factors. The production and degradation rate of eDNA, the habitat of the species, the sampling strategy and the following laboratory procedures can all influence the probability of detecting a target species at a given sampling point. These factors can lead to false negative results. The use of site occupancy models can help overcome this issue by accounting for imperfect detections and calculating species detection probabilities. The major goal of this chapter is to assess the effects of different environmental variables, as well as the volume of water filtered, on the probability of detection of amphibians, using eDNA methods.

Chapter 2: Comparison of eDNA capture and species detection methods in turbid waters

2.1 Introduction

eDNA methods can be applied across a wide range of aquatic ecosystems. Previous studies have demonstrated the applicability of this method to stagnant water bodies, such as ponds (e.g. Dejean et al., 2012) or lakes (e.g. Jerde et al., 2013), as well as running waters, such as streams (e.g. Goldberg et al., 2011) or rivers (e.g. Minamoto et al., 2012). Nevertheless, the efficiency of eDNA studies in turbid waters remains unknown and few studies have addressed the difficulties of biodiversity assessment in these environments (Egeter et al., 2018).

Species detection and the assessment of biodiversity using eDNA methods in turbid waters poses a unique set of challenges. Due to the high-sediment loads, filters become easily clogged and filtration time may increase significantly (Hinlo et al., 2017; Robson et al., 2016) compared to sampling in low turbidity waters. The use of large-pore filters (Robson et al., 2016), multiple filters (Hinlo et al., 2017) or precipitation and centrifugation methods (Williams et al., 2017; Ahmad et al., 2011) are some of the solutions that may help overcome these problems. Due to their large surface area, capsules allow the filtration of large volumes (Valentini et al., 2016) and could help overcome the clogging problem as well. Nevertheless, given its recent application in eDNA studies, a comparison of the performance of high-surface-area capsules with more common eDNA methods is missing.

Regardless of the high-sediment loads, amphibian species often inhabit turbid environments (Lobos et al., 2013; Schmutzer et al., 2008), such as agricultural ponds (Knutson et al., 2004) or shallow prairie lakes (Jackson & Moquin, 2011). Sediments play an important role in eDNA preservation, by binding to DNA molecules and protecting them from degradation (Williams et al., 2017; Barnes et al., 2014). Previous studies have demonstrated that DNA is more concentrated in aquatic sediments than in the water column (Turner et al., 2015), thus theoretically increasing species detection in turbid waters.

The detection of species-specific DNA is a common application of eDNA studies. This is particularly useful for rare and invasive species, with applications in conservation and eradication projects, respectively (e.g. Rees et al., 2014). The species detection assay applied to a given set of eDNA samples will affect the outcome of any eDNA study as different assays will have different sensitivities (see section 1.5.3). Assay sensitivity could therefore affect the outcome of studies comparing eDNA capture methods and is an important consideration.

The need to overcome the challenges associated with turbid waters and to identify the best method for species detection and monitoring in high-turbidity systems, which can harbour high amphibian biodiversity levels, make turbid environments a good system for eDNA studies. This chapter aims to compare three eDNA capture methods – precipitation, disc filters and capsules – across a gradient of turbidity and evaluate their performance on a) eDNA capture and b) species detection, using both qPCR and HTS and the fire salamander, *Salamandra salamandra*, as the study organism.

2.2 Material and methods

2.2.1 Target species and site selection

The target species for this study was the fire salamander, *Salamandra salamandra gallaica*, a larviparous urodele lineage commonly found across western Iberian Peninsula. Pregnant females deliver up to 90 larvae into water ponds (i.e. ponds, puddles and streams) during the reproductive periods (Autumn and Spring), where they stay until they complete metamorphosis. In order to obtain a range of sites sharing a common species, so that eDNA capture methods could be compared for their efficacy in species detection, and due to its abundance in the surrounding area of Porto district, the initial criterion for site selection was the presence of salamanders. Diurnal surveys were conducted in a range of sites throughout Porto in late March 2018, and an initial set of sites based on species presence was selected.

Next, sites were selected based on water turbidity. Turbidity is an important parameter of water quality, which is a measure of the cloudiness of the water (Myre & Shaw, 2006). The turbidity tube, an adaption from the Secchi disc, is an inexpensive technique, easy to use and adaptable to the field, requiring neither calibration nor a power source, making it a suitable method when funds are limited (Myre & Shaw, 2006). This technique measures water clarity, which is directly related to turbidity (Anderson & Davic, 2004). Water is slowly poured into the tube until the disc at the bottom is no longer visible and the water level in the tube is recorded (Myre & Shaw, 2006). A turbidity tube was built using a cylindrical acrylic tube, with a diameter of 90 mm and a height of 160 cm, and a Secchi disc was attached to the bottom of the tube. Clarity was measured at each site and sites were then selected in order to have a gradient of turbidity. In total, nine sites were selected (fig. 5, supplementary material fig. 21).

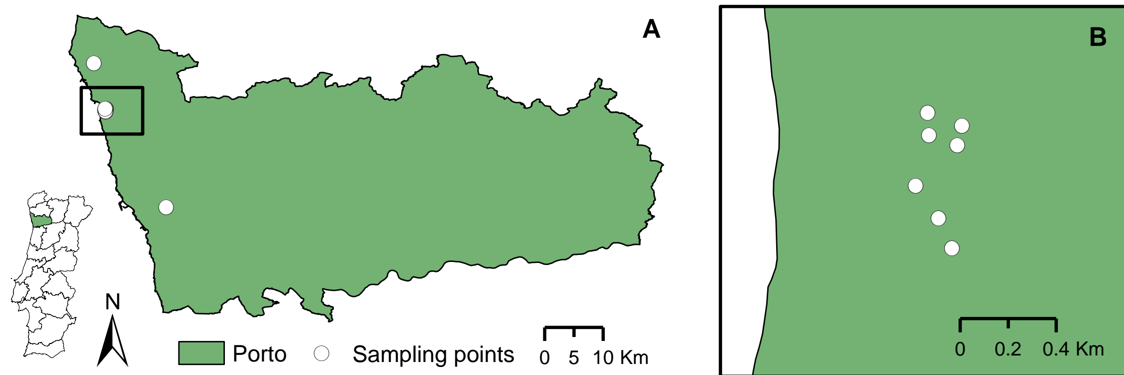


Fig. 5 Selected sampling points for comparison of methods in turbid waters.

2.2.2 Water sampling

Water collection was performed using the three different capture methods: precipitation, disc filters and capsules. Disc filters with a surface area of approximately 17.4 cm^2 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^2. Both filters had a polyethersulfone hydrophilic membrane and a pore size of 0.45 µm.

At each site, surface water was collected at two different sampling points, selected randomly. At each sampling point all three methods were employed, giving a total of 18 samples for each method. For capsules and disc filters, water was pumped through the units using a peristaltic pump (Solinst 410, Solinst Canada Ltd.), powered by a portable car battery. Silicon tubes (Solinst Canada Ltd.) were used to connect the pump to the filtering units. The water was filtered until the filter membrane clogged. The volume filtered with each method was recorded (supplementary material table 8) and the filters were stored in sterile bags. Regarding precipitation, a sterile 50 ml falcon was filled with 15 ml of water collected from the surface. Immediately after collection, 1.5 ml of sodium acetate 3M and 33.5 ml of absolute ethanol were added to the 15 ml water aliquots (Ficetola et al., 2008). All the samples were transported at room temperature to the laboratory and stored at -20ºC until DNA extraction. Time between field sampling and placement in storage was less than five hours. Any equipment being re-used across sites (i.e. silicon tubes and filtering cups) was cleaned in 10% bleach for at least 30 minutes and rinsed with distilled water to remove any bleach residues.

Two negative control were collected at each site, to monitor for possible contamination. This was carried out before collecting the samples, to ensure that equipment was decontaminated prior to commencing fieldwork. For the first, 15 ml of distilled water (brought form the laboratory) was added to a 50 ml falcon tube, along with 1.5 ml of

sodium acetate 3M and 33.5 ml of absolute ethanol. For the second, to ensure that all tubing and other reusable filtering apparatus was clean prior to starting sampling, 100 ml of distilled water was pumped through a filtering unit with a disc filter, which was subsequently stored as for other disc filters.

Clarity values were obtained using a turbidity tube and information on larvae abundance and pond size was recorded as well (supplementary material table 8).

2.2.3 DNA extraction

All DNA extractions were performed in a low-copy DNA laboratory, equipped with UV radiation. Strict protocols were followed, including disposable lab wear, UV sterilization of all equipment before entering the lab and cleaning workbenches and all the material needed for extraction with 60% 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.

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. The buffer was then poured into a clean container and filtered through a 0.45 µm 47 mm disc filter.

Disc filters were cut into small pieces and placed into a 15 ml falcon with 2 ml n-lauroylsarcosine based buffer (Maudet et al., 2004).

DNA extraction of precipitated samples followed the protocol by Ficetola et al. 2008, with minor modifications. Samples were centrifuged at 4000 rpm for 45 minutes, at 10ºC. The supernatant was discarded and 2 ml of n-lauroylsarcosine based buffer was added to the tubes.

All tubes with n-lauroylsarcosine based buffer were kept at 54ºC for 0.5 h. From here the E.Z.N.A.® Tissue DNA kit (Omega Bio-tek), following the manufacturer´s instructions, but using 300 µl BL Buffer, 300 µl ethanol and 50 µl Elution Buffer. A negative control was performed with each batch of extractions to monitor for possible contamination. In total 78 samples were extracted, including field and extraction negatives.

2.2.4 DNA quantification

Double-stranded DNA was quantified by fluorometry (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes, Eugene, Oregon, USA) in a 96-well black polystyrene microplate (OptiPlate™-96, Perkin Elmer). Briefly, DNA samples were diluted 1:100 with

TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and 100 µl of diluted Picogreen was added to the wells, following manufacturer's instructions. Two blanks were prepared, as well as five standards ranging from 0 to 20 µg/ml, and fluorescein values were read on a fluorescent plate reader (1420 Multilabel Counter, Victor™ 3, Perkin Elmer).

Using the software Wallac 1420 Workstation (Perkin Elmer), a linear regression was calculated with the fluorescein values obtained from the standards and their known concentration. The equation obtained was then used to calculate the concentration (µg/ml) in the remaining wells. Readings were performed three times and an average was obtained for each sample. Each time, R^2 values were inspected to ensure accuracy (ranged from 0.9997 – 0.9998). Lastly, the volume of each sample was measured with a micropipette (Eppendorf Research, Hamburg, Germany) to calculate the total mass of DNA (ng) captured.

2.2.5 qPCR

Species-specific primers and probes were designed using AlleleID 7 software. For this, available COI sequences from all amphibian species in Portugal were used as input, specifying *Salamandra salamandra* as the target (table 1). Due to the amplicon length (advised to be between 80 and 150 bp) and the high difference between melting temperatures of both primers (which should be within 1-2ºC of each primer) (Thornton & Basu, 2011), primer pair A was discarded. The recommendation to avoid guanine (G) repeats, as they might reduce amplification efficiency (Bustin & Huggett, 2017), precluded primer pair B from further analysis.

Species-specific primers C and D were chosen for further optimisation *in vitro* using extracted DNA from both *S. salamandra* and other non-target amphibian species known to occur in the study area (table 2), at similar concentrations. 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, were included as non-targets during PCR optimisation. Probes were labelled with the reporter dye FAM at the 5' end of the sequence and the quencher dye BHQ at the 3' end.

A gradient PCR including target and non-target DNA was conducted for both primers. In addition, eDNA samples collected from sites were *S. salamandra* doesn't occur were also included. Primer pair D outperformed primer pair C due to its higher specificity and it was chosen for further optimisation.

Table 1 Details of primers designed and trialled for qPCR analysis.

Primer set	Primer	Sequence (5' – 3')	Length (bp)	Melting temperature (ºC)
Salamander_ A	forward	CTTCACCCAACCTGGTCTAA	187	63.2
	reverse	TGATAACTGCAACTAATGAGATAA		59.8
	probe	TTGCCGGAATACCACGACGTTA		70.6
Salamander_ B	forward	TTCATT**GGGG**TTAATCTAACAT	148	59.3
	reverse	TGATAACTGCAACTAATGAGATA		59.1
	probe	TTCAGATTATCCAGACGCTTACACA		68.1
Salamander_ C	forward	ACACTTCACCCAACCTGGTC	135	66.3
	reverse	TGTGTAAGCGTCTGGATAATCTG		64.3
	probe	CCTTGCCGGAATACCACGACGTT		73.6
Salamander_ D	forward	CACCCTTATTCGTATGATCTGTC	112	64.6
	reverse	GTAGTGTTTAGGTTTCGATCTG		62.7
	probe	ACCGCAATCCTACTCCTCCTATCTCT		72.2

Table 2 Amphibian species used to optimise qPCR primers.

Order	Family	Species
Anura	Alytidae	*Alytes obstetricans*
		Discoglossus galganoi
	Pelodytidae	*Pelodytes atlanticus*
	Pelobatidae	*Pelobates cultripes*
	Bufonidae	*Epidalea calamita*
		Bufo spinosus
Urodela	Salamandridae	*Lissotriton boscai*
		Lissotriton helveticus
		Triturus marmoratus
		Salamandra salamandra

To determine the sensitivity and calculate the limit of detection of this assay, a set of standards were made, following methods similar to Sint et al. (2012). A PCR product produced by the Salamander_D primer pair was visualized on a 2% agarose gel stained with GelRed (Biotium), manually excised, and cleaned with the QIAquick® gel extraction kit (Qiagen), following manufacturer's instructions. The purified DNA was quantified on Qubit™ using the dsDNA high sensitivity assay kit (Invitrogen by ThermoFisher

Scientific), following the manufacturer's instructions and the number of copies was calculated using the software DNA CALCULATOR (Sint et al. 2012). A range of seven 10-fold dilutions from 3.00E+06 to 3.00E+00 copies$^{-\mu l}$ were performed.

Using the standards, four primer concentrations were tested – 0.3, 0.6, 0.9 and 1 µM – always maintaining the same primer/probe ratio. For each combination of primer concentration and standard, PCRs were conducted in triplicate. Based on the efficiency and R^2 values observed, a 0.6 µM of primer concentration was chosen.

Final qPCR conditions were performed in a total volume of 10 µl, including 5 µl of TaqMan Environmental Master Mix 2.0 (ThermoFisher Scientific), 0.6 µM of each primer, 0.17 µM of probe, 2.63 µl of H_2O and 1 µl of extracted DNA. PCRs were conducted in low profile unskirted 96-well plates (MLL-9601, BioRad), covered with microseal B adhesive seals (MSB-1001, BioRad), on a C1000 Touch™ thermal cycler, CFX96™ Real-Time System (BioRad). PCR cycles were as follows: 10 minutes of denaturation at 95ºC, followed by 45 cycles of denaturation at 95ºC for 30s, annealing at 54ºC for 30s and extension at 60ºC for 60s. All eDNA samples were conducted in triplicate with standards and negative controls included on each plate, also in triplicate.

qPCR results were analysed with Bio-Rad CFX Manager 3.1 software. Within the "Plate Setup" option, the fluorophore was selected to FAM, plate type to "BR clear" and wells were selected as "Unknown" for eDNA samples, "NTC" for negative controls and "Standard" for the six amplicon standards. The values of copies$^{-\mu l}$ were also included for each standard. The Cq threshold was defined as 10 times the fluorescence value of the average standard deviation of baseline cycles (e.g. Barletta et al., 2004; Sails et al., 2003). qPCR reactions were considered positive if a sample's fluorescence intersected the threshold line (coded as 1), and negative otherwise (coded as 0). Only samples that were positive in two out of three qPCR replicates were considered for downstream data analysis.

To further ensure that positive results corresponded to amplification of *S. salamandra*, 25 of the positive eDNA amplicons were randomly selected and sequenced by capillary electrophoresis. qPCR products were cleaned with ExoSAP (ExoSAP-IT® PCR Product Cleanup and FastAP Thermosensitive Alkaline Phosphatase, ThermoFisher Scientific). Sequencing reactions were carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), in a total volume of 10 µl, including 0.5 µl of BigDye®, 1 µl of BigDye® Buffer, 0.5 µl of primer, 7 µl of H_2O and 1 µl of cleaned qPCR product. Reactions were purified with Sephadex (GE Healthcare Bio-Sciences, Sweden) and sequenced by capillary electrophoresis using an ABI 3130xl

Genetic Analyzer Sequencer (Applied Biosystems, Foster City, Ca, USA). Resultant sequences were BLASTed (Zhang et al., 2000) against the NCBI Nucleotide database to validate species identification.

