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Using Environmental DNA to Characterize Amphibian Communities at Sites Infected with *Batrachochytrium salamandrivorans* in the Netherlands

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ABSTRACT.—*Batrachochytrium salamandrivorans* (*Bsal*) fungus has the potential to cause high mortality rates in some European salamanders and newts (urodelans) and is in the process of expanding its invasive range in Europe. Therefore, monitoring its distribution and better understanding both the species threatened and the mechanics of infection are essential in mitigating damage *Bsal* may cause. Environmental DNA has emerged as a promising noninvasive method for detecting both this fungal pathogen and amphibian communities in infected areas. We applied these methods in the province of Gelderland, Netherlands, where the pathogen has previously been detected and is expanding its range, with the goal of characterizing the natural amphibian community present. We sampled 27 bodies of water in the region surrounding the known outbreak sites, determined the presence or absence of *Bsal* using a targeted quantitative polymerase chain reaction assay, and applied an environmental DNA metabarcoding approach to characterize the amphibian communities using two different primer sets. The 12S vertebrate primer set outperformed the 16S amphibian primer set and detected all expected amphibians in the study area: *Bufo bufo*, *Lissotriton vulgaris*, *Pelobates fuscus*, *Pelophylax* spp., *Rana temporaria* and *Triturus cristatus*. *Bsal* was detected at 8 of 27 ponds. A distance-based redundancy analysis found a weak but significant relationship between *Bsal* presence and composition of amphibian communities. This study may provide a basis for future studies on *Bsal* and its relationship with amphibian communities in Europe, highlighting the need for further research into mechanisms of persistence and transmission between bodies of water.

Amphibians are the most threatened vertebrate class with 40.7% of species classified as threatened according to the International Union for Conservation of Nature's Red List of Threatened Species (Luedtke et al., 2023). Among the causes of this decline are deforestation, habitat loss, and climate change, alongside pathogens like ranavirus and the virulent fungal disease chytridiomycosis. *Batrachochytrium dendrobatidis* was, until relatively recently, thought to be the only fungal disease causing chytridiomycosis. In 2010, a dramatic mortality event of Fire Salamanders (*Salamandra salamandra*) started in the Netherlands and left just 0.01% of the Dutch population remaining by 2016 (Spitzen-van der Sluijs et al., 2016). The superficial skin lesions and deep epidermal ulcerations found on these salamanders were caused by a second highly pathogenic chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*; Martel et al., 2013). While most European urodelans have been shown to be susceptible to *Bsal* infection, interspecific differences, such as those in the skin microbiome, have an impact on infection severity and, ultimately, mortality (Bletz et al., 2018; Smith et al., 2018). Environmental factors such as temperature have also been shown to impact the severity of infection (Carter et al., 2021). However, many pathways of transmission in natural settings are still largely unknown (Blaustein et al., 2018).

Since 2010, the fungus has been detected within the Netherlands at four new locations outside of the original infection site in Limburg. Thought to have been introduced from Asia to Europe via the pet trade, *Bsal* has now been found in wild urodelan populations in four European countries: Germany, the Netherlands, Belgium, and Spain. (Martel et al., 2014, 2020; Laking et al., 2017; Fitzpatrick et al., 2018; Castro Monzon et al., 2022). *Bsal* is likely to be more widespread in Europe than is currently known because there is limited surveillance of the disease. Although individuals can be tested reliably using skin swabs and quantitative polymerase chain reaction (qPCR), this assay is only generally done after a decline in a population is recorded or dead individuals are found (Bloom et al., 2013). Current active and passive surveillance techniques are not efficient because *Bsal* has a widespread and 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; Erens et al., 2023). However, a recent paper from Spitzen-van der Sluijs et al. (2020) detailed a new technique utilizing environmental DNA (eDNA) to overcome many of the issues associated with monitoring *Bsal*. 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 analyzed using a variety of new and emerging techniques, broadly categorized into single-species or metabarcoding approaches, to determine the species present

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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., 2012).

