Using eDNA to construct community assemblages of amphibians at sites infected with *Batrachochytrium salamandrivorans* in the Netherlands.

Anna Marie Davison

The University of Salford

School of Science, Engineering and Environment

Submitted on Fulfilment of the Requirements for the Award of the Degree of MSc (By Research) Biological Sciences.

April 2022

Table of Contents

List of Tables and Figures

Abstract

Amphibians worldwide are in decline with one of the main culprits being the fungal disease chytridiomycosis. This fungus affects the skin of amphibians, leading to excessive skin shedding or skin lesions which, in many cases, result in death. The recently discovered *Batrachochytrium salamandrivorans* (*Bsal*) fungus, which only affects salamanders and newts (urodelans), has currently only spread to a few countries in Europe past its native range in Asia. However, *Bsal* has been shown to cause high mortality rates in some European urodelans and has the potential to spread much further across the continent. For this reason, monitoring the spread of the disease and the species threatened with infection is essential in mitigating the damage *Bsal* may cause. Utilising environmental DNA (eDNA) metabarcoding is a relatively new and effective method of monitoring biodiversity, especially elusive species. Thus the aims of this study are to 1.) validate the use of two different metabarcoding primers and single-species approaches in detecting amphibian species with eDNA in the Netherlands and 2.) investigate the relationship between *Bsal* and the amphibian species present at a site in Gelderland, Netherlands. *Bufo bufo, Lissotriton vulgaris, Pelobates fuscus, Pelophylax spp. and Rana temporaria* were detected by both the *12S-V5* vertebrate and the 16S amphibian universal primer sets used in this study with the *12S-V5* primers also detecting *Triturus cristatus.* The detection of amphibians by these two primer sets were compared, with the *12S-V5* vertebrate primers proving more suitable for use in this capacity. The vertebrate primers were then compared to single-species qPCR approaches for two protected amphibian species: *Triturus cristatus* (great crested newt) and *Pelobates fuscus* (spadefoot toad). The single-species approach outperformed the metabarcoding primer set, detecting both species more often. After testing the vertebrate primer set data with distance-based redundancy analysis, no significant relationship between *Bsal* presence and the amphibian community composition was found but further data is needed to confirm this finding. Potential amphibian or waterbird vectors of *Bsal* are explored and future avenues for research are discussed. This research provides the foundation for future studies on *Bsal* and its relationship with amphibian communities and helps inform researchers on correct primer selection for work with amphibians in Europe.

Introduction

1.1 Chytridiomycosis and the Decline of Amphibians

Amphibians are essential components of a functioning ecosystem and yet are poorly studied when compared to other vertebrates (Halliday, 2008), with 16.4% of known amphibian species being data deficient on the IUCN's Red List (IUCN, 2022). Due to their presence in both terrestrial and aquatic habitats, amphibians can provide an important energetic link between these areas and their dramatically different larval and adult stages mean that a single species can fill the equivalent ecological role of two species (Whiles et al., 2006). Amphibians are also of a direct benefit to humans, from providing a traditional food source to medical research models (Tyler et al., 2007; Hocking and Babbitt, 2013).

However, amphibians worldwide are currently facing an extinction crisis as deforestation, habitat loss and climate change alongside ranavirus and the virulent fungal disease chytridiomycosis, have combined to devastating effect for many species. Of the amphibian species assessed by the IUCN Red List, 41% are threatened with extinction and 9.2% are listed as critically endangered (IUCN, 2022). A study from 2019 estimated that chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) alone has led to the extinction of 90 species and the decline of 501, 39% of which are still in decline (Scheele et al., 2019).

Chytridiomycosis was discovered to be causing declines in anurans in Australia and Central America in 1998 (Berger *et al.,* 1998) and the subsequent year, *Bd* was identified as the cause (Longcore *et al.,* 1999). This fungus infects the skin of amphibians, causing excessive shedding of the epidermis and disrupting its function as a permeable membrane for transport of ions and respiratory gases, often leading to death (Berger *et al.,* 1998; Voyles *et al.,* 2009). Motile spores are deposited into the environment by an infected host and can persist in water for weeks (Johnson and Speare, 2003). *Bd* spores can be translocated to new locations via moist media such as bird feathers and soil (Johnson and Speare, 2005) or via vector species such as crustaceans and fish (Prahl *et al.,* 2020). However, at the global scale, current evidence suggests that global trade in amphibians is the primary method of translocation (Fisher and Garner, 2007).

Since its discovery, *Bd* has been shown to be widespread on all continents except Antarctica (where there are no amphibian hosts) and is infectious to not only anurans, but also urodelans (Ohst *et al.,* 2011;

Chatfield *et al.,* 2012; Bales *et al.,* 2015) and caecilians (Doherty-Bone *et al.,* 2013). This, coupled with *Bd*'s propensity to cause mass mortality events in a large number of species, has attracted huge scientific attention to the fungus with the aim of preventing further extinctions.

1.2 Overview of *Batrachochytrium salamandrivorans*

Bd was, until recently, thought to be the only fungal disease causing chytridiomycosis. A dramatic mortality event of fire salamanders (*Salamandra salamandra*) in the Netherlands, which started in 2010 and left just 0.01% of the Dutch population remaining by 2016 (Spitzen-van der Sluijs *et al.,* 2016), uncovered a second highly pathogenic chytrid fungus (Martel et al., 2013). The lethal skin infections found on these salamanders were caused by *Batrachochytrium salamandrivorans* (*Bsal*) (Martel et al., 2013). The symptoms of *Bsal*-driven chytridiomycosis include superficial skin lesions and deep epidermal ulcerations which are then subsequently infected with secondary bacteria leading to septicaemia and death in worst case scenarios.

Subsequently, *Bsal* has been shown to only infect urodelans (Martel et al., 2013; 2014), although it is evident from laboratory trials and observations that there are interspecific differences in the response to *Bsal* infection. A study by Smith *et al.* (2018) suggested that interspecific differences in salamander mucosomes could be responsible for this differing susceptibility to *Bsal*. All subspecies of *S. salamandra* appear to be highly susceptible to *Bsal* which has manifested in large declines across infected wild populations (Martel *et al.,* 2013; Spitzen-van der Sluijs et al. 2013; Sabino-Pinto *et al.,* 2015; Wagner *et al.,* 2020). Newt species can also develop chytridiomycosis from *Bsal* (Martel *et al.,* 2020) but death from the fungal infection is not as prevalent as in *S. salamandra*, suggesting that newt species should be considered as lower risk (Martel *et al.,* 2014).

Although *Bsal* and *Bd* are superficially similar in their cause of lethal skin erosion, studies show that potential urodelan hosts show different reactions to each fungus when exposed (Martel *et al.,* 2013; 2014). Furthermore, there is a deficit in the knowledge of the pathogenic mechanisms of *Bsal*. Compared to what is known about *Bd*, the transmission dynamics of *Bsal* and the influence of biotic and abiotic factors on pathogen response is largely undocumented (Blaustein *et al.,* 2018). Current *Bsal* experimental studies

have used a restricted range of doses, focused on just one pathogen isolate and limited source populations for the species tested which, given the differing response of *Bd* hosts to changes in these variables, should be expanded upon in future research (Blaustein *et al.,* 2018).

Thought to have been introduced from Asia to Europe via the pet trade, *Bsal* has now been found in captive populations in five European countries of which Germany, the Netherlands and Belgium also host wild populations of infected urodelans (Martel et al., 2014; Spitzen-van der Sluijs et al., 2016; Laking *et al.,* 2017; EFSA Panel on Animal Health and Welfare, 2018). Recently, a mass mortality of marbled newts (*Triturus marmoratus*) in Spain has been attributed to *Bsal* infection, expanding its known range (Martel *et al.,* 2020). Legislation currently regulates the importation of urodelans to the EU from non-EU territories but trade in anurans, which can act as potential *Bsal* vectors, remains unregulated (Gilbert *et al.,* 2020). Screenings have also been conducted in captive and wild populations in a number of European countries including Austria, Croatia, Czech Republic, France, Italy, Montenegro, Poland, Slovakia, Slovenia, Sweden and Switzerland (Gimeno *et al.,* 2015; Parrott *et al*., 2017; Baláž *et al*., 2018; EFSA Panel on Animal Health and Welfare, 2018; Sabino-Pinto *et al.,* 2018; Grasselli *et al.*, 2019; Lastra González *et al.,* 2019) but have not yielded any conclusive positive results.

Screening of urodelans in the Americas has also currently found no evidence of *Bsal* (Klocke *et al.,* 2017; Waddle *et al.,* 2020; Hill *et al.,* 2021) and increased restrictions on the trade of salamanders have been enforced to attempt to avert a biodiversity crisis (Yap *et al.,* 2015; U.S. Fish and Wildlife Service, 2016). It is uncertain how American urodelans would respond to infection with *Bsal*, but modelling studies predict that *Bsal* could flourish in many locations (Yap, 2016; Basanta *et al.,* 2019). Lab studies suggest that many American species can develop chytridiomycosis after exposure to *Bsal* and those species that don't develop the disease still display a stress response after exposure (Carter *et al.,* 2019; Barnhart *et al.,* 2020). Efforts to control and study the fungus in Europe could be key to preventing massive loss of species if *Bsal* ever reaches the Americas.

I. Transport Via Passive Carriers

There has been some evidence to suggest that *Bsal* can not only spread via infected urodelans but also via passive carriers such as anurans and birds (Nguyen *et al.,* 2017; Stegen *et al.,* 2017; Lötters *et al.,* 2020). *Bsal* was found on small-webbed fire-belly toads (*Bombina microdeladigitora*) imported from Vietnam (Nguyen *et al.,* 2017) and more recently on a wild common frog (*Rana temporaria*) in Germany (Lötters *et al.,* 2020). Experimental evidence has shown that *Bsal* could also adhere to the legs of geese to transfer larger distances between ponds (Stegen *et al.,* 2017). More tolerant urodelans can also act as vectors and reservoirs for the fungus, such as the Alpine newt (*Ichthyosaura alpestris*) which could be exacerbating the decline of susceptible local *S. salamandra* populations according to a recent study (Beninde *et al.,* 2021).

The scientific opinion on *Bsal* transmission detailed in a paper from the EFSA Panel on Animal Health and Welfare (2018) suggests that the most important vectors of transmission within a subpopulation is via active and passive amphibian carriers. Water flow and substrates can also contribute to *Bsal* spread on a relatively small scale (Martel *et al.,* 2013; 2014; Stegen *et al.,* 2017). However transfer via amphibian-related human activities and wild waterbirds have the most potential when considering larger distances of transfer between metapopulations (Zhu *et al.,* 2014; EFSA Panel on Animal Health and Welfare, 2018). There are also a number of other vectors of transmission which have been confirmed for *Bd* and therefore could also be vectors for *Bsal* spread such as via invertebrates, fish, reptiles and wild mammals (Kilburn *et al.,* 2011; McMahon *et al.,* 2013; Liew *et al.,* 2017; EFSA Panel on Animal Health and Welfare, 2018).

II. European Monitoring of *Batrachochytrium salamandrivorans*

Bsal is likely to be more widespread through Europe than currently known as there is limited surveillance of the disease. Although individuals can be tested reliably using skin swabs and qPCR (quantitative real-time polymerase chain reaction), this is only generally done after a decline in a population is recorded or dead individuals are found. Active studies which seek to determine whether *Bsal* has entered an area could miss the fungus in low concentrations, thereby limiting the reliability of such studies (EFSA Panel on Animal Health and Welfare, 2018). *Bsal* has the propensity to spread further via anthropogenic or natural means and rapidly extirpate susceptible urodelans (Stegen et al., 2017; Fitzpatrick et al., 2018). European climatic conditions are largely suitable for *Bsal* and Western Palearctic urodelans have been shown to be highly

susceptible to infection (Martel et al., 2014; Feldmeier *et al.,* 2016; EFSA Panel on Animal Health and Welfare, 2018). These factors combine to create a situation in which it is both essential to monitor the spread of this disease, but also very difficult and costly to do so. Current active and passive surveillance techniques are not efficient as *Bsal* has a widespread scattered distribution, can be present in low concentrations and sick or dead individuals can be difficult to find (Spitzen-van der Sluijs et al., 2016; 2018; Dalbeck et al., 2018). However, a recent paper from Spitzen-van der Sluijs et al. (2020) detailed a new technique utilising environmental DNA (eDNA) to overcome many of the issues associated with monitoring *Bsal*.

1.3 eDNA Monitoring Methods

eDNA is DNA shed from organisms as they interact with the environment and can be found in samples of water, soil and air (Roh *et al.,* 2006; Ficetola *et al.,* 2008; Lynggaard *et al.,* 2022). This eDNA can then be analysed using a variety of new and emerging techniques, broadly categorised into single-species or metabarcoding approaches, to determine the species present at a sample's site of origin. The single-species approach is also known as a species-specific or targeted approach and is used to determine the presence of eDNA from a single species of interest within a sample using species-specific primers and probes (Thomsen *et al.,* 2012b). This technique was developed from methods used in ancient sediment macro-organism diversity assessments (Willerslev *et al.,* 2003) and has only arose in the last 20 years. There has been an initial focus on detection of aquatic species such as amphibians (Ficetola *et al.,* 2008; Thomsen *et al.,* 2012b) and fishes (Jerde *et al.,* 2011; Thomsen *et al.,* 2012a) with primers targeting a short fragment of mitochondrial DNA, which are generally of greater number in environmental samples than nuclear DNA or longer fragments (Herbert and Gregory, 2005; Rees *et al.,* 2014a). The use of single-species detection has continued to expand with more recent studies on using the technique to quantify species biomass and abundance (Takahara *et al.,* 2012; Lacoursière-Roussel *et al*., 2016; Wilcox *et al.,* 2016) and for routine use in biodiversity monitoring programs (Rees *et al.,* 2014b; Biggs *et al.,* 2015; Ruppert *et al.,* 2017).

A single-species approach can be used for the detection of *Bsal* and utilises quantitative real-time polymerase chain reaction (qPCR) (Spitzen-van der Sluijs *et al.,* 2020). eDNA is first extracted from the sample and, for the *Bsal* protocol, twelve replicates per sample alongside one extraction control and one

negative control are amplified using qPCR (Spitzen-van der Sluijs *et al.,* 2020). The results of the qPCR can then be analysed, quantifying the number of target DNA fragments in each replicate and therefore determining whether the target species is present in the sample (Kubista *et al.,* 2006; Klymus *et al.,* 2020). eDNA provides a much more feasible option for monitoring *Bsal* than other established methods as, instead of needing to take samples from a high number of individuals in a water body to determine if the fungus is present, this approach simply requires a water sample taken from the top of the water, where the *Bsal* spores float (Spitzen-van der Sluijs *et al.,* 2020).

The potential for eDNA to inform research on *Bsal* is not limited to single-species monitoring of the fungus itself. eDNA metabarcoding has the capacity to detect full communities of species and could therefore be utilised to determine the community of amphibians present in a *Bsal* infected area (Charvoz *et al.,* 2021; Svenningsen *et al.,* 2022). The metabarcoding approach works using a primer set which amplifies a sequence common to a taxonomic group with enough variation to determine the community of species present (Valentini *et al.,* 2016). After the DNA is extracted from the sample, it is amplified using this metabarcoding primer set in a polymerase chain reaction (PCR) (Alberdi *et al.,* 2018). The number of replicates for each sample varies between studies but they range from two to over ten (Ficetola *et al.,* 2015) and are accompanied by extraction blanks, negative controls and positive controls to reduce the impact of contamination and false positives (Ficetola *et al.,* 2016). All replicates, blanks and controls are combined into a library which is prepared for sequencing by removing primer dimer and attaching adapters before sequencing on a high-throughput machine (Deiner *et al.,* 2015). The results of the sequencing can then be processed using a bioinformatics pipeline to determine the range of species present within the samples and the number of sequences detected for each of these species (Coissac *et al.,* 2012). Attaining information on the full amphibian community present at sites of *Bsal* infection using metabarcoding is beneficial for a number of reasons including both identifying the species at risk and the potential vector species. Furthermore, through establishment of a chronosequence or multiple sampling events over time, changes to the amphibian community composition as a result of *Bsal* and *Bsal*-caused population declines can be identified.

Metabarcoding has been successfully used to detect amphibians in Europe in many studies and is highly successful during the spring mating season when amphibians are mostly aquatic (Dufresnes *et al.,* 2019; Harper *et al.,* 2020a; Svenningsen *et al.,* 2022). However, as with most eDNA studies, there is a bias towards research conducted in temperate regions despite the high value of data on full amphibian communities and rare species in megadiverse regions such as the tropics (Lopes *et al.,* 2016; 2020; Sasso *et al.,* 2017; Carvalho *et al.,* 2021). Regardless of location, use of well refined metabarcoding primer sets utilising markers with enough variation to allow species-level discrimination and a comprehensive sequence reference database is essential for accurate results (Coissac *et al.,* 2012; Deagle *et al.,* 2014). As there is no perfect universal primer, marker choice is study specific and informed decisions must be made to ensure accurate data (Riaz *et al.,* 2011; Deagle *et al.,* 2014). There are a variety of primers used in amphibian eDNA metabarcoding studies, with most targeting the 12S or 16S mitochondrial rRNA region, which are split into those which target vertebrates and those which target just amphibians (Riaz *et al.,* 2011; Valentini *et al.,* 2016; Bálint *et al.,* 2018; Egeter *et al.,* 2019; Sakata *et al.,* 2022). Determining whether there is any difference in the amphibian data produced by vertebrate and amphibian primer sets is a question not yet addressed in the literature but would help inform optimal primer selection in future studies.

The use of eDNA in ecological monitoring is a relatively new technique (Ficetola *et al.*, 2008) with clear benefits for biodiversity monitoring including its non-invasive approach, highly sensitive detection of rare or elusive species and the potential to easily detect entire species assemblages (Goldberg *et al.,* 2015; Garlapati *et al.,* 2019). Metabarcoding is increasingly being used in biodiversity studies but, in the sparse comparison studies for targeted and metabarcoding approaches, the latter has been shown to be less sensitive than a targeted approach (Harper *et al.,* 2018; Bylemans *et al.,* 2019). Although many studies have compared eDNA methods with traditional survey techniques and found eDNA to be more accurate and cost-effective than traditional methods (Davy *et al.,* 2015; Smart *et al.,* 2015; Boussarie *et al.,* 2018; Sales *et al.,* 2020a; 2020b), its use for surveying is still relatively sparse with only a few species regularly surveyed using eDNA worldwide (Fediajevaite *et al.,* 2021). There are still a number of issues which need to be overcome such as the need for standardisation, issues with quantifying abundance and the spatial and temporal dynamics of eDNA to name a few (Beng and Corlett, 2020; Fediajevaite *et al.,* 2021). However,

the field of eDNA is constantly expanding and the current level of expertise is more than adequate for use detecting *Bsal* and the amphibian communities present at infected sites.

1.4 RAVON and *Batrachochytrium salamandrivorans* in the Netherlands

RAVON (Reptielen Amfibieën Vissen Onderzoek Nederland) is a non-governmental organisation which is responsible for national monitoring networks and distribution research for reptiles, amphibians and fish in the Netherlands. As such, RAVON has been heavily involved in the monitoring of *Bsal* in the Netherlands since its discovery in South Limburg in 2013 (Martel *et al.,* 2013) and was involved with the initial eDNA monitoring studies. Since then, *Bsal* has spread in South Limburg to Kerkrade and has also been found at four new locations: Berg en Dal, Wormdal, Pepinusbeekdal and a site in the Gelderland province (RAVON, 2021). Of these new sites, Pepinusbeekdal was not re-sampled but both Berg en Dal and Wormdal were re-sampled and tested negative for *Bsal* in 2015 and 2017 respectively (RAVON, 2021). The two private ponds in Gelderland have remained positive for *Bsal* and are currently the focus of a re-sampling project, the data from which will form the basis of this study.

