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Effective Amphibian Conservation Monitoring and Habitat Assessment Using eDNA

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ABSTRACT

The European green toad (*Bufotes viridis*) is currently in decline and considered endangered across the northern extent of its native range, with large investments in ongoing conservation and translocation efforts. To assist conservation efforts, survey methods must be established that are cost-effective, non-invasive, and rapidly deployable. Here we evaluated the effectiveness of eDNA metabarcoding for amphibian conservation across three objectives: (1) Test *B. viridis* probability of detection before and after translocation efforts in 3 ponds in Öland, Sweden. (2) Assess pond biodiversity and biotic interactions across Öland and Kalmar using eDNA metabarcoding. (3) Determine which surveyed sites are suitable for future translocation efforts. We found that the detection probability of *B. viridis* increased 100% 24 h after the translocation was initiated, whereby they were undetected prior to release. Additionally, we detected 11 fish species, 14 bird species, 9 mammal species, and 4 amphibian species across the translocated sites. The results from the 37 pond eDNA surveys resulted in the detection of 15 fish species, 38 bird species, 8 amphibian species, and 17 mammal species. Species richness of the surveyed ponds ranged from 1 to 24, with an average richness of 8. Co-occurrence analysis found significant associations between several species, including a significant negative association between amphibian occurrence and cattle and gray heron and positive associations with duck and common crane. Multi-Criteria Decision Analysis (MCDA) suggests 6 sites had consistent lower site rankings, indicating them as more favorable locations for future amphibian translocation efforts. Overall, these findings showcase eDNA high-throughput sequencing as a viable means to non-invasively assess European green toads and simultaneously assess wider community dynamics that may help evaluate the sustainability of reintroduced and endemic populations.

[Correction added on 30 October 2025, after first online publication: the spelling of the author names Försäter and Niklasson has been corrected.]

1 | Introduction

Biodiversity conservation is essential for maintaining species diversity and plays a key role in ensuring ecological stability and healthy ecosystems. Conservation translocations, the intentional movement and release of endangered species, are increasingly recognized as highly valuable in modern conservation efforts (Gaywood et al. 2022). To ensure long-term success, effective management plans for translocations require several key components, including stakeholder involvement, establishment of breeding programs, pre-reintroduction habitat assessment, reintroduction planning and implementation, ongoing monitoring of target populations, and adaptive population management (Yoccoz et al. 2001). Successful translocation strategies have been implemented for reintroducing and managing several notable species, including wolves (*Canis lupus*) (Ripple and Beschta 2012), kākāpō (*Strigops habroptilus*) (Jamieson 2015), beavers (*Castor fiber*) (Auster et al. 2021), and black-footed ferrets (*Mustela nigripes*) (Jachowski et al. 2011). Broadly, such programs were able to obtain success due to the dedicated efforts to monitor released individuals using devices such as radio collars, banding, or camera tracking to monitor and ensure individuals remained healthy. Amphibian translocations and reintroductions are also widespread (Griffiths and Pavajeau 2008), including the Wyoming toad (*Anaxyrus baxteri*) (Linhoff and Donnelly 2022), corroboree frogs (*Pseudophryne corroboree*) (Rojahn et al. 2018), natterjack toad (*Epidalea calamita*) (Rannap et al. 2024), pool frog (*Pelophylax lessonae*) (Sainsbury et al. 2017), and great crested newt (*Triturus cristatus*) (Edgar et al. 2005). Monitoring of translocated populations also needs to ensure population sizes remain large enough to ensure successful establishment and long-term stability, with mitigation measures taken during the establishment process to avoid re-introduction failure. However, smaller or cryptic species (e.g., amphibians), which make up the bulk of conservation species of interest, are often elusive and difficult to monitor post-reintroduction (Morant et al. 2020). Without developing and adapting effective monitoring or population assessment practices for such species, conservation strategies may then be prohibitively expensive or may potentially fail (Morris et al. 2021; Seymour and Smith 2023).

Post-release monitoring for amphibian related translocations typically relies on call counts, egg/larval surveys, torchlight/bottle-trapping, and mark-recapture, which are labor-intensive, seasonally constrained, characterized by low and variable detection probailities and may cause habitat disturbance (Griffiths and Pavajeau 2008; Biggs et al. 2015). For instance, the reintroduction of the Wyoming toad (*Anaxyrus baxteri*) has faced repeated setbacks due to difficulties in locating individuals post-release, challenges in accurately estimating survival rates, and uncertainty in detecting breeding success (Linhoff and Donnelly 2022). Similarly, corroboree frog (*Pseudophryne corroboree*) translocations have been hampered by low detectability in the wild and complex habitat requirements, complicating assessment of establishment and population viability (Rojahn et al. 2018). In Europe, conservation efforts for the natterjack toad (*Epidalea calamita*) and pool frog (*Pelophylax lessonae*) have encountered problems due to unreliable monitoring, leading to underestimation of population size and failure to promptly identify declining trends or emerging threats (Rannap et al. 2024; Sainsbury et al. 2017). A Swedish translocation project for the great crested newt (*Triturus cristatus*) highlighted how inadequate pre-translocation surveys led to underestimates of source population size and subsequent challenges in evaluating translocation success. Although a population was eventually established in the recipient site, a large proportion of translocated newts initially disappeared, underscoring both the difficulty of monitoring and the need for robust, long-term post-release assessments (Gustafson et al. 2016). These examples highlight the necessity for spatial planning and strategic, adaptive monitoring to maximize conservation success and ensure the efficient use of limited resources. As we look to improve amphibian monitoring programs, recent advancements in environmental DNA (eDNA) offer a promising avenue for enhancing the detection and management of elusive and conservation-important species.