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 can therefore be utilized to determine the community of amphibians present in a *Bsal*-infected area (Charvoz et al., 2021; Svenningsen et al., 2022). Recent research has increased evidence supporting the hypothesis that amphibian community composition in the immediate infection area can influence severity and transmission of infection (Stegen et al., 2017; Beninde et al., 2021), with the EFSA Panel on Animal Health and Welfare (2018) suggesting that the most important vectors of transmission within a subpopulation are active and passive amphibian carriers. Several species of anuran have been shown to carry the fungus in the captive pet trade (Nguyen et al., 2017) and in infection trials (Stegen et al., 2017), but currently only *Rana temporaria* has been shown to do so in the wild outside the native range of *Bsal* (EFSA Panel on Animal Health and Welfare 2018; Lötters et al., 2020). More tolerant urodelans can also act as vectors and reservoirs for the fungus, such as Alpine Newts (*Ichthyosaura alpestris*), which could be exacerbating the decline of susceptible local *S. salamandra* populations according to a recent study (Beninde et al., 2021). Therefore, attaining information on the full amphibian community present at sites of *Bsal* infection using eDNA metabarcoding is beneficial for a number of reasons, including identification of both the species at risk and other key species in the area that can either spread or exacerbate *Bsal*. Furthermore, declines in *Bsal*-susceptible species could potentially cause changes to the wider amphibian community as other species fill the empty niche (Hirzel and Le Lay, 2008). This has the potential to either reduce the impact of *Bsal* or increase it, depending on changes in the number of tolerant, reservoir, and vector species (Holt and Pickering, 1985; Brannelly et al., 2021). While not much is known about amphibian community composition dynamics in the presence of *Bsal* in the wild, it could potentially be a very important factor in predicting spread and severity of outbreaks.

In this study, our first objective was to determine the extent of *Bsal* prevalence in ponds in the Netherlands using an eDNA-targeted approach with qPCR. After characterizing the amphibian communities present at these same sites using eDNA metabarcoding, our second objective was to determine whether the composition of these amphibian communities was associated with presence of *Bsal* at a fine spatial scale to investigate possible means of transmission.

MATERIALS AND METHODS

Two ponds were identified that have been confirmed to contain *Bsal* (RAVON, 2021). Twenty-seven bodies of water within a 2 km radius of the original two sites were subject to one session of eDNA sampling in mid-April 2021. This sampling did not include the original two bodies of water. Exact locations are anonymized to obscure the location of protected species and because many sites are on private property.

For each body of water, 20 systematic samples of 100 mL of water were taken with a single-use 100 mL sterile water

sampling ladle while standing on the bank (as per Spitzen-van der Sluijs et al., 2020). These samples were collected from the top 5 cm of the water column, decanted into a 2 L self-supporting sterile Whirl-Pak® bag, shaken to homogenize the liquid, and the full 2 L of water was filtered through a VigiDNA® 0.45 µM cross-flow filtration capsule. The filtration capsule was then filled with 80 mL of CL1 conservation buffer to preserve the filter. eDNA extractions were performed following the procedure described in Pont et al. (2018). After DNA extraction, samples were tested for inhibition by qPCR following the protocol described in Biggs et al. (2015). qPCR was performed in a final volume of 25 µL, using 3 µL of template DNA, 3 µL of 10–3 ng/µLDNA of a synthetic gene, 12.5 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 3.5 µL of ddH₂O, 1 µL of each specific primer for the synthetic gene (10 µM), and 1 µL of probe (2.5 µM) under thermal cycling 50 °C for 5 min and 95 °C for 10 min, followed by 55 cycles of 95 °C for 30 s and 52 °C for 1 min. All samples were analyzed in duplicate. If at least one replicate showed a different threshold cycle (Ct) value than expected (at least 2 Ct), the sample would be considered inhibited and diluted fivefold. None of the samples showed evidence of inhibition.