The re-sampling project involves using eDNA to test for *Bsal* presence in the original two ponds and in 27 surrounding water bodies found within a 2km radius to track the spread of the fungus. All laboratory work was conducted by SPYGEN and every sample was also tested using a targeted approach to detect great crested newt (*T. cristatus*) and spadefoot toad (*Pelobates fuscus*). These two species are protected in the Netherlands (Council Directive 1992/43/EEC) and *T. cristatus* have been found infected with *Bsal* at two ponds in Gelderland (RAVON, 2021). Alongside *S. salamandra* and *T. cristatus*, *I. alpestris* and *Lissotriton vulgaris* (smooth newt) have also been found to be infected with *Bsal* in the Netherlands (RAVON, 2021) meaning that determining the communities of amphibians present at these sites would be beneficial to determining which species are spreading, or at risk of population decline from, *Bsal*.

1.5 The Dilution Effect Hypothesis

While the presence of hosts and potential vectors of *Bsal* can serve to grow and spread the fungus, the dilution effect hypothesis (DEH) argues that higher biodiversity can also serve to dilute the effect of the disease and reduce transmission. Dilution occurs when an increase in the diversity of species leads to less contact between host and vector species and therefore less transmission. This hypothesis was introduced

in 2000 (Ostfeld and Keesing, 2000) and has since been hotly debated with many papers arguing its significance in a world experiencing large loss of biodiversity and an accompanying increase in disease (Civitello *et al.,* 2015; Halsey, 2019; Halliday *et al.,* 2020). Despite criticism, around 80% of studies that address the diversity-disease relationship found evidence for the dilution effect (Huang *et al.,* 2016), including a number of studies on *Bd* (Searle *et al.,* 2011; Venesky *et al*, 2014; Becker *et al.,* 2014).

However this relationship is not simple, the presence of specific species can disproportionately increase or decrease infection loads (Becker *et al.,* 2014) and the host species must remain when species diversity declines for the dilution effect to be strong (Huang *et al.,* 2016). Currently there is no research on the dilution effect with *Bsal* as the focal disease and its superficial similarities to *Bd* aren't enough to conclude on the influence the dilution effect may have (Martel *et al.,* 2013; 2014).

1.6 Project Aims

This study has two main aims. Firstly, to validate the use of two different metabarcoding primers and single-species approaches in detecting amphibian species with eDNA in the Netherlands. This can be summarised into two research objectives:

1. Determine whether the 12S vertebrate or 16S amphibian metabarcoding primer set performs better when detecting amphibian communities in the Netherlands.
2. Determine whether a metabarcoding or single-species approach performs better when detecting two protected amphibian species: *Triturus cristatus* and *Pelobates fuscus*.

The second aim of this study is to investigate the relationship between *Bsal* and the amphibian species present at a site in Gelderland, Netherlands. This can be summarised into two research objectives:

3. Determine the relationship between amphibian community composition and *Bsal* presence.
4. Explore the potential association of individual amphibian species and waterbirds (as a group) with *Bsal* positive sites.

Methods

2.1 Field Sampling

Two ponds were identified by RAVON in Gelderland, Netherlands which have been confirmed to contain *Bsal.* Twenty seven water bodies within a 2km radius of the original two sites were subject to one session of eDNA sampling in mid-April 2021. This did not include the original two sites. Figure 1 shows the location of the study area within the Netherlands but the exact location is anonymised to obscure the location of protected species and as many sites are on private property.

FIGURE 1 – LOCATION OF THE STUDY SITE WITHIN THE NETHERLANDS

Another sampling occasion later in Spring was planned but COVID-19 restrictions prevented fieldwork from being conducted abroad. For each pond, 20 systematic samples of 100ml of water were taken standing on the banks of the pond using a 100ml sterile water sampling ladle. These water samples were collected from the top 5cm of the water, decanted into a 2-litre self-supporting sterile Whirl-Pak® bag and shook to homogenise the liquid. A sterile 100ml syringe was then used to push the full 2 litres of water through a VigiDNA® 0.45 µM cross-flow filtration capsule. The filtration capsule was then filled with 80ml of CL1

Conservation buffer to preserve the filter. All equipment was provided by SPYGEN. Throughout this process, latex gloves were worn to prevent contamination. Gloves were changed before adding the buffer and between ponds alongside a full decontamination of kit and boots between ponds to prevent the spread of *Bsal* (RAVON, 2020).

2.2 eDNA Extraction and Metabarcoding

Samples were then stored at room temperature and sent from RAVON to SPYGEN for single-species eDNA analysis detecting *Bsal*, *P. fuscus* and *T. cristatus*. The DNA was extracted from the samples in a room prepared for water DNA extraction with UV treatment, positive air pressure and frequent air renewal. Laboratory personnel changed into full protective clothing before entering the extraction room comprising a disposable body suit with hood, mask, laboratory shoes, overshoes and gloves. Full details of the DNA extraction methods are given in Spitzen-van der Sluijs *et al.* (2020). After the DNA extraction, the samples were tested for inhibition by qPCR following the protocol described in Biggs *et al.* (2015) and those found to be inhibited were diluted two-fold before amplification with the primer and probes.

The qPCR for all three single-species assays are performed in a final volume of 25µl with 1 µl of both forward and reverse primers (10 µM), 1 µl of probe (2.5 µM), 3 µl of template DNA, 12.5 µl of TaqMan Environmental Master Mix 2.0 (Life Technologies) and 6.5 µl of ddH2O. For the detection of *Bsal*, samples were amplified using primers and probes designed in Spitzen-van der Sluijs *et al.* (2020) (Bsal_F 5' CACATTGCACTCTACTTT 3'; Bsal_R 5' AAGACAAGGAAATGAATTAAA 3'; Bsal_Pr 6-FAM-TGATTCTCAAACAGGCATACTCTAC-BHQ-1) which amplify a short fragment of 54 base pairs (bp). The qPCR process entailed thermal cycling at 50°C for 5 min and 95°C for 10 min, followed by 50 cycles at 95°C for 30 s and 53.3°C for 1 min. For the detection of *T. cristatus*, samples were amplified using primers designed in Thomsen *et al.* (2012b) and validated in Biggs *et al.* (2015) (TCCBL 5' CGTAAACTACGGCTGACTAGTACGAA 3'; TCCBR 5' CCGATGTGTATGTAGATGCAAACA 3'; TCCB_Probe FAM-CATCCACGCTAACGGAGCCTCGC-BHQ1) which amplify an 81 bp fragment. The qPCR process entailed thermal cycling at 50 °C for 5 min and 95 °C for 10 min, followed by 55 cycles of 95 °C for 30 s and 56.3 °C for 1 min. Primers and probes for *P. fuscus* alongside the qPCR process have been developed by SPYGEN and are as yet unpublished.

Further details on these methods are contained in Spitzen-van der Sluijs *et al.* (2020) and Biggs *et al.* (2015). The remaining extracted DNA was then sent on to the University of Salford for use in this project, no field or extraction blanks were included.

Following their arrival at the University of Salford, the 27 extractions were stored at -20^{o}C until PCR amplification. The samples were amplified on two separate occasions using two different metabarcoding primer sets. The first was the vertebrate *12S-V5* primer set (*12S-V5*_F 5' TAGAACAGGCTCCTCTAG 3'; *12S-V5*_R 5' TTAGATACCCCACTATGC 3'; Riaz *et al*., 2011) which amplifies a short fragment (~98 bp) of the 12S rRNA region with sample-specific multiplex identifier (MIDs) tags. 24 unique MIDs tags for both forward and reverse primers were used to differentiate between samples. During PCR for these primers, the samples were denatured for 5min at 95° followed by 35 cycles of 15s at 95°, 30s at 57° and 30s at 72° with a final elongation of 5min at 72°. The second was the 16S amphibian primer set which targets a 150 bp fragment in the 16S ribosomal RNA region of the mitochondrial genome (BA-4445-F 5'-RACCGTGCRAAGGTAGCR-3; BA-178-R 5'-CCATRGGGTCYTCTCGTCT-3'; Bálint *et al.,* 2018) with sample specific MIDs tags. 24 unique MIDs tags for both forward and reverse primers were used to differentiate between samples. The PCR amplification for these primers followed an adapted protocol with samples denatured for 15min at 95° followed by 35 cycles of 30s at 94°, 1min 30s at 55° and 1min at 72° with a final elongation of 30min at 60°.

During PCR for both primer sets, each sample had three replicates to reduce bias in individual reactions. Three replicates of a positive control sample from another project involving captive *Agalychnis lemur* (lemur leaf frog), a central American species, were included on the plate for each primer set alongside four negative control replicates. The positive control is used to identify tag jumping for subsequent data filtering steps and the negative control is used to identify cross sample contamination during the PCR process. Amplification was confirmed using 1.2% agarose gel electrophoresis stained with GelRed (Cambridge Bioscience), PCR products were then pooled into two separate libraries (one for each primer set) and bp length of amplified fragments was confirmed using the Tapestation.

RATIO Agencourt AMPure XP (Beckman Coulter) was used to perform a left-sided size selection with a bead ratio of 1:1.5 for the *12S-V5* vertebrate primers library and 1:0.9 for the 16S amphibian primers library. The KAPA HyperPrep kit (KapaBiosystems) was used to add Dual-Index adapter 4 to each library. The NEBNext qPCR quantification kit (Biolabs) was then used to quantify each library by qPCR in order to dilute correctly to a concentration of 9pM in preparation for sequencing. An Illumina MiSeq Reagent Kit v2 Nano was then used to sequence each library on separate sequencing runs.

2.3 Bioinformatic Analysis

Once the sequencing was complete, the files were downloaded from *BaseSpace* (basespace.illumina.com). The bioinformatic analysis was conducted using the *OBITools* metabarcoding package (Boyer *et al.,* 2016) followed by a direct blast against the GenBank nucleotide database (NCBI) to assign taxonomy, all conducted on a Linux server (*Ubuntu 18.04.6 LTS*). The quality of the reads were first assessed using *FASTQ* (Andrews, 2015) and then the forward and reverse reads were merged with *illuminapairedend*. Demultiplexing was conducted with the *ngsfilter* command and then *obigrep* was used to select fragments within a suitable range and remove reads with ambiguous bases. For both primer sets, a wider range of fragment sizes were retained in an initial run and then re-run with a tighter range informed by the results of the first run. For the *12S-V5* vertebrate primers, the range was initially 50-250bp and the final run was 85-120bp. For the 16S amphibian primers, the range was initially 80-250bp and the final run was 110-120bp. The data was dereplicated using *obiuniq*, then chimera removal was conducted using the *uchime denovo* method (Edgar et al. 2011) in *VSEARCH* (Rognes et al. 2016) with a *minh* value of 0.9. Sequences were then clustered using *SWARM* (Mahé et al., 2015) and exported to be used in the taxonomic assignment process.

Before taxonomic assignment, singletons (MOTUs represented by only one sequence read) were removed to aid in computation time. Taxonomic assignment of MOTUs was carried out using *blastn* against the GenBank database. A maximum of 25 hits were returned with the sequences requiring at least 80% identity and 90% alignment, then the most common taxid was assigned. MOTUs required at least 98% identity to be assigned at species level and all other assignments were filtered out in subsequent steps (Sales *et al.,* 2020a).

The results were filtered using an adapted version of the method used in Broadhurst *et al.* (2021) with read counts at each stage recorded in Table 1. First, reads were removed proportional to the number of positive control reads found in samples other than the positive control to account for tag jumping (Schnell *et al.,* 2015). Then the maximum number of reads within the negative control samples were removed from all read counts. Reads from domestic animals and humans were removed. MOTUs assigned at species level and with more than five reads were kept to produce a file containing the vertebrate community. Subsequently, the amphibian community was isolated in a separate file. For the amphibian primers, no vertebrate community file was created but the same steps were followed. *Pelophylax lessonae* was replaced with *Pelophylax spp.* as the mitochondrial DNA of *P. lessonae* and the hybrid *Pelophylax kl. esculentus* are indistinguishable using eDNA methods (Holsbeek and Jooris, 2010), although the hybrid is more common.

2.4 Statistical Analysis

All statistical analyses were conducted in *R v4.1.2* and maps were made in *ArcGIS Pro 2.9* and *Adobe Illustrator 26.0*. First, bubble charts were created using *ggplot2* to show the proportional read count of each amphibian species at each site for both primer sets. Subsequent analyses were conducted with the data converted into presence/absence and site 22 removed as no data for this site was attained, likely due to an error at the PCR stage. Box and whisker diagrams were constructed to show the species richness detected across sites by the amphibian and vertebrate primer sets separately. A species accumulation curve was constructed using the *iNEXT* function to extrapolate the amphibian species richness data to 50 sites for each primer set and the combined primer data.

Maps of the amphibian communities present at each site as generated by the two primer sets were constructed to show their distribution alongside the presence of *Bsal*. To compare the communities produced by each primer set site by site, Sørensen's Index values (with 0 signifying no common species and 1 signifying a complete species match) were calculated from the two communities (one for each primer set) produced for each site and then represented in a box and whisker plot. Sites where both primer sets produced zero amphibian species were given a Sorensen's Index value of 1. The vertebrate primer set was subsequently selected to move forward with the analyses (see Results).

A further map was constructed to visually represent the detection of *T. cristatus* and *P. fuscus* by a single-species approach (data provided by RAVON and SPYGEN) compared with the vertebrate primer data. To examine whether the approach selected had an effect on whether each species was detected at each site, two McNemar's tests were conducted using the mid-*P* method (Pembury Smith and Ruxton, 2020).

Sites were split into those which tested positive and those which tested negative for *Bsal* and a box and whisker plot constructed based on the amphibian species richness values from the vertebrate primer set data for each category. An NMDS plot was constructed to determine whether the amphibian communities detected by the vertebrate primers were related to *Bsal* presence or absence. The Jaccard index was used as a measure of community similarity and sites with no species detected were removed (sites 2, 16, 17 and 19). This left 15 *Bsal* negative sites and 7 *Bsal* positive sites. A two-dimensional approach was used after examining the stress test to ensure it was within an acceptable range. The *adonis* function was then used to conduct a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations to determine whether *Bsal* presence was significantly associated with certain amphibian communities. The assumption of no difference in dispersion between *Bsal* positive and negative amphibian communities was tested using a permutational analysis of multivariate dispersions (PERMDISP) with 999 permutations. As the PERMANOVA is not as powerful with a significant PERMDISP result, a distance-based redundancy analysis (dbRDA) plot was also constructed using the Jaccard Index with 1000 permutations to determine the strength of *Bsal* as an explanatory variable of amphibian community composition. The initial NMDS plot was retained to demonstrate the PERMDISP results and the species associations with *Bsal* positive sites.

The proportion of sites in the *Bsal* positive and *Bsal* negative categories containing each amphibian species were calculated alongside the same data for waterbirds as a combined category. This was then displayed on a bar chart to determine whether there were any visible differences in the proportions which could be explored in future studies.

Results

3.1 Overview

The Miseq sequencing run yielded around 1.6 million raw reads for the *12S-V5* vertebrate primers and 1 million reads for the 16S amphibian primers. Table 1 shows the reads for each primer dataset remaining at the end of the bioinformatics process (first line) and after each stage of the filtering process. After the negative control step, the number of reads assigned to each class for the vertebrate primer set data (all reads were in phylum Chordata) and each phylum for the amphibian primer data are shown in Appendixes 1 and 2 respectively. Non-target reads at that stage comprise just 1.3% for the vertebrate primer data but most reads sequenced in the amphibian primer dataset were not amphibian as shown by the dramatic reduction in reads at the final filtering step (Table 1). The reads for the amphibian primers were dominated by Rotifera (27.4%), Arthropoda (25.5%) and non-assigned reads (44.5%). Average read depth for amphibian species per sample is 19165 for the vertebrate primer set and 105 for the amphibian primer set.

TABLE 1 - READ COUNTS FOR EACH PRIMER SET AT EACH STAGE OF THE POST-BIOINFORMATIC FILTERING PROCESS.

	12S-V5 Vertebrate primer set	16S Amphibian Primer set
Total reads before filtering criteria	1370905	542354
After accounting for tag-switching	1368503	542354
After removing positive control	1366027	530744
After removing negative control	1365335	521921
After removing all human reads	998859	521423
After removing domestic animals (*Bos, Canis, Felis, Ovis and Sus*)	963799	521423
MOTUs with minimum identity >0.98 and less than 5 reads removed	945831	99420
After removing all non-amphibian reads	498302	2848
Tag-switching proportion	0.0004	0

The vertebrate community produced by the vertebrate primers was extracted just before removing all non-amphibian reads in the filtering process and is shown in Table 2. All three replicates for site 22 failed and it was subsequently removed from the data. Thirteen species of birds were detected including five waterbirds: mallard (*Anas platyrhynchos*), grey heron (*Ardea cinerea*), Eurasian coot (*Fulica atra*), white stork (*Ciconia Ciconia*) and great cormorant (*Phalacrocorax carbo*). One of the bird species detected was the Siberian accentor (*Prunella montanella*), a rare migrant to Western Europe. The sequence has 234 reads at one site and was a 100% match with *P. montanella* and *Prunella fulvescens* but the former was deemed more feasible. Eight mammal species were detected with most species occurring at just one site except the beech marten (*Martes foina*), wood mouse (*Apodemus sylvaticus*) and roe deer (*Capreolus capreolus*) which occur at two sites. Four fish species were detected but only the asp (*Leuciscus aspius*) is native in the wild in Europe and therefore the other three species are assumed to have been introduced into sites as decorative pond species. The results from RAVON and SPYGEN showed that eight of the 27 sites tested positive for *Bsal* and these are also displayed in Table 2.

TABLE 2 - TABLE WITH GREEN BOXES INDICATING DETECTION OF A SPECIES BY THE *12S-V5* VERTEBRATE PRIMER SET. *BSAL* POSITIVE SITES ARE INDICATED IN RED AND SITE 22 IS SHADED GREY DUE TO FAILURE. SPECIES IN RED ARE NON-NATIVE IN EUROPE.

			Site																								
Class	Species	Common name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Amphibia	*Lissotriton vulgaris*	Smooth Newt																											
Amphibia	*Pelobates fuscus*	Spadefoot Toad																											
Amphibia	*Bufo bufo*	Common Toad																											
Amphibia	*Pelophylax sp.*	Pool/Edible Frog																											
Amphibia	*Rana temporaria*	Common Frog																											
Amphibia	*Triturus cristatus*	Great Crested Newt																											
Aves	*Anas platyrhynchos*	Mallard																											
Aves	*Columba palumbus*	Common Wood Pigeon																											
Aves	*Ardea cinerea*	Grey Heron																											
Aves	*Corvus corax*	Common Raven																											
Aves	*Parus major*	Great Tit																											
Aves	*Garrulus glandarius*	Eurasian Jay																											
Aves	*Sturnus vulgaris*	Common Starling																											
Aves	*Fulica atra*	Eurasian Coot																											
Aves	*Fringilla coelebs*	Common Chaffinch																											
Aves	*Dendrocopos major*	Great Spotted Woodpecker																											
Aves	*Ciconia ciconia*	White Stork																											
Aves	*Phalacrocorax carbo*	Great Cormorant																											
Aves	*Prunella montanella*	Siberian Accentor																											
Mammalia	*Martes foina*	Beech Marten																											
Mammalia	*Apodemus sylvaticus*	Wood Mouse																											
Mammalia	*Capreolus capreolus*	Roe Deer																											
Mammalia	*Lepus europaeus*	European Hare																											
Mammalia	*Myodes glareolus*	Bank Vole																											
Mammalia	*Oryctolagus cuniculus*	European Rabbit																											
Mammalia	*Sciurus vulgaris*	Red Squirrel																											
Mammalia	*Rattus norvegicus*	Brown Rat																											
Actinopteri	*Leuciscus aspius*	Asp																											
Actinopteri	*Pimephales promelas*	Fathead Minnow																											
Actinopteri	*Carassius auratus*	Goldfish																											
Actinopteri	*Lepomis gibbosus*	Pumpkinseed																											

3.2 Metabarcoding Primer Comparison

For comparison of the primer sets, just the amphibian species detected are included. When comparing the two primer sets, there is an obvious deficiency in data from the amphibian primers, producing just 1% of the reads for amphibians produced by the vertebrate primers. Furthermore, the vertebrate primers detected six amphibian species where the amphibian primers only detected five, missing *T. cristatus* which is a species of concern for these sites. The proportion of reads which constitutes each species at each site for both primer sets is shown in Figure 2. There are some similarities in the proportion of each species detected by both primer sets, with a few sites showing almost the same proportion and composition for both primer sets such as 8 and 24. *Lissotriton vulgaris* clearly dominates the reads and the two species of interest (*T. cristatus* and *P. fuscus*) are the least detected for both primer sets.