2.2.6 High-throughput sequencing

2.2.6.1 Library preparation

Amplicons for the Illumina Miseq platform were generated by means of a two-step PCR. The first PCR was conducted with primer pair 12S-V5-1 (Riaz et al., 2011), which targets a 105 bp region of the mitochondrial 12S rRNA gene. This primer was chosen based on a pilot study (data not shown), which demonstrated that the primer amplified *S. salamandra* DNA from eDNA samples with good resolution. PCRs were performed using a total volume of 10 µl, including 5 µl of Master Mix (Qiagen), 0.3 nM of each primer, 2.4 µl of H_2O and 2 µl of template DNA. Both forward and reverse primers used included the Illumina overhang adapters for downstream addition of sample indexes and flow cell sequencing adapters. All 78 samples were done in duplicate and a negative control was included in each reaction to monitor for possible contamination. Optimised PCR conditions were as follows: 15 minutes of denaturation at 95ºC, followed by 40 cycles of denaturation at 95ºC for 30s, annealing at 47ºC for 30s and extension at 72ºC for 30s, followed by a final extension step at 72ºC for 10 minutes. PCR products were visualized on a 2% agarose gel stained with GelRed (Biotium) to validate amplicon size and amplification success.

The second PCR was conducted to incorporate sample-specific indexes and Illumina adaptors to the amplicons from the first PCR. PCRs were performed in a total volume of 10 µl, including 5 µl of 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 1 µl of unique indexing primer combinations (Gansauge & Meyer, 2013; Kircher et al., 2012), 2 µl of water and 2 µl of the previous PCR product diluted 1:4 with diH_20. PCR cycling conditions consisted of an initial denaturation at 95ºC for 3 minutes, then 10 cycles of: denaturation at 95ºC for 30s; annealing at 55ºC for 30s; and extension at 72ºC for 30s, with a final extension step at 72ºC for 5 minutes. To validate the increase in amplicon size, indicating that indexes and adaptors had been incorporated, two random samples per plate were visualised on a 2% agarose gel stained with GelRed (Biotium).

Indexed PCR products were cleaned with 0.9x AMPure XP beads (Beckman Coulter) using a Magnetic Bead Extractor for 96-well microplates (V&P Scientific, Inc.) and eluted in a final volume of 25 µl Tris 10 mM. Cleaned PCR products were then quantified by spectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific), normalized to 15 nM using diH_20, before being pooled together by combining 5 µl of each sample.

The concentration of the pool was assessed with Qubit™ (Robin et al., 2016) using the dsDNA high sensitivity assay kit (Invitrogen by ThermoFisher Scientific), following manufacturer's instructions. Additionally, to validate fragment sizes, amplicons were analysed in a 2200 TapeStation (Agilent Technologies). The pool was then diluted to 4 nM with Tris 10mM 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).

2.2.6.2 Sequence data

Before analysing the generated sequence data, a 12S nucleotide database was created with sequences from species known to occur in the study areas (chapter 2 and chapter 3). For this, 33 sequences from ten different species were retrieved from NCBI (supplementary material table 9). Due to a lack of sequences in the regions of interest for four species (*Pelophylax perezi*, *Lissotriton boscai*, *Lissotriton helveticus*, *Pelodytes atlanticus*), four additional sequences previously obtained through capillary electrophoresis using the selected primers were added to the database. For this, PCR products were sequenced with both primers forward and reverse, twice, and a consensus sequence was created using Geneious v4.8.5 (see supplementary material for DNA sequences).

Reads produced on the MiSeq platform (Illumina) were demultiplexed according to the sample-specific indexes using BASESPACE (basespace.illumina.com). With the OBITools package (Boyer et al., 2016; Ficetola et al., 2010), paired-end reads were aligned and alignments with a quality score < 40 were removed. Singletons (sequences with a read count of 1) and reads with length < 75 bp and > 120 bp were removed, based on expected amplicon size. The resulting sequences were blasted against the 12S amphibian database using MEGABLAST algorithm (Zhang et al., 2000). BLAST results were assigned to taxa using MEGAN Community Edition 6.10.8 (Huson et al., 2016). Only hits with 99% identity were considered and sequences were only assigned to species level if there were no hits to other species that had BLAST scores within 1 % of the top hit. Additionally, sequences with reads counts < 20 (based on PCR controls) and ≤ 3% of the total read count of the respective sample were removed. This threshold was based on the presence of a small number of reads of one species in samples outside the species distribution range. Finally, sequences that appeared in only one PCR replicate were not considered. Samples where *S. salamandra* was detected after

bioinformatic filtering were considered positive (coded as 1). Otherwise, samples were treated as negative (coded as 0).

2.2.7 Data analyses

Statistical analyses were performed using R (R Development Core Team, 2008).

DNA quantification data, i.e., the total mass of eDNA captured, was analysed using the "lmer" function from the package lme4 1.1-17 (Bates et al., 2015). To assess the effects of measured variables, eDNA quantity was treated as a continuous response, whereas water clarity and volume filtered were treated as a continuous factor and method as a categorical factor. The quantity of eDNA captured per litre of water processed (always 15 ml for precipitation) was analysed as well, treated as a continuous response, with only method as a categorical factor. When using volume as a continuous response, water clarity and method were treated as above, and analysed using the same function. Due to high correlation between volume filtered and method, relationships between water clarity and volume filtered were examined for each method separately. Normality (tested with Shapiro-Wilk test and quantile-quantile plots on residuals of the models), homoscedasticity (verified with fitted values vs residuals plots) and autocorrelation (confirmed with a residual autocorrelation plot) were assessed each time and response variables were transformed when one of the assumptions was violated.

Species detection data from qPCR and HTS were analysed using the "glmer" function for binary data, from the same package. To assess the effects of measured variables, species detection was treated as a binary response, while water clarity, volume filtered and eDNA quantity were treated as a continuous factor and method as a categorical factor.

Significance values were assessed with the "anova" function from the package car 3.0-0 (Fox & Weisberg, 2011). When a significant relationship among methods was detected, "emmeans" function from package emmeans 1.2.3 (Lenth, 2018) was applied to compare p-values for each comparison of levels.

In order to account for non-independence of sampling points within sites, site was included in all linear models as a random factor. Random factors can be included in the models in four different ways, either affecting only the intercept, only the slope, both correlated or both uncorrelated. Site was included as random in the models in all four different ways each time and the best model was selected based on AIC values.

2.3 Results

2.3.1 Effect of filtering method and water clarity on volume of water filtered

The volume of water filtered was significantly different ($p < 0.0001$) between capsules ($\bar{x} = 7.89$ L, SE = 6.79) and disc filters ($\bar{x} = 1.10$ L, SE = 1.03), with capsules filtering on average 2.11 ln(L) more than disc filters (fig. 6).

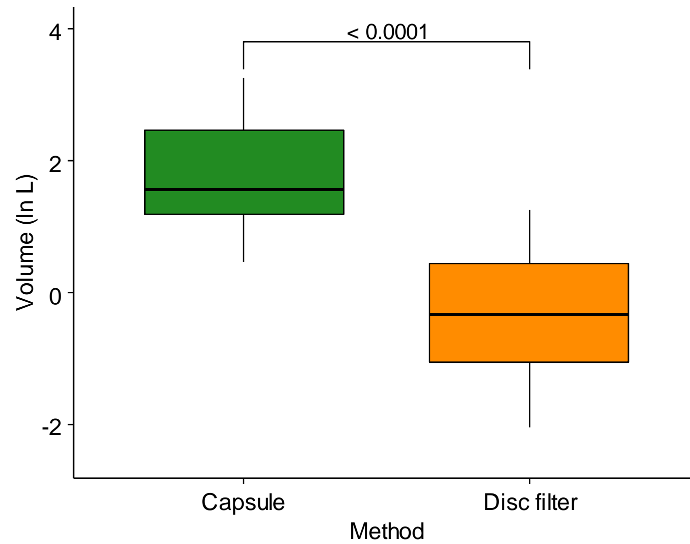


Fig. 6 Volume of water filtered with capsules and disc filters.

Additionally, although there was not a significant relationship between water clarity and volume of water filtered with capsules (supplementary material fig. 22), there was a significant relationship using disc filters ($p < 0.001$) (fig. 7). For every one cm of clarity, there was an increase of 0.03 ln(L) volume.

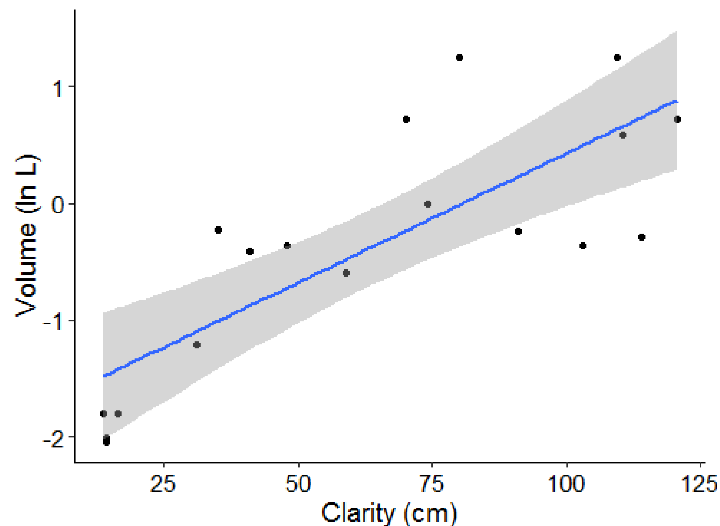


Fig. 7 Relationship between volume filtered and water clarity using disc filters.

2.3.2 Effect of filtering method, water clarity and volume filtered on eDNA capture

There was a significant difference ($p < 0.0001$) in the quantity of eDNA captured between methods (capsules: $\bar{x} = 366.61$ ng, SE = 261.66; disc filters: $\bar{x} = 194.29$ ng, SE = 113.74; precipitation: $\bar{x} = 69.91$ ng, SE = 74.22). Capsules captured on average 0.61 ln(ng) more DNA than disc filters ($p < 0.01$) and 1.83 ln(ng) more than precipitation ($p < 0.0001$), while disc filters captured on average 1.22 ln(ng) more DNA than precipitation ($p < 0.0001$) (fig. 8A).

Regarding the quantity of eDNA captured per litre of water processed, the opposite pattern was observed (capsules: $\bar{x} = 112.32$ ng/L, SE = 144.59; disc filters: $\bar{x} = 413.54$ ng/L, SE = 475.28; precipitation: $\bar{x} = 4660.91$ ng/L, SE = 4947.93), with significant differences among methods ($p < 0.0001$). Precipitation captured on average 2.60 ln(ng/L) more DNA than disc filters ($p < 0.0001$) and 4.10 ln(ng/L) more than capsules ($p < 0.0001$), while disc filters captured on average 1.50 ln(ng/L) more than capsules ($p < 0.01$) (fig. 8B).

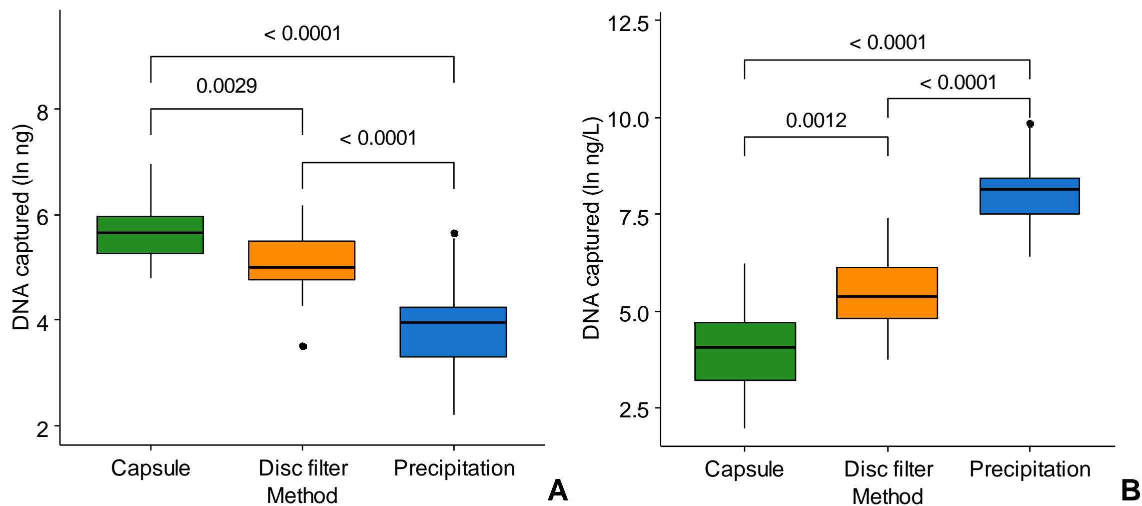


Fig. 8 Mass of eDNA captured **(A)** and mass of eDNA captured per litre of water processed **(B)** for each method.

Neither water clarity nor volume filtered had a significant relationship with eDNA quantity captured.

2.3.3 Effect of filtering method, water clarity and volume filtered on qPCR detection

The final qPCR assays exhibited 92-100% efficiency, R^2 between 0.993-0.998 and slope between -3.33 and -3.53. All 25 randomly selected qPCR positives, sequenced by capillary electrophoresis, were identified as *S. salamandra*, confirming the specificity of the primers developed. The limit of detection observed was at 30 copies$^{-\mu l}$, i.e., all qPCR replicates of the 30 copies/ul standard and above amplified (and crossed Cq threshold) successfully.

There was a significant relationship between water clarity and the detection of *S. salamandra* for capsule-collected eDNA ($p < 0.001$), with higher water clarity resulting in higher rates of species detection (slope = 1.13) (fig. 9A). No relationship was detected between water clarity and the other two methods.

The volume of water filtered with capsules had a significant relationship with qPCR detection as well ($p < 0.05$), with species detection being higher at larger volumes (slope = 1.26) (fig. 9B). No significant relationship was observed for disc filters.

There was a significant difference in qPCR detection between methods ($p < 0.01$), with species detection being statistically different between precipitation and the other two methods (fig. 10).

No relationship between species detection and eDNA quantity captured was observed for any of the methods.

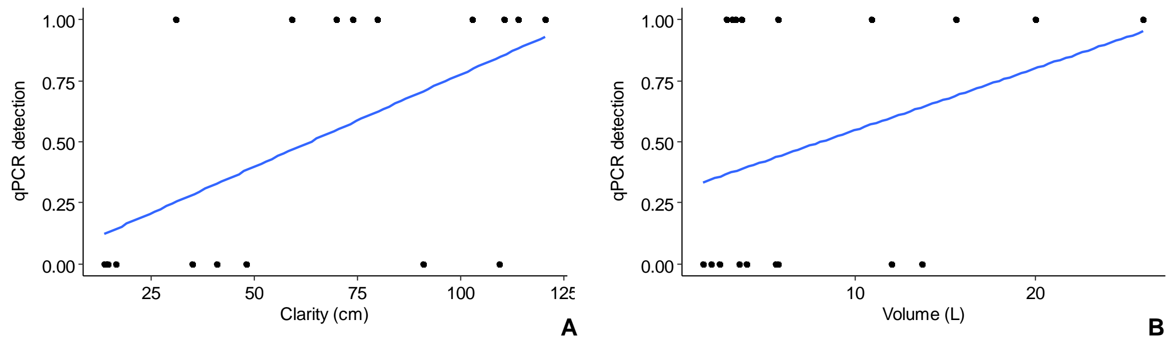


Fig. 9 The relationship between qPCR detection (0/1) of *S. salamandra* and water clarity **(A)** and volume filtered **(B)** for capsule-captured eDNA.

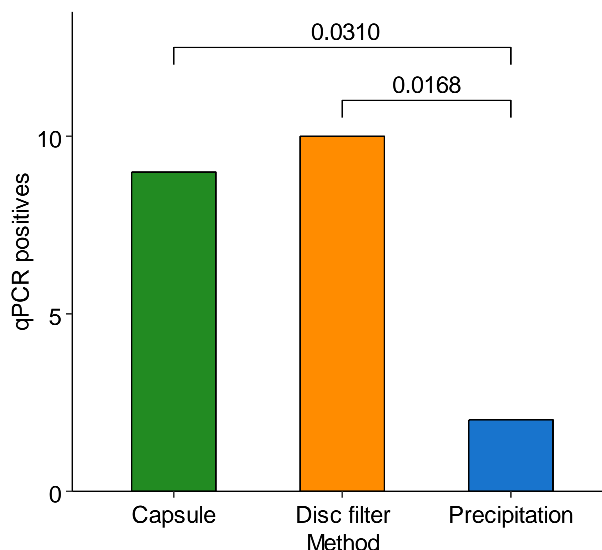


Fig. 10 Number of samples in which *S. salamandra* was detected by qPCR (n = 18 for each method).

2.3.4 Effect of filtering method, water clarity and volume filtered on HTS detection

In total, 2,107,127 reads were produced prior to bioinformatic filtering. After filtering, 303,609 reads were identified as amphibian species, of which 69,080 reads corresponded to *S. salamandra.* A multi-species comparison between capture methods was not conducted, given that the pilot study for primer selection showed the primers do not amplify all amphibians present in the study area.

Similar to qPCR detection, volume of water filtered had a significant relationship with the detection of *S. salamandra* for capsules ($p < 0.05$), but not for disc filters or precipitation. Once more, higher volumes of water filtered favoured species detection with HTS (slope = 0.39) (fig. 11).