For single-species qPCR assays to detect *Bsal*, reactions were carried out using the protocol described in Spitzen-van der Sluijs et al. (2020) in a final volume of 25 µL, containing 3 µL of template DNA, 12.5 µL of TaqMan Environmental Master Mix 2.0 (Life Technologies), 6.5 µL of ddH₂O, 1 µL of forward primer (*Bsal_F*: CACATTGCACCTACTTT, 10 µM), 1 µL of reverse primer (*Bsal_R*: AAGACAAGGAAATGAATTA, 10 µM), and 1 µL of probe (*Bsal_Pr*: 6-FAM-TGATTCTCAAACAGGCATACACTCTAC-BHQ-1, 2.5 µM). The qPCR mixture was heated 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. A dilution series of DNA ranging from 1.83×10^3 GE/µL to 18.3 GE/µL was used as the qPCR standard (with 3 replicates each) in each qPCR plate. The genome equivalents were obtained from *Bsal* DNA which was extracted from a five-day-old culture containing both sporangia and zoospores, extracted during the study of Spitzen-van der Sluijs et al. (2020). Samples were run on a BIO-RAD® CFX96 Touch™ Real-Time PCR Detection System in a room dedicated to amplified DNA analysis with negative air pressure and physically separated from the DNA extraction room. After the qPCR analyses for *Bsal*, a sample was considered positive if 1 of up to 12 replicates was positive and only if the observed Ct value was lower than 40 cycles. The remaining extracted DNA was then sent on to the University of Salford, UK, on dry ice for the eDNA metabarcoding analyses as described below.

We amplified extracted DNA samples 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' TTAGATACCCACTATGC 3'; Riaz et al., 2011) which amplifies a short fragment (~98 bp) of the 12S rRNA region with sample-specific multiplex identifier (MID) tags. During polymerase chain reaction (PCR) for these primers, the samples were denatured for 5 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C with a final elongation of 5 min at 72 °C. The second was the 16S amphibian primer set which targets a 150 bp fragment in the 16S rRNA region of the mitochondrial genome (BA-4445-F 5'-RACCGTGCRAAGGTAGCR-3; BA-178-R 5'-CCATRGGGTCYTCTCGTCT-3'; Bálint et al., 2018) with sample-specific MID tags. PCR amplification for these primers followed an adapted protocol with samples denatured

for 15 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 1 min 30 s at 55 °C, and 1 min at 72 °C with a final elongation of 30 min at 60 °C. Hereafter, these primer sets will be referred to as 12S vertebrate and 16S amphibian throughout. Amplification with amphibian-specific batra 12S primers (Valentini et al., 2016) was also attempted but because of nontarget amplification in a significant number of these samples, this was not pursued further. Twenty-four unique MID tags were used for each primer set, for both forward and reverse primers, to differentiate between samples. During PCR for both primer sets, each sample had three replicates to reduce biases in individual reactions. Three replicates of a positive control sample from another project involving captive *Agalychnis lemur*, a Central American species, were included on the plate for each primer set to identify tag jumping for subsequent data filtering steps. Four negative control replicates were included to identify potential 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.

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 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 to dilute correctly to a concentration of 9pM with 1% PhiX™ in preparation for sequencing. An Illumina MiSeq® Reagent v2 2x150-bp nano kit was then used to sequence each library on separate sequencing runs. The bioinformatic analysis was conducted using the OBITools metabarcoding package (Boyer et al., 2016) followed by a direct blast against the GenBank nucleotide database (National Center for Biotechnology Information) to assign taxonomy. For the taxonomic assignment, molecular operational taxonomic units required at least 98% identity to be assigned at species level, retained more than five reads, and all other assignments were filtered out in subsequent steps (Sales et al., 2020).

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 S1. 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 negative control samples were removed from all read counts. Reads from domestic animals and humans were removed. Molecular operational taxonomic units assigned at species level and with more than five reads were kept and compiled to a file containing the vertebrate community. Subsequently, the amphibian community was isolated in a separate file. For the 16S amphibian primers, the same steps were followed but no vertebrate community file was created. *Pelophylax lesssonae* was replaced with *Pelophylax spp.* because the mitochondrial DNA of *P. lesssonae* and the hybrid *Pelophylax kl. esculentus* are indistinguishable using eDNA methods (Holsbeek and Jooris, 2010), although the hybrid is more common.

All statistical analyses were conducted in R version 4.1.2 (R Core Team, 2021) and maps were made in ArcGIS Pro 2.9 (Environmental Systems Research Institute, 2021) and Adobe Illustrator 26.0 (Adobe, 2021). Subsequent analyses were conducted with data converted into presence-absence, and site 22 was removed because no data for this site was attained, likely

due to an error at the PCR stage. A species accumulation curve was constructed using the iNEXT function (Hsieh et al., 2016), extrapolating amphibian species richness data to 50 sites for each primer set and combined primer data to determine their effectiveness.