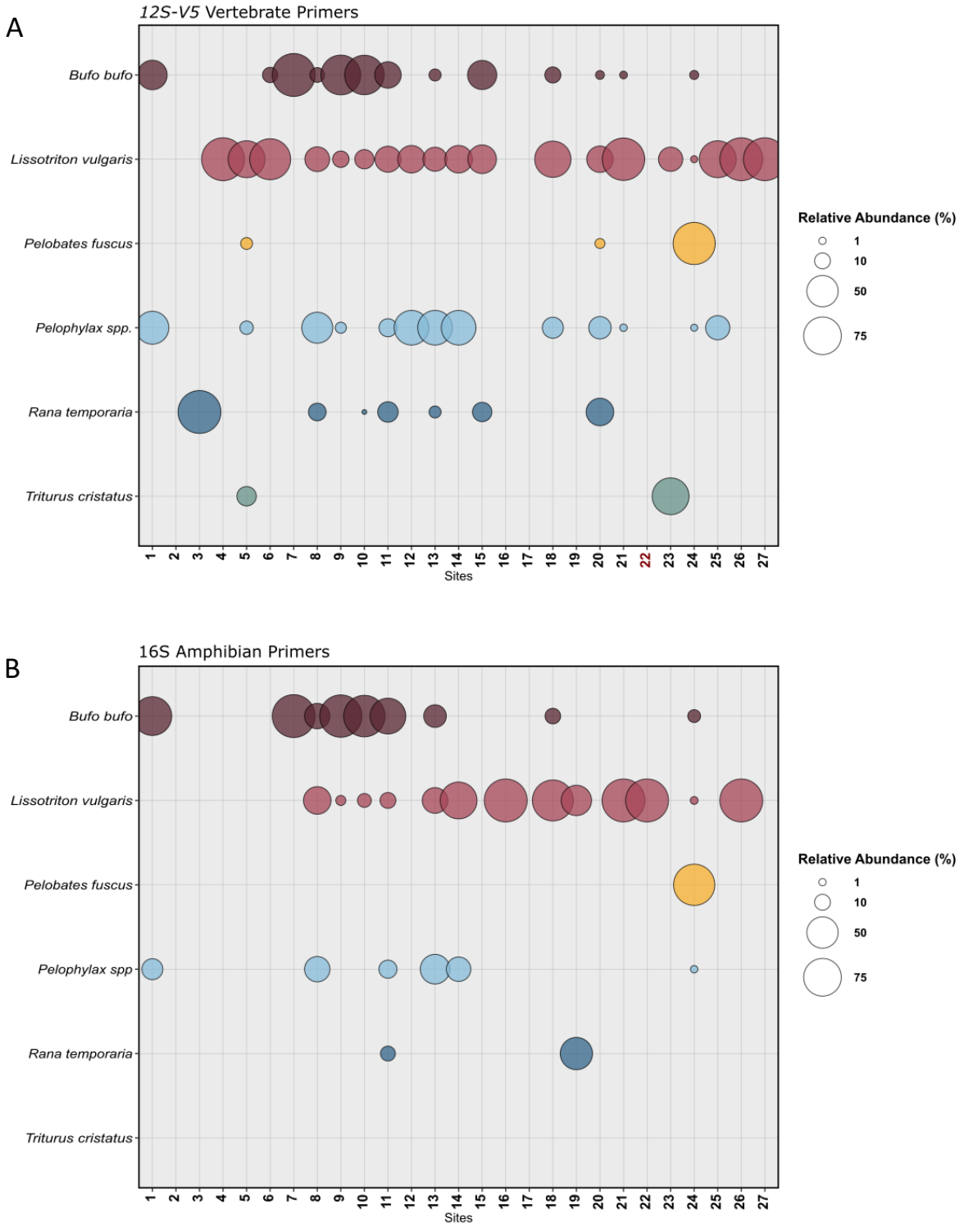

FIGURE 2 – RELATIVE ABUNDANCE OF THE READS FOR EACH SPECIES AT EACH SITE DETECTED USING THE A.) *12S-V5* VERTEBRATE PRIMER SET B.) 16S AMPHIBIAN PRIMER SET. SITE 22 (RED) FAILED FOR THE VERTEBRATE PRIMER SET AND IS SUBSEQUENTLY REMOVED FROM THE DATA.

Site amphibian species richness values were combined for each primer set and are shown in Figure 3. The vertebrate primer set has a larger range of species richness values, reaching a maximum of five, than the amphibian primer set which only reaches four. Overall, the vertebrate primer set has generally higher species richness across sites than the amphibian primer set. Figure 4 further shows how the vertebrate primer set has been much more successful at detecting the species richness of the sites, reaching a plateau within the number of sites surveyed in this study. Furthermore, the vertebrate primer data almost independently drives the combined data, making the contribution of the amphibian primer data essentially redundant.

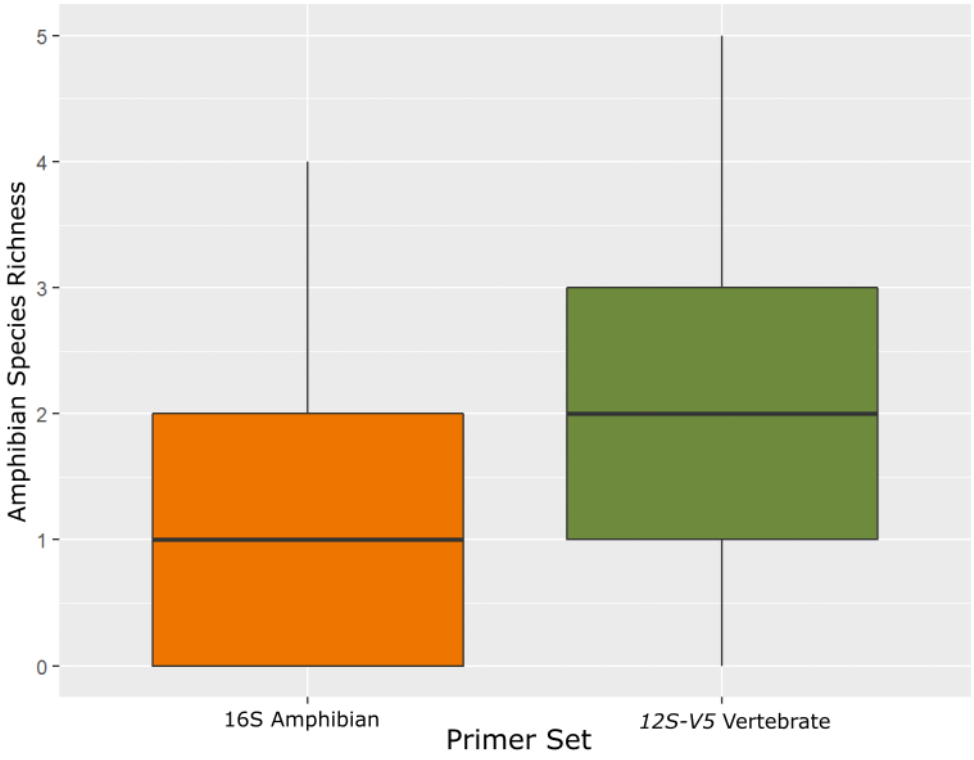

FIGURE 3 - AMPHIBIAN SPECIES RICHNESS DETECTED BY THE 16S AMPHIBIAN AND *12S-V5* VERTEBRATE PRIMER SETS PER SITE.

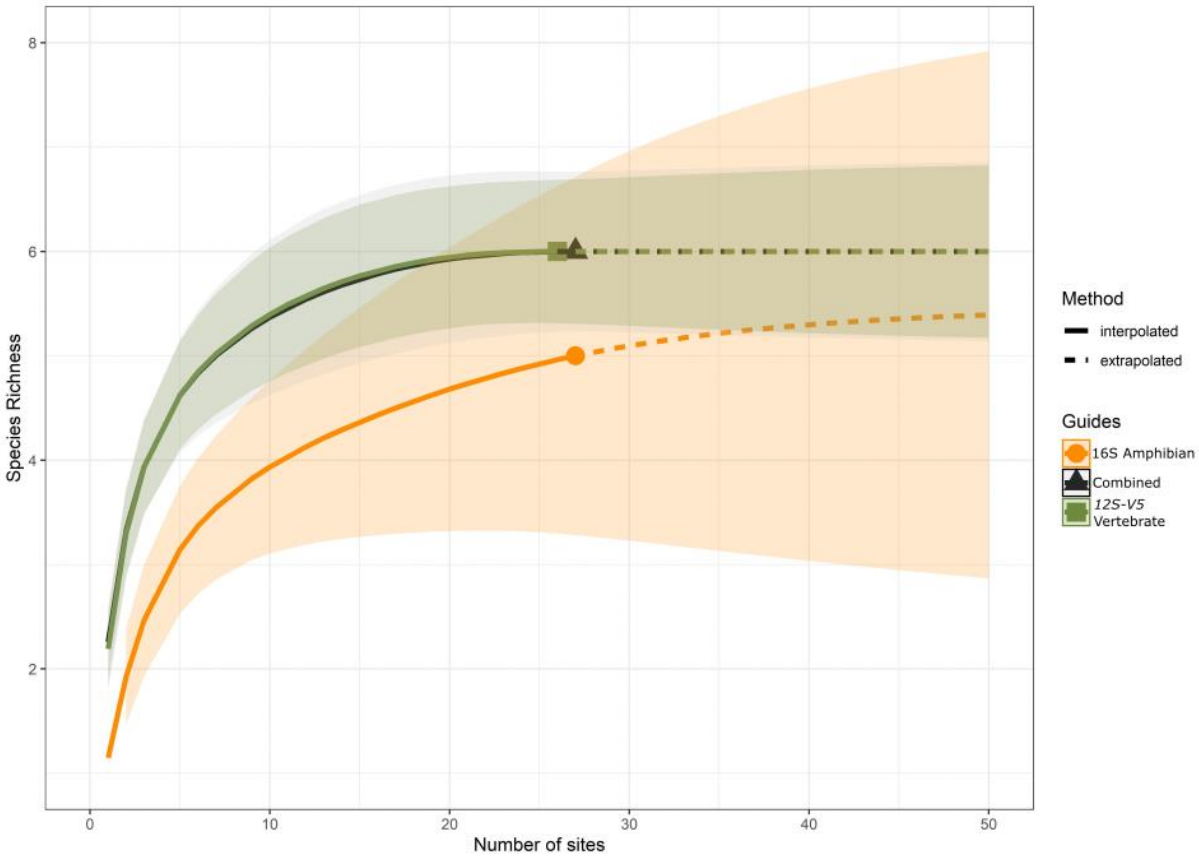

FIGURE 4 - SPECIES ACCUMULATION CURVE SHOWING THE INTERPOLATED SPECIES RICHNESS DETECTED BY EACH PRIMER SET AND BY BOTH PRIMER SETS COMBINED ALONGSIDE THE EXTRAPOLATED RICHNESS TO 50 SITES. VERTEBRATE PRIMER DATA HAS SITE 22 REMOVED DUE TO FAILURE AND THEREFORE HAS 26 SITES.

Maps were constructed displaying the amphibian communities at each site as detected by the vertebrate (Figure 5) and amphibian (Figure 6) primer sets alongside the *Bsal* data, with 8 sites found to be *Bsal* positive and 19 to be *Bsal* negative. Figures 5 and 6 further show the deficiency of the amphibian primer data as it contains ten sites with no amphibian reads where the vertebrate primers detected amphibians. However, of the four sites where nothing was detected by the vertebrate primers, the amphibian primers detected species at three. At site 22 the amphibian primers detected *L. vulgaris* but, due to an error during the lab process (most likely at the PCR stage), no DNA at all was detected for this site by the vertebrate primers. At site 16, the amphibian primers detected *L. vulgaris* and at site 19 it also detected *R. temporaria* where the vertebrate primers detected nothing. However, all of these detections by the amphibian primer set have a low number of reads, ranging between 8 and 23 after filtering.

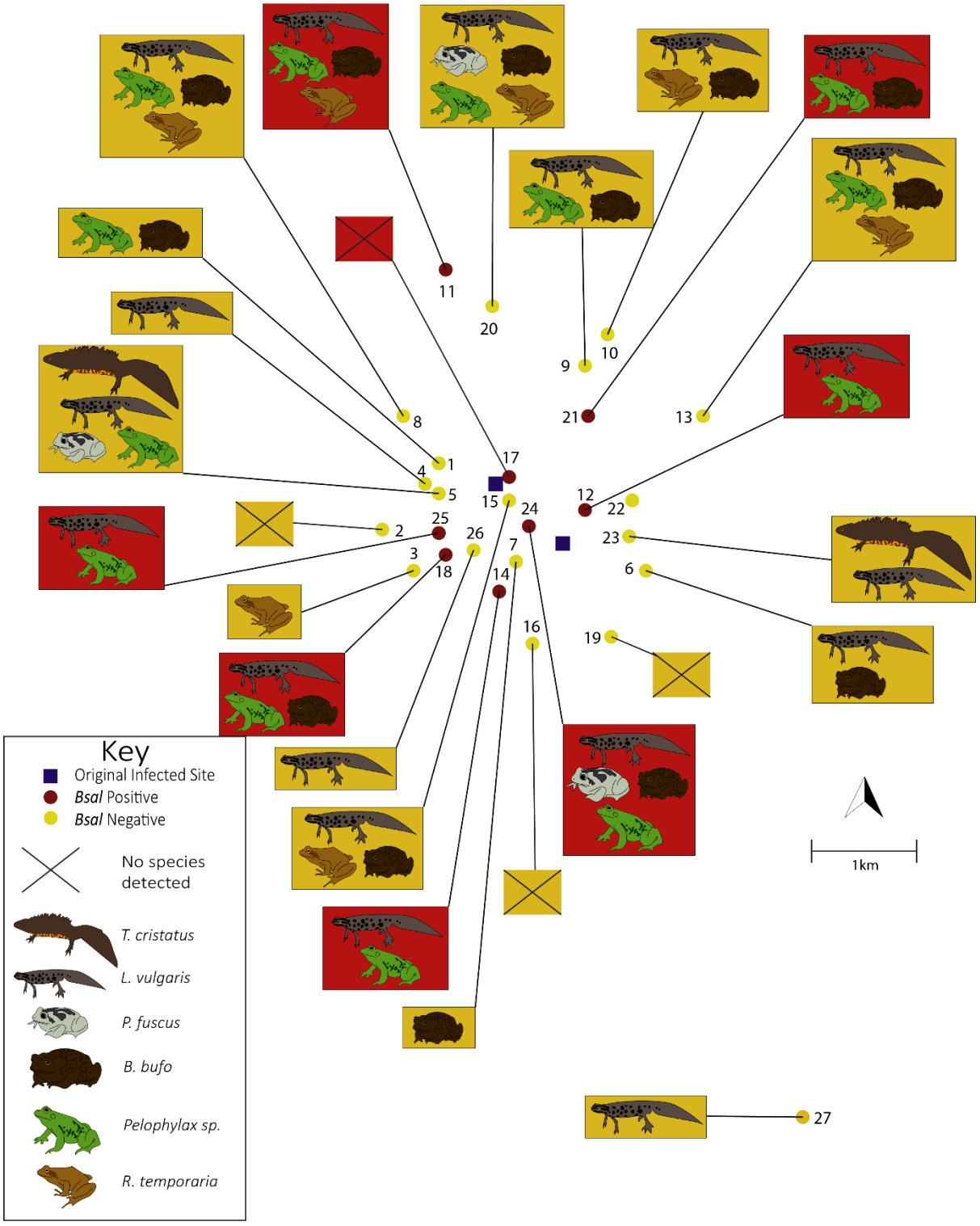

FIGURE 5 - MAP DISPLAYING THE AMPHIBIAN COMMUNITIES DETECTED USING THE *12S-V5* VERTEBRATE PRIMER SET AT *BSAL* POSITIVE AND NEGATIVE SITES. THE ORIGINAL TWO SITES WHERE *BSAL* WAS DETECTED ARE INCLUDED IN BLUE. SITE 22 FAILED SO NO RESULTS ARE SHOWN FOR THIS SITE.

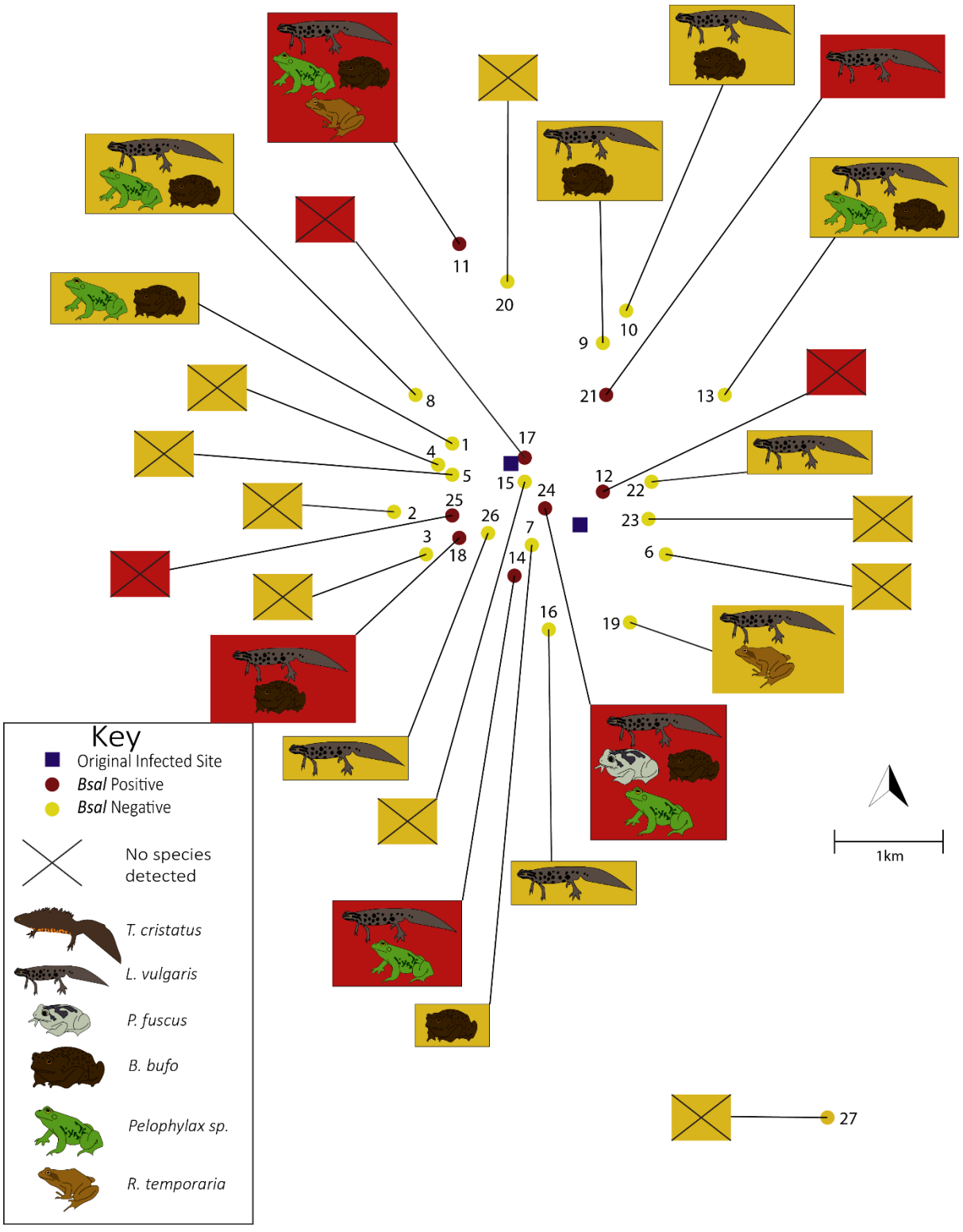

FIGURE 6 - MAP DISPLAYING THE AMPHIBIAN COMMUNITIES DETECTED USING THE 16S AMPHIBIAN PRIMER SET AT *BSAL* POSITIVE AND NEGATIVE SITES. THE ORIGINAL TWO SITES WHERE *BSAL* WAS DETECTED ARE INCLUDED IN BLUE.