No significant relationship between species detection and either water clarity or eDNA quantity was observed for any of the methods.

A significant difference in species detection between methods was observed ($p < 0.05$), between precipitation and capsules (fig. 12).

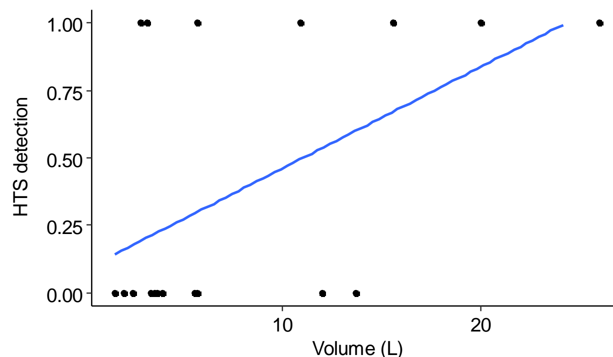


Fig. 11 The relationship between HTS detection (0/1) of *S. salamandra* and volume filtered for capsule-captured eDNA.

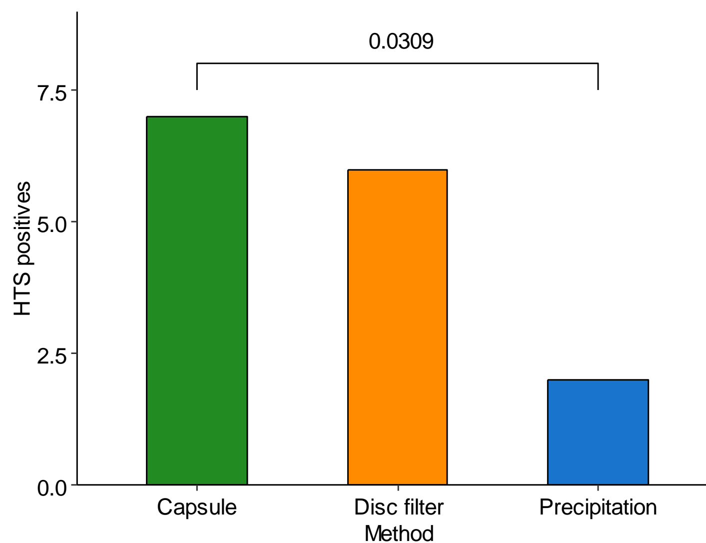


Fig. 12 Number of samples where *S. salamandra* was detected by HTS (n = 18 for each method).

2.3.5 Species detection with qPCR vs HTS

Only disc filters exhibited a significant difference in species detection between qPCR and HTS ($p < 0.01$) (fig. 13), with *S. salamandra* detection being higher with qPCR.

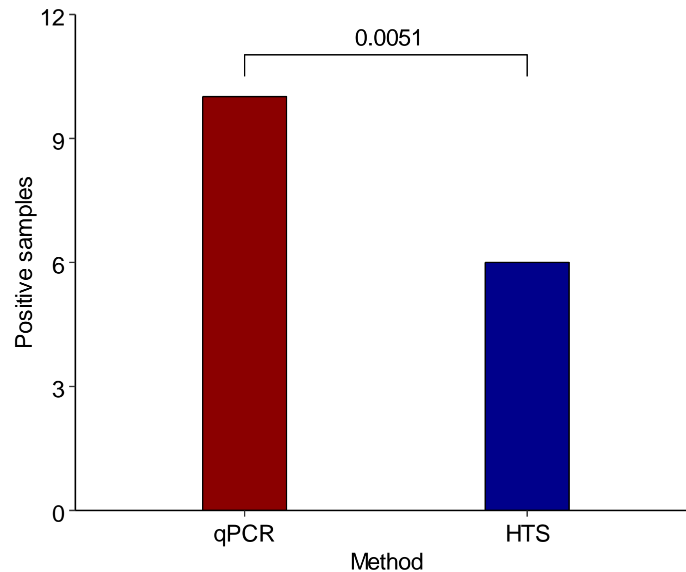


Fig. 13 Number of samples where *S. salamandra* was detected by qPCR and HTS with disc filters (n = 18 for each assay).

2.3.6 Overview of results

The results obtained are summarized in table 3.

Capsules filtered significantly higher volumes of water than disc filters. Water clarity was positively related to the volume filtered for disc filters, but not for capsules.

The quantity of eDNA captured was significantly different across the three capture methods. Capsules captured the most, followed by disc filters, then precipitation. However, this trend was reversed when considering the quantity of eDNA captured per litre of water processed. qPCR species detection rates were significantly lower for precipitation than for the other two eDNA capture methods (which were similar). HTS species detection rates were only significantly different between capsule and precipitation methods, the latter having a lower detection rate.

Both the volume of water filtered and water clarity were positively related to qPCR species detection rates when using capsules, but not using the other two methods. The volume of water filtered was only positively related to HTS species detection rates when using capsules. Overall, species detection was only significantly different between the two detection assays when using disc filters, with qPCR having a greater detection rate than HTS.

Table 3 Summary of results obtained for eDNA methods comparison (Cap – capsules; Disc – disc filters; Prec – precipitation). X and Y represent the independent and dependent variables in each research question, respectively. Significance values are represented by stars: p<0.05 (*), p<0.01 (**), p<0.001 (***) and p<0.0001 (****). The lack of significance is represented by "ns" and comparisons not performed by a dash (-).

X \ Y	*Volume*	*ng*	*ng/L*	*qPCR detection*	*HTS detection*	*qPCR vs HTS*
Disc vs Cap	****	**	**	ns	ns	-
Disc vs Prec	-	****	****	*	ns	-
Cap vs Prec	-	****	****	*	*	-
Cap	-	-	-	-	-	ns
Disc	-	-	-	-	-	**
Prec	-	-	-	-	-	ns
Clarity (Cap)	ns	ns	-	***	ns	-
Clarity (Disc)	***	ns	-	ns	ns	-
Clarity (Prec)	-	ns	-	ns	ns	-
Volume (Cap)	-	ns	-	*	*	-
Volume (Disc)	-	ns	-	ns	ns	-
ng (Cap)	-	-	-	ns	ns	-
ng (Disc)	-	-	-	ns	ns	-
ng (Prec)	-	-	-	ns	ns	-

2.4 Discussion

The small size of the puddles and ponds sampled in this study, together with the confirmed presence and relatively high abundance of the target species during eDNA sampling, constitute a good study system to evaluate the efficiency of different capture and sequencing methods and to provide recommendations for eDNA-based surveys in turbid waters. This study identified that: 1) filtration techniques outperformed precipitation, generating higher species detections and captured eDNA; 2) *S. salamandra* eDNA detection was significantly better with qPCR than HTS when using disc filters; and 3) disc filters seem to be a more suitable choice for *S. salamandra* eDNA detection when sampling in turbid ecosystems, as they are cheaper and no differences in detection rates between filtering methods was observed.

2.4.1 Effect of volume of water filtered in eDNA-based surveys

The chances of DNA fragments being captured increases as more water is sampled, theoretically increasing the probability of detecting the target species (Herder et al.,

2014). In this study, a relationship between volume of water filtered and species detection using both qPCR and HTS was observed. Nevertheless, only for capsule-collected eDNA the relationship was significant. This might be explained by the larger volumes of water filtered with this method, significantly higher than the volumes filtered with disc filters.

The higher performance of capsules regarding the volume of water filtered was somewhat expected, due to their larger surface area. Previous studies have demonstrated the capacity of filtering large volumes with this type of filters in low turbidity systems, such as 20L (Vences et al., 2016), 45 L (Civade et al., 2016), and even 100 L (Valentini et al., 2016). Nevertheless, filtering higher water volumes may not always be advantageous, as it might increase the concentration of inhibitors in the sample (Herder et al., 2014), constraining subsequent laboratory procedures. Aditionally, in this study, despite the lower volumes filtered, disc filters displayed higher levels of mass DNA per litre.

Regarding the volume of water filtered, capsules are clearly a more suitable choice for eDNA studies. However, their lower levels of mass DNA per L, the possibility of capturing more inhibitors, and their high costs question their efficiency as a standard for eDNA surveys. Consequently, capsules may be more appropriate for running waters, as 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 stagnant water bodies, were DNA is less diluted (Herder et al., 2014).

2.4.2 Comparison of eDNA recovery and species detection between capture methods

Filtration and precipitation are currently the two main approaches to capture eDNA in aquatic ecosystems (Hinlo et al., 2017; Herder et al., 2014; Li et al., 2018). Filtration is more common with disc filters, while capsules have only recently been applied in eDNA studies (Lopes et al., 2017; Civade et al., 2016). Consequently, the number of studies comparing high-surface-area capsules with other eDNA methods is missing. To the best of our knowledge, only one study compared the performance of capsules with high surface area with common filtration methods (Vences et al., 2016). Nonetheless, the authors noted that their sampling design, with only two capsules, was not suitable for statistical comparison.

In our study, the choice of capture method clearly influenced DNA recovery and species detection, with filtration methods (i.e., capsules and disc filters) capturing more DNA 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 (Piggott, 2016; Hinlo et al., 2017; Eichmiller et al., 2016). The greater amounts of DNA captured and species detection observed for filtration methods were likely associated to their higher sample volumes (Raemy & Ursenbacher, 2018).

When using precipitation, all eDNA present in the water sample is captured and isolated (Herder et al., 2014), theoretically increasing the probability of detecting the target species. Nevertheless, although precipitation is more efficient when considering the quantity of eDNA captured per litre of water processed, this method captures less DNA and provides lower species detection than filtration methods, likely due to the low water volume that can be processed.

Capsules and disc filters had similar performances in terms of species detection. Nevertheless, capsules captured significantly more DNA than disc filters. Even though the volume of water filtered was significantly higher with capsules than disc filters, it did not influence the quantity of eDNA captured, thus making it unlikely to explain the observed differences. As the pore size, membrane material and extraction method of capsules and disc filters were similar, it is possible that some other inherent characteristic of this method contributed to the results. Capsules might reduce the exposure to environmental stressors since capture of eDNA takes place inside the filter capsule, protecting DNA from degradation (Spens et al., 2017).

The low quantity of captured DNA and low species detection rates using the precipitation method make it an ineffective approach for eDNA studies. Within filtration methods, capsules exhibited slightly better performances than disc filters. Moreover, capsules decrease the risk of contamination and DNA degradation due to reduced exposure to physical and biogenic stressors, given that capture, storage and extraction of eDNA occurs inside the capsule (Spens et al., 2017). Nevertheless, disc filters are cheaper than capsules, making this a suitable method when funds are limited.

2.4.3 Effect of turbidity in eDNA studies

As demonstrated in previous studies, DNA is highly concentrated in aquatic sediments (Turner et al., 2015) and sampling in turbid waters might be expected to result in high DNA recoveries and consequently higher species detection.

Contrary to expected, our results show no link between turbidity and the quantity of DNA captured with any of the methods. This might be explained by the absence of a post-extraction step to remove PCR inhibitors, that might influence downstream analyses (Goldberg et al., 2015). The only effects of turbidity detected were regarding qPCR

detection for capsules and volume of water filtered for disc filters, where more turbid waters resulted in lower detection rates and less volume filtered, respectively.

In a recent study, Egeter et al. (2018) reported that survey in turbid water bodies was severely constrained due to clogging of filters and could not be carried out as originally anticipated. The authors hypothesized that encapsulated filters might provide an efficient alternative to disc filters in high-turbid systems due to their large surface area. Nevertheless, despite being influenced by turbidity, disc filters appear suitable when sampling in turbid ecosystems. Species detection with this method is possible even in highly turbid waters (supplementary material table 10) and it is not influenced by water turbidity. Combined with pre-filtering steps (Robson et al., 2016) and/or increased number of filter replicates (Hinlo et al., 2017), which can help overcome clogging issues, common disc filters appear to be an efficient solution for filtering in high-turbidity systems.

Recently, Spens et al. (2017) recognized that further research is needed in order to identify optimal procedures for filtering in highly turbid waters. This study is an additional step towards that goal. Despite being limited by the rather low sample size, we demonstrated that species detection in highly turbid waters is possible with disc filters. In our study system, focusing on one species that was found in relatively high abundance, capsules did not offer a clear advantage, despite being more expensive. However, the effects of the capture methods on multi-species detections were outside the scope of this study and such differences may exist. Further research is thus needed in the future in order to validate the performance of disc filters in turbid waters. Ultimately, this will provide an extended application of eDNA studies to turbid ecosystems, which often harbour high biodiversity levels.

2.4.4 qPCR vs HTS comparison for eDNA detection of *Salamandra salamandra*

Species detection with eDNA methods can be accomplished with either a single-species or a multiple-species approach. Single species detection is particularly useful for endangered (e.g. Piggott, 2016) or invasive species (e.g. Hunter et al., 2015), where the knowledge on species distribution allows the development of proper conservation or eradication measures, respectively. Species detection can be achieved using different assays, such as conventional PCR, quantitative PCR (qPCR) and HTS. Conventional PCR is usually less sensitive and specific (Herder et al., 2014), and consequently less appropriate for species-specific studies (Piggott, 2016). Comparisons between qPCR and HTS assays are essential in order to provide an informed decision of the most suitable method for species detection. In this study, only disc filters exhibited a significant difference between both methods, with qPCR providing higher species detection than

HTS. Even though species detection might have been influenced by larvae abundance, which varied among sites, the same set of sites was used for both detection assays, thus decreasing its influence on the observed results. Moreover, in a few cases, one or two methods failed even when abundance was high.

Few studies so far have compared the efficiency between both detection assays for a target species. As partially opposed to our results, Murray et al. (2011) demonstrated that qPCR and HTS approaches displayed very similar results when attempting to detect four prey species from penguin scats. Even though no statistical differences were detected between detection methods, a slight difference was apparent. The authors explained the observed pattern by highlighting the different fragment sizes and specificity of the primers used for each method. The fragment sizes used in our study were very similar, differing by only 7 bp (qPCR target fragment: 112 bp; HTS target fragment: 105 bp), making it unlikely to explain the observed differences. Nevertheless, the specificity of our primers was somewhat different. qPCR primers were designed to specifically target *S. salamandra*, while the HTS primers used vertebrates in general as target. This discrepancy between primer specificity might explain the better performance of qPCR over HTS when using disc filters.

Considering the above, qPCR appears to be more advantageous than HTS if the goal of the study is a species-specific approach, targeting *S. salamandra*, and using disc filters. In fact, previous studies have been successfully applying qPCR methods for detecting this species (Preißler et al., 2018). While HTS is often more advantageous and cost-efficient in high species diversity systems (Thomsen & Willerslev, 2015), single species detection with qPCR is generally cheaper 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).

To the best of our knowledge, no other study besides Murray et al. (2011) compared qPCR and HTS for species-specific detection. The differences in the observed results between their study and ours highlight the importance of testing the most suitable approach for each study and target species, as well as the need for further research on this topic. With this study, we provide important information on the most appropriate method for *S. salamandra* detection. This will allow an informed decision regarding the detection method selected in future eDNA studies targeting this species, thus providing a better knowledge of its distribution and a closer monitoring, important for this species conservation (Preißler et al., 2018).

2.4.5 Future research for eDNA-based surveys in turbid waters

eDNA methods have become a common procedure nowadays and the number of studies applying this technique continues to increase. Notwithstanding, there are still some gaps to fill in order to fully understand this technique's potential. Particularly, knowledge about the efficiency of eDNA methodologies in turbid waters remains limited (e.g. Egeter et al., 2018; Williams et al., 2017) and further effort should be allocated in order to understand the dynamics between aquatic sediments and eDNA.

Identifying the best capture method is essential for accurate biodiversity surveys using eDNA techniques. This study is the first to compare high-surface-area capsules with common eDNA methods such as precipitation and filtration with disc filters, with the same pore size and in a gradient of turbidity, paving the way for a better understanding on the efficiency of eDNA methodologies in high-turbidity ecosystems. We were able to demonstrate that species detection is possible in turbid environments using two filtration methods. Such information can be highly advantageous for future eDNA studies in turbid waters, allowing an informed decision regarding the capture method to use. Nevertheless, further research with larger sample sizes and a multi-species approach should be conducted in order to proper validate their performance in high-turbidity waters. Ultimately, this will provide an application of amphibian eDNA studies in turbid ecosystems.

Chapter 3: Factors influencing species detection in Mediterranean ponds

3.1 Introduction

Monitoring of biodiversity is crucial to identify species' distribution and abundance and to detect conservation needs. Nonetheless, all types of faunal surveys have imperfect detections and it is likely that only a fraction of species present at the site will be detected (Kéry & Schmidt, 2008). This leads to an under-estimation of species distribution, possibly delaying the application of conservation measures. The combination of statistical methods that account for imperfect detection (e.g. Goldberg et al., 2018), and new powerful methodologies, such as eDNA, capable of outperforming traditional surveys regarding species detection (e.g. Valentini et al., 2016; Sigsgaard et al., 2015), are essential for developing accurate biodiversity monitoring.