Sites were split into those which tested positive and those which tested negative for *Bsal*. Sites with no amphibian species detected were removed (sites 2, 16, 17, and 19), which left 15 *Bsal*-negative sites and 7 *Bsal*-positive sites. 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 and a significant difference was found ($F = 10.44$, $P = 0.005$) (Oksanen et al., 2019). Because permutational multivariate analysis of variance is not as powerful with a significant PERMDISP result, a distance-based redundancy analysis (dbrDA) was performed instead, using the Jaccard index matrix as the response variable. This was done to determine the strength of *Bsal* as an explanatory variable of amphibian community composition. Significance was assessed with an analysis of variance (function ANOVA in the R package vegan) with 1,000 permutations.

RESULTS

The *Bsal* qPCR assay identified 8 *Bsal*-positive sites and 19 -negative sites. The MiSeq sequencing runs yielded ~1.7 million raw reads for the 12S vertebrate primers and ~1 million reads for the 16S amphibian primers prior to data filtering. Table S1 shows the reads for each primer data set remaining at the end of the bioinformatics process and after each stage of the filtering process. Nontarget reads (nonvertebrate) comprised just 1.3% of the 12S vertebrate primer data, with 498,302 reads belonging to the class Amphibia (Table S1). See the Supplementary Data and Table S2 for a breakdown on the vertebrate communities found at each site. The vast majority of reads sequenced in the 16S amphibian primer data set were not amphibian as shown by the dramatic reduction in reads at the final filtering step (Table S1). The reads for the 16S amphibian primers were dominated by Rotifera (27.4%), Arthropoda (25.5%), and nonassigned reads (44.5%), with only ~1% of the reads belonging to the class Amphibia. Average read depth for amphibian species per sample was 19,165 for the 12S vertebrate primer set and only 105 for the 16S amphibian primer set.

The 12S vertebrate primers detected six amphibian species where the 16S amphibian primers only detected five, with the latter missing *Triturus cristatus*, which is a species of concern for these sites. *Lissotriton vulgaris* clearly dominated the reads, and the two species of interest due to their higher conservation concern (*T. cristatus* and *Pelobates fuscus*) were the least frequently detected species for both primer sets. See the Supplementary Data and Figure S1 for a breakdown of the proportion of reads for each amphibian as detected by each primer set.

A map was constructed displaying the amphibian communities at each site as detected by the 12S vertebrate and 16S amphibian primer sets alongside the *Bsal* data, with 8 sites found to be *Bsal*-positive and 19 to be *Bsal*-negative (Fig. 1). Figure 1 further shows the deficiency of the 16S amphibian primer data as it contains ten sites with no amphibian reads whereas the 12S vertebrate primers detected amphibians. However, of the