To compare the sites directly, the amphibian community produced by each primer set was used to produce Sørensen's Index values for each site. There was a clustering of values around 0 and around 1 with a mean value of 0.5236 showing the almost bisection of the data into sites with zero agreement between amphibian communities and almost complete or complete agreement.

The *12S-V5* vertebrate primer set outperformed the 16S amphibian primer set by producing more comprehensive amphibian communities. Henceforth, the analyses will be conducted using just the data produced using the vertebrate primer set.

3.3 Single-Species versus Metabarcoding

When compared to the single-species data on *T. cristatus* and *P. fuscus*, the vertebrate metabarcoding primers detected these species of interest less often (Figure 7). The largest number of sites where the two approaches agreed (13) held neither of the species. Both approaches detected *P. fuscus* at three sites and *T. cristatus* at one site. The vertebrate primers detected *T. cristatus* at one site where it was not detected in the single-species data but the vast majority of *T. cristatus* detections were made only by the single-species approach. Despite the relatively low number of discordant pairs, the results from the McNemar's test showed that the approaches produce significantly different results for both *T. cristatus* (p=0.0391) and *P. fuscus* (p=0.0313). The single-species approach is therefore more likely to detect both species than the metabarcoding approach.

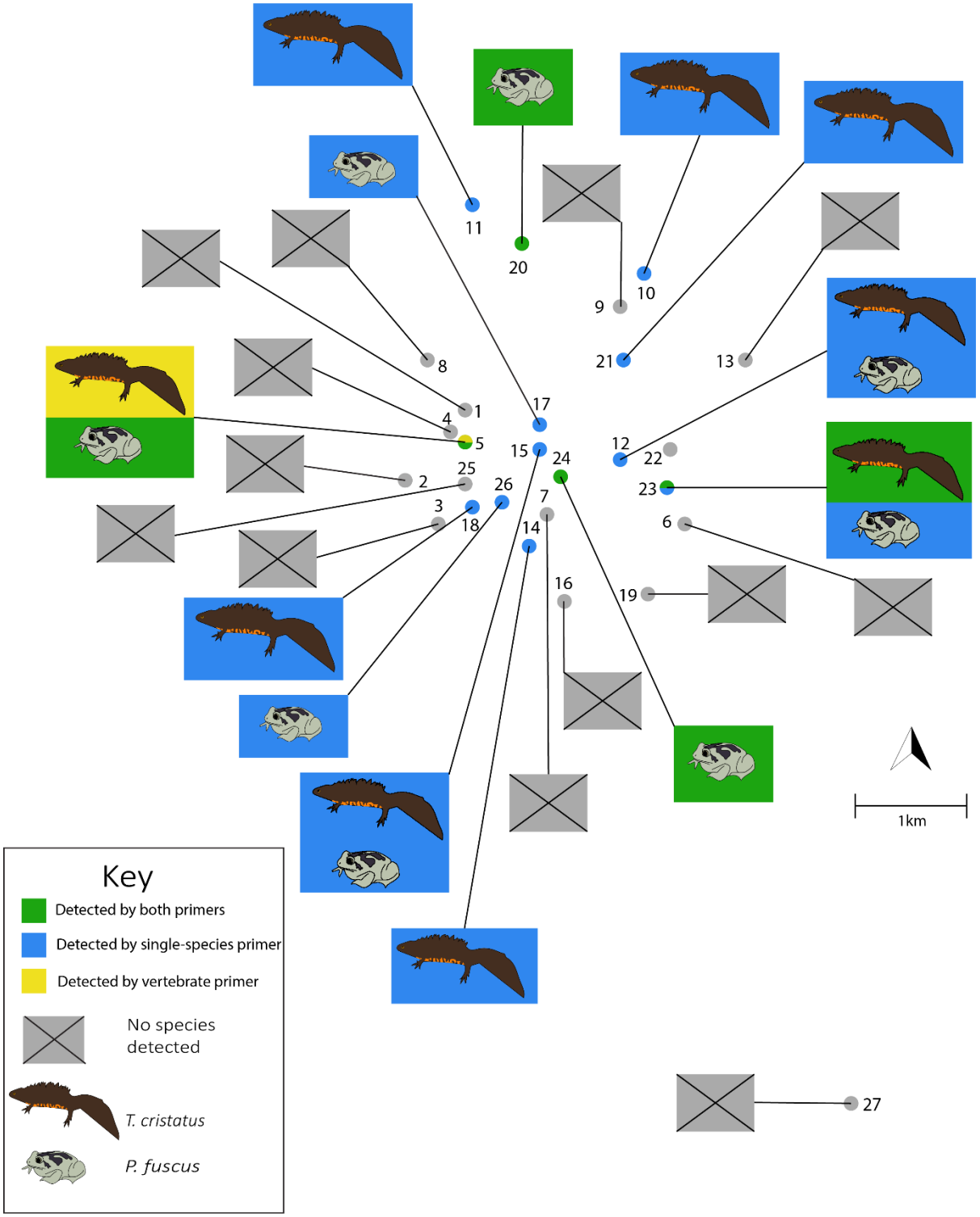

FIGURE 5 - MAP COMPARING THE DETECTION OF *T. CRISTATUS* AND *P. FUSCUS* VIA SINGLE-SPECIES AND METABARCODING (*12S-V5* VERTEBRATE PRIMERS) APPROACHES. SITE 22 IS NOT INCLUDED AS IT IS REMOVED FROM THE VERTEBRATE PRIMER DATA.

3.4 Amphibian Communities and *Bsal*

Sites were split into those which tested positive for *Bsal* and those that tested negative and species richness per site for the two groups were plotted in a box and whisker plot (Figure 8). The number of sites in each category is not equal, with 18 negative sites and 8 positive sites, which is likely the cause of the apparent difference in species richness in Figure 8.

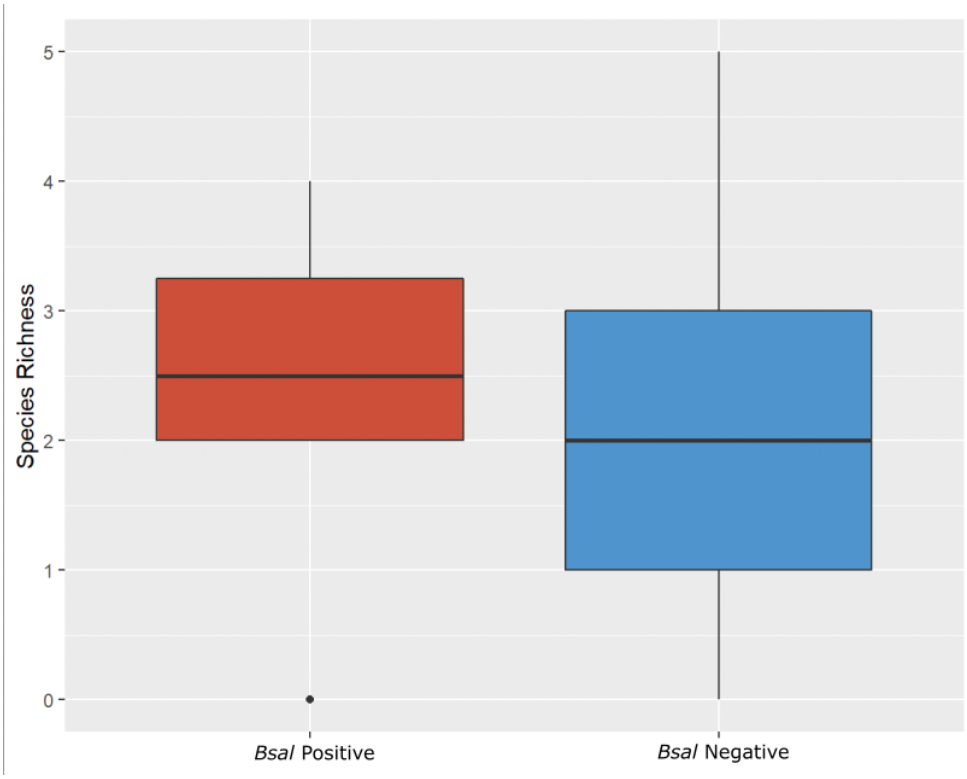

FIGURE 6 - AMPHIBIAN SPECIES RICHNESS DETECTED BY THE *12S-V5* VERTEBRATE PRIMER SET FOR *BSAL* POSITIVE AND *BSAL* NEGATIVE SITES.

To explore the amphibian communities present at the sites and their association with *Bsal* presence, an NMDS plot was constructed (Figure 9) with a stress value of 0.112. *Bsal* positive sites appear clustered within the middle but there is no distinct separation from negative sites. When a PERMANOVA was conducted, *Bsal* presence was found to have a non-significant explanatory power over amphibian communities ($F = 1.994$, $p = 0.157$). A PERMDISP test was conducted and the difference in distribution between *Bsal* positive and negative sites is significantly different ($F = 10.44$, $p = 0.005$). The non-significant PERMANOVA means that centroids of the two groups are not significantly different but there is a significant difference in their variance (PERMDISP), meaning that sites within the *Bsal* positive category have more similar communities than those in the *Bsal* negative category.

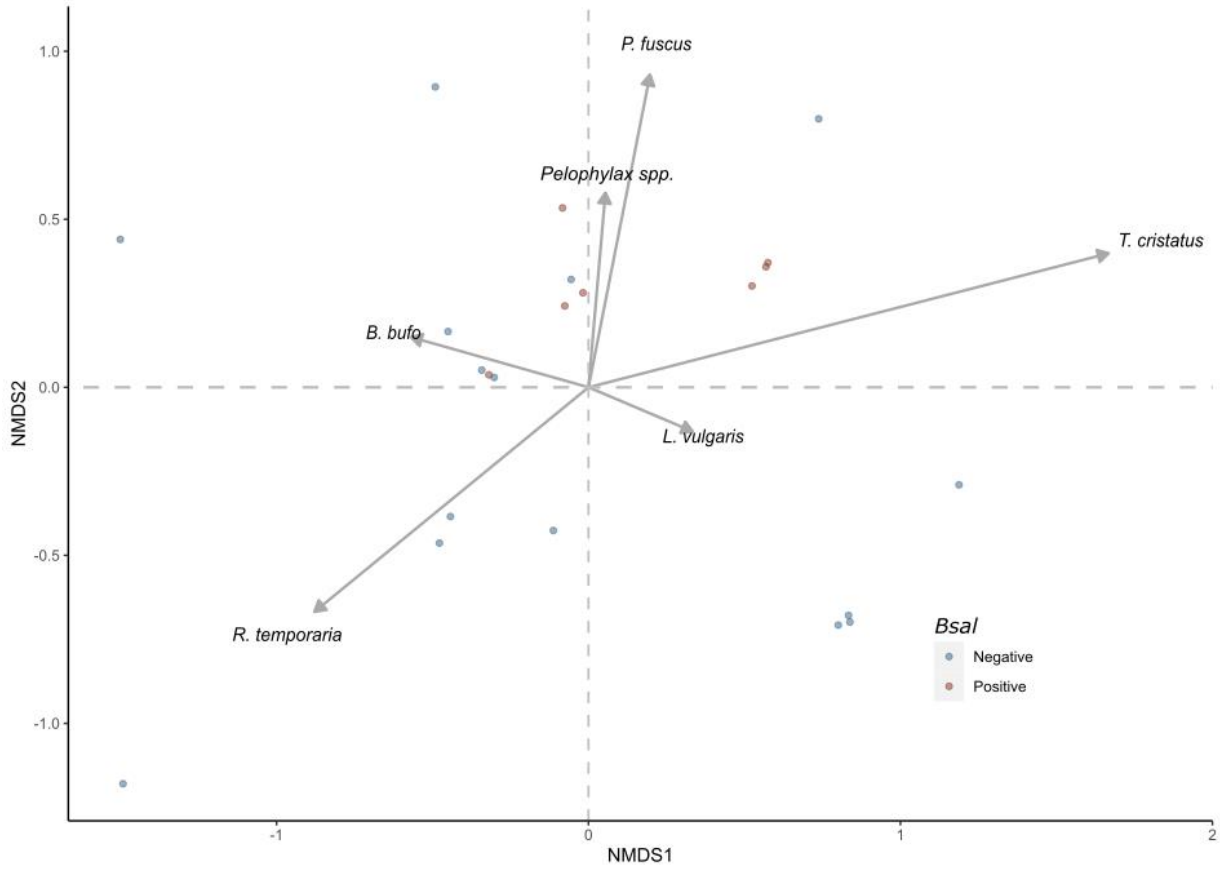

FIGURE 7 - NMDS PLOT SHOWING THE SIMILARITY OF AMPHIBIAN COMMUNITIES DETECTED BY THE *12S-V5* VERTEBRATE PRIMER SET AT *BSAL* POSITIVE AND NEGATIVE SITES OVERLAID WITH THE SPECIES ASSOCIATIONS. POINTS ARE JITTERED FOR VISIBILITY.

As the PERMANOVA is not as powerful with a significant PERMDISP result, a dbRDA plot was created to determine whether *Bsal* is significant as an explanatory variable for amphibian community composition. The dbRDA result (Figure 10) was significant but without much explanatory power (R^2adj = 0.0569, p=0.0497). The first axis CAP1 explained 10.18% of the variation and the second axis MDS1 explained 36.69%. The low adjusted R^2 value suggests that the effect of *Bsal* on the differences between amphibian communities present is no more significant than any other random value and that other variables not recorded in this study likely have a much larger explanatory power. This is also evidenced by the high explanatory power of the second axis over the first, which is driven solely by *Bsal*.

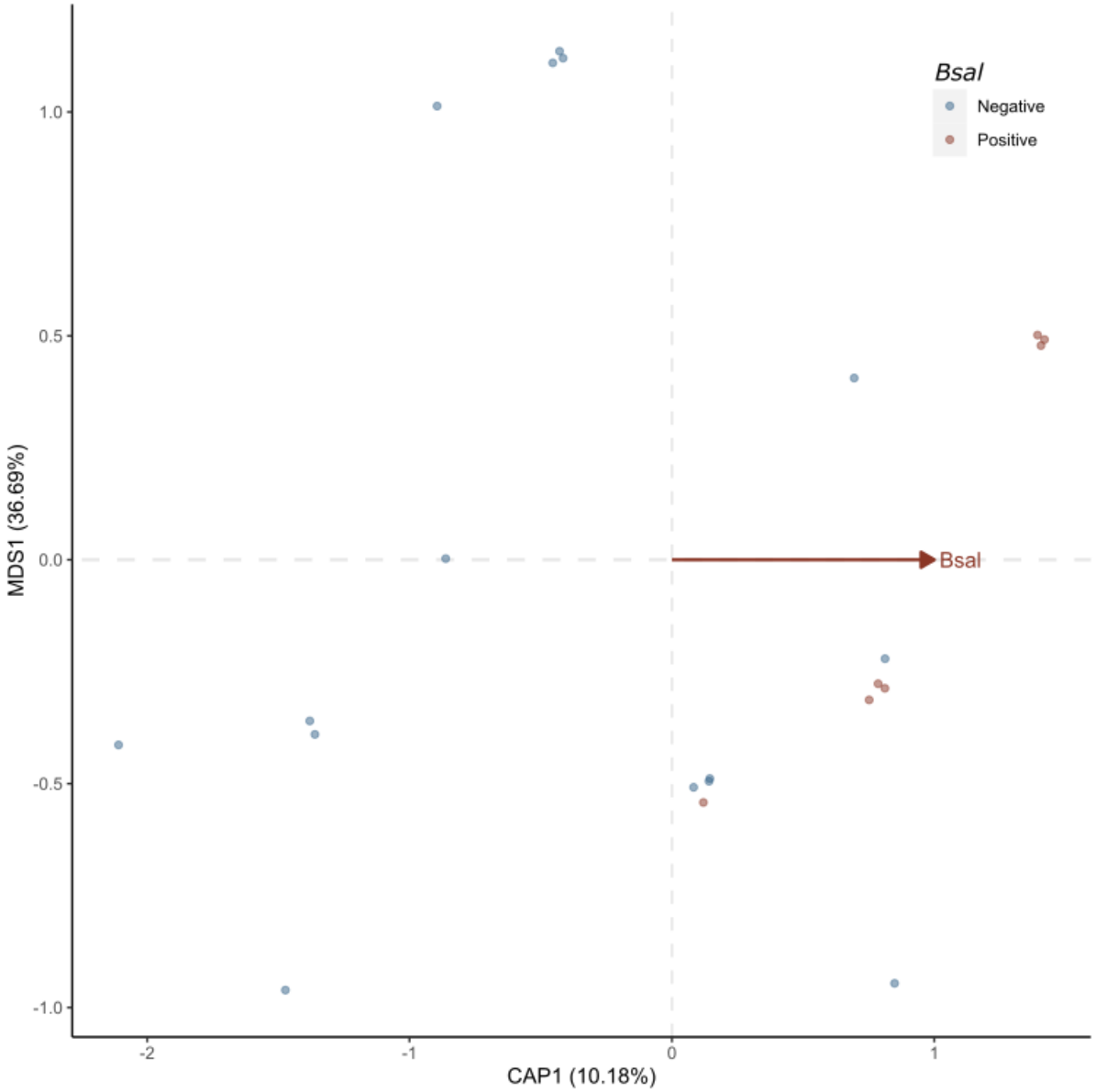

FIGURE 8 - DBRDA PLOT SHOWING THE INFLUENCE OF *BSAL* ON THE DIFFERENCES IN AMPHIBIAN COMMUNITY COMPOSITION AS DETECTED BY THE VERTEBRATE *12S-V5* PRIMER SET. POINTS ARE JITTERED FOR VISIBILITY.

When the proportion of sites within the *Bsal* positive and *Bsal* negative categories containing each amphibian species and waterbirds was investigated, most had very similar values regardless of category (Figure 11). Waterbird species included mallard (*A. platyrhynchos*), grey heron (*A. cinerea*), Eurasian coot (*F. atra*), white stork (*C. ciconia*) and great cormorant (*P. carbo*) but were dominated by mallard. This study does not have enough data to produce statistically significant results considering the relative lack of *Bsal* sites and restriction to just presence/absence data so no further statistical analyses were conducted. However, Figure 11 highlights *Pelophylax spp.* and potentially *R. temporaria* as species with potential links to *Bsal* presence. Due to the presence of *T. cristatus* at *Bsal* positive sites in the single-species data, the lack of *T. cristatus* at *Bsal* positive sites in Figure 11 is not considered significant and is likely due to a deficiency in the vertebrate primer data.