Despite its generally higher detection probability when compared to traditional approaches, even at very low population densities (e.g. Ficetola et al. 2008), the probability of detecting eDNA of a target organism is not consistent across sites nor across species and is influenced by a range of ecological features (Herder et al., 2014). The production and degradation rate of eDNA, the habitat of the species, the sampling strategy as well as laboratory procedures are some of the factors that may influence eDNA detectability and generate false negative results.

The production rate of eDNA can be affected by different biological traits of the target organism, such as their diet (Klymus et al., 2015) or reproductive status (Spear et al., 2015; de Souza et al., 2016), with increased eDNA production during the breeding period. Amphibian larvae are highly abundant in water bodies during the reproductive season, shedding large amounts of eDNA (Herder et al., 2014). Moreover, the abundant mucus produced by amphibian epidermal cells is known to be an important source of DNA (Livia et al., 2006) increasing the detection probability of this taxonomic group. Production rates can also differ between species (Herder et al., 2014) and even between individuals of the same species (Pilliod et al., 2014; Strickler et al., 2015; Klymus et al., 2015) due to intrinsic biological traits such as their size or sex (Goldberg et al., 2015)

Once released into the environment, DNA degradation can occur either by biotic (microbial activity, endonucleases) or abiotic factors (e.g. UV radiation, temperature, pH, etc.) (Herder et al., 2014), generally degrading faster in warm or acidic aquatic habitats (Strickler et al., 2015). Previous research conducted under controlled mesocosm experiments showed that eDNA is detectable from two weeks (Thomsen et al., 2012) to nearly one month (Dejean et al., 2011) in aquatic environments after the removal of the species source of DNA. Similarly, in natural conditions, DNA could only be detected from a few hours (Dell'Anno & Corinaldesi, 2004) to nearly one month (Dejean et al., 2011),

with large running waters providing lower detectability due to high dispersion and dilution of DNA (Herder et al., 2014).

The volume of water filtered can also influence species detection. Filtering larger volumes theoretically increases detection probabilities, as the chances of DNA fragments being captured increases (Herder et al., 2014). Capsules are known to be able to filter large volumes of water (Valentini et al., 2016; chapter 2 of this master thesis). Nevertheless, due to its recent application in eDNA research, the number of studies testing this method is still scarce (Spens et al., 2017; Vences et al., 2016) and its potential is not fully understood. For example, the question remains if filtering small volumes several times provides similar results to filtering a single large volume. As DNA is not homogenously distributed in the water (Herder et al., 2014), filtering in multiple sites within the study area is often recommended (Goldberg et al., 2016). Nevertheless, processing higher volumes can also increase species detection (Lopes et al., 2017; chapter 2 of this master thesis) and might require fewer sampling sites.

In short, the non-detection of the target species (i.e. the occurrence of false negatives) does not imply its absence. The use of site occupancy models (Mackenzie et al., 2002) can help overcome this uncertainty associated with eDNA methods (Goldberg et al., 2016; Hunter et al., 2015; Herder et al., 2014). Such models account for imperfect detection and use the information on presence/absence data to calculate species detection probabilities. As an example, Schmidt et al. (2013) obtained a more reliable estimation of occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* from aquatic eDNA samples by using site occupancy models. Likewise, Hunter et al. (2015) were able to estimate for the first time the occurrences and detection probabilities of giant constrictor snakes in southern USA. More recently, Goldberg et al. (2018) used these models to better understand how degradation and dispersion processes influence the detection of amphibians in wetlands.

Following the global trend, Mediterranean amphibian species have experienced recent population declines, mainly due to invasive species (Cruz et al., 2008; Rebelo et al., 2010), infectious diseases (Rosa et al., 2013; Rosa et al., 2017) and chemical contamination (Marques et al., 2008). Temporary Mediterranean ponds are important sources of amphibian diversity, sustaining important populations of some species. These habitats are crucial for amphibian reproduction, but habitat destruction and fragmentation due to agricultural intensification have resulted in the decline of Mediterranean amphibian communities (Beja & Alcazar, 2003; Curado et al., 2011; Ferreira & Beja, 2013).

eDNA methods might help monitoring amphibian species, but the effects of abiotic factors on species detectability, as well as the effects of methods themselves (e.g. volume filtered), should be accounted for if such studies are to be trusted. Site occupancy models are capable of coping with imperfect species detection and can incorporate environmental variables in order to assess their influence on species detection rates. The combination of this tool with eDNA-based surveys has the potential to provide better estimates of species distribution, critical for conservation and management actions.

Using a set of temporary ponds in southwest Portugal where several Mediterranean amphibians occur, this chapter aims to: 1) use site occupancy approaches to measure the probability of detection of amphibian species according to several environmental variables and 2) compare detection probabilities of amphibian species processing either low volumes of water at multiple sampling points (obtained with disc filters) or a single large volume of water (obtained with capsules).

3.2 Material and methods

3.2.1 Study area and target species

Fieldwork was conducted in 16 sites within a natural park (Parque Natural do Sudoeste Alentejano e Costa Vicentina - PNSACV) on the coastal plain of southwest Portugal (fig. 14, supplementary material fig. 23). This region is characterized by a Mediterranean climate, with an oceanic influence. The landscape is predominantly flat, mainly used for agriculture and livestock production (Beja & Alcazar, 2003; Ferreira & Beja, 2013). Small, shallow depressions are filled with water in the winter and dried in the summer, constituting temporary ponds that are used by breeding amphibians. These ponds vary from each other in several physical, chemical and ecological variables (Beja & Alcazar, 2003). Despite the intense agriculture and grazing activities representative of the region, a large diversity of taxa can be observed within the ponds, such as small crustaceans, aquatic insects, vascular plants and amphibians (Beja & Alcazar, 2003).

Concerning amphibian diversity, three species of Caudata and seven species of Anura are known to occur in the study area from previous studies (Ferreira & Beja, 2013). Even though breeding seasons vary among species, amphibian reproduction is highest between April and May. In this period, amphibian activity increases, with different reproductive cycles present in the water at the same time (i.e. mating, egg deposition and larvae development). Therefore, sampling was conducted from 24th April to 7th May 2018, concentrating efforts on the period with highest amphibian activity.

This study system was chosen because species occurrence data was already available for the region and multiple species are known to occur at each site. Moreover, the low dispersion and dilution of DNA typical of small stagnant water bodies are expected to provide relatively high eDNA concentrations, making detections more frequent, which is essential for the computation of robust and reliable site occupancy models.

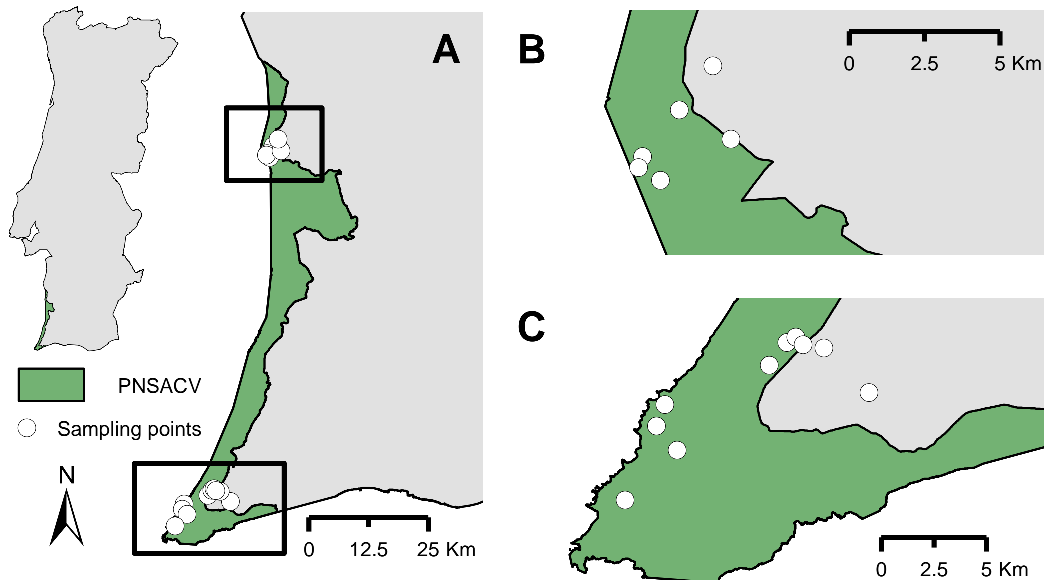


Fig. 14 Study area and sampling points for site occupancy models.

3.2.2 Water sampling

Water collection was performed using disc filters and capsules, with the same features as in chapter 2. Based on the results of chapter 2, the precipitation method was not used. At each site, surface water was filtered using disc filters in five different sampling points, selected in order to maximize the geographic coverage along the pond edge. Capsules were only used in one of the five sampling points. For both capture methods, water was pumped through the filtering units using a peristaltic pump (Solinst 410, Solinst Canada Ltd.), powered by a portable car battery. Silicon tubes (Solinst Canada Ltd.) were used to connect the pump to the filtering units. The water was filtered until the filter membrane clogged. The volume filtered with each method was recorded (supplementary material table 11) and both the disc filters and capsules were stored in sterile bags. All samples were transported at ambient temperature and placed in freezer storage (c. -20ºC) at the local field station within ten hours of sampling. At the end of the field trip, samples were transported to the laboratory with frozen ice packs and transferred to a -20ºC freezer until DNA extraction. Any equipment being re-used across sites (i.e. silicon tubes and filtering cups) was sterilized in plastic tanks using a chlorine solution for at least 30 minutes and rinsed with bottled water to remove any chlorine residues.

To monitor for possible contamination of tubes and filtering apparatus, one negative control was collected at each site prior to sample collection, to ensure that equipment was properly decontaminated. This was collected by filtering 100 ml of bottled water using a disc filter. Gloves were used during handling of samples and changed between sites or whenever they come into contact with a potential contaminant.

Based on evidence from the literature that they are likely to influence eDNA detectability, the following variables were measured on the day of sampling: water temperature, pH and conductivity were measured with a waterproof portable meter (model HI 98130, HANNA instruments); clarity values were obtained using a turbidity tube (see section 2.2.1); pond area was obtained by a complete walk around the pond with a portable GPS; pond depth was measured at the centre of each pond (after sample collection, to avoid contamination) (supplementary material table 11).

3.2.3 Amphibian surveys

Amphibians were sampled using sweep sampling, targeting mainly larvae, as this is a common and efficient method for inventorying amphibians (Beja & Alcazar, 2003). Depending on the size of the pond, three to four 30' sweeps were conducted using dipnets and covering all habitats in each sampling site (fig. 15A) (Ferreira & Beja, 2013; Beja & Alcazar, 2003). The specimens were identified to species level based on morphological traits and number of larvae for each species was recorded. At the end of each sampling session, individuals were released to their sampling point. Adults of some species were sometimes seen or heard during the fieldwork and therefore recorded as present in the site as well. When some of the common species known to occur in the region were not found during diurnal sampling further nocturnal surveys were conducted, consisting of a walk through the pond and setting up a funnel trap (fig. 15B).

Fig. 15 Amphibian surveys conducted using sweeps **(A)** and funnel traps **(B)**.

3.2.4 DNA extraction

DNA extractions from disc filters and capsules were performed using the same precautions and protocols described in chapter 2 (see section 2.2.3). In total, 117 samples were extracted, including field and extraction negatives.

3.2.5 High-throughput sequencing

3.2.5.1 Primer selection

COI, 12S and 16S are commonly used genetic markers for vertebrate eDNA studies. Initially, a literature review was conducted in order to select primers targeting these markers that have been previously used to detect amphibian species (table 4). Next, the available sequences from all Portuguese amphibian species for these three markers were retrieved from NCBI and BOLD and a small database was created for each one (COI database comprised 15 sequences, while 12S and 16S databases contained 17 sequences). Finally, an *in silico* PCR was performed using the ecoPCR tool from the OBITools package (Boyer et al., 2016; Ficetola et al., 2010), allowing a maximum of two mismatches per primer. This tool uses as input the databases previously created and the primers selected, and outputs a list of species that the primers can amplify. This was performed for each set of primers. Based on the number of Portuguese amphibian species amplified *in silico* (data not shown), primers were selected for *in vitro* optimisation.

PCR conditions (i.e. annealing time and temperature) were optimised using extracted DNA from all Portuguese amphibian species (table 5). PCR products were sequenced by capillary electrophoresis, as described in chapter 2 (see section 2.2.5), to validate species identification. Due to its low resolution, primer pair BA (Bálint et al., 2017) was discarded from the analysis. The remaining three primer sets were then tested with eDNA samples and primer pair EGETER-FROG-16S-F3/R3 was discarded due to low amplification success in these samples. Final PCRs for library preparation were thus conducted with primer pairs 12S-V5-1 and 12S-V5-2 (Riaz et al., 2011). These primer pairs exhibited taxonomic resolution to species level and good amplification success with eDNA samples. Even though amplification of some species was exclusive to each primer pair, when combined, these primers exhibited amplification for all species and were thus used together. Primer pairs 12S-V5-1 and 12S-V5-2 target the mitochondrial 12S rRNA gene, amplifying a region of 105 and 100 bp respectively. They share the same reverse primer and the resulting amplicons overlap over most of their length.

Table 4 Set of primers tested during *in silico* PCR. Those highlighted in bold were chosen for further optimization.

Marker	***Primer forward***	***Primer reverse***	***Target***	***Reference***
16S	EGETER-FROG-16S-F1	EGETER-FROG-16S-R1	Amphibians	Not published
16S	EGETER-FROG-16S-F2	EGETER-FROG-16S-R2	Amphibians	Not published
16S	**EGETER-FROG-16S-F3**	**EGETER-FROG-16S-R3**	**Amphibians**	**Not published**
16S	Vert-16S-eDNAF1	Vert-16SeDNA-R1	Fish and amphibians	(Vences et al., 2016)
16S	**BA-4445-F**	**BA-178-R**	**Amphibians**	**(Bálint et al., 2017)**
16S	L2513	H2714	Vertebrates	(Kitano et al., 2007)
16S	16Smam1	16Smam2	Mammals	(Ficetola et al., 2010)
16S	16Sr (1)	16Sr (1)	Vertebrates	(Riaz et al., 2011)
16S	16Sr (2)	16Sr (2)	Vertebrates	(Riaz et al., 2011)
12S	L1085	H1259	Vertebrates	(Kitano et al., 2007)
12S	Am12s_F	Am12s_R	Amphibians	(Evans et al., 2016)
12S	batra_F	batra_R	Amphibians	(Valentini et al., 2016)
12S	**12S-V5 (1)**	**12S-V5 (1)**	**Vertebrates**	**(Riaz et al., 2011)**
12S	**12S-V5 (2)**	**12S-V5 (2)**	**Vertebrates**	**(Riaz et al., 2011)**
12S	12S-V5 (3)	12S-V5 (3)	Vertebrates	(Riaz et al., 2011)
COI	UniMinibarR1	UniMinibarF1	Eukaryotes	(Meusnier et al., 2008)

Table 5 Amphibian species used for HTS primer optimisation.

Order	Family	Species
Anura	Ranidae	*Pelophylax perezi*
		Rana iberica
	Bufonidae	*Bufo spinosus*
		Epidalea calamita
	Hylidae	*Hyla molleri*
		Hyla meridionalis
	Pelobatidae	*Pelobates cultripes*
	Alytidae	*Alytes obstetricans*
		Alytes cisternasii
		Discoglossus galganoi
	Pelodytidae	*Pelodytes atlanticus*
		Pelodytes ibericus
Urodela	Salamandridae	*Triturus marmoratus*
		Triturus pygmaeus
		Lissotriton boscai
		Lissotriton helveticus
		Salamandra salamandra
		Pleurodeles waltl
		Chioglossa lusitanica

3.2.5.2 Library preparation and sequence data

Library preparation and sequence data analysis followed the same protocols described in chapter 2 (see sections 2.2.6.1 and 2.2.6.2)

3.2.6 Data analyses

Statistical analyses were performed separately for each species using R (R Development Core Team, 2008).

Initially, the package unmarked 0.12-2 (Fiske & Chandler, 2011) was used to generate a saturated detection model, including all variables measured in the field. Due to the restricted sample size, the best occupation model was not calculated and occupancy probabilities were kept constant, thus assuming the same probability of occupancy for every pond. Species presence, as determined by both sweep sampling and eDNA, was included in the saturated model. The function “dredge” from the package MuMIn v1.42.1 (Barton, 2018) was then used to generate and compare a set of models with all possible

combinations of variables. To allow a comparison between capture methods, the variable "Method" was fixed in order to be present in all models. Detection models were ranked based on AIC and the model with lowest AIC value was selected for each species (supplementary material tables 12-14).