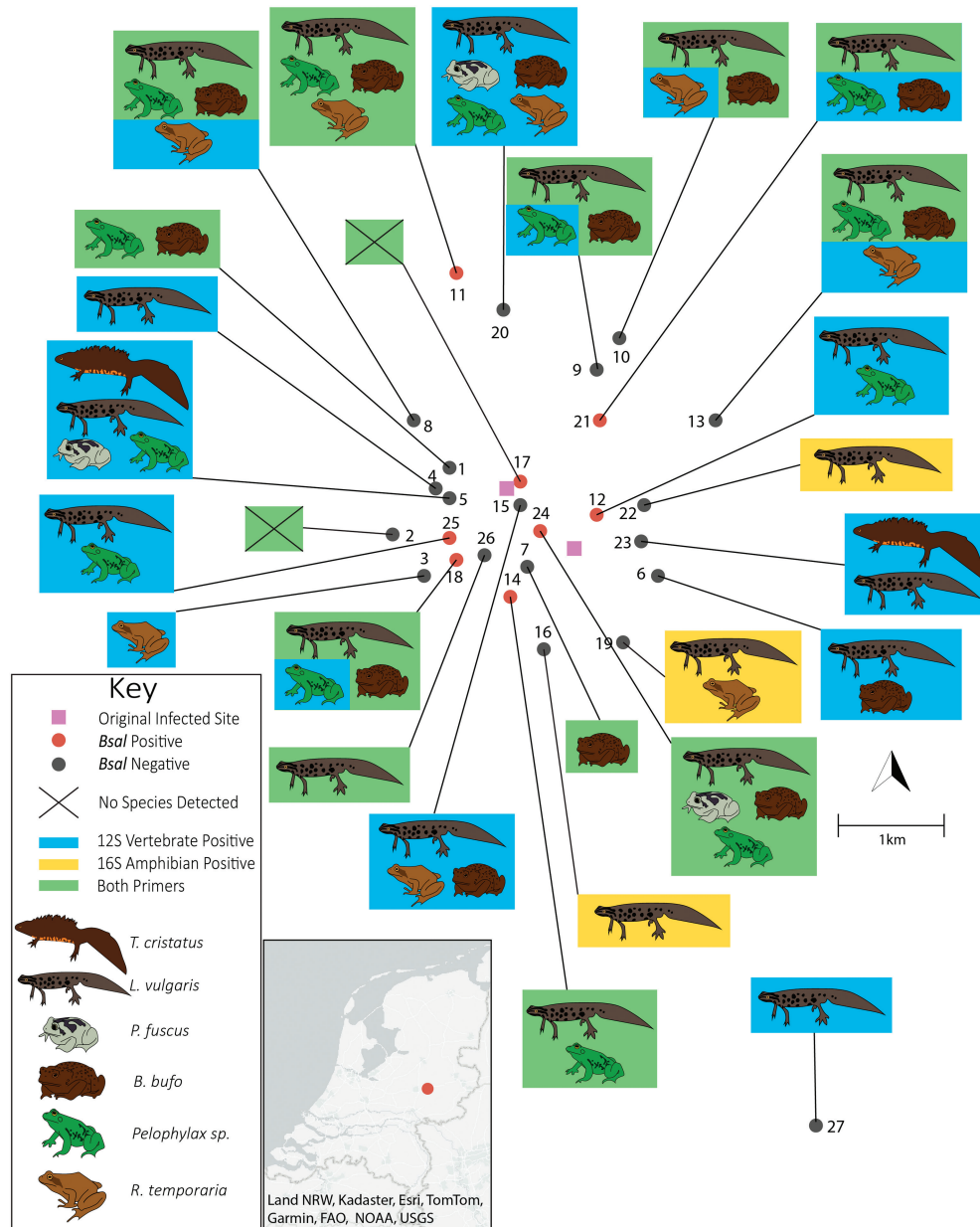


FIG. 1 Map displaying the amphibian communities detected using the 12S vertebrate and 16S amphibian primer sets at *Batrachochytrium salamandrivorans*-positive and -negative sites (underlying map not shown because ponds are on private properties). The original two sites where *Bsal* was detected are included in blue squares. Approximate location of the study site within the Netherlands is shown at the bottom of the figure.

four sites where no amphibian species were detected by the 12S vertebrate primers, the 16S amphibian primers detected species at three. At site 22, the 16S amphibian primers detected *L. vulgaris* but no sequence data was returned for this site by the 12S vertebrate primers. At site 16, the 16S amphibian primers detected *L. vulgaris*, and at site 19 they also detected *R. temporaria*, whereas the 12S vertebrate primers detected no amphibian species. However, all of these detections by the 16S amphibian primer set have a low number of reads, ranging between only 8 and 23 reads after filtering.

Overall, the 12S vertebrate primer set detected a generally higher species richness across sites than the 16S amphibian primer set with a median of two detected species for the former and one detected species for the latter. The 12S vertebrate primer set was much more successful at detecting species richness of the sites, reaching a plateau within the number of sites

surveyed (27) in this study (Fig. 2). Furthermore, the 12S vertebrate primer data almost independently drives the combined data in Figure 2, making the contribution of the 16S amphibian primer data essentially redundant. Therefore, the following analyses on associations between amphibian communities and *Bsal* presence was only conducted using the 12S vertebrate primer set.