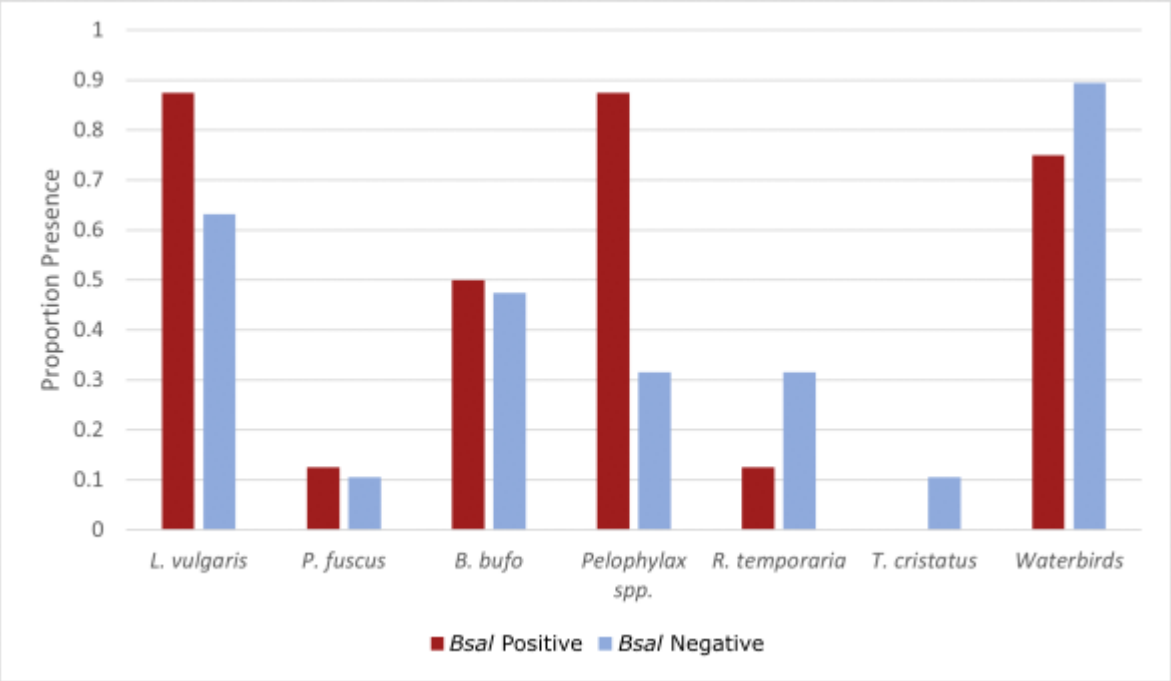

FIGURE 9 - THE PROPORTION OF SITES WITHIN *BSAL* POSITIVE AND NEGATIVE CATEGORIES CONTAINING EACH AMPHIBIAN SPECIES/WATERBIRDS AS DETECTED USING THE VERTEBRATE *12S-V5* PRIMER SET.

Discussion

This study has successfully detected the expected range of amphibian species at sites in Gelderland alongside a component of the wider vertebrate community present at these sites. The species accumulation curve for the *12S-V5* vertebrate primer set (Figure 4) demonstrates that the sampling effort in this study was sufficient to detect all amphibian species present at the study area. The single-species data attained from RAVON demonstrates the spread of *Bsal* from the initial two sites to eight further sites without a clear path of spread as some of the closest sites remain uncontaminated (Figures 5 and 6). However, it should be noted that the chronological contamination of sites by *Bsal* is unknown as only two sites were sampled originally (RAVON, 2021). The lack of recorded infections of sub-populations of *T. cristatus* at other ponds at the time of original sampling could suggest that, even if *Bsal* was present at other sites, it was not as well established as within the original sites. However, there is a chance that *Bsal* could already have extirpated *T. cristatus* from these sites unnoticed.

From the literature, there are several ways the infection could have spread including via active urodelan carriers, passive carriers such as anurans and waterbirds or via humans (EFSA Panel on Animal Health and Welfare, 2018). The *12S-V5* vertebrate metabarcoding primers enabled the detection of five species of waterbirds alongside eight other species of bird, eight species of mammals and four fish species. Although not pertinent to this study, one of the species detected was the Siberian accentor (*Prunella montanella*), a rare migrant to Western Europe and potentially the first finding of this species in the Netherlands since 2016 (Waarneming.nl, 2021). Such a finding demonstrates the value of using eDNA to monitor species populations as it has the ability to detect rare or cryptic species (Pfleger *et al.,* 2016; Nester *et al.,* 2020; Neice and McRae, 2021). Furthermore, the use of a vertebrate primer set is highly beneficial as the data generated on non-target species can form the basis of further studies without the need for additional field or laboratory work.

4.1 Metabarcoding Primer Comparison

Comparison between the 16S amphibian and *12S -V5* vertebrate metabarcoding primer sets revealed that the former consistently failed to detect the full range of amphibian species detected by the latter at each site. The amphibian primers did not detect *T. cristatus,* consistently detected a lower amphibian species

richness at each site and only contained 1% of the amphibian reads detected by the vertebrate primers. Therefore, future research into amphibians in Europe should opt for the *12S-V5* vertebrate primer set (Riaz *et al*, 2011) as it not only provides a much more comprehensive dataset on the amphibian communities present, but it also provides valuable extra data on the other vertebrate species present. This is in agreement with the existing literature as the *12S-V5* primers are among the most highly used metabarcoding primers for detecting amphibians alongside the *batra* 12S amphibian primers (Valentini *et al.,* 2016) and primers developed for specific study areas (Egeter *et al.,* 2019; Charvoz *et al.,* 2021).

The 16S amphibian primers have not yet been used in any published peer-reviewed works except the original Bálint *et al.,* (2018) study. It is likely that, as this primer set is developed for use within tropical biodiverse ecosystems and developed from anuran sequences from South America, it is not optimised for use detecting European amphibian species. However, it could be argued that the 16S amphibian primers should simply not be recommended for use in any study. This is due to the large quantity of Rotifera, Arthropoda and completely unidentified DNA fragments which dominated the reads in the output file after the bioinformatics stage and also dominated the reads when the primer set was used in other recent projects at the University of Salford (A. McDevitt, *pers. comm*.). Changes can be made to increase the number of reads of 'rare' amphibian species detected by the 16S amphibian primer set, such as increasing the number of replicates at the PCR stage or using a sequencing kit with a larger read depth (Grey *et al.,* 2018; Shirazi *et al.,* 2021; Ushio *et al.,* 2022). However, a less costly and more streamlined solution would be to simply select an alternative amphibian targeting primer set, such as the *batra* 12S primers, or use the *12S-V5* vertebrate primers.

The *batra* primer set was not used in this study as the PCR process had not yet been optimised in the laboratory at the University of Salford. Following the original cycling parameters given by Valentini *et al.* (2016) resulted in strong amplification of a non-target fragment, which was ~30 bp larger than the target fragment when the PCR product was tested on the Tapestation. The initial annealing temperate of 55° was increased to 60° and 65° in separate, small-scale PCR trials to attempt to remove the non-target fragment (Bronnenhuber and Wilson, 2013) but this was unsuccessful. The volumes of the reagents in the

amplification mixture followed the same ratio as used for the *12S-V5* vertebrate and 16S amphibian primers in this study which differs from those used in Valentini *et al.* (2016). This change, in addition to the lack of human-blocking primers used in the original paper (Valentini *et al.,* 2016), could have contributed to the amplification of this non-target fragment but time pressures for completion of laboratory work prevented further trials testing modifications to the amplification mixture.

Despite this difficulty at the PCR stage, a comparison study of the *batra* 12S primers alongside the *12S-V5* primers is still needed to facilitate informed choices in future amphibian eDNA research. Studies which compare the *12S-V5* primers to metabarcoding primers targeting more constrained taxonomic groups have mixed results. The *12S-V5* primers have the capacity to detect terrestrial mammals (Harper *et al.,* 2019) and have performed well detecting rare Amazonian species when compared to a mammal COI metabarcoding primer set (Kocher *et al.,* 2017a; Coutant *et al.,* 2021). However, when used for studies on fish, the *12S-V5* primers have had difficulty distinguishing between closely related families and species when compared to 16S and 12S *Mifish* primers (Hänfling *et al.,* 2016; Doble *et al.,* 2019). Although the diversity of amphibian species in this study is relatively low, the vertebrate primers did struggle to detect some of the rarer species. Using an amphibian targeting primer set, such as the *batra* 12S primers, could provide a greater read depth for amphibian species as only amphibian eDNA is amplified, increasing the chances of detecting rare species (Shirazi *et al.,* 2021). The *batra* primer set has been successfully utilized in studies on amphibians in China (Li *et al.,* 2021a; 2021b), Brazil (Sasso *et al.,* 2017; Lopes *et al.,* 2021) and, more importantly, Switzerland (Dufresnes *et al.,* 2019; 2020) where the amphibian communities contain many of the same species as in this study. Therefore, the 12S *batra* primers are likely a better contender than the 16S amphibian primers for providing reliable and comprehensive amphibian community data.

Despite this, the *12S-V5* vertebrate primers performed well in this study and have the additional benefit of producing the full vertebrate community, not just amphibians, which includes species of ecological significance, such as predator species or, in this case, potential disease vectors. The *12S-V5* primer set is also very versatile and has detected amphibians using a variety of methods besides eDNA including through carnivore diet analysis (Shehzad *et al.,* 2012; Kumari *et al.,* 2019; Harper *et al.,* 2020b; Shao *et al.,* 2021),

invertebrate gut contents (Kocher *et al.,* 2017b; Lynggaard *et al.,* 2019) and, most recently, from air samples (Lynggaard *et al.,* 2022). The *12S-V5* primers have also successfully detected a wide-range of European amphibian species including all genera found in the Netherlands (Kumari *et al.,* 2019; Brys *et al.,* 2020; Harper *et al.,* 2020a; 2020b; Peixoto *et al.,* 2020; Svenningsen *et al.,* 2022) (Appendix 3) reinforcing the reliability of the results found in this study. Therefore, in the absence of data on the *batra* 12S amphibian primers, the *12S-V5* is still a suitable primer set for use in amphibian eDNA studies in Europe.

4.2 Single Species versus Metabarcoding

The results of the McNemar's test determined that there was a significant difference between the data produced by the two approaches for both *T. cristatus* and *P. fuscus* and that the single-species approach was more likely to detect both species. This is in line with the literature as a targeted approach is widely considered better than metabarcoding for amphibians and many other organisms as metabarcoding can mask species at low abundances (Brandon-Mong *et al*., 2015; Evans *et al.,* 2016; Harper *et al.,* 2018). However, as metabarcoding methods become a more financially competitive option, the extra data produced for the wider species community will make it a better option for monitoring purposes in many cases (Harper *et al.,* 2018).

Some differences in the metabarcoding methods adopted could further the detection of rare species in these samples. The number of replicates differs greatly between the metabarcoding protocol adopted in this study and the single-species protocol from SPYGEN with the former using three replicates and the latter using twelve. This increase in replicates makes rare species far more likely to be detected as there is a larger quantity of DNA from the sample being analysed (Grey *et al.,* 2018; Ushio *et al.,* 2022). However, the risk of false positives also increase with further replicates and a suitably rigorous method for removing them must be adopted in order to not also remove true positives of rare species (Ficetola *et al.,* 2015). This study used an Illumina MiSeq Reagent Kit v2 Nano which has a supposed maximum of around 1 million reads for the library, although the vertebrate dataset included much more without becoming overclustered (Illumina, 2022). The amphibian read depth per sample in this study for the *12S-V5* vertebrate primer was 19165 which is relatively low (Harper *et al.,* 2019; Dully *et al.,* 2021). A larger kit, such as the MiSeq Reagent Kit v2 which generates around 15 million reads for two libraries run together (Illumina, 2022), could

increase the chances of detecting rarer species by providing a greater read depth (Shirazi *et al.,* 2021). However increasing the number of replicates and size of the sequencing kit are costly changes and the magnitude of the positive impact they have depends heavily on the study parameters so care should be taken when considering employing them (Shirazi *et al.,* 2021).

4.3 Amphibian Communities and *Bsal*

Having a record of the full amphibian communities present at sites of *Bsal* infection is valuable as the species at risk of infection and the potential vector species can then be determined. Furthermore, it is not yet known whether *Bsal* has an impact on the amphibian community as a whole. *Bd*-induced chytridiomycosis has caused huge declines in host species and extirpation for many (Lips *et al.,* 2006; Scheele *et al.,* 2019) which then leaves an empty niche which can be utilised by other species (Hirzel and Le Lay, 2008). A similar outcome, but on a smaller scale, could occur at sites of *Bsal* infection. If a *Bsal*-tolerant species fills this niche, it could lead to an acceleration of *Bsal* transmission but if a *Bsal*-resistant species fills the niche, it can act to slow the spread of the fungus (Holt and Pickering, 1985; Brannelly *et al.,* 2020). In this study, *L. vulgaris* can be considered a tolerant species (Bates *et al.,* 2019) and anurans are considered resistant (Martel *et al.,* 2013).

When the potential trend between *Bsal* presence and the community composition of amphibians was investigated using a dbRDA, it was found that there was a significant, but not highly influential, trend between them. Such a low adjusted R^2 value suggests that *Bsal* is no more influential than any other random variable and the larger percentage of explanatory power on the y-axis (Figure 9) indicates that, as a driver of differences in amphibian community composition, there are other much more influential drivers than *Bsal*. Well known drivers such as habitat and microhabitat availability suitable for amphibian breeding activities are likely to be much more influential on amphibian community composition than *Bsal* here (Werner *et al.,* 2007; Vági *et al.,* 2013; Konowalik *et al.,* 2020). However, the significant difference in the intra-group variability, with *Bsal* positive sites containing amphibian communities which are more similar (Figure 9), could potentially show a homogenisation of communities due to *Bsal* or that these similar communities are more likely to be successfully infected with the fungus (Smith *et al.,* 2009). This could also simply be an artefact of a comparative lack of *Bsal* positive sites compared to negative sites.

There are a number of reasons why, with further data, *Bsal* may yet be found to be a significant driver of amphibian community composition. Firstly, this study contained relatively few sites and only one sampling occasion. Larger spatial and temporal resolution would provide the volume of data needed to accurately reveal the relationship between *Bsal* and amphibian community composition (Beentjes *et al.,* 2019). As most amphibians in this study vacate to terrestrial refuges during the winter, sampling times are still advised within the amphibian breeding season to ensure sufficient eDNA present in the water but sampling over many years could track changes in communities as they occur. Detection of amphibian communities across *Bsal* positive sites in the Netherlands, ideally also including *Bsal* positive sites in Germany, Belgium and Spain, would provide the most comprehensive view of which species are exposed to the pathogen in its invasive range. It would also allow more in-depth analyses into the dynamics of the amphibian communities within *Bsal* positive areas and potentially enable the construction of a chronosequence to provide further insight into community development over time.

Another reason for the lack of a relationship with *Bsal* could be due to the low number of sites where *T. cristatus* was detected by the metabarcoding primers. This meant that the main species of concern was not included as heavily in the analyses as perhaps it should be, given the single-species results. Methods suggested in the previous section to increase detection of rare species by the *12S-V5* vertebrate primers could be utilised in future studies to overcome this issue. Finally, *Bsal* may not be well enough established at the sites sampled within this study to have influenced a change in the community yet.

It is not known whether *T. cristatus* sub-populations have experienced any significant decline due to *Bsal* in this area yet. However, evidence from other studies have shown *T. cristatus* experiences a moderate mortality rate when exposed to *Bsal* and declines have been observed in wild German sub-populations (Bates *et al.,* 2019; Lötters *et al.,* 2020). If *T. cristatus* numbers do significantly decline due to *Bsal*, future monitoring will detect fewer *T. cristatus* reads or they could be absent from sites where it was previously detected (Lötters *et al.,* 2020). It is as yet unclear whether another amphibian species would increase in population to fill the empty niche produced by *T. cristatus* decline. However, if this decline has already begun, the association of *Pelophylax spp.* with *Bsal* positive sites in this study (Figure 11) may indicate

utilisation of this niche. As a *Bsal* resistant species, this would be beneficial for *T. cristatus* populations in the area as the spread of the fungus is slowed. *Lissotriton vulgaris* is another likely candidate as it is highly associated with *T. cristatus*, sharing certain prey species and hibernacula (Roşca *et al.,* 2013; Dervo *et al.,* 2018). As a tolerant species, *L. vulgaris* could help drive *T. cristatus* decline by acting as a reservoir, constantly providing a source of new infection for *T. cristatus* populations (Brannelly *et al.,* 2020; Lötters *et al.,* 2020). It is unclear as to whether *L. vulgaris* could also decrease in population significantly due to *Bsal*-induced chytridiomycosis but there is research to suggest that the species is relatively resistant to extreme cases of the disease (Bates *et al.,* 2019) and dramatic reductions in wild populations are yet to be recorded.

Abundance data would have added another dimension to this community analysis and perhaps revealed differences in the populations of species at *Bsal* positive and negative sites. It could also have revealed the status of *T. cristatus* populations, with the opportunity to track any declines if more sampling sessions are conducted in the future. One shortcoming of eDNA is the current difficulty with using the data to determine the abundance of species within a site or study area. Several studies have shown that read count is positively correlated with abundance or biomass, with fish being by far the most studied taxa (e.g. Takahara *et al.,* 2012; Evans *et al.,* 2016; Lacoursière-Roussel *et al*., 2016; Wilcox *et al.,* 2016) and amphibians only having a few studies (Evans *et al.,* 2016; Li *et al.,* 2021a). A recent meta-analysis of published works found fish are currently the only vertebrates with enough published research to safely conclude this positive correlation with abundance or biomass (Carvalho *et al.,* 2021). However, this trend is much stronger in laboratory studies than in field studies making eDNA an imperfect tool for estimating abundance or biomass under uncontrolled conditions (Evans *et al.,* 2016; Carvalho *et al.,* 2021).

There are a variety of factors in field studies other than abundance which affect the amount of eDNA in a sample and therefore lead to this weakened positive correlation between read count and abundance (Beng and Corlett, 2020). Factors influencing the eDNA input into the system include rates of DNA shed by an individual which is dependent on condition, size, species, age and seasonal activity (Pilliod *et al.,* 2013; Spear *et al.,* 2015; Li *et al.,* 2021a). Once the eDNA is shed into the water, it will begin to decay with such

factors as levels of ultra-violet light and microbiology influencing its persistence (Strickler *et al.,* 2015; Barnes and Turner, 2016). Furthermore, sampling and processing protocols, such as primer selection, will all have an effect on the abundance of eDNA detected within samples (Pilliod *et al.,* 2013; Brannock and Halanych, 2015; Deiner *et al.,* 2015; Piñol *et al.,* 2019).

Although there is still a long way to go before measuring abundance using eDNA is reliable enough for routine use (Deiner *et al.,* 2017; Dickie *et al.,* 2018), it is a promising development for future studies. Measures of abundance produced with eDNA will still be limited compared to the array of population measures provided by traditional methods, but the former is much less time intensive and less invasive for the individuals being surveyed (Dalbeck *et al.,* 2018). Furthermore, for future *Bsal* projects, the lack of intense contact with infected individuals compared to traditional methods means that eDNA also aids in reducing any inadvertent transmission of the fungus by researchers (EFSA Panel on Animal Health and Welfare, 2018). Therefore, although not yet available for *Bsal* and amphibian community studies in the immediate future, developments in quantifying abundance will be crucial in providing data on the status of host populations and changes in the wider amphibian community.

4.4 Amphibian Species and *Bsal* Transmission

As expected, the rarest species detected in this study were those protected under the European Habitats Directive (Council Directive 1992/43/EEC): *Triturus cristatus* (Annex II and IV) and *Pelobates fuscus* (Annex IV). *Lissotriton vulgaris* dominates the reads in this study and is present at most sites which was to be expected as it is a common species and syntopic with *T. cristatus*. The proportions of *Bsal* positive and negative sites hosting each amphibian species and waterbirds as a group were examined but this study did not contain enough *Bsal* positive sites for further meaningful statistical analysis (Figure 11). Even so, this work can be considered a preliminary study providing the groundwork for future research and provides a few potential lines of investigation.