The detection probabilities were then calculated for each variable present in the best model of detection. For this, the function "predict" from the package unmarked 0.12-2 (Fiske & Chandler, 2011) computed the detection probabilities within the range of values recorded for each variable, based on the model selected. The effects of each variable were plotted using the package effects v4.0-1 (Fox, 2003). Lastly, the detection probabilities of both sampling methods (five disc filters vs one capsule) were calculated based on the coefficients of the best detection model.

3.3 Results

From the 16 sites sampled, pond size ranged from 190 – 3791 m^2 ($\bar{x}$ = 1430.9 m^2) and pond depth from 20 – 72 cm ($\bar{x}$ = 41.3 cm). Clarity values varied from 3 – 93 cm ($\bar{x}$ = 24.7 cm), temperature from 15.1 – 28.4 ^oC ($\bar{x}$ = 21.9 ^oC), pH form 5.7 – 8.3 ($\bar{x}$ = 7.3) and conductivity from 0.12 – 2.4 mS ($\bar{x}$ = 0.8 mS). The volume of water filtered with disc filters was between 15 and 1250 ml ($\bar{x}$ = 243.7 ml), whereas with capsules it ranged from 600 to 5300 ml ($\bar{x}$ = 1781.3 ml). Species detection levels in the field ranged from 1 – 6 species per site ($\bar{x}$ = 3.7). All the values recorded for each variable and the list of species detected in each site are summarized in table 11, in supplementary material.

HTS produced 6,289,009 reads prior to bioinformatic filtering. After filtering, 1,431,499 reads were identified as amphibian species. All PCR negatives (n = 10) and extraction negatives (n = 5) were clean after bioinformatic filtering, indicating that precautions taken during laboratory work were effective. One field control out of 16 was contaminated. To avoid an over-estimation of results, the species detected in this field control was removed from the samples of the respective site.

Of the ten species observed in the field, only six were detected with HTS. Three of the six species were detected in a low number of sites and eDNA samples (table 6) and weren't considered for analysis to avoid unreliable results. Detection models were thus calculated for *Hyla meridionalis*, *Pelobates cultripes* and *Pleurodeles waltl*.

Table 6 Number of sites where species were detected through sweep sampling and eDNA (total number of sites = 16) as well as the number of samples, including both disc filters and capsules (total number of samples = 96). Species highlighted in bold were used for calculating detection models.

Species	***Sweeps (sites)***	***eDNA (sites)***	***eDNA (samples)***
Bufo spinosus	1	0	0
Lissotriton boscai	1	0	0
Discoglossus galganoi	2	1	1
Pelodytes atlanticus	4	3	4
Epidalea calamita	4	2	2
Triturus pygmaeus	7	0	0
Pelophylax perezi	8	0	0
Pleurodeles waltl	**9**	**7**	**11**
Pelobates cultripes	**11**	**8**	**38**
Hyla meridionalis	**11**	**12**	**35**

3.3.1 *Hyla meridionalis*

Species detection was best explained by clarity (fig. 16A) and volume filtered (fig. 16B) (supplementary material table 12), even though only clarity had a significant relationship ($p < 0.01$). The higher the values of clarity and volume filtered, the greater the probability of detecting *H. meridionalis*.

Detection probability was higher with five disc filters (65.9%) than one capsule (8.6%) (fig. 17). In fact, the same volume filtered with five disc filters provides a greater detection probability than with capsules (fig. 16B).

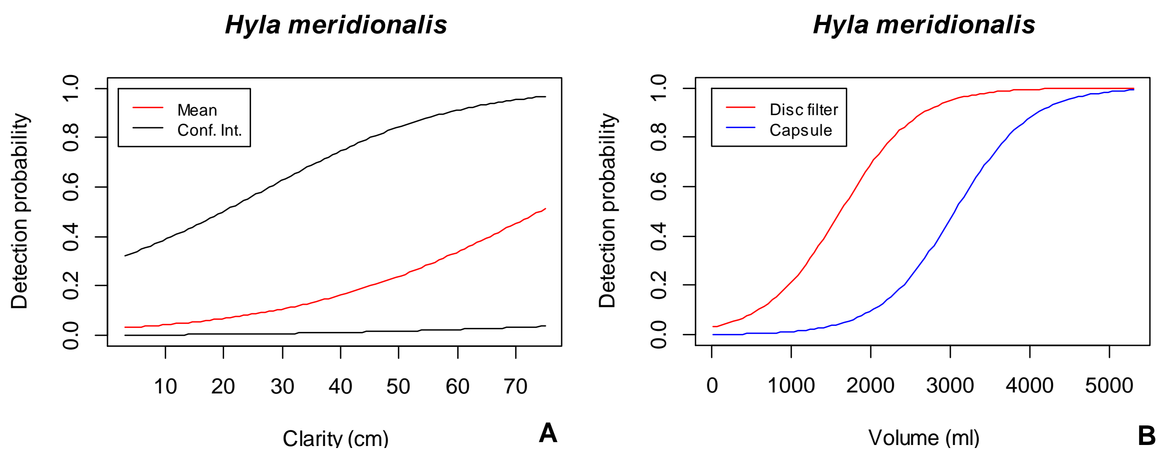


Fig. 16 Detection probability of *Hyla meridionalis* according to clarity **(A)** and volume filtered **(B)**.

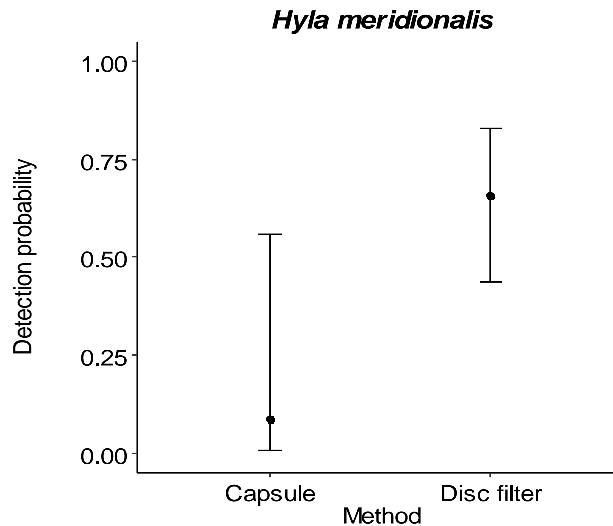
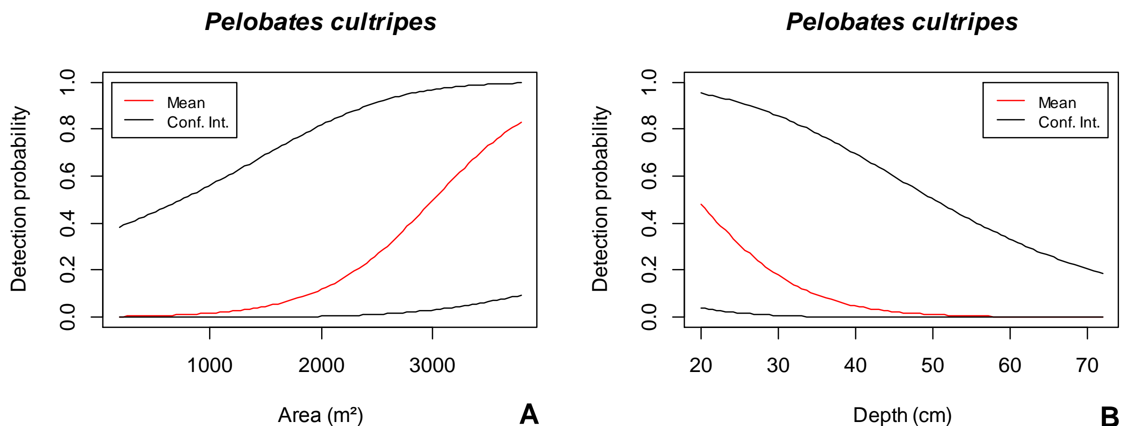


Fig. 17 Probability of detecting *Hyla meridionalis* (at sites where this species is confirmed) with either one capsule or five disc filters per site. Error bars indicate 95% confidence intervals.

3.3.2 *Pelobates cultripes*

Apart from temperature, the best model of detection contained all variables measured in the field: area (fig. 18A), depth (fig. 18B), conductivity (fig. 18C), pH (fig. 18D), clarity (fig. 18E) and volume (fig. 18F) (supplementary material table 13). All variables had a significant relationship with species detection, except for volume. With increasing values of area ($p < 0.05$), pH ($p < 0.01$) and volume filtered, the probability of detecting *P. cultripes* increases. In contrast, lower values of depth ($p < 0.01$), conductivity ($p < 0.01$) and clarity ($p < 0.001$) increase species detection.

Detection probability of *P. cultripes* was higher using five replicates of disc filters (66.6%) than just one capsule (4.1%) (fig. 19). The same volume filtered with five disc filters provided higher species detection than with capsules (fig. 18F).



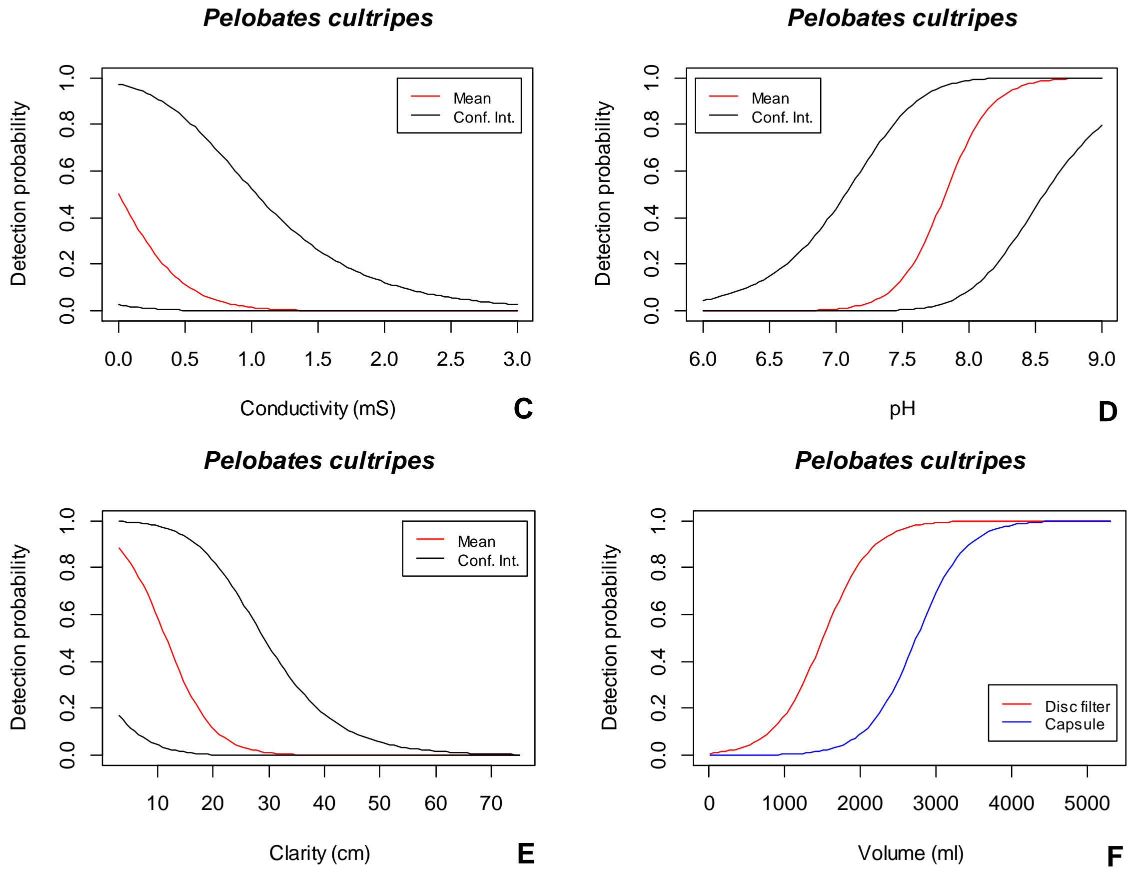


Fig. 18 Detection probability of *Pelobates cultripes* according to pond area **(A)**, depth **(B)**, conductivity **(C)**, pH **(D)**, clarity **(E)** and volume filtered **(F)**.

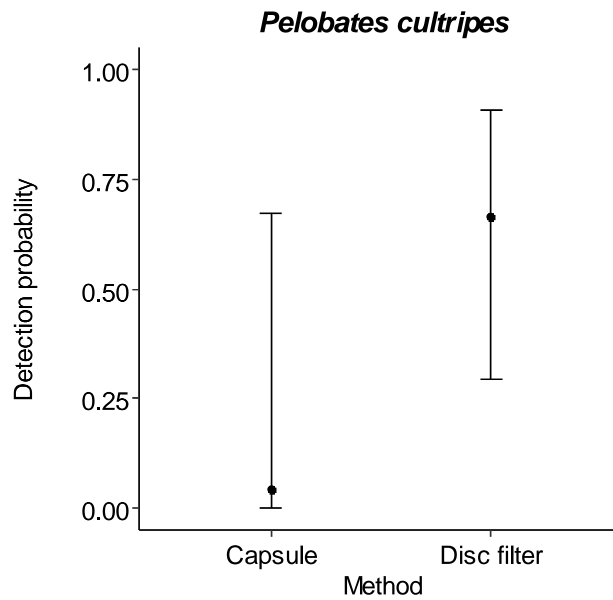


Fig. 19 Probability of detecting *Pelobates cultripes* (at sites where this species is confirmed) with either one capsule or five disc filters per site. Error bars indicate 95% confidence intervals.

3.3.3 *Pleurodeles waltl*

For *P. waltl*, none of the variables measured were present in the best model (supplementary material table 14). Nevertheless, the probability of detection was higher with five disc filters (19.9%) than one capsule (9.99%) (fig. 20).

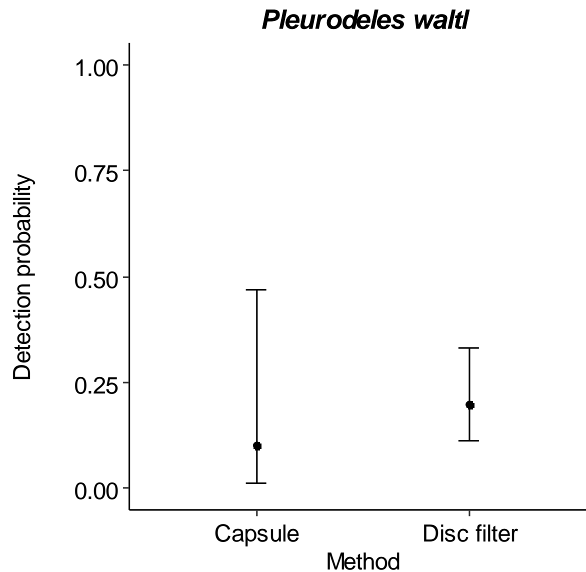


Fig. 20 Probability of detecting *Pleurodeles waltl* (at sites where this species is confirmed) with either one capsule or five disc filters per site. Error bars indicate 95% confidence intervals.

3.3.4 Overview of results

The results obtained are summarized in table 7.

Only water clarity and volume of water filtered were present in more than one of the final three detection models. For water clarity, the observed patterns were contradictory, with increased clarity values favouring species detection for *H. meridionalis,* while decreasing detection probabilities for *P. cultripes*. For both these species, the greater the volume of water filtered, the higher the detection probability, however the relationships were not significant.

Increased values of pond area and pH increased species detection probability for *P. cultripes*. On the contrary, higher values of conductivity and depth decreased detection probabilities. Only temperature was not present in any of the final detection models. For *P. waltl*, none of the measured variables were present in the best detection model.

Detection probabilities were consistently higher using five disc filters than using one capsule.

Table 7 Summary of environmental variables and their influence on species detection probabilities. Abbreviations indicate each variable's significance (S – significant; NS – not significant) and its effect (+ or - whether increased values favour or decrease detection, respectively). NA indicates variables not present in the best detection model.

	H. meridionalis	*P. cultripes*	*P. waltl*
Clarity	S / +	S / -	NA
Volume	NS / +	NS / +	NA
Area	NA	S / +	NA
pH	NA	S / +	NA
Conductivity	NA	S / -	NA
Depth	NA	S / -	NA
Temperature	NA	NA	NA

3.4 Discussion

In this study, the relatively low number of sampling sites and the low detection rate of some species, both using sweep sampling and HTS methods, precluded the estimation of detection probabilities for all species present in the study area. Nevertheless, detection models were possible to compute for three species: *Hyla meridionalis*, *Pelobates cultripes* and *Pleurodeles waltl*. A significant influence on species detection was observed for the majority of the variables measured, but not across all species. The only variable influencing detection in more than one species was water clarity, exhibiting opposite patterns. Regarding sampling method, detection probabilities were consistently higher using five disc filters than using one capsule.