Sites where amphibian species were detected were split into those that tested positive for *Bsal* and those that tested negative. The number of sites in each category was not equal, with 15 negative sites and 7 positive sites. A PERMDISP test was conducted, and the difference in distribution between *Bsal*-positive and -negative sites was found to be significantly different ($F = 10.44$, $P = 0.005$). This means that sites within the *Bsal*-positive category have more intragroup similarity than those in the *Bsal*-negative category. A dbRDA plot was created to explore

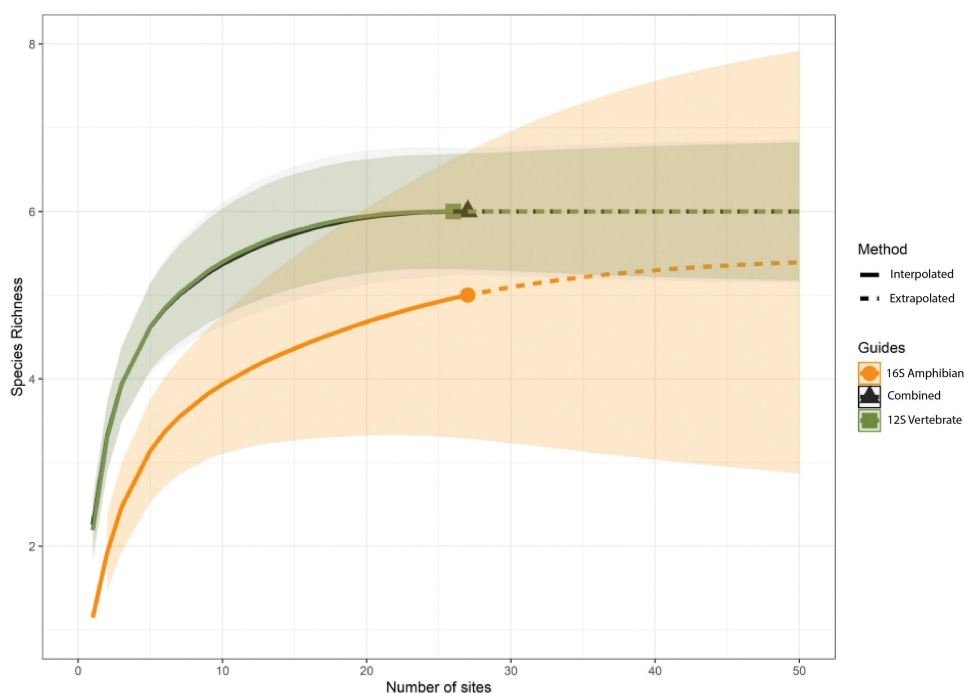


FIG. 2 Species accumulation curve showing the interpolated species richness detected by each primer set and by both primer sets combined alongside the extrapolated richness to 50 sites. Shaded regions display the 95% confidence interval. 12S vertebrate primer data has site 22 removed due to failure and therefore has 26 sites.

the contribution of the binary *Bsal* infection presence variable to the species community composition observed. The dbRDA (Fig. 3) was found to be significant ($R^2_{adj} = 0.0569$, $P = 0.0497$). The maximum constrained variance in the data set was 10.2%, but the more conservative adjusted R^2 value suggests that the binary *Bsal* infection presence variable explains just 5.7% of the total variance in amphibian community composition.

DISCUSSION

This study has successfully detected the expected range of amphibian species at sites in Gelderland, the Netherlands, in addition to a component of the wider vertebrate community present at these sites (Table S2). The species accumulation curve for the 12S vertebrate primer set (Fig. 2) demonstrates that overall sampling effort in this study was sufficient to detect all amphibian species expected in the study area. As expected, the rarest species detected in this study were those protected under the European Habitats Directive (Council Directive 1992/43/EEC): *T. cristatus* (Annex II and IV) and *P. fuscus* (Annex IV). *L. vulgaris* dominates the read count 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 *Bsal*-targeted qPCR demonstrates that *Bsal* has spread from the initial two sites to eight further sites without a clear path of spread as some of the closest sites to the original two sites remain uncontaminated (Fig. 1). However, it should be noted that the chronological contamination of sites with *Bsal* is unknown as only two sites were sampled originally (RAVON, 2021).