One such interesting observation in the data is the high proportion of *Pelophylax spp.* at *Bsal* positive sites compared to negative sites alongside its association with the clustering of *Bsal* positive sites in the NMDS plot (Figure 9). As anurans have been shown to be passive carriers of *Bsal*, *Pelophylax spp.* could be

contributing to the dispersion of *Bsal* from the original two sites, both of which are close to recorded *Pelophylax spp.* populations in this study. However, this could also be due to *Pelophylax spp.* populations increasing where *T. cristatus* has declined due to *Bsal* and left empty niches as hypothesised in the previous section (Holt and Pickering, 1985; Brannelly *et al.,* 2020).

One issue with *Pelophylax spp.* acting as a vector for *Bsal* is that this species complex has high site fidelity and are only likely to move between nearby ponds within a few hundred metres (Holenweg Peter *et al.,* 2001). This means that the transmission hypothesis could only explain *Bsal* spread to site 17 and perhaps 24, due to the presence of a nearby stream, if the two original sites are also considered as the first to be infected. Even if the infection did not originate at the two originally sampled sites, *Pelophylax spp.* could not cross the distance from any site to sites 11 and 21 and therefore the presence of *Bsal* at these locations cannot be attributed to this species. Anurans more likely to facilitate the spread of *Bsal* across long distances are *B. bufo* and *R. temporaria* which are well known to travel several kilometres overland (Kovar *et al.,* 2009), with *B. bufo* travelling up to 5km (van Gelder *et al.,* 1986). However, a lower proportion of *R. temporaria* were present at *Bsal* positive sites than negative sites in Figure 11 which, although not statistically significant, doesn't support the hypothesis of this species as a significant *Bsal* vector. Furthermore, these distances are mostly travelled during the spring when individuals migrate from winter hibernacula to breeding ponds and it is mostly juveniles which travel between ponds (Kovar *et al.,* 2009). This hypothesis of anuran transmission of *Bsal* between sites relies upon the persistence of the fungus over winter in hibernacula and on the skin of vector or host species. Although a direct study testing overwintering amphibians for the presence of *Bsal* has yet to be conducted, *Bsal* has been shown to thrive at low temperatures on terrestrial species (*S. salamandra*; Martel *et al.,* 2013) and therefore hypothesised by experts to be capable of spreading through overwintering and subsequent migration (EFSA Panel on Animal Health and Welfare, 2018). However, anurans are known to have lower infection loads compared to urodelans and therefore validation that *Bsal* can indeed persist on overwintering anurans is needed.

Despite the capacity for a higher infection load (EFSA Panel on Animal Health and Welfare, 2018), *L. vulgaris* is unlikely to be acting as an effective vector for *Bsal* as it generally travels much shorter distances than the

anuran species detected here (Kovar *et al.,* 2009). However, *L. vulgaris* is widespread throughout the study area, making it a potential reservoir for *Bsal* once introduced into the immediate environment. The little research done on *L. vulgaris* and *Bsal* suggests that the species is not as prone to developing lethally high loads of the fungus as *T. cristatus* and is more likely to fully recover (Bates *et al.,* 2019). As mentioned previously, the two species are highly associated, sharing certain prey species and hibernacula (Roşca *et al.,* 2013; Dervo *et al.,* 2018) and *Bsal* is likely to spread between them despite the difference in their aquatic spatial niches (Dolmen and Koksvik, 1983; Covaciu-Marcov *et al.,* 2010) as spores are released into the environment (Stegen *et al.,* 2017). Although unclear whether *L. vulgaris* is acting as a significant *Bsal* vector, this species could still be a key component of *Bsal* transmission and persistence in the Netherlands, acting in much the same way as the *Triturus anatolicus* population is in the newly discovered *Bsal* outbreak in Spain (Martel *et al.,* 2020).

In order to determine to what extent anurans are acting as vectors for *Bsal*, quantifying the *Bsal* load present on each species while overwintering in hibernacula would provide valuable insight into which species has the highest capacity for transmission. This can be conducted using either an experimental or observational approach, with the former being logistically easier but lacking the ecosystem and environmental dynamics present in the latter. For an observational study, potential hibernacula around sites can be searched or perhaps more sophisticated options such as using tagging and harmonic direction finding (Rowley and Alford, 2007) would aid in the discovery of hibernacula. These can then be surveyed at the beginning and end of winter with swab samples taken to identify *Bsal* and quantify load using qPCR. Not only would the composition of species sharing hibernacula in this area be determined, but the ability of *Bsal* to persist outside of water over a long time period and on different wild hosts would be quantified.

An experimental study directly involving the amphibian species observed in contact with *Bsal* at sites in the Netherlands would shed light on whether and how they transmit *Bsal*. To date, experimental studies have focused on highly susceptible species, such as *S. salamandra* (Martel *et al.,* 2013; Blooi *et al.,* 2015; Sabino-Pinto *et al.,* 2015; Smith *et al.,* 2018), but further research on tolerant and resistant species is needed to gain greater understanding of how they contribute to *Bsal* transmission (Stegen *et al.,* 2017). Current *Bsal*

experimental studies are limited in other ways, for example, with the exception of a handful of papers, only one isolate from the original *Bsal* outbreak has currently been used in experimental studies (Kelly *et al.,* 2021). Experimental studies have been key in broadening understanding of the mechanics of *Bd* transmission and persistence in the wild (e.g. Blaustein *et al.,* 2005; McMahon *et al.,* 2012; Jani and Briggs, 2014). Further experimental studies on *Bsal*, informed by observation in the field, will be essential in devising effective plans for mitigation (Gilbert *et al.,* 2020) and potentially eradication from wild sites (Blooi *et al.,* 2015).

4.5 Long-Distance *Bsal* Vectors

Although the contribution of amphibian-amphibian transmission cannot be discounted, barriers to amphibian movement, such as roads and buildings, are very common in the study area making long-distance travel less likely. Transport across these longer distances could be facilitated by human activities or via waterbirds with the *Bsal* fungus attached to their legs (Stegen *et al.,* 2017). However, it is difficult to quantify the effect humans have on transmission and the data within this study was insufficient to determine whether individual waterbirds are travelling between sites. White storks and grey herons are present at a number of sites, including at least two *Bsal* positive sites for each species (Table 2), and it is feasible, due to their relatively large foraging radius, that individuals visited multiple sites. Mallards are widespread across sites although it is difficult to determine whether individuals are visiting multiple sites as foraging radius is largely dependent upon resource availability (Schoener, 1983; Bengtsson *et al.,* 2014). Tracking technologies could be used to determine the movement patterns of waterbirds in the area and determine whether they are acting as vectors of transmission but this is resource intensive and costly. Advancing eDNA technologies have made quantifying population genetics and potentially identifying individuals possible from a water sample but there are still many challenges to be overcome before this method becomes widespread and could be utilised in this capacity (Adams *et al.,* 2019; Tsuji *et al.,* 2020).

The majority of the sites sampled in this study are on private land making it likely that humans come into contact with the water sources yet difficult to quantify the extent to which this occurs. The impact of interactions with the sites by RAVON when sampling or visiting was minimised by thorough decontamination of the equipment using protocols deemed to be sufficient to prevent spread (Thomas *et*

al., 2019; RAVON, 2020). Questionnaires could be issued to the private owners to attempt to attain an idea of how each site is used and the likelihood of spread via spores attaching to human clothing or equipment (EFSA Panel on Animal Health and Welfare, 2018). However, the full extent of interaction is unlikely to be reflected in the results of these questionnaires as they are either forgotten or unknown to the owner.

Conclusion

To summarise, eDNA is a suitable method for detecting amphibians in the Netherlands and the full range of amphibian species expected in the study area were detected using metabarcoding primers during this study. The results of the comparison between the two metabarcoding primer sets used in this study demonstrates that the *12S-V5* vertebrate primer set is suitable for amphibian eDNA studies in Europe but the 16S amphibian primer set is not and therefore is not recommended for use. However further research comparing the *12S-V5* primers to the *batra* 12S amphibian primers is required to ensure fully informed decisions on metabarcoding primer selection can be made for future studies. Despite the success of the *12S-V5* metabarcoding primers in detecting a large quantity of amphibian eDNA, the single-species approach was found to be more effective in detecting both *T. cristatus* and *P. fuscus* when the results were compared. However, metabarcoding approaches are still valued for their ability to produce data on communities of species. Efforts to increase the quantity of DNA from rare species reaching the sequencing stage may therefore be necessary to increase the performance of metabarcoding primers in future studies which include rare species of interest.

No significant relationship was discovered between *Bsal* presence and amphibian community composition. The dbRDA plot determined that *Bsal* was significant as an explanatory variable for amphibian community composition but not as influential as other factors which likely include suitable habitat and microhabitat availability. However further data is needed to confirm this conclusion due to the small number of *Bsal* positive sites in this study. *Lissotriton vulgaris* and *Pelophylax spp.* have been identified as species which may potentially increase in number to fill available niches in the event that *T. cristatus* populations experience decline. The former could be acting as a reservoir for *Bsal* and exacerbating its effect on *T. cristatus* populations but validation with further studies is needed. Anuran species which travel long distances from hibernacula to spring breeding ponds could be acting as vectors of *Bsal* (*B. bufo, R. temporaria*) and may have led to its spread to eight further sites from the two original sites. Humans and waterbirds may also play a role in the spread of *Bsal* to sites of new contamination which are widely spread and contain barriers to amphibian movement.

Further studies of this nature alongside experimental studies to validate these hypotheses are essential in understanding the transmission of *Bsal* and its impact on the amphibian species exposed to it. Future studies with a larger spatial scale, potentially including sites of infection in Germany, Belgium and Spain, and a larger temporal scale, will aid in capturing the impact of *Bsal* on wild amphibian communities. Once the transmission of *Bsal* is understood, new sites of contamination could be predicted and measures can be taken to slow the spread and prevent further mass mortality events.

Acknowledgements

I would like to first thank my supervisors, Allan McDevitt, Ilaria Coscia and Naiara Sales for their support and guidance throughout this project. I would also like to thank the members of the McDevitt lab for all their advice and help with the laboratory work, bioinformatics and statistical analyses. I would especially like to thank Matthew O'Donnell and Andhika Prasetyo for completing the laboratory work for the amphibian 16S primers, Andhika Prasetyo for his help both in the lab and with bioinformatics and Sam Browett for his extensive bioinformatics expertise.

I would like to thank my collaborator RAVON, with special mention to Jelger Herder, Annemarieke Spitzen-van der Sluijs and Jesper Berndsen, for allowing access to their samples and data for this project. I would also like to thank SPYGEN for coordinating the sample delivery.

Finally, I would like to thank Nick Pruijn for his constant support and encouragement throughout this process. I am spurred on to ever greater things by his belief in me.

References

Adams, C. I. M., Knapp, M., Gemmell, N. J., Jeunen, G-J., Bunce, M., Lamare, M. D. and Taylor, H. R. 2019. Beyond Biodiversity: Can Environmental DNA (eDNA) Cut It as a Population Genetics Tool? *Genes.* **10**, 192.

Alberdi, A., Aizpurua, O., Gilbert, M. T. P. and Bohmann, K. 2018. Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*. **9**(1), 134-147.

Andrews, S. 2015. *FASTQC A Quality Control tool for High Throughput Sequence Data*. [Accessed: 30/09/2021]. Available from: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Baláž, V., Solský, M., González, D. L., Havlíková, B., Zamorano, J. G., Sevilleja, C. G., Torrent, L. and Vojar, J. 2018. First survey of the pathogenic fungus *Batrachochytrium salamandrivorans* in wild and captive amphibians in the Czech Republic. *Salamandra.* **54**(1), 87-91.

Bales, E. K., Hyman, O. J., Loudon, A. H., Harris, R. N., Lipps, G., Chapman, E., Roblee, K., Kleopfer, J. D. and Terrell, K. A. 2015. Pathogenic Chytrid Fungus *Batrachochytrium dendrobatidis*, but Not *B. salamandrivorans*, Detected on Eastern Hellbenders. *PLOS ONE.* **10**(2), e0116405.

Bálint, M., Nowak, C., Márton, O., Pauls, S. U., Wittwer, C., Aramayo, J. L., Schulze, A., Chambert, T., Cocchiararo, B. and Jansen, M. 2018. Accuracy, limitations and cost efficiency of eDNA-based community survey in tropical frogs. *Molecular Ecology Resources*. **18**(6), 1415-1426.

Barnes, M. A. and Turner, C. R. 2016. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*. **17**(1), 1-17.

Barnhart, K., Bletz, M. C., LaBumbard, B., Tokash-Peters, A., Gabor, C. R. and Woodhams, D. C. 2020. *Batrachochytrium salamandrivorans* elicits acute stress response in spotted salamanders but not infection or mortality. *Animal Conservation*. **23**(5), 533-546.

Basanta, M. D., Rebollar, E. A. and Parra-Olea, G. 2019. Potential risk of *Batrachochytrium salamandrivorans* in Mexico. *PLOS ONE.* **14**(2), e0211960.

Bates, K. A., Shelton, J. M., Mercier, V. L., Hopkins, K. P., Harrison, X. A., Petrovan, S. O. and Fisher, M. C. 2019. Captivity and infection by the fungal pathogen *Batrachochytrium salamandrivorans* perturb the amphibian skin microbiome. *Frontiers in Microbiology*. 10, 1834.

Becker, C. G., Rodriguez, D., Toledo, L. F., Longo, A. V., Lambertini, C., Corrêa, D. T., Leite, D. S., Haddad, C. F. and Zamudio, K. R. 2014. Partitioning the net effect of host diversity on an emerging amphibian pathogen. *Proceedings of the Royal Society B: Biological Sciences*. **281**(1795), 20141796.

Beentjes, K. K., Speksnijder, A. G., Schilthuizen, M., Hoogeveen, M. and van der Hoorn, B. B. 2019. The effects of spatial and temporal replicate sampling on eDNA metabarcoding. *PeerJ*. **7**, p.e7335.

Beng, K. C. and Corlett, R. T. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*. **29**, 2089-2121.

Bengtsson, D., Avril, A., Gunnarsson, G., Elmberg, J., Söderquist, P., Norevik, G., Tolf, C., Safi, K., Fiedler, W., Wikelski, M., Olsen, B. and Waldenström, J. 2014. Movements, Home-Range Size and Habitat Selection of Mallards during Autumn Migration. *PLOS ONE.* **9**(6), e100764.

Beninde, J., Keltsch, F., Veith, M., Hochkirch, A. and Wagner, N. 2021. Connectivity of Alpine newt populations (*Ichthyosaura alpestris*) exacerbates the risk of *Batrachochytrium salamandrivorans* outbreaks in European fire salamanders (*Salamandra salamandra*). *Conservation Genetics*. **22**(4), 653-659.

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyatt, A. D., McDonald, K. R. and Hines, H. B. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences*. **95**(15), 9031-9036.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R. A., Foster, J., Wilkinson, J. W., Arnell, A., Brotherton, P., Williams, P. and Dunn, F. 2015. Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*. **183**, 19-28.

Blaustein, A. R., Romansic, J. M., Scheessele, E. A., Han, B. A., Pessier, A. P. and Longcore, J. E. 2005. Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conservation Biology*. **19**(5), 1460-1468.

Blaustein, A. R., Urbina, J., Snyder, P. W., Reynolds, E., Dang, T., Hoverman, J. T., Han, B., Olson, D. H., Searle, C. and Hambalek, N. M. 2018. Effects of emerging infectious diseases on amphibians: a review of experimental studies. *Diversity*. **10**(3), 81.

Blooi, M., Martel, A., Haesebrouck, F., Vercammen, F., Bonte, D. and Pasmans, F. 2015. Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. *Scientific Reports*. **5**(1), 1-4.

Boussarie, G., Bakker, J., Wangensteen, O. S., Mariani, S., Bonnin, L., Juhel, J. B., Kiszka, J. J., Kulbicki, M., Manel, S., Robbins, W. D. and Vigliola, L, 2018. Environmental DNA illuminates the dark diversity of sharks. *Science Advances*. **4**(5), eaap9661.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., and Coissac, E. 2016. Obitools: aunix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*. **16**(1), 176-182.

Brandon-Mong, G. J., Gan, H. M., Sing, K. W., Lee, P. S., Lim, P. E. and Wilson, J. J. 2015. DNA metabarcoding of insects and allies: an evaluation of primers and pipelines. *Bulletin of Entomological Research.* **105**(6), 717-727.

Brannelly, L. A., McCallum, H. I., Grogan, L. F., Briggs, C. J., Ribas, M. P., Hollanders, M., Sasso, T., Familiar López, M., Newell, D. A. and Kilpatrick, A. M. 2021. Mechanisms underlying host persistence following amphibian disease emergence determine appropriate management strategies. *Ecology Letters*. **24**(1), 130-148.

Brannock, P. M. and Halanych, K. M. 2015. Meiofaunal community analysis by high-throughput sequencing: Comparison of extraction, quality filtering, and clustering methods. *Marine Genomics*. **23**, 67-75.

Broadhurst, H. A., Gregory, L. M., Bleakley, E. K., Perkins, J. C., Lavin, J. V., Bolton, P., Browett, S. S., Howe, C. V., Singleton, N., Tansley, D. and Sales, N.G. 2021. Mapping differences in mammalian distributions and diversity using environmental DNA from rivers. *Science of the Total Environment*. **801**, 149724.

Bronnenhuber, J. E. and Wilson, C. C. 2013. Combining species-specific COI primers with environmental DNA analysis for targeted detection of rare freshwater species. *Conservation Genetics Resources*. **5**(4), 971-975.

Brys, R., Haegeman, A., Halfmaerten, D., Neyrinck, S., Staelens, A., Auwerx, J. and Ruttink, T. 2020. Monitoring of spatiotemporal occupancy patterns of fish and amphibian species in a lentic aquatic system using environmental DNA. *Molecular Ecology*. **30**(13), 3097-3110.

Bylemans, J., Gleeson, D. M., Duncan, R. P., Hardy, C. M. and Furlan, E. M. 2019. A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environmental DNA.* **1**(4), 402-414.

Carter, E. D., Miller, D. L., Peterson, A. C., Sutton, W. B., Cusaac, J. P. W., Spatz, J. A., Rollins-Smith, L., Reinert, L., Bohanon, M., Williams, L. A., Upchurch, A. and Gray, M. J. 2019. Conservation risk of *Batrachochytrium salamandrivorans* to endemic lungless salamanders. *Conservation Letters.* **13**(1), e12675.

Carvalho, C. S., De Oliveira, M. E., Rodriguez-Castro, K. G., Saranholi, B. H. and Galetti Jr, P. M. 2021. Efficiency of eDNA and iDNA in assessing vertebrate diversity and its abundance. *Molecular Ecology Resources* doi: 10.1111/1755-0998.13543.

Charvoz, L., Apothéloz-Perret-Gentil, L., Reo, E., Thiébaud, J. and Pawlowski, J. 2021. Monitoring newt communities in urban area using eDNA metabarcoding. *PeerJ.* **9**, p.e12357.

Chatfield, M. W. H., Moler, P., Richards-Zawacki, C. L. 2012. The Amphibian Chytrid Fungus, *Batrachochytrium dendrobatidis*, in Fully Aquatic Salamanders from Southeastern North America. *PLOS ONE*. **7**(9), e44821.

Civitello, D. J., Cohen, J., Fatima, H., Halstead, N. T., Liriano, J., McMahon, T. A., Ortega, C. N., Sauer, E. L., Sehgal, T., Young, S. and Rohr, J. R. 2015. Biodiversity inhibits parasites: broad evidence for the dilution effect. *Proceedings of the National Academy of Sciences*. **112**(28), 8667-8671.

Coissac, E., Riaz, T. and Puillandre, N. 2012. Bioinformatic challenges for DNA metabarcoding of plants and animals. *Molecular Ecology*. **21**(8), 1834-1847.

Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. [Online]. [Accessed: 21/03/2022]. Available from: https://eur-lex.europa.eu/

Coutant, O., Richard-Hansen, C., de Thoisy, B., Decotte, J. B., Valentini, A., Dejean, T., Vigouroux, R., Murienne, J. and Brosse, S. 2021. Amazonian mammal monitoring using aquatic environmental DNA. *Molecular Ecology Resources*. **21**(6), 1875-1888.

Covaciu-Marcov, S. D., Cicort-Lucaciu, A. Ş., Mitrea, I., Sas, I., Căus, A. V. and Cupşa, D. 2010. Feeding of three syntopic newt species (*Triturus cristatus*, *Mesotriton alpestris* and *Lissotriton vulgaris*) from Western Romania. *North-Western Journal of Zoology*. **6**(1), 95-108.

Dalbeck, L., Düssel-Siebert, H., Kerres, A., Kirst, K., Koch, A., Lötters, S., Ohlhoff, D., Sabino-Pinto, J., Preißler, K., Schulte, U., Schulz, V., Steinfartz, S., Veith, M., Vences, M., Wagner, N. and Wegge, J. 2018. Die Salamanderpest und ihr Erreger *Batrachochytrium salamandrivorans* (*Bsal*): aktueller Stand in Deutschland. *Zeitschrift für Feldherpetologie*. **25**, 1-22.

Davy, C. M., Kidd, A. G. and Wilson, C. C. 2015. Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. *PLOS ONE*. **10**(7), e0130965.

Deiner, K., Walser, J. C., Mächler, E. and Altermatt, F. 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation*. **183**, 53-63.

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N. and Pfrender, M. E. 2017. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*. **26**(21), 5872-5895.

Dervo, B. K., Museth, J. and Skurdal, J. 2018. Assessing the Use of Artificial Hibernacula by the Great Crested Newt (*Triturus cristatus*) and Smooth Newt (*Lissotriton vulgaris*) in Cold Climate in Southeast Norway. *Diversity*. **10**(3), 56.

Dickie, I. A., Boyer, S., Buckley, H. L., Duncan, R. P., Gardner, P. P., Hogg, I. D., Holdaway, R. J., Lear, G., Makiola, A., Morales, S. E. and Powell, J. R. 2018. Towards robust and repeatable sampling methods in eDNA-based studies. *Molecular Ecology Resources*. **18**(5), 940-952.

Doble, C. J., Hipperson, H., Salzberg, W., Horsburgh, G. J., Mwita, C., Murrell, D. J. and Day, J. J. 2019. Testing the performance of environmental DNA metabarcoding for surveying highly diverse tropical fish communities: A case study from Lake Tanganyika. *Environmental DNA*. **2**(1), 24-41.

Doherty-Bone, T. M., Gonwouo, N. L., Hirschfeld, M., Ohst, T., Weldon, C., Perkins, M., Kouete, M. T., Browne, R. K., Loader, S. P., Gower, D. J. and Wilkinson, M. W. 2013. *Batrachochytrium dendrobatidis* in amphibians of Cameroon, including first records for caecilians. *Diseases of Aquatic Organisms*. **102**(3), 187-194.

Dolmen, D. and Koksvik, J. I. 1983. Food and feeding habits of *Triturus vulgaris* (L.) and *T. cristatus* (Laurenti)(Amphibia) in two bog tarns in central Norway. *Amphibia-Reptilia*. **4**(1), 17-24.

Dufresnes, C., Déjean, T., Zumbach, S., Schmidt, B. R., Fumagalli, L., Ramseier, P. and Dubey, S. 2019. Early detection and spatial monitoring of an emerging biological invasion by population genetics and environmental DNA metabarcoding. *Conservation Science and Practice*. **1**(9), e86.

Dufresnes, C., Golay, J., Schuerch, J., Dejéan, T. and Dubey, S. 2020. Monitoring of the last stronghold of native pool frogs (*Pelophylax lessonae*) in Western Europe, with implications for their conservation. *European Journal of Wildlife Research*. **66**, 1-10.

Dully, V., Wilding, T. A., Mühlhaus, T. and Stoeck, T. 2021. Identifying the minimum amplicon sequence depth to adequately predict classes in eDNA-based marine biomonitoring using supervised machine learning. *Computational and Structural Biotechnology Journal.* **19**, 2256-2268.

Edgar, R. .C, Haas, B. .J, Clemente, J. C., Quince, C. and Knight, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*. **27**(16), 2194–2200.

EFSA Panel on Animal Health and Welfare, More, S., Miranda, M. A., Bicout, D., Bøtner, A., Butterworth, A., Calistri, P., Depner, K., Edwards, S., Garin-Bastuji, B., Good, M., Michel, V., Raj, M., Nielsen, S. S., Sihvonen, L., Spoolder, H., Stegeman, J. A., Thulke, H-H., Velarde, A., Willeberg, P., Winckler, C., Baláž, V., Martel, A., Murray, K., Fabris, C., Munoz-Gajardo, I., Gogin, A., Verdonck, F. and Schmidt, C. G. 2018. Risk of survival, establishment and spread of *Batrachochytrium salamandrivorans* (*Bsal*) in the EU. *EFSA Journal*. **16**(4), e05259.

Egeter, B., Roe, C., Peixoto, S., Puppo, P., Easton, L. J., Pinto, J., Bishop, P. J. and Robertson, B. C. 2019. Using molecular diet analysis to inform invasive species management: A case study of introduced rats consuming endemic New Zealand frogs. *Ecology and Evolution*. **9**(9), 5032-5048.

Evans, N. T., Olds, B. P., Renshaw, M. A., Turner, C. R., Li, Y., Jerde, C. L., Mahon, A. R., Pfrender, M. E., Lamberti, G. A. and Lodge, D. M. 2016. Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Molecular Ecology Resources*. **16**(1), 29-41.

Fediajevaite, J., Priestley, V., Arnold, R. and Savolainen, V. 2021. Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. *Ecology and Evolution*. **11**(9), 4803-4815.

Feldmeier, S., Schefczyk, L., Wagner, N., Heinemann, G., Veith, M and Lötters, S. 2016. Exploring the Distribution of the Spreading Lethal Salamander Chytrid Fungus in Its Invasive Range in Europe – A Macroecological Approach. *PLOS ONE.* **11**(10), e0165682.

Ficetola, G. F., Miaud, C., Pompanon, F. and Taberlet, P. 2008. Species detection using environmental DNA from water samples. *Biology Letters*. **4**(4), 423-425.

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., Pompanon, F. and Rayé, G. 2015. Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*. **15**(3), 543-556.

Ficetola, G. F., Taberlet, P. and Coissac, E. 2016. How to limit false positives in environmental DNA and metabarcoding? *Molecular Ecology Resources*. **16**(3), 604-607.

Fisher, M. C. and Garner, T. W. 2007. The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews*. **21**(1), 2-9.

Fitzpatrick, L. D., Pasmans, F., Martel, A and Cunningham, A. A. 2018. Epidemiological tracing of *Batrachochytrium salamandrivorans* identifies widespread infection and associated mortalities in private amphibian collections. *Scientific Reports*. **8**, 13845.

Garlapati, D., Charankumar, B., Ramu, K., Madeswaran, P. and Murthy, M. R. 2019. A review on the applications and recent advances in environmental DNA (eDNA) metagenomics. *Reviews in Environmental Science and Bio/Technology*. **18**(3), 389-411.

van Gelder, J. J., Aarts, H. M. J. and Staal, H-J. W. M. 1986. Routes and speed of migrating toads (*Bufo bufo* L) a telemetric study. *The Herpetological Journal*. **1**(3), 111-114.

Gilbert, M. J., A. M. Spitzen-van der Sluijs, S. Canessa, J. Bosch, A. Cunningham, E. Grasselli, A. Laudelout, S. Lötters, C. Miaud, S. Salvidio, M. Veith, A. Martel & F. Pasmans. 2020. Mitigating *Batrachochytrium salamandrivorans* in Europe: *Batrachochytrium salamandrivorans* emergency action plan for European urodeles. [Online]. Nijmegen: European Commission.[Accessed: 10 December 2021]. Available from: http://*Bsal*europe.com/

Gimeno, A., Meikl, M., Pitt, A., Winkler, M. and Berninger, U. G. 2015. Testing of Fire Salamanders around Salzburg for *Batrachochytrium salamandrivorans* within a school project. *Journal on Protected Mountain Areas Research and Management*. **7**, 72-76.

Goldberg, C. S., Strickler, K. M. and Pilliod, D. S. 2015. Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation*. **183**, 1-3.

Grasselli, E., Bianchi, G., Dondero, L., Marchianò, V., Carafa, M., Perrone, M. and Salvidio, S. 2019. First screening for *Batrachochytrium salamandrivorans* (*Bsal*) in wild and captive salamanders from Italy. *Salamandra*. **55**(2), 124-126.

Grey, E. K., Bernatchez, L., Cassey, P., Deiner, K., Deveney, M., Howland, K. L., Lacoursière-Roussel, A., Leong, S. C. Y., Li, Y., Olds, B. and Pfrender, M. E., 2018. Effects of sampling effort on biodiversity patterns estimated from environmental DNA metabarcoding surveys. *Scientific Reports*. **8**(1), 1-10.

Halliday, T. R. 2008. Why amphibians are important. *International Zoo Yearbook*. **42**(1), 7-14.

Halliday, F. W., Rohr, J. R. and Laine, A-L. 2020. Biodiversity loss underlies the dilution effect of biodiversity. *Ecology Letters*. **23**(11), 1611-1622.

Halsey, S. 2019. Defuse the dilution effect debate. *Nature Ecology & Evolution*. **3**(2), 145-146.

Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., Blackman, R. C., Oliver, A. and Winfield, I. J. 2016. Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology*. **25**(13), 3101-3119.

Harper, L. R., Handley, L. L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., Lewis, E., Adams, I. P., Brotherton, P., Phillips, S. and Hänfling, B. 2018. Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution*. **8**(12), 6330-6341.

Harper, L. R., Handley, L. L., Carpenter, A. I., Ghazali, M., Di Muri, C., Macgregor, C. J., Logan, T. W., Law, A., Breithaupt, T., Read, D. S. and McDevitt, A. D. 2019. Environmental DNA (eDNA) metabarcoding of pond water as a tool to survey conservation and management priority mammals. *Biological Conservation*. **238**, 108225.

Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Lewis, E., Adams, I. P., Brotherton, P., Phillips, S. and Hänfling, B. 2020a. Generating and testing ecological hypotheses at the pondscape with environmental DNA metabarcoding: A case study on a threatened amphibian. *Environmental DNA*. **2**(2), 184-199.

Harper, L. R., Watson, H. V., Donnelly, R., Hampshire, R., Sayer, C. D., Breithaupt, T. and Hänfling, B. 2020b. Using DNA metabarcoding to investigate diet and niche partitioning in the native European otter (*Lutra lutra*) and invasive American mink (*Neovison vison*). *Metabarcoding and Metagenomics*. **4**, 113-133.

Hebert, P. D. and Gregory, T. R. 2005. The promise of DNA barcoding for taxonomy. *Systematic Biology*. **54**(5), 852-859.

Hill, A. J., Hardman, R. H., Sutton, W. B., Grisnik, M. S., Gunderson, J. H. and Walker, D. M. 2021. Absence of *Batrachochytrium salamandrivorans* in a Global Hotspot for Salamander Biodiversity. *Journal of Wildlife Diseases.* **57**(3), 553-560.

Hirzel, A. H. and Le Lay, G. 2008. Habitat suitability modelling and niche theory. *Journal of Applied Ecology.* **45**(5), 1372-1381.

Hocking, D. J. and Babbitt, K. J. 2013. Amphibian contributions to ecosystem services. *Herpetological Conservation and Biology*. **9**(1), 1-17.

Holenweg Peter A-K., Reyer H-U., Tjetje G. A. 2001. Homing behavior of *Rana lessonae*, *Rana ridibunda*, and their hybridogenetic associate *Rana esculenta* after experimental displacement. *Amphibia-Reptilia*. **22**, 475–480

Holsbeek, G. and Jooris, R. 2010. Potential impact of genome exclusion by alien species in the hybridogenetic water frogs (*Pelophylax esculentus* complex). *Biological Invasions*. **12**(1), 1-13.

Holt, R. D. and Pickering, J. 1985. Infectious disease and species coexistence: a model of Lotka-Volterra form. *The American Naturalist*. **126**(2), 196-211.

Huang, Z. Y. X., Van Langevelde, F., Estrada-Peña, A., Suzán, G. and De Boer, W.F. 2016. The diversity–disease relationship: evidence for and criticisms of the dilution effect. *Parasitology*. **143**(9), 1075-1086.

Illumina. 2022. *Cluster Generation and Sequencing Reagents*. [Online]. [Accessed: 10/03/2022]. Available from: https://www.illumina.com/

IUCN. 2022. *Summary Statistics*. [Online]. [Accessed: 26/05/2021]. Available from: https://www.iucnredlist.org/

Jani, A. J. and Briggs, C. J. 2014. The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proceedings of the National Academy of Sciences*. **111**(47), E5049-E5058.

Jerde, C. L., Mahon, A. R., Chadderton, W. L. and Lodge, D. M. 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conservation Letters*. **4**(2), 150-157.

Johnson, M. L. and Speare, R. 2003. Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. *Emerging Infectious Diseases*. **9**(8), 922.

Johnson, M. L. and Speare, R. 2005. Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Diseases of Aquatic Organisms*. **65**(3), 181-186.

Kelly, M., Pasmans, F., Muñoz, J. F., Shea, T. P., Carranza, S., Cuomo, C. A. and Martel, A. 2021. Diversity, multifaceted evolution, and facultative saprotrophism in the European *Batrachochytrium salamandrivorans* epidemic. *Nature Communications*. **12**, 6688.

Kilburn, V. L., Ibanez, R. and Green, D. M. 2011. Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama. *Diseases of Aquatic Organisms.* **97**, 127-134.

Klocke, B., Becker, M., Lewis, J., Fleischer, R. C., Muletz-Wolz, C. R., Rockwood, L., Aguirre, A. A. and Gratwicke, B. 2017. *Batrachochytrium salamandrivorans* not detected in U.S. survey of pet salamanders. *Scientific Reports.* **7**, 13132.

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M. and Piaggio, A. J. 2020. Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*. **2**(3), 271-282.

Kocher, A., de Thoisy, B., Catzeflis, F., Huguin, M., Valière, S., Zinger, L., Bañuls, A. L. and Murienne, J. 2017a. Evaluation of short mitochondrial metabarcodes for the identification of Amazonian mammals. *Methods in Ecology and Evolution*. **8**(10), 1276-1283.

Kocher, A., de Thoisy, B., Catzeflis, F., Valière, S., Bañuls, A-L. and Murienne, J. 2017b. iDNA screening: Disease vectors as vertebrate samplers. *Molecular Ecology*. **26**(22), 6478-6486.

Konowalik, A., Najbar, A., Konowalik, K., Dylewski, Ł., Frydlewicz, M., Kisiel, P., Starzecka, A., Zaleśna, A. and Kolenda, K. 2020. Amphibians in an urban environment: a case study from a central European city (Wrocław, Poland). *Urban Ecosystems*. **23**(2), 235-243.

Kovar, R., Brabec, M., Bocek, R. and Vita, R. 2009. Spring migration distances of some Central European amphibian species. *Amphibia-Reptilia.* **30**, 367-378.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L. and Ståhlberg, A. 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine*. **27**(2-3), 95-125.

Kumari, P., Dong, K., Eo, K. Y., Lee, W-S., Kimura, J. and Yamamoto, N. 2019. DNA metabarcoding-based diet survey for the Eurasian otter (*Lutra lutra*): Development of a Eurasian otter-specific blocking oligonucleotide for 12S rRNA gene sequencing for vertebrates. *PLOS ONE*. **14**(12), e0226253.

Lacoursière-Roussel, A., Côté, G., Leclerc, V. and Bernatchez, L. 2016. Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. *Journal of Applied Ecology* **53**(4), 1148-1157.

Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A. and Nguyen, T. T. 2017. *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports*. **7**, 44443.

Lastra González, D., Baláž, V., Solský, M., Thumsová, B., Kolenda, K., Najbar, A., Najbar, B., Kautman, M., Chajma, P., Balogová, M. and Vojar, J. 2019. Recent findings of potentially lethal salamander fungus *Batrachochytrium salamandrivorans*. *Emerging Infectious Diseases*. **25**(7), 1416.

Li, W., Hou, X., Xu, C., Qin, M., Wang, S., Wei, L., Wang, Y., Liu, X. and Li, Y. 2021a. Validating eDNA measurements of the richness and abundance of anurans at a large scale. *Journal of Animal Ecology*. **90**(6), 1466-1479.

Li, W., Song, T., Hou, X., Qin, M., Xu, C. and Li, Y. 2021b. Application of eDNA Metabarcoding for Detecting Anura on a Tropical Island. *Diversity*. **13**(9), 440.

Liew, N., Moya, M. J. M., Wierzbicki, C. J., Hollinshead, M., Dillon, M. J., Thornton, C. R., Ellinson, A., Cable, J., Fisher, M. C. and Mostowy, S. 2017. Chytrid fungus infection in zebrafish demonstrates that the pathogen can parasitize non-amphibian vertebrate hosts. *Nature Communications*. **8**, 15048.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. and Collins, J. P. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences*. **103**(9), 3165-3170.

Longcore, J. E., Pessier, A. P. and Nichols, D. K. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*. **91**(2), 219-227.

Lopes, C. M., Sasso, T., Valentini, A., Dejean, T., Martins, M., Zamudio, K. R. and Haddad, C. F. 2017. eDNA metabarcoding: a promising method for anuran surveys in highly diverse tropical forests. *Molecular Ecology Resources*. **17**(5), 904-914.

Lopes, C. M., Baêta, D., Valentini, A., Lyra, M. L., Sabbag, A. F., Gasparini, J.L., Dejean, T., Haddad, C. F. B. and Zamudio, K. R. 2021. Lost and found: Frogs in a biodiversity hotspot rediscovered with environmental DNA. *Molecular Ecology*. **30**(13), 3289-3298.

Lopes, C. M., Baêta, D., Sasso, T., Vanzetti, A., Raquel Zamudio, K., Taberlet, P. and Haddad, C. F. B. 2021. Power and limitations of environmental DNA metabarcoding for surveying leaf litter eukaryotic communities. *Environmental DNA*. **3**(3), 528-540.

Lötters, S., Wagner, N., Albaladejo, G., Böning, P., Dalbeck, L., Düssel, H., Feldmeier, S., Guschal, M., Kirst, K., Ohlhoff, D., Preissler, K., Reinhardt, T., Schlüpmann, M., Schulte, U., Schulz, V., Steinfartz, S., Twietmeyer, S., Veith, M., Vences, M. and Wegge, J. 2020. The amphibian pathogen *Batrachochytrium* salamandrivorans in the hotspot of its European invasive range: past – present – future. *Salamandra*. **56**(3), 173-188.

Lynggaard, C., Nielsen, M., Santos-Bay, L., Gastauer, M., Oliveira, G. and Bohmann, K. 2019. Vertebrate diversity revealed by metabarcoding of bulk arthropod samples from tropical forests. *Environmental DNA.* **1**(4), 329-341.

Lynggaard, C., Bertelsen, M. F., Jensen, C. V., Johnson, M. S., Frøslev, T. G., Olsen, M. T. and Bohmann, K. 2022. Airborne environmental DNA for terrestrial vertebrate community monitoring. *Current Biology*. **32**, 1-8.

Mahé, F., Rognes, T., Quince, C., De Vargas, C., and Dunthorn, M. 2015. Swarm v2: highly-scalable and high-resolution amplicon clustering. *PeerJ*. **3**, e1420.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. and Pasmans, F. 2013. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*. **110**(38), 15325-15329.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., Farrer, R. A., Schmidt, B. R., Tobler, U., Goka, K., Lips, K. R., Muletz, C., Zamudio, K. R., Bosch, J., Lötters, S., Wombwell, E., Garner, T. W. J., Cunningham, A. A., Spitzen-van der Sluijs, A., Salvidio, S., Ducatelle, R., Nishikawa, K., Nguyen, T. T., Kolby, J. E., Van Bocxlaer, V., Bossuyt, F. and Pasmans, F. 2014. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*. **346**(6209), 630-631.