3.4.1 Effects of environmental variables on amphibian species detection

The efficiency of eDNA methods in aquatic systems can be affected by several physiological, ecological and hydrological processes. In this study, low values of conductivity were associated to higher species detection for *P. cultripes*. Conductivity is a measure of water's ability to conduct electricity and is positively related to the concentration of ions present in the water. Exposure to ions has been shown to promote changes in the double-helix structure of DNA, such as major and minor grooves, local unwinding, disordering regions and changes in diameter (Dong et al., 2010), which might influence the way DNA degrades and behaves within the water, during the filtration process or even during subsequent laboratory procedures. Few studies have addressed the effect of water conductivity on eDNA detectability, although it was shown to be negatively correlated to fish eDNA concentration in another study (Takahara et al., 2012), supporting our results. Conversely, in a recent study Goldberg et al. (2018) failed to

observed a significant relationship between water conductivity and amphibian species detection.

pH levels also influenced detection probabilities for *P. cultripes*, with more alkaline environments favouring species detection. Acidic conditions favour the activity of hydrolytic enzymes capable of degrading DNA (Alaeddini et al., 2010; Lindahl, 1993). Previous studies assessing the influence of pH on DNA degradation rates have demonstrated that lower pH levels accelerate DNA degradation and decrease species detection (Strickler et al., 2015; Seymour et al., 2018; Goldberg et al., 2018), supporting our observations.

Pond depth and area influenced detection probabilities as well for *P. cultripes*. The observed pattern is likely a result of species habitat preferences. Previous studies demonstrated that the presence of *P. cultripes* in Mediterranean ponds declined with pond depth and that amphibian richness in Mediterranean temporary ponds increased with pond area (Beja & Alcazar, 2003). This might result in higher abundance of the species in shallow and large ponds, thus increasing the chance of detecting the species in habitats with those features. In fact, this hypothesis was to some extent reflected in sweep data.

Regarding water clarity, opposite patterns were observed for *H. meridionalis* and *P. cultripes*, with species detection increasing and decreasing with clarity, respectively. Once more this might be a result of habitat preference. It is possible that *H. meridionalis* prefers low turbidity environments, increasing its abundance and, therefore, its detection probability in these ecosystems. On the contrary, *P. cultripes* might prefer turbid waters, where its detection probabilities were greater. Few studies have addressed the effect of water turbidity on amphibian presence. Nevertheless, a previous study assessing amphibian species occurrence failed to observe a significant relationship with water turbidity for both *H. meridionalis* and *P. cultripes* (Jakob et al., 2003).

The only variable that failed to exhibit a correlation with species detection was water temperature. Previous studies using a one-off measurement, identical to our study design, have already documented higher degradation rates with increasing temperature (Tsuji et al., 2017; Strickler et al., 2015; Goldberg et al., 2018) and therefore it would be expected that detection probabilities increased at lower temperatures. The lack of an effect on species detection could be explained by the low spectrum observed in this study, with temperatures ranging only from 15.1ºC to 28.4ºC. The above-mentioned studies experienced larger ranges of water temperature, namely 20ºC (Tsuji et al., 2017),

25ºC (Goldberg et al., 2018) and 30ºC (Strickler et al., 2015), which may facilitate the observation of a pattern.

It seems clear that ecological variables influence the detection of amphibians in Mediterranean temporary ponds. Understanding the processes limiting eDNA detection is crucial in eDNA-based surveys. Accounting for those factors in optimized sampling designs will provide better estimates of species distribution and increase the efficiency of eDNA biodiversity monitoring.

3.4.2 Comparison of eDNA detectability between filtration methods

Filtration methods can differ regarding several characteristics. Previous studies have established that attributes such as pore size (e.g. Li et al., 2018) and filter membrane (e.g. Hinlo et al., 2017) can influence eDNA recoveries. As such, accurate comparisons between filtration methods should involve identical properties.

In this study, detection probabilities were higher with multiple disc filters than capsules. This was observed for all three species from which detection models were calculated, but to a lesser extent for *P. waltl*. The smaller differences observed for this species might be related to the lower number of samples where the species was detected, which might hamper the calculation of a reliable probability.

Both filtration methods tested had identical membranes and pore sizes. However, surface area differed among them, with capsules offering a much larger area than disc filters. This attribute provides filtration of larger volumes (e.g. Valentini et al., 2016), increasing captured DNA (Herder et al. 2014) and thus detection probabilities (e.g. Goldberg et al., 2018).

Despite the higher surface area and ability to filter larger volumes of water typical of capsules, employing multiple disc filters demonstrated higher species detection. As the volume of water filtered did not have a significant relationship with species detection, even though it was present in the best detection model for both *H. meridionalis* and *P. cultripes,* it seems that volume filtered explained a relatively minor portion of the variation in eDNA detectability for these species.

While disc filters were used multiple times within the study area, covering all habitats within the pond, capsules were only used once. The number of sampling points is likely to have played an important role in species detection, where filtering multiple sites within the pond increased detection probabilities. In fact, as DNA is not homogenously distributed in the water (Herder et al., 2014), a field replicate approach is usually

recommended for eDNA-based surveys in order to increase species detection (Goldberg et al., 2016).

3.4.3 Limitations of the study and recommendations for the future

In this study, the low number of sampling sites and the low detection rates, both using sweep sampling and HTS methods, precluded the application of these models for all amphibian species known to occur in the study area.

The relatively low sample size might justify why none of the variables explained variation in species detection for *P. waltl.* Perhaps this species is more generalist than *H. meridionalis* and *P. cultripes* and a greater number of sites would be needed in order to detect an influence of environmental variables on species detection. Further sampling should be considered in the future and will likely lead to better estimates of species detection probabilities (Herder et al., 2014).

The low detection rates observed for sweep sampling and HTS data possibly resulted from the unique temporal sampling session. As breeding seasons change among amphibian species (Ferreira & Beja, 2013), sampling only in April/May might miss early-breeding species, with highest activity in previous months. Since seasonal activity of species is also known to influence DNA availability (de Souza et al., 2016; Franklin et al., 2018), with reproduction periods being associated with higher DNA levels (Spear et al., 2015), the different life cycles of each species likely introduced biases in species detection with HTS as well. For instance, detection in the field of *H. meridionalis* and *P. cultripes* was almost exclusively through larvae, in high abundances. As larvae are constantly in the water, they shed high amounts of DNA and thus species detection with HTS was frequent. Conversely, field detection of *P. perezi* was only possible using audio and visual observation of adults. As they spend less time in water, they shed less DNA thus hampering species detection. To overcome this bias, multiple sampling sessions would be needed to cover all amphibians´ reproductive cycle occurring in these temporary ponds.

Additional explanations can be hypothesized to explain the low detection rate observed with HTS data. The use of strict filtering (e.g. only considering a species when hits were 99% or higher), to ensure reliable results, likely reduced overall detection rates. However, relaxed bioinformatic filters can induce incorrect taxonomy assignments, generate false positive results and add unreliability to a study's conclusions. A trade-off between strict bioinformatics and species detection exists. Although outside the scope of this study, the data generated in this thesis offers the potential to further investigate less stringent bioinformatic protocols, and the effects of these warrant further study.

A comparison of species detection probabilities between traditional surveys (sweep sampling) and eDNA (capsules and disc filters) was not performed in this study. Nevertheless, it should be considered for further exploration as previous studies demonstrated opposite results regarding the best method to use, depending on the target species.

3.4.4 Implications for amphibian conservation and eDNA research

The methodology developed in this study helps to elucidate how environmental variables affect amphibian eDNA detection in Mediterranean temporary ponds. At the same time, this study is the first to compare capsules performance against multiple replicates of common disc filters, which provided greater detection probabilities. Accounting for those factors will allow optimized capture methods and provide better estimates of species distribution in aquatic habitats, increasing the efficiency of eDNA biodiversity monitoring.

Many studies compare biodiversity across sites based on eDNA results. However, sites are not directly comparable as detection probabilities will be affected by environmental variables specific of each location. Any studies comparing diversity across different sites using eDNA are probably fundamentally flawed, unless they account for detection probabilities. eDNA studies generally do not take this into account and doing so could globally improve current research.

This becomes particularly useful for a declining and threatened group such as amphibians. The large diversity of this vertebrate group and their ecological importance requires the application of powerful and improved survey methods to increase the knowledge of its distribution. Only when environmental factors in eDNA research can be accounted for, will it be possible to apply efficient conservation measures, that can mitigate amphibian decline.

Chapter 4: Final remarks

This study addressed key questions using eDNA methodologies with a focus on amphibian species detection in turbid waters. The questions were met by targeting key steps in eDNA studies in the field, in the laboratory and during data analysis.

The work developed in this study is the first to provide comparisons between high-surface-area capsules and common eDNA sampling methods such as disc filters and precipitation. Precipitation methods proved to be much less effective for capturing DNA and for species detection. This is attributed to the low volume they are limited to, given that based on the quantity of DNA extracted per volume processed, precipitation was highly efficient. Capsules provided higher volume of water filtered and DNA captured when compared to disc filters. Species detection with disc filters was possible even in highly turbid waters and this technique appears suitable for sampling in turbid ecosystems, especially when funds are limited. However, capsules require less handling in the field and less field equipment, lowering the risk of contamination.

In the lab, we evaluated the most appropriate method for *Salamandra salamandra* detection. qPCR was slightly more sensitive for disc filters, providing higher species detection than HTS. Additionally, we developed a new set of species-specific primers and probe for this species. Together, this offers an improved detection assay for future eDNA studies targeting *Salamandra salamandra*, allowing a better knowledge of its distribution and a closer monitoring, important for this species conservation (Preißler et al., 2018). We further demonstrated the efficiency of a novel DNA extraction method for capsules, without the need for high speed centrifugation of the buffer poured from the capsule, as described in previous studies applying this method. This step is often hampered if laboratories are not equipped with a centrifuge capable of spinning 50 ml tubes at 15000 g, thus offering an important alternative for recovering capsule-collected eDNA in future studies.

The methodology developed in this study further provides evidence of the effects of ecological variables in amphibian detection in Mediterranean temporary ponds, investigated for the first time for Portuguese amphibians. The application of site occupancy models revealed a significant influence on species detection observed for most of the variables measured, but not in a consistent pattern among species. This result brings implications for future eDNA research. Many studies compare biodiversity across sites based on eDNA results. Nevertheless, as demonstrated in this study, sites are not directly comparable as detection probabilities will be affected by environmental variables specific of each location. For example, even if DNA of a target species is present in two sites at equal concentrations, the site with more favourable eDNA

conditions will increase species detection, and thus results are not directly comparable. eDNA studies to date may have been overlooking these effects and taking this into account could improve current research.

This work has addressed some of the key outstanding questions regarding field, laboratory and data analysis methods for eDNA techniques. It has also helped to identify new research areas and highlight that previous studies may be overlooking important aspects potentially leading to erroneous conclusions. It is clear that further research is needed within eDNA methodology to better understand the optimal sampling and detection methods for each specific habitat and research question. Before eDNA methods can be reliably used to help combat global amphibian declines effectively, these methodological challenges need to be better understood.

Chapter 5: References

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Chapter 6: Supplementary material

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Fig. 21 Images of the nine sampling sites sampled for the second chapter. Numbering corresponds to supplementary material table 8.

Table 8 Summary of sampling sites for the second chapter, including salamander abundance observed in the field (number of individuals per pond length), volume filtered with capsules and disc filters, and the size of the pond. Larvae abundance was obtained using a transect sampling approach (see section 1.2).

Site	*Coordinates*	*Larvae abundance*	*Volume (L)*		*Clarity (cm)*	*Size (m)*		
			Capsule	**Disc filter**		**Length**	**Width**	**Depth**
1	41°23'53.90"N 8°45'5.16"W	0.7	12 13.7	0.79 3.5	91 109.5	13.5	1.5	0.35
2	41°10'37.92"N 8°38'24.24"W	2	20 26	1.8 2.05	110.5 120.7	5	2	0.40
3	41°19'40.44"N 8°44'1.32"W	2.2	5.6 5.7	0.13 0.135	14.3 14.1	33	3.5	0.08
4	41°19'33.60"N 8°44'3.12"W	1.1	2 1.6	0.165 0.165	13.7 16.4	14	3	0.07
5	41°19'41.69"N 8°43'56.85"W	0.5	2.5 3.2	0.7 1	48 74	11	8	0.20
6	41°19'39.07"N 8°43'57.49"W	0.8	15.6 10.9	3.5 2.05	80 70	6	4	0.20
7	41°19'25.12"N 8°43'58.23"W	3.8	2.9 3.4	0.7 0.75	103 114	16	12	0.25
8	41°19'29.18"N 8°44'0.02"W	0.8	3.7 5.7	0.55 0.3	59 31	13	2	0.05
9	41°19'43.44"N 8°44'1.49"W	1.4	3.6 4	0.8 0.665	35 41	11	3	0.07

Table 9 Sequences retrieved from NCBI used for 12S database.

Species name	***Accession number***
Alytes obstetricans	AJ440759.1
Alytes obstetricans	AY585337.1
Alytes obstetricans	DQ283112.1
Alytes obstetricans	JQ626651.1
Alytes obstetricans	KJ858769.1
Alytes obstetricans	KJ858770.1
Alytes obstetricans	KJ858771.1
Alytes obstetricans	KJ858772.1
Alytes obstetricans	KJ858773.1
Alytes obstetricans	KJ858774.1
Alytes obstetricans	KJ858775.1
Alytes obstetricans	KJ858776.1
Alytes obstetricans	KJ858777.1
Alytes obstetricans	KJ858778.1
Bufo spinosus	AY325988.1
Bufo spinosus	DQ158438.1
Discoglossus galganoi	AY585339.1
Discoglossus galganoi	JQ626648.1
Discoglossus galganoi	JQ626649.1
Discoglossus galganoi	JQ626650.1
Epidalea calamita	U52726.1
Epidalea calamita	EU938400.1
Hyla meridionalis	AY819370.1
Hyla meridionalis	EF566953.1
Pelobates cultripes	AJ871086.1
Pleurodeles waltl	DQ283445.1
Pleurodeles waltl	EU880330.1
Salamandra salamandra	AY928619.1
Salamandra salamandra	EU880331.1
Salamandra salamandra	KX094979.1
Triturus marmoratus	EU880337.1
Triturus marmoratus	HQ697279.1
Triturus pygmaeus	HQ697280.1

Consensus sequences (excluding primer binding sites) generated as part of this study, for incorporation into the 12S database. Sequences were obtained by Sanger sequencing amplicons produced from DNA extracted from tissue samples of these species, using the 12SV5-1 primer pair.

Pelophylax perezi
5' ACTATGCCCAGCCGTAAACAATTAACTTACATCAACCACGCCAGGGAATTACGA GCAATGCTTAAAACCCAAAGGACTTGACGGTGTCCCACCCAC 3'

Lissotriton boscai
5' ACTATGCCCAGCCATAAACTTTGATTTATCCGCCAGAGTACTACGAGCCACAGC TTAAAACTCAAAGGACTTGGCGGTGCTCTACACCCCC 3'

Lissotriton helveticus
5' ACTATGCCCAGCCATAAACTTTGACCTATCCGCCAGAGTACTACGAGCAACAGC TTAAAACTCAAAGGACTTGGCGGTGCCCTATACCCAC 3'

Pelodytes atlanticus
5' ACTATGCTTAGCCGTAAACTTTAATACTTACAATAAACATTCGCCAGGGTACTAC GAGCGTTAGCTTAAAACCCAAAGGACTTGGCGGTGCCCCAAACCCAC 3'

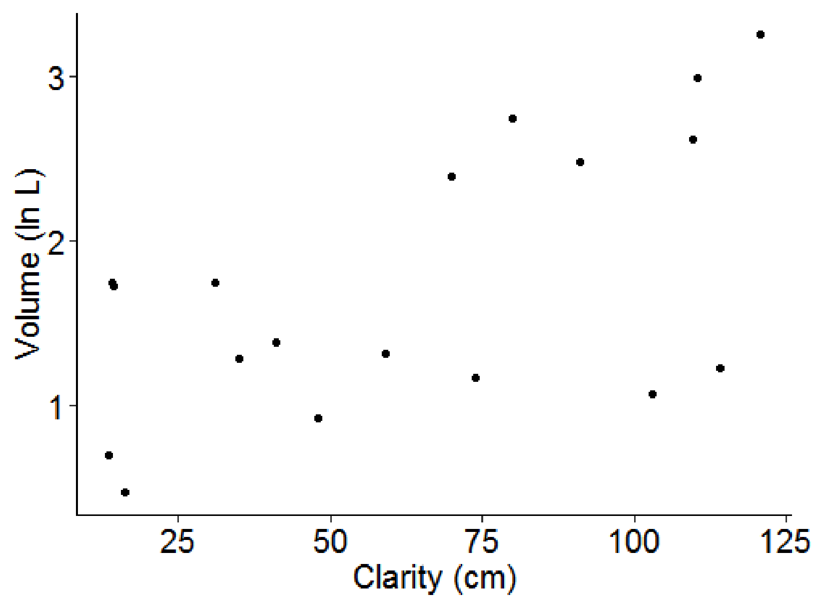

Fig. 22 Relationship between volume filtered and water clarity using capsules.