Comparisons between the 16S amphibian and 12S 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 16S amphibian primers did not detect *T. cristatus*, consistently detected a lower

amphibian species richness at each site, and contained <1% of the amphibian reads detected by the 12S vertebrate primers. The 16S amphibian primers have not yet been used in any published peer-reviewed studies except the original Bálint et al. (2018) study. It is likely that, as this primer set was developed for use within biodiverse tropical ecosystems and developed from anuran sequences from South America, it was not optimized for use in detecting European amphibian species. However, of greater concern is that this primer set leads to a large proportion of nontarget amplification, with large quantities of Rotifera and Arthropoda being amplified (Table S1), which likely hinders its ability to optimally target amphibians (Collins et al., 2019). The 12S vertebrate primers did detect all six of the expected amphibian species in the study area, with an average of 2.2 species per site, which is in line with a previous study in the Netherlands where one to five reproducing species were found per pond (Stumpel and van der Voet, 1998). However, this could possibly be an underestimation of the species present at each site, with some of the rarer species being detected less frequently. For example, one of the main species of concern, *T. cristatus*, was not detected as often as perhaps it should have been given the known populations of *T. cristatus* in the area (Spitzen-van der Sluijs, personal observation). A previous study in the UK demonstrated that while these general 12S vertebrate primers are less sensitive than a species-specific qPCR assay for *T. cristatus*, they did provide similar proportions of detections when more conservative detection thresholds were applied (Harper et al., 2018). However, a more targeted approach (either with a species-specific qPCR assay or amphibian-focused 12S or 16S metabarcoding primers; Valentini et al., 2016; Sakata et al., 2022) would be warranted in future studies. It is not currently known whether *T. cristatus* subpopulations have experienced any significant decline due to *Bsal* in this area. However, evidence from other studies has shown *T. cristatus* experience a moderate

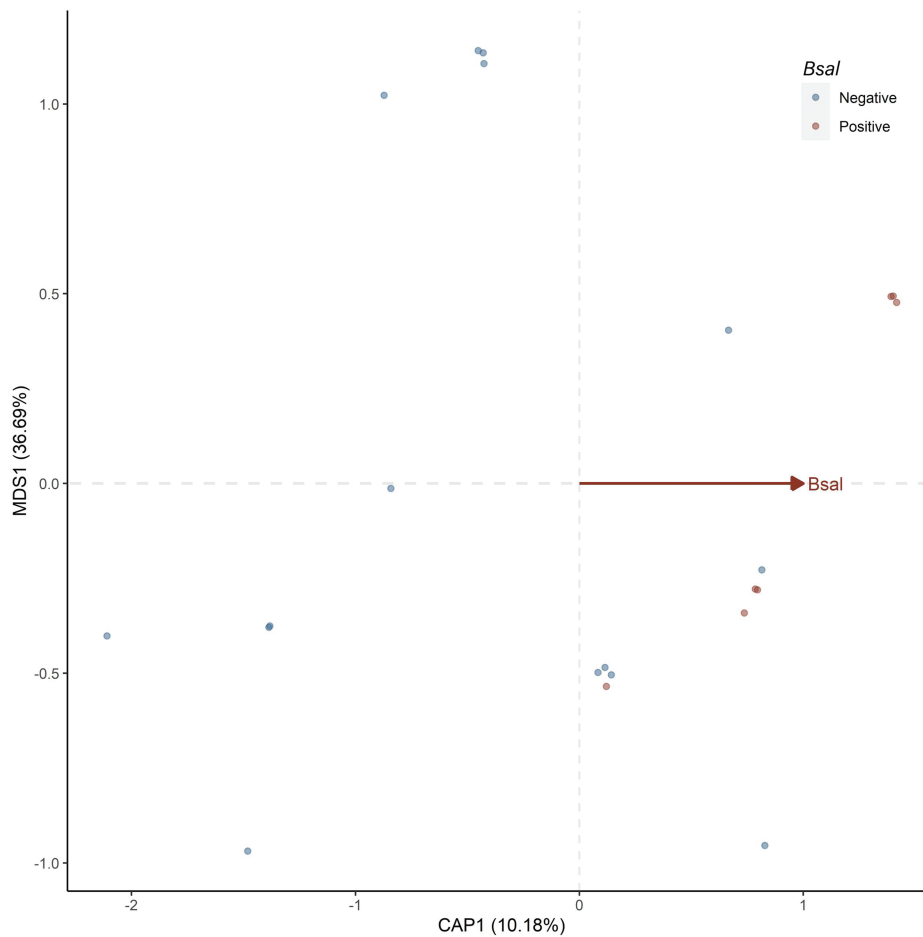


FIG. 3 Distance-based redundancy analysis plot showing the influence of *Batrachochytrium salamandrivorans* on the differences in amphibian community composition as detected by the 12S vertebrate primer set. CAP1 is the first constrained ordination axis and explains the maximum variation associated with the binary *Bsal* variable. MDS1 is the first unconstrained ordination axis and explains the general dissimilarities among samples. The influence of the binary *Bsal* variable on the CAP1 axis is displayed with the loading arrow. Points are jittered for visibility.