Martel, A., Vila-Escale, M., Fernández-Giberteau, D., Martinez-Silvestre, A., Canessa, S., Van Praet, S., Pannon, P., Chiers, K., Ferran, A., Kelly, M. and Picart, M., 2020. Integral chain management of wildlife diseases. *Conservation Letters*, 13(2), p.e12707.

McMahon, T. A., Brannelly, L. A., Chatfield, M. W. H., Johnson, P. T. J., Joseph, M. B., McKenzie, V. J., Richards-Zawacki, C. L., Venesky, M. D. and Rohr, J. R. 2013. Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. *Proceedings of the National Academy of Sciences.* **110**, 210-215.

Neice, A. A. and McRae, S. B. 2021. An eDNA diagnostic test to detect a rare, secretive marsh bird. *Global Ecology and Conservation*. **27**, 01529.

Nester, G. M., De Brauwer, M., Koziol, A., West, K. M., DiBattista, J. D., White, N. E., Power, M., Heydenrych, M. J., Harvey, E. and Bunce, M. 2020. Development and evaluation of fish eDNA metabarcoding assays facilitate the detection of cryptic seahorse taxa (family: Syngnathidae). *Environmental DNA*. **2**(4), 614-626.

Nguyen, T. T., Nguyen, T. V., Ziegler, T., Pasmans, F. and Martel, A. 2017. Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. *Amphibia-Reptilia*. **38**(4), 554-556.

Ohst, T, Gräser, Y, Mutschmann, F. and Plötner, J. 2011. Neue erkenntnisse zur gefährdung Europäischer amphibien durch den hautpilz *Batrachochytrium dendrobatidis*. *Zeitschrift für Feldherpetologie*. **18**(1), 1-17.

Ostfeld, R. S. and Keesing, F. 2000. Biodiversity and disease risk: the case of Lyme disease. *Conservation Biology*. **14**(3), 722-728.

Parrott, J. C., Shepack, A., Burkart, D., LaBumbard, B., Scimè, P., Baruch, E. and Catenazzi, A. 2017. Survey of pathogenic chytrid fungi (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) in salamanders from three mountain ranges in Europe and the Americas. *EcoHealth*. **14**(2), 296-302.

Peixoto, S., Chaves, C., Velo-Antón, G., Beja, P. and Egeter, B. 2020. Species detection from aquatic eDNA: Assessing the importance of capture methods. *Environmental DNA.* **3**(2), 435-448.

Pembury Smith, M. Q. R. and Ruxton, G. D. 2020. Effective use of the McNemar test. *Behavioural Ecology and Sociobiology.* **74**, 133.

Pfleger, M. O., Rider, S. J., Johnston, C. E. and Janosik, A. M. 2016. Saving the doomed: Using eDNA to aid in detection of rare sturgeon for conservation (Acipenseridae). *Global Ecology and Conservation*. **8**, 99-107.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S. and Waits, L. P. 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences*. **70**(8), 1123-1130.

Piñol, J., Senar, M. A. and Symondson, W. O. 2019. The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. *Molecular Ecology*. **28**(2), 407-419.

Prahl, J., Wilson, T. P., Giles, D. and Craddock, J. H. 2020. An overview of research regarding reservoirs, vectors and predators of the chytrid fungus *Batrachochytrium dendrobatidis*. *Acta Herpetologica*. **15**(1), 39-45.

RAVON. 2020. *Desinfectie protocol veldwerk*. [Online]. [Accessed:03/03/2022]. Available from: https://www.ravon.nl/Portals/

RAVON. 2021. *Bsal.* [Online]. [Accessed: 09/06/2021]. Available from: https://www.ravon.nl/*Bsal*

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. and Gough, K. C. 2014a. The detection of aquatic animal species using environmental DNA–a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*. **51**(5), 1450-1459.

Rees, H. C., Bishop, K., Middleditch, D. J., Patmore, J. R., Maddison, B. C. and Gough, K. C. 2014b. The application of eDNA for monitoring of the Great Crested Newt in the UK. *Ecology and Evolution*. **4**(21), 4023-4032.

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P. and Coissac, E. 2011. ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*. **39**(21), e145.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*. **4**, e2584.

Roh, C., Villatte, F., Kim, B. G. and Schmid, R. D. 2006. Comparative study of methods for extraction and purification of environmental DNA from soil and sludge samples. *Applied Biochemistry and Biotechnology*. **134**(2), 97-112.

Roşca, I., Gherghel, I., Strugariu, A. and Zamfirescu, Ş. R. 2013. Feeding ecology of two newt species (*Triturus cristatus* and *Lissotriton vulgaris*) during the reproduction season. *Knowledge and Management of Aquatic Ecosystems*. **408**, 5.

Rowley, J. J. L. and Alford, R. A. 2007. Techniques for tracking amphibians: The effects of tag attachment, and harmonic direction finding versus radio telemetry. *Amphibia-Reptilia*. **28**(3), 367-376.

Ruppert, K. M., Kline, R. J. and Rahman, M. S. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*. **17**, e00547.

Sabino-Pinto, J., Bletz, M., Hendrix, R., Perl, R. G. B., Martel, A., Pasmans, F., Lötters, S., Mutschmann, F., Schmeller, D. S., Schmidt, B. R., Veith, M., Wagner, N., Vences, M. and Steinfartz, S. 2015. First detection of the emerging fungal pathogen *Batrachochytrium salamandrivorans* in Germany. *Amphibia-Reptilia.* **36**(4), 411-416.

Sabino-Pinto, J., Veith, M., Vences, M. and Steinfartz, S. 2018. Asymptomatic infection of the fungal pathogen *Batrachochytrium salamandrivorans* in captivity. *Scientific Reports.* **8**, 11767.

Sakata, M. K., Kawata, M. U., Kurabayashi, A., Kurita, T., Nakamura, M., Shirako, T., Kakehashi, R., Nishikawa, K., Hossman, M. Y., Nishijima, T., Kabamoto, J., Miya, M. and Minamoto, T. 2022. Development and evaluation of PCR primers for environmental DNA (eDNA) metabarcoding of Amphibia. *Metabarcoding and Metagenomics*. **6**, p.e76534.

Sales, N. G., Kaizer, M. D. C., Coscia, I., Perkins, J. C., Highlands, A., Boubli, J. P., Magnusson, W. E., Da Silva, M. N. F., Benvenuto, C. and McDevitt, A. D. 2020a. Assessing the potential of environmental DNA metabarcoding for monitoring Neotropical mammals: a case study in the Amazon and Atlantic Forest, Brazil. *Mammal Review*. **50**(3), 221-225.

Sales, N. G., McKenzie, M. B., Drake, J., Harper, L. R., Browett, S. S., Coscia, I., Wangensteen, O. S., Baillie, C., Bryce, E., Dawson, D. A., Ochu, E., Hanfling, B., Lawson Handley, L., Mariani, S., Lambin, X., Sutherland, C. and McDevitt, A.D. 2020b. Fishing for mammals: Landscape-level monitoring of terrestrial and semi-aquatic communities using eDNA from riverine systems. *Journal of Applied Ecology.* **57**(4), 707-716.

Sasso, T., Lopes, C. M., Valentini, A., Dejean, T., Zamudio, K. R., Haddad, C. F. and Martins, M. 2017. Environmental DNA characterization of amphibian communities in the Brazilian Atlantic forest: potential application for conservation of a rich and threatened fauna. *Biological Conservation*. **215**, 225-232.

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Martel, A., Beukema, W., Acevedo, A. A., Burrowes, P. A., Carvalho, T., Catenazzi, A., De la Riva, I., Fisher, M. C., Flechas, S. V., Foster, C. N., Frías-Álvarez, P., Garner, T. W. J., Gratwicke, B., Guayasamin, J. M., Hirschfeld, M., Kolby, J. E., Kosch, T. A., La Marca, E., Lindenmayer, D. B., Lips, K. R., Longo, K. R., Maneyro, R., McDonald, C. A., Mendelson III, J., Palacios-Rodriguez, P., Parra-Olea, G., Richards-Zawaki, C. L., Rödel, M-O., Rovito, S. M., Soto-Azat, C., Toledo, L. F., Voyles, J., Weldon, C., Whitfield, S. M., Wilkinson, M., Zamudio, K. R. and Canessa, S. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*. **363**(6434), 1459-1463.

Schnell, I. B., Bohmann, K. and Gilbert, M. T. P. 2015. Tag jumps illuminated - reducing sequence-to-sample misidentifications in metabarcoding studies. *Molecular Ecology Resources*. **15**(6), 1289-1303.

Schoener, T. W. 1983. Simple Models of Optimal Feeding-Territory Size: A Reconciliation. *The American Naturalist*. **121**(5), 608-629.

Searle, C. L., Gervasi, S. S., Hua, J., Hammond, J. I., Relyea, R. A., Olson, D. H. and Blaustein, A. R. 2011. Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. *Conservation Biology*. **25**(5), 965-974.

Shao, X., Lu, Q., Liu, M., Xiong, M., Bu, H., Wang, D., Liu, S., Zhao, J., Li, S. and Yao, M. 2021. Generalist carnivores can be effective biodiversity samplers of terrestrial vertebrates. *Frontiers in Ecology and the Environment*. **19**(10), 557-563.

Shehzad, W., Riaz, T., Nawaz, M. A., Miquel, C., Poillot, C., Shah, S. A., Pompanon, F., Coissac, E. and Taberlet, P. 2012. Carnivore diet analysis based on next-generation sequencing: application to the leopard cat (*Prionailurus bengalensis*) in Pakistan. *Molecular Ecology*. **21**(8), 1951-1965.

Shirazi, S., Meyer, R. S. and Shapiro, B. 2021. Revisiting the effect of PCR replication and sequencing depth on biodiversity metrics in environmental DNA metabarcoding. *Ecology and Evolution*. **11**(22), 15766-15779.

Smart, A. S., Tingley, R., Weeks, A. R., Van Rooyen, A. R. and McCarthy, M. A. 2015. Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications*. **25**(7), 1944-1952.

Smith, K. G., Lips, K. R. and Chase, J. M. 2009. Selecting for extinction: nonrandom disease-associated extinction homogenizes amphibian biotas. *Ecology Letters*. **12**(10), 1069-1078.

Smith, H. K., Pasmans, F., Dhaenens, M., Deforce, D., Bonte, D., Verheyen, K., Lens, L. and Martel, A. 2018. Skin mucosome activity as an indicator of *Batrachochytrium salamandrivorans* susceptibility in salamanders. *PLOS ONE.* **13**(7), e0199295.

Spear, S. F., Groves, J. D., Williams, L. A. and Waits, L. P. 2015. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. *Biological Conservation*. **183**, 38-45.

Spitzen-van der Sluijs, A., Spikmans, F., Bosman, W., de Zeeuw, M., van der Meij, T., Goverse, E., Kik, M., Pasmans, F. and Martel, A. 2013. Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra)* to the edge of extinction in the Netherlands. *Amphibia-Reptilia*. **34**(2), 233-239.

Spitzen-van der Sluijs, A., Martel, A., Asselberghs, J., Bales, E. K., Beukema, W., Bletz, M. C., Dalbeck, L., Goverse, E., Kerres, A., Kinet, T., Kirst, K., Laudelout, A., da Fonte, L. F. M., Nöllert, A., Ohlhoff, D., Sabino-Pinto, J., Schmidt, B. R., Speybroeck, J., Spikmans, F., Steinfartz, S., Veith, M., Vences, M., Wagner, N., Pasmans, F. and Lötters, S. 2016. Expanding Distribution of Lethal Amphibian Fungus *Batrachochytrium salamandrivorans* in Europe. *Emerging Infectious Diseases*. **22**(7), 1286-1288.

Spitzen-van der Sluijs, A., Stegen, G., Bogaerts, S., Canessa, S., Steinfartz, S., Janssen, N., Bosman, W., Pasmans, F. and Martel, A. 2018. Post-epizootic salamander persistence in a disease-free refugium suggests poor dispersal ability of *Batrachochytrium salamandrivorans*. *Scientific Reports*. **8**, 3800.

Spitzen-van der Sluijs, A., Stark, T., DeJean, T., Verbrugghe, E., Herder, J., Gilbert, M., Janse, J., Martel, A., Pasmans, F. and Valentini, A. 2020. Using environmental DNA for detection of *Batrachochytrium salamandrivorans* in natural water. *Environmental DNA*. **2**(4), 565-571.

Stegen, G., Pasmans, F., Schmidt, B. R., Rouffaer, L. O., Van Praet, S., Schaub, M., Canessa, S., Laudelout, A., Kinet, T., Adriaensen, C., Haesebrouck, F., Bert, W., Bossuyt, F. and Martel, A. 2017. Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. *Nature*. **544**(7650), 353-356.

Strickler, K. M., Fremier, A. K. and Goldberg, C. S. 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*. **183**, 85-92.

Svenningsen, A. K. N., Pertoldi, C. and Bruhn, D. 2022. eDNA Metabarcoding Benchmarked towards Conventional Survey Methods in Amphibian Monitoring. *Animals*. **12**(6), 763.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. and Kawabata, Z. I. 2012. Estimation of fish biomass using environmental DNA. *PLOS ONE*. **7**(4), e35868.

Thomas, V., Wang, Y., Van Rooij, P., Verbrugghe, E., Baláž, V., Bosch, J., Cunningham, A. A., Fisher, M. C., Garner, T. W., Gilbert, M. J. and Grasselli, E. 2019. Mitigating *Batrachochytrium salamandrivorans* in Europe. *Amphibia-Reptilia*. **40**(3), 265-290.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P. R., Rasmussen, M. and Willerslev, E. 2012a. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLOS ONE*. **7**(8), e41732

Thomsen, P. F., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L. and Willerslev, E. 2012b. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*. **21**(11), 2565-2573.

Tsuji, S., Miya, M., Ushio, M., Sato, H., Minamoto, T. and Yamanaka, H. 2020. Evaluating intraspecific genetic diversity using environmental DNA and denoising approach: A case study using tank water. *Environmental DNA*. **2**, 42-52

Tyler, M. J., Wassersug, R. and Smith, B. 2007. How frogs and humans interact: Influences beyond habitat destruction, epidemics and global warming. *Applied Herpetology*. **4**(1), 1-18.

U.S. Fish and Wildlife Service. 2016. *Listing Salamanders as Injurious Due to Risk of Salamander Chytrid Fungus (January 12, 2016)*. [Online]. [Accessed: 08/06/2021]. Available from: https://www.fws.gov/

Ushio, M., Furukawa, S., Murakami, H., Masuda, R. and Nagano, A. J. 2022. [Pre-print]. An efficient early-pooling protocol for environmental DNA metabarcoding. *bioRxiv*. DOI: https://doi.org/10.1101/2022.02.15.480497

Vági, B., Kovács, T., Băncilă, R., Hartel, T. and Anthony, B. P. 2013. A landscape-level study on the breeding site characteristics of ten amphibian species in Central Europe. *Amphibia-Reptilia*. **34**(1), 63-73.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F. and Gaboriaud, C. 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*. **25**(4), 929-942.

Venesky, M. D., Liu, X., Sauer, E. L. and Rohr, J. R. 2014. Linking manipulative experiments to field data to test the dilution effect. *Journal of Animal Ecology*. **83**(3), 557-565.

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. and Speare, R. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*. **326**(5952), 582-585.

Waarneming.nl. 2021. Bergheggenmus: *Prunella montanella* (Pallas, 1776). [Online]. [Accessed: 25/02/2021]. Available from: https://waarneming.nl/species/

Waddle, J. H., Grear, D. A. and Winzeler, M. E. 2020. *Batrachochytrium salamandrivorans* (*Bsal*) not detected in an intensive survey of wild North American amphibians. *Scientific Reports.* **10**, 13012.

Wagner, N., Lötters, S., Dalbeck, L., Düssel., H., Guschal, M., Kirst, K., Ohlhoff, D., Wegge, J., Reinhardt, T. and Veith, M. 2020. Long-term monitoring of European fire salamander populations (*Salamandra salamandra)* in the Eifel Mountains (Germany): five years of removal sampling of larvae. *Salamandra.* **56**(3), 243-253.

Werner, E. E., Skelly, D. K., Relyea, R. A. and Yurewicz, K. L. 2007. Amphibian species richness across environmental gradients. *Oikos*. **116**(10), 1697-1712.

Whiles, M. R., Lips, K. R., Pringle, C. M., Kilham, S. S., Bixby, R. J., Brenes, R., Connelly, S., Colon-Guad, J. C., Hunte-Brown, M., Huryn, A. D., Montgomery, C. and Peterson, S. 2006. The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. *Frontiers in Ecology and the Environment*. **4**(1), 27-34.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Sepulveda, A. J., Shepard, B. B., Jane, S. F., Whiteley, A. R., Lowe, W. H. and Schwartz, M. K. 2016. Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char *Salvelinus fontinalis*. *Biological Conservation*. **194**, 209-216.

Willerslev, E., Hansen, A. J., Binladen, J., Brand, T. B., Gilbert, M. T. P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D. A. and Cooper, A. 2003. Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science.* **300**(5620), 791-795.

Yap, T. A., Koo, M. S., Ambrose, R. F., Wake, D. B. and Vredenburg, V. T. 2015. Averting a North American biodiversity crisis. *Science.* **349**(6247), 481-482.

Yap, T. A. 2016. *Assessing the threat of two deadly fungal pathogens (Batrachochytrium dendrobatidisand Batrachochytrium salamandrivorans) to amphibian biodiversity and the impacts of human-mediated movement of an invasive carrier species and climate change.* PhD, University of California.

Zhu, W., Xu, F., Bai, C., Liu, X., Wang, S., Gao, X., Yan, S., Li, X., Liu, Z. and Li, Y. 2014. A survey for *Batrachochytrium salamandrivorans* in Chinese amphibians. *Current Zoology*. **60**(1), 729-735.

Appendix

APPENDIX 1 – NUMBER OF READS ASSIGNED TO EACH CLASS FOR THE VERTEBRATE PRIMER AFTER THE POSITIVE CONTROL STAGE IN THE FILTERING PROCESS (TABLE 1).

Class	Reads
Actinopteri	253073
Amphibia	498344
Aves	191519
Mammalia	404665
No Assignment	17734

APPENDIX 2 - NUMBER OF READS ASSIGNED TO EACH PHYLUM FOR THE AMPHIBIAN PRIMER AFTER THE POSITIVE CONTROL STAGE IN THE FILTERING PROCESS (TABLE 1).

Phylum	Reads
Arthropoda	132996
Chordata	8940
Mollusca	25
Platyhelminthes	3123
Porifera	120
Proteobacteria	6
Rotifera	142932
Saccharibacteria	83
Streptophyta	1596
No Assignment	232100

APPENDIX 3 – STUDIES DETAILING THE DETECTION OF AMPHIBIAN GENERA FOUND IN THE NETHERLANDS BY THE *12S-V5* PRIMER.

Genera	Detected by
Bufo	Harper *et al.,* 2020a; Harper *et al.,* 2020b; Peixoto *et al.,* 2020; Svenningsen *et al.,* 2022
Lissotriton	Brys *et al.,* 2020; Harper *et al.,* 2020a; Peixoto *et al.,* 2020; Svenningsen *et al.,* 2022
Pelobates	Peixoto *et al.,* 2020
Pelophylax	Kumari *et al.,* 2019; Harper *et al.,* 2020b
Rana	Kumari *et al.,* 2019; Harper *et al.,* 2020b; Svenningsen *et al.,* 2022
Salamandra	Peixoto *et al.,* 2020
Tritutus	Brys *et al.,* 2020; Harper *et al.,* 2020a; Svenningsen *et al.,* 2022