Table 10 qPCR and HTS detection of *S. salamandra* with the 3 capture methods tested. Species detection is coded as 1 and non-detection as 0 (n = 18 for each combination of capture method and assay).

Clarity (cm)	Capsule		Disc filters		Precipitation	
	qPCR	HTS	qPCR	HTS	qPCR	HTS
13.7	0	0	1	0	0	0
14.1	0	0	0	0	0	0
14.3	0	0	0	0	0	0
16.4	0	0	1	0	0	0
31	1	1	0	1	0	0
35	0	0	0	0	0	0
41	0	0	0	0	0	0
48	0	0	1	0	1	1
59	1	0	1	1	0	0
70	1	1	0	1	0	0
74	1	1	0	0	0	0
80	1	1	1	1	0	0
91	0	0	0	0	0	0
103	1	1	1	0	0	0
109.5	0	0	1	0	0	0
110.5	1	1	1	1	0	0
114	1	0	1	0	0	0
120.7	1	1	1	1	1	1
Total	**9**	**7**	**10**	**6**	**2**	**2**

G21

G35

G16

G09

G07

G60

G03

VB01

VB02

G40

206b

B06a

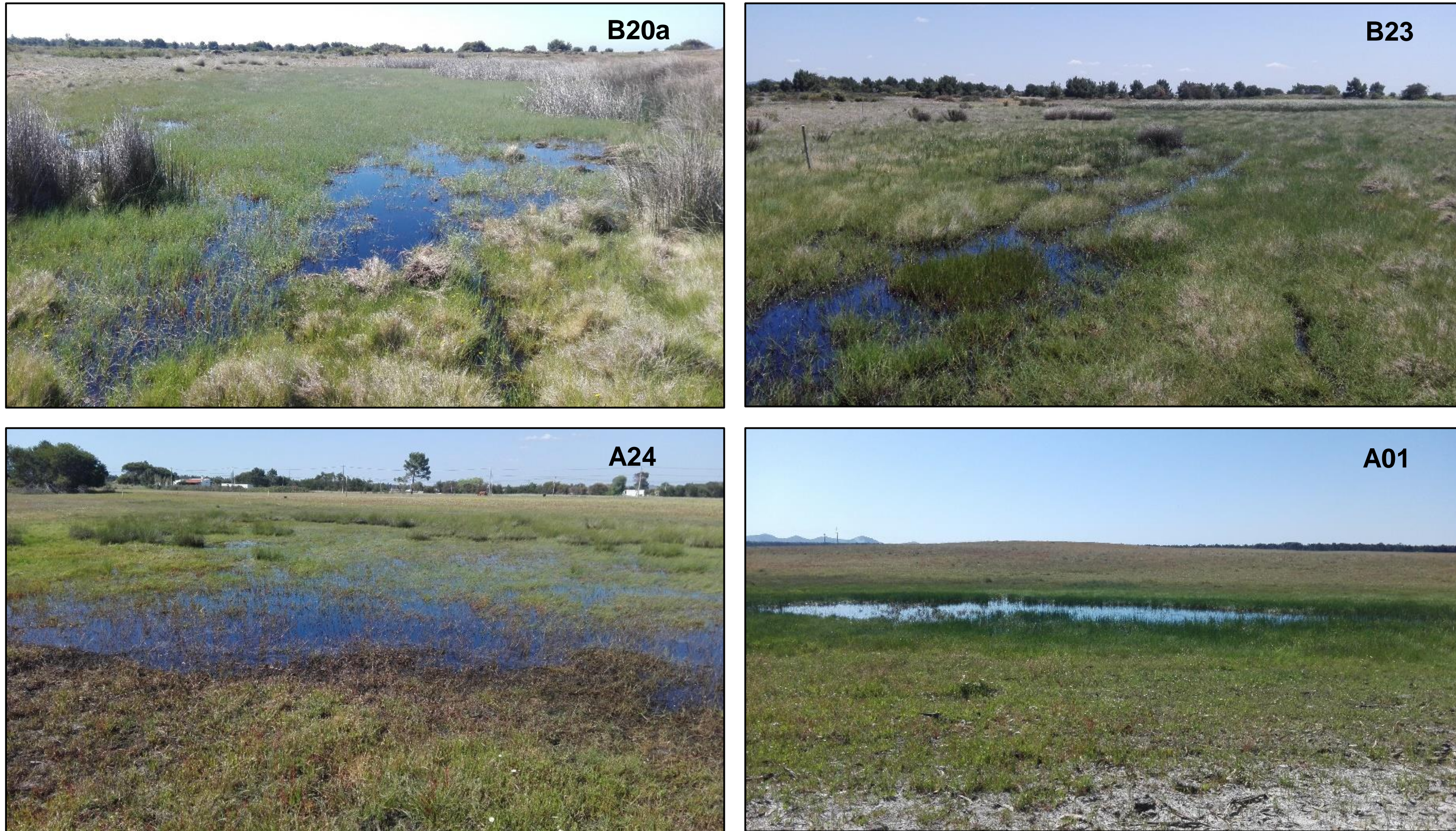

Fig. 23 Images of the 16 sampling sites sampled for the third chapter. Numbering corresponds to supplemenatry material table 11.

Table 11 Summary of sampling sites for the third chapter, including the variables measured in each site, the volume filtered with both disc filters and capsules, and the list of species observed with sweep sampling in the field and with eDNA (NA: no amplification).

Site	***Coordinates***	***Area (m^2)***	***Depth (cm)***	***Clarity (cm)***	***Temperature (°C)***	***pH***	***Conductivity (mS)***	***Volume (ml)***		***Species detected***		
								Disc filter	***Capsule***	***Disc filter***	***Capsule***	***Sweeps***
G21	37° 6'32.42"N 8°54'22.10"W	1050	55	25	16.7	7.4	0.87	550	1700	*H. meridionalis P. waltl*	*H. meridionalis*	*H. meridionalis P. perezi P. cultripes P. waltl*
				19	17.3	7.3	0.84	300		*H. meridionalis*		
				28	16.5	7.3	0.82	450		*H. meridionalis P. waltl*		
				37	15.8	7.3	0.92	750		*H. meridionalis*		
				33	19.7	7.3	0.83	450		*H. meridionalis*		
G35	37° 7'6.99"N 8°53'55.11"W	2250	60	47	23.8	8	0.29	350	1200	*H. meridionalis*	*NA*	*H. meridionalis P. cultripes P. waltl T. pygmaeus*
				45	25.4	8.1	0.29	300		*H. meridionalis P. cultripes P. waltl*		
				50	26.6	8	0.29	300		*H. meridionalis P. cultripes*		
				40	27.4	8.3	0.33	350		*H. meridionalis P. cultripes*		
				48	28.4	8.2	0.32	550		*H. meridionalis P. cultripes*		

G16	37° 5'50.13"N 8°51'48.77"W	2067	27	37	19.4	7.7	0.14	200	800	*H. meridionalis*	*NA*	*H. meridionalis P. cultripes T. pygmaeus P. waltl B. spinosus P. perezi*
				35	19.4	6.8	0.16	100		*H. meridionalis P. cultripes P. waltl*		
				63	20	6.9	0.16	100		*H. meridionalis P. cultripes P. waltl*		
				50	20.4	7.5	0.12	50		*H. meridionalis P. cultripes*		
				49	19.5	7.6	0.14	100		*H. meridionalis P. waltl*		
G09	37° 5'31.89"N 8°57'2.81"W	690	37	29	26	7.7	0.68	50	1300	*P. atlanticus*	*NA*	*P. waltl H. meridionalis P. atlanticus T. pygmaeus P. perezi*
				28	27	7.7	0.58	75		*NA*		
				27	27.1	7.8	0.58	50		*H. meridionalis*		
				32	27.7	7.7	0.62	50		*NA*		
				25	26.6	7.6	0.65	50		*NA*		
G07	37° 4'58.59"N 8°57'15.49"W	820	23	10	27.4	8.1	0.67	50	1800	*NA*	*H. meridionalis P. atlanticus*	*P. atlanticus H. meridionalis P. perezi E. calamita*
				14	27.2	8.2	0.65	50		*NA*		
				13	26.6	8.2	0.66	50		*NA*		
				15	27	8.1	0.68	40		*NA*		
				18	27	8	0.83	70		*NA*		

G60	37° 4'21.98"N 8°56'43.67"W	510	64	5	15.8	7.75	0.77	25	1100	*NA*	*NA*	*T. pygmaeus*
				4	16.7	7.7	0.77	20		*NA*		
				3	16.5	7.75	0.78	23		*NA*		
				4	16.1	7.75	0.81	15		*NA*		
				3	16.5	7.71	0.78	15		*NA*		
G03	37° 3'4.94"N 8°58'3.92"W	930	20	11	25.7	7.8	2.3	185	1000	*P. cultripes E. calamita*	*P. cultripes*	*P. cultripes E. calamita*
				12	23.4	7.8	2.16	175		*P. cultripes*		
				14	25	7.8	2.4	150		*P. cultripes H. meridionalis*		
				6	24.1	7.8	2.33	75		*NA*		
				12	24	7.8	2.17	150		*P. cultripes*		
VB01	37° 6'58.82"N 8°52'58.14"W	198	26	26	15.5	6.8	0.36	200	2400	*NA*	*NA*	*P. atlanticus P. waltl*
				15	15.4	6.5	0.38	350		*NA*		
				10	15.1	6.5	0.36	150		*NA*		
				33	15.3	6.4	0.38	200		*P. atlanticus*		
				38	15.4	6.4	0.38	350		*P. atlanticus P. waltl*		

VB02	37° 7'15.33"N 8°53'41.19"W	2340	53	65	23	7.3	0.7	350	3000	*H. meridionalis*	*H. meridionalis*	*D. galganoi* *H. meridionalis* *T. pygmaeus* *P. cultripes*
				68	23.2	7.6	0.81	575		*H. meridionalis*		
				93	24.1	7.9	0.81	500		*H. meridionalis*		
				85	24.7	7.7	0.64	600		*H. meridionalis*		
				64	24.7	7.9	0.57	350		*NA*		
G40	37° 7'3.40"N 8°53'29.61"W	190	72	58	22.9	8.1	0.87	1100	5300	*NA*	*NA*	*P. atlanticus* *P. perezi* *P. waltl*
				59	22.8	7.9	0.87	1250		*P. waltl*		
				57	23.2	7.8	0.87	1150		*NA*		
				37	23.4	7.7	0.86	1100		*NA*		
				21	24.6	7.5	1	425		*NA*		
206b	37°45'54.89"N 8°47'1.47"W	597	50	13	18.4	7.61	0.6	40	2000	*ND*	*P. cultripes*	*P. cultripes* *P. perezi*
				12	19.1	7.59	0.6	47.5		*P. cultripes*		
				11	18.7	7.4	0.59	40		*P. cultripes*		
				12	18.1	7.59	0.6	37.5		*P. cultripes*		
				11	19.2	7.49	0.59	37.5		*P. cultripes*		
B06a	37°44'39.34"N 8°47'21.96"W	890	46	11	23.2	6.44	1.21	100	600	*NA*	*NA*	*H. meridionalis* *L. boscai* *P. cultripes*
				9	24.6	6.44	1.04	80		*NA*		
				9	25.4	6.46	1.04	40		*NA*		

				8	22.2	6.22	1.03	80		*NA*		
				7	21.8	6.22	1.03	50		*H. meridionalis*		
B20a	37°45'4.82"N 8°47'41.06"W	3133	48	17	19.2	6.89	0.88	190	2500	*NA*	*P. cultripes*	*P. perezi* *P. cultripes* *H. meridionalis* *T. pygmaeus* *D. galganoi* *P. waltl*
				21	20.5	6.59	0.88	250		*P. cultripes*		
				21	17.8	6.62	0.93	380		*NA*		
				14	18.1	6.54	0.83	165		*P. cultripes* *H. meridionalis*		
				13	17.9	6.24	0.74	350		*P. cultripes*		
B23	37°44'53.06"N 8°47'45.20"W	3791	29	5	24.8	6.23	1.16	65	700	*P. cultripes*	*H. meridionalis* *P. cultripes* *D. galganoi*	*H. meridionalis* *P. cultripes* *E. calamita* *P. perezi*
				7	23	6.42	1.14	90		*P. cultripes* *P. waltl*		
				8	17.5	5.74	0.85	110		*NA*		
				9	18.7	6.3	0.63	265		*H. meridionalis* *P. cultripes*		
				7	23.6	6.41	0.97	80		*P. cultripes*		
A24	37°45'23.41"N 8°46'5.67"W	1237	20	10	27.7	6.91	0.61	165	1500	*P. cultripes* *H. meridionalis*	*P. cultripes* *H. meridionalis*	*P. cultripes* *H. meridionalis* *P. waltl*
				15	27.1	6.99	0.57	250		*P. cultripes* *H. meridionalis*		
				18	26	6.73	0.53	375		*P. cultripes*		

				11	25.9	6.76	0.7	100		*P. cultripes* *E. calamita*		
				15	25	6.81	0.64	300		*P. cultripes* *H. meridionalis*		
A01	37°46'42.28"N 8°46'25.60"W	2201	30	13	21	6.98	0.6	160	1600	*H. meridionalis* *P. cultripes*	*P. cultripes* *P. waltl*	*H. meridionalis* *T. pygmaeus* *P. cultripes* *P. waltl* *E. calamita*
				10	21.4	6.65	0.6	125		*H. meridionalis* *P. cultripes* *P. waltl*		
				19	21	6.78	0.6	160		*P. cultripes*		
				10	21	6.65	0.59	200		*H. meridionalis* *P. cultripes*		
				12	21.9	6.58	0.6	40*		*P. cultripes*		

.

Table 12 Candidate detection models for *Hyla meridionalis*, ranked based on AIC value. Abbreviations for variables are as follows: Clr – clarity; Mth – method; Vol – volume; Cnd – conductivity; Tmp – temperature; Ph – pH; Dpt – depth; Are – area.

Detection model	***AIC***	***delta***	***weight***	***df***	***logLik***
p(Clr + Mth + Vol)	91.7	0	0.054	5	-40.865
p(Clr + Cnd + Mth + Vol)	91.9	0.13	0.051	6	-39.929
p(Clr + Mth + Tmp + Vol)	92.4	0.66	0.039	6	-40.192
p(Clr + Cnd + Mth + Tmp + Vol)	92.5	0.73	0.038	7	-39.23
p(Clr + Mth + Tmp)	92.8	1.1	0.031	5	-41.414
p(Clr + Mth + Ph + Vol)	92.9	1.2	0.03	6	-40.466
p(Cnd + Mth + Vol)	93	1.24	0.029	5	-41.483
p(Clr + Cnd + Mth + Tmp)	93.3	1.55	0.025	6	-40.642
p(Clr + Mth)	93.5	1.8	0.022	4	-42.763
p(Clr + Cnd + Mth + Ph + Vol)	93.6	1.83	0.022	7	-39.779
p(Clr + Dpt + Mth + Vol)	93.6	1.83	0.022	6	-40.779
p(Cnd + Mth + Tmp + Vol)	93.7	1.94	0.021	6	-40.836
p(Are + Clr + Cnd + Mth + Tmp + Vol)	93.7	1.99	0.02	8	-38.858
p(Are + Clr + Mth + Vol)	93.7	1.99	0.02	6	-40.86
p(Clr + Cnd + Dpt + Mth + Vol)	93.7	1.99	0.02	7	-39.861
p(Clr + Dpt + Mth + Tmp + Vol)	93.8	2.05	0.019	7	-39.89
p(Are + Clr + Cnd + Mth + Vol)	93.8	2.1	0.019	7	-39.913
p(Clr + Cnd + Dpt + Mth + Tmp + Vol)	93.8	2.1	0.019	8	-38.916
p(Are + Clr + Mth + Tmp + Vol)	94.1	2.39	0.016	7	-40.059
p(Clr + Cnd + Mth)	94.1	2.41	0.016	5	-42.068
p(Cnd + Mth + Ph + Tmp + Vol)	94.2	2.43	0.016	7	-40.078
p(Clr + Mth + Ph + Tmp + Vol)	94.3	2.61	0.015	7	-40.167
p(Clr + Cnd + Mth + Ph + Tmp + Vol)	94.4	2.68	0.014	8	-39.202
p(Clr + Dpt + Mth + Ph + Vol)	94.5	2.78	0.013	7	-40.256
p(Clr + Mth + Ph)	94.5	2.79	0.013	5	-42.26
p(Are + Clr + Mth + Tmp)	94.6	2.87	0.013	6	-41.302
p(Are + Clr + Mth + Ph + Vol)	94.6	2.89	0.013	7	-40.31
p(Are + Clr + Cnd + Mth + Tmp)	94.7	2.94	0.012	7	-40.335
p(Clr + Dpt + Mth + Tmp)	94.7	2.98	0.012	6	-41.354
p(Cnd + Dpt + Mth + Vol)	94.7	3	0.012	6	-41.365
p(Cnd + Mth + Ph + Vol)	94.8	3.06	0.012	6	-41.394
p(Clr + Mth + Ph + Tmp)	94.8	3.1	0.012	6	-41.413
p(Are + Cnd + Mth + Vol)	94.9	3.18	0.011	6	-41.457
p(Are + Cnd + Mth + Tmp + Vol)	95	3.25	0.011	7	-40.487
p(Clr + Cnd + Dpt + Mth + Tmp)	95.1	3.39	0.01	7	-40.562
p(Are + Clr + Cnd + Dpt + Mth + Tmp + Vol)	95.1	3.39	0.01	9	-38.562
p(Are + Clr + Cnd + Mth + Ph + Vol)	95.2	3.43	0.01	8	-39.581