mortality rate when exposed to *Bsal*, and declines have been observed in wild German subpopulations (Bates et al., 2019; Lötters et al., 2020).

Having a record of the full amphibian communities present at sites of *Bsal* infection is valuable as it not only provides information on the species at risk of infection but also the potential vector species in the area. Furthermore, it is not yet known whether *Bsal* has an impact on the amphibian community as a whole. Chytridiomycosis induced by *Batrachochytrium dendrobatidis* has caused huge declines in host species and the extirpation of many (Lips et al., 2006; Scheele et al., 2019), which then leaves an empty niche that can be utilized 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., 2021). In this study, *L. vulgaris* can be considered a tolerant species (Bates et al., 2019) and anurans are considered resistant (Martel et al., 2013). The potential trend between *Bsal* presence and the community composition of amphibians was investigated using a dbRDA, which concluded that the *Bsal* infection explained only a small proportion of the variance in the amphibian community composition detected here. Well-known drivers such as the availability of habitats and microhabitats 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 intragroup variability, with *Bsal*-positive sites containing amphibian communities that are more similar, could potentially show a homogenization 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 is clearly an avenue which requires further investigation.

There are several reasons why, with further data, *Bsal* may yet be found to be an important driver of amphibian community composition. First, there is the caveat that the eDNA metabarcoding approach used here may be underestimating the amphibian community in the area. Second, 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 compositions (Beentjes et al., 2019). Because most amphibians in this study vacate the water outside of spring and early summer, sampling times should be within the amphibian breeding season to ensure sufficient eDNA present in the water, but even yearly sampling is highly valuable for tracking the progression of community change. 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 for more in-depth analyses into the dynamics of the amphibian communities within *Bsal*-positive areas and potentially enable the construction of a chronosequence in the absence of long-term studies to provide further insight into community development over time.

Abundance data would also have added another dimension to this community analysis and perhaps revealed differences in 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 difficulty in consistently determining 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 being the subject of only a few studies (Evans et al., 2016; Li et al., 2021). However, a recent meta-analysis found that fish are currently the only vertebrates where it can be reliably concluded that this positive correlation with abundance or biomass exists (Carvalho et al., 2021).

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 movement less likely. This is in line with the current research as transfer via amphibian-related human activities and wild waterbirds are considered to have the most potential when considering larger distances of transfer between metapopulations (Zhu et al., 2014; EFSA Panel on Animal Health and Welfare, 2018). For example, experimental evidence has shown that *Bsal* can adhere to the legs of geese to transfer over larger distances between ponds (Stegen et al., 2017). However, while the proportion of *Bsal*-positive and -negative sites hosting each amphibian species and waterbirds as a group were examined, this study did not contain enough *Bsal*-positive sites for further meaningful statistical analysis (data not shown). While the methods used in this study are not sufficient to determine the influence of waterbirds on *Bsal* transmission without the ability to identify individuals, it is an important avenue of research on the road to understanding *Bsal* transmission.

In conclusion, the dbRDA determined that *Bsal* was significant as an explanatory variable for amphibian community composition, but further investigations incorporating variables such as suitable habitat and microhabitat availability are clearly warranted. This study further demonstrated eDNA metabarcoding as a suitable method for detecting amphibians in the Netherlands, and the full range of amphibian species expected in the study area were detected. While the data set in this study is small, we hope to inspire further studies using eDNA in different locations and ideally conducted over multiple years to add to the data on amphibian community composition and *Bsal*. As knowledge on *Bsal* transmission increases, new infection sites could be predicted, and measures can be taken to slow the spread and prevent further mass mortality events.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online alongside the manuscript.