p(Clr + Cnd + Mth + Ph + Tmp)	95.2	3.44	0.01	7	-40.584
p(Clr + Cnd + Dpt + Mth + Ph + Vol)	95.3	3.58	0.009	8	-39.653
p(Are + Clr + Mth)	95.4	3.7	0.009	5	-42.713
p(Clr + Dpt + Mth)	95.4	3.7	0.009	5	-42.716
p(Are + Clr + Dpt + Mth + Vol)	95.5	3.8	0.008	7	-40.766
p(Are + Clr + Dpt + Mth + Tmp + Vol)	95.6	3.83	0.008	8	-39.778
p(Clr + Cnd + Mth + Ph)	95.6	3.86	0.008	6	-41.797
p(Cnd + Dpt + Mth + Tmp + Vol)	95.6	3.89	0.008	7	-40.809
p(Clr + Dpt + Mth + Ph + Tmp + Vol)	95.7	3.96	0.007	8	-39.843
p(Are + Clr + Cnd + Mth + Ph + Tmp + Vol)	95.7	3.97	0.007	9	-38.849
p(Are + Clr + Cnd + Dpt + Mth + Vol)	95.7	3.98	0.007	8	-39.852
p(Clr + Cnd + Dpt + Mth + Ph + Tmp + Vol)	95.8	4.04	0.007	9	-38.887
p(Are + Clr + Mth + Ph + Tmp + Vol)	95.9	4.13	0.007	8	-39.93
p(Are + Cnd + Mth + Ph + Tmp + Vol)	96	4.24	0.007	8	-39.983
p(Clr + Cnd + Dpt + Mth)	96	4.29	0.006	6	-42.011
p(Are + Clr + Cnd + Mth)	96.1	4.4	0.006	6	-42.063
p(Cnd + Dpt + Mth + Ph + Tmp + Vol)	96.1	4.4	0.006	8	-40.067
p(Are + Clr + Dpt + Mth + Ph + Vol)	96.2	4.47	0.006	8	-40.099
p(Are + Clr + Mth + Ph)	96.4	4.67	0.005	6	-42.198
p(Are + Clr + Dpt + Mth + Tmp)	96.5	4.76	0.005	7	-41.243
p(Are + Clr + Cnd + Dpt + Mth + Tmp)	96.5	4.76	0.005	8	-40.246
p(Are + Clr + Mth + Ph + Tmp)	96.5	4.79	0.005	7	-41.258
p(Clr + Dpt + Mth + Ph)	96.5	4.79	0.005	6	-42.258
p(Cnd + Dpt + Mth + Ph + Vol)	96.6	4.86	0.005	7	-41.296
p(Are + Cnd + Dpt + Mth + Vol)	96.7	4.93	0.005	7	-41.327
p(Are + Clr + Cnd + Mth + Ph + Tmp)	96.7	4.94	0.005	8	-40.335
p(Clr + Dpt + Mth + Ph + Tmp)	96.7	4.97	0.005	7	-41.351
p(Are + Cnd + Mth + Ph + Vol)	96.8	5.06	0.004	7	-41.394
p(Are + Cnd + Dpt + Mth + Tmp + Vol)	96.9	5.18	0.004	8	-40.456

Table 13 Candidate detection models for *Pelobates cultripes*, ranked based on AIC value. Abbreviations for variables are the same as in table 12.

Detection model	*AIC*	*delta*	*weight*	*df*	*logLik*
p(Are + Clr + Cnd + Dpt + Mth + Ph + Vol)	59.2	0	0.455	9	-20.607
p(Are + Clr + Cnd + Dpt + Mth + Ph)	60.4	1.23	0.246	8	-22.224
p(Are + Clr + Cnd + Dpt + Mth + Ph + Tmp + Vol)	61.2	1.94	0.172	10	-20.579
p(Are + Clr + Cnd + Dpt + Mth + Ph + Tmp)	62.3	3.1	0.097	9	-22.156
p(Are + Clr + Dpt + Mth + Ph)	67.6	8.41	0.007	7	-26.813
p(Clr + Cnd + Dpt + Mth + Ph + Tmp + Vol)	68	8.8	0.006	9	-25.009
p(Are + Clr + Dpt + Mth + Ph + Vol)	68.1	8.88	0.005	8	-26.046
p(Clr + Cnd + Dpt + Mth + Ph + Vol)	69	9.8	0.003	8	-26.505
p(Are + Clr + Dpt + Mth + Ph + Tmp)	69.5	10.32	0.003	8	-26.765
p(Are + Clr + Dpt + Mth + Ph + Tmp + Vol)	70	10.78	0.002	9	-25.997
p(Clr + Cnd + Dpt + Mth + Ph)	70.6	11.35	0.002	7	-28.281
p(Clr + Cnd + Dpt + Mth + Ph + Tmp)	71.6	12.4	0.001	8	-27.805
p(Are + Clr + Cnd + Mth + Ph + Tmp)	72.4	13.22	0.001	8	-28.215
p(Are + Clr + Cnd + Mth + Ph)	72.6	13.34	0.001	7	-29.278
p(Are + Clr + Cnd + Mth + Ph + Tmp + Vol)	74.2	15.03	0	9	-28.121
p(Are + Clr + Cnd + Mth + Ph + Vol)	74.6	15.34	0	8	-29.278
p(Are + Clr + Mth + Ph)	77	17.77	0	6	-32.49
p(Are + Clr + Mth + Ph + Tmp)	77.2	17.98	0	7	-31.596
p(Clr + Dpt + Mth + Ph)	77.5	18.25	0	6	-32.733
p(Clr + Cnd + Dpt + Mth + Tmp + Vol)	77.5	18.29	0	8	-30.753
p(Clr + Dpt + Mth + Ph + Vol)	77.7	18.46	0	7	-31.839
p(Clr + Dpt + Mth + Ph + Tmp + Vol)	78.6	19.36	0	8	-31.288
p(Are + Clr + Cnd + Dpt + Mth + Tmp + Vol)	78.8	19.63	0	9	-30.423
p(Clr + Dpt + Mth + Ph + Tmp)	78.9	19.67	0	7	-32.44
p(Are + Clr + Mth + Ph + Vol)	79	19.75	0	7	-32.483
p(Are + Clr + Mth + Ph + Tmp + Vol)	79.1	19.84	0	8	-31.527
p(Clr + Cnd + Dpt + Mth + Tmp)	79.6	20.39	0	7	-32.801
p(Clr + Cnd + Dpt + Mth + Vol)	80.1	20.85	0	7	-33.031
p(Are + Clr + Cnd + Dpt + Mth + Tmp)	80.2	20.94	0	8	-32.079
p(Clr + Cnd + Dpt + Mth)	80.4	21.23	0	6	-34.224
p(Are + Clr + Cnd + Dpt + Mth)	81.1	21.9	0	7	-33.555
p(Are + Clr + Cnd + Dpt + Mth + Vol)	81.2	21.98	0	8	-32.596
p(Clr + Cnd + Mth + Ph)	81.7	22.46	0	6	-34.836
p(Clr + Cnd + Mth + Ph + Tmp)	81.7	22.5	0	7	-33.856
p(Are + Clr + Dpt + Mth)	82	22.74	0	6	-34.979
p(Clr + Dpt + Mth)	82.4	23.18	0	5	-36.195
p(Are + Clr + Dpt + Mth + Tmp)	82.6	23.39	0	7	-34.304

p(Are + Clr + Dpt + Mth + Vol)	82.9	23.69	0	7	-34.451
p(Clr + Dpt + Mth + Vol)	83	23.78	0	6	-35.498
p(Clr + Cnd + Mth + Ph + Tmp + Vol)	83.2	23.94	0	8	-33.579
p(Clr + Dpt + Mth + Tmp)	83.3	24.08	0	6	-35.647
p(Are + Clr + Dpt + Mth + Tmp + Vol)	83.4	24.14	0	8	-33.677
p(Clr + Dpt + Mth + Tmp + Vol)	83.6	24.38	0	7	-34.798
p(Clr + Cnd + Mth + Ph + Vol)	83.6	24.39	0	7	-34.803
p(Dpt + Mth)	83.9	24.7	0	4	-37.957
p(Cnd + Dpt + Mth)	84.5	25.27	0	5	-37.244
p(Are + Clr + Cnd + Mth + Tmp)	84.9	25.69	0	7	-35.451
p(Are + Dpt + Mth)	85	25.74	0	5	-37.475
p(Dpt + Mth + Ph)	85.1	25.87	0	5	-37.54
p(Are + Clr + Mth + Tmp)	85.1	25.9	0	6	-36.559
p(Are + Dpt + Mth + Ph)	85.4	26.2	0	6	-36.706
p(Clr + Cnd + Mth + Tmp)	85.4	26.2	0	6	-36.708
p(Cnd + Dpt + Mth + Ph)	85.6	26.4	0	6	-36.809
p(Dpt + Mth + Vol)	85.6	26.41	0	5	-37.813
p(Dpt + Mth + Tmp)	85.7	26.48	0	5	-37.846
p(Are + Cnd + Dpt + Mth)	86	26.82	0	6	-37.015
p(Are + Clr + Mth)	86.2	26.95	0	5	-38.083
p(Cnd + Dpt + Mth + Tmp)	86.2	26.95	0	6	-37.084
p(Cnd + Dpt + Mth + Vol)	86.2	26.96	0	6	-37.088
p(Clr + Mth + Ph)	86.3	27.05	0	5	-38.132
p(Are + Clr + Cnd + Mth + Tmp + Vol)	86.3	27.12	0	8	-35.167
p(Clr + Mth + Ph + Tmp)	86.4	27.21	0	6	-37.21
p(Are + Cnd + Dpt + Mth + Ph)	86.6	27.36	0	7	-36.287
p(Clr + Cnd + Mth + Tmp + Vol)	86.7	27.44	0	7	-36.329
p(Are + Dpt + Mth + Tmp)	86.7	27.52	0	6	-37.365
p(Are + Clr + Mth + Tmp + Vol)	86.8	27.57	0	7	-36.394

Table 14 Candidate detection models for *Pleurodeles waltl*, ranked based on AIC value. Abbreviations for variables are the same as in table 12.

Detection model	*AIC*	*delta*	*weight*	*df*	*logLik*
p(Mth)	62.5	0	0.038	3	-28.272
p(Clr + Mth + Tmp)	62.6	0.07	0.037	5	-26.308
p(Mth + Tmp)	62.6	0.09	0.036	4	-27.318
p(Cnd + Mth)	63.1	0.52	0.029	4	-27.534
p(Cnd + Mth + Tmp)	63.2	0.67	0.027	5	-26.607
p(Clr + Mth)	63.2	0.69	0.027	4	-27.617
p(Mth + Ph + Tmp)	63.3	0.72	0.027	5	-26.63
p(Clr + Mth + Ph)	63.6	1.07	0.022	5	-26.808
p(Are + Clr + Mth + Tmp)	63.7	1.13	0.022	6	-25.839
p(Are + Mth + Ph + Tmp)	64.1	1.59	0.017	6	-26.067
p(Clr + Dpt + Mth + Tmp)	64.2	1.71	0.016	6	-26.125
p(Dpt + Mth + Ph + Tmp)	64.3	1.74	0.016	6	-26.144
p(Clr + Cnd + Mth + Tmp)	64.3	1.78	0.016	6	-26.163
p(Cnd + Mth + Ph + Tmp)	64.4	1.82	0.015	6	-26.183
p(Clr + Dpt + Mth)	64.4	1.88	0.015	5	-27.211
p(Mth + Ph)	64.5	1.94	0.014	4	-28.244
p(Clr + Mth + Tmp + Vol)	64.5	1.95	0.014	6	-26.244
p(Are + Mth)	64.5	1.96	0.014	4	-28.254
p(Dpt + Mth)	64.5	1.98	0.014	4	-28.263
p(Mth + Vol)	64.5	2	0.014	4	-28.271
p(Are + Mth + Tmp)	64.6	2.03	0.014	5	-27.289
p(Clr + Mth + Ph + Tmp)	64.6	2.06	0.014	6	-26.302
p(Dpt + Mth + Tmp)	64.6	2.09	0.013	5	-27.317
p(Mth + Tmp + Vol)	64.6	2.09	0.013	5	-27.318
p(Clr + Cnd + Mth)	64.7	2.12	0.013	5	-27.333
p(Are + Clr + Mth)	64.8	2.25	0.012	5	-27.396
p(Cnd + Dpt + Mth + Tmp)	64.8	2.26	0.012	6	-26.401
p(Are + Dpt + Mth + Ph + Tmp)	64.8	2.26	0.012	7	-25.404
p(Cnd + Mth + Vol)	64.9	2.32	0.012	5	-27.433
p(Are + Cnd + Mth + Tmp)	64.9	2.36	0.012	6	-26.451
p(Are + Cnd + Mth)	64.9	2.37	0.012	5	-27.458
p(Cnd + Dpt + Mth)	65	2.43	0.011	5	-27.485
p(Cnd + Mth + Ph)	65	2.49	0.011	5	-27.517
p(Cnd + Mth + Tmp + Vol)	65.1	2.51	0.011	6	-26.528
p(Mth + Ph + Tmp + Vol)	65.1	2.56	0.011	6	-26.554
p(Clr + Mth + Vol)	65.1	2.59	0.01	5	-27.568
p(Are + Cnd + Mth + Ph + Tmp)	65.3	2.78	0.009	7	-25.661

p(Are + Clr + Mth + Ph)	65.4	2.82	0.009	6	-26.683
p(Are + Clr + Dpt + Mth + Tmp)	65.4	2.91	0.009	7	-25.724
p(Clr + Cnd + Mth + Ph)	65.5	2.97	0.009	6	-26.756
p(Are + Clr + Cnd + Mth + Tmp)	65.5	2.99	0.009	7	-25.767
p(Clr + Dpt + Mth + Ph)	65.5	3	0.008	6	-26.771
p(Clr + Mth + Ph + Vol)	65.6	3.04	0.008	6	-26.791
p(Are + Clr + Mth + Ph + Tmp)	65.6	3.07	0.008	7	-25.805
p(Are + Clr + Mth + Tmp + Vol)	65.7	3.13	0.008	7	-25.839
p(Are + Clr + Dpt + Mth)	66	3.5	0.007	6	-27.021
p(Clr + Dpt + Mth + Ph + Tmp)	66.1	3.56	0.006	7	-26.049
p(Are + Mth + Ph + Tmp + Vol)	66.1	3.59	0.006	7	-26.064
p(Cnd + Dpt + Mth + Ph + Tmp)	66.2	3.7	0.006	7	-26.121
p(Clr + Cnd + Dpt + Mth + Tmp)	66.2	3.71	0.006	7	-26.125
p(Clr + Dpt + Mth + Tmp + Vol)	66.2	3.71	0.006	7	-26.125
p(Dpt + Mth + Ph + Tmp + Vol)	66.3	3.72	0.006	7	-26.131
p(Clr + Cnd + Mth + Ph + Tmp)	66.3	3.75	0.006	7	-26.146
p(Are + Cnd + Dpt + Mth + Tmp)	66.3	3.76	0.006	7	-26.154
p(Clr + Cnd + Mth + Tmp + Vol)	66.3	3.78	0.006	7	-26.161
p(Are + Dpt + Mth + Ph + Tmp + Vol)	66.3	3.79	0.006	8	-25.167
p(Dpt + Mth + Ph)	66.3	3.8	0.006	5	-28.173
p(Cnd + Mth + Ph + Tmp + Vol)	66.4	3.82	0.006	7	-26.183
p(Are + Clr + Cnd + Mth)	66.4	3.84	0.006	6	-27.19
p(Are + Mth + Ph)	66.4	3.84	0.006	5	-28.192
p(Clr + Dpt + Mth + Vol)	66.4	3.85	0.006	6	-27.194
p(Clr + Cnd + Dpt + Mth)	66.4	3.88	0.005	6	-27.211
p(Are + Cnd + Mth + Tmp + Vol)	66.4	3.89	0.005	7	-26.219
p(Mth + Ph + Vol)	66.5	3.94	0.005	5	-28.244
p(Clr + Mth + Ph + Tmp + Vol)	66.5	3.94	0.005	7	-26.244
p(Are + Mth + Vol)	66.5	3.95	0.005	5	-28.247