Environmental DNA (eDNA) is DNA isolated from environmental samples (i.e., water, soil or air) whereby the target species are absent with the DNA being extracted from cellular material left behind by the target species (e.g., skin, hair, feces) (Deiner et al. 2017; Seymour 2019). eDNA-based species monitoring has been effective in monitoring several elusive species, including the great crested newt (Biggs et al. 2015) and the Hula painted frog (*Latonia nigriventer*) (Perl et al. 2022). Utilizing eDNA for species monitoring greatly benefits conservation efforts by reducing the need for direct visual or physical contact with the species, thereby enhancing the efficiency and accuracy of monitoring programs (Seymour and Smith 2023). Combined with high-throughput metabarcoding, eDNA bioassessment also enables simultaneous assessment of conservation species and their biological interactions, providing managers ecosystem-level data from a single non-invasive environmental sample (Harper et al. 2020; Seymour et al. 2020). As such, eDNA-based assessment is of particular importance for improving conservation management plans of elusive species, including the European green toad (*Bufotes viridis*).

The European green toad (*B. viridis*) is a widespread amphibian species found across central and Eastern Europe, extending to the northern regions of southern Sweden and Estonia (Sillero et al. 2014). In Sweden, its distribution is limited to a few fragmented populations in the southernmost provinces (Fohrman 2025; Wirén 2006). These populations are geographically isolated from the European mainland, potentially leading to a reduced gene pool and associated problems such as diminished adaptive potential and parasite resistance (Höglund et al. 2022; Rogell et al. 2011). Over the past 50 years, *B. viridis* populations in Sweden have experienced a rapid decline, making it the most threatened amphibian species in the country according to the Swedish Red List (Eide et al. 2020). Efforts to conserve *B. viridis* have included habitat restoration efforts, along with the release of wild-collected and laboratory-reared eggs, tadpoles, and young toads at restored habitat sites. However, despite the release of hundreds of thousands of tadpoles and toadlets, very few have returned as breeding adults (Fohrman 2025). This low success rate may be partially attributed to biological factors, including predation pressure, interspecies competition, and chytrid fungal infection. As such, development and implementation of eDNA-metabarcoding based monitoring of *B. viridis*, along with other amphibians, would greatly aid reintroduction efforts and overall routine habitat assessment.

The primary objective of this study was to evaluate the utility of eDNA metabarcoding for improving conservation management of amphibian populations. Using the European green toad (*B. viridis*) as a model species, we applied eDNA metabarcoding to quantify shifts in community diversity following introductions, evaluate reintroduction success, and assess how ecological communities responded to management interventions. We estimated changes in detection probabilities to enhance the accuracy of monitoring for elusive amphibians, thereby improving population assessments in conservation programs and ecological studies. We also conducted a landscape-level survey of amphibian habitats to provide a broader understanding of aquatic biota and amphibian species distributions and habitat associations. Additionally, we assessed suspected ponds for *Batrachochytrium dendrobatidis* (Bd), which has been previously unrecorded across the study area. Finally, we performed co-occurrence analyses to identify biotic interactions among amphibians and other taxonomic groups, yielding insights into community-level dynamics. This multifaceted approach provides evidence for the broader applicability of eDNA-based methods to infer ecological dynamics and supports the development of more effective conservation strategies for amphibians.

2 | Methods

2.1 | Translocation Sampling

In May 2021, the Nordens Ark foundation, in co-operation with the Kalmar County Board, translocated *B. viridis* tadpoles into three ponds near the northern shore of Öland, Sweden. Prior assessments confirmed that these ponds did not have green toads before the translocation occurred. To determine whether eDNA could effectively determine the presence and absence of *B. viridis* in the field, eDNA samples were collected from each pond 3 h before the tadpoles were released and again the day after the release.

eDNA sampling included triplicate 1 L water samples using buckets that had been soaked in 10% bleach for 1 h prior to use. For each eDNA sampling point, 5 L of water were collected in the form of sub-samples, which were combined into a composite sample for more reliable results (Harper et al. 2018). Sample collectors wore sterile gloves and face masks to prevent sample contamination. Water was manually filtered using a 100 mL sterile syringe through 5 μm GF/0.8 μm PES encapsulated filter units (NatureMetrics Ltd., UK). Fixation with 96% ethanol (molecular grade 200 proof) followed the protocol according to Spens et al. (2017).

2.2 | Survey Sampling

Additionally, 31 freshwater bodies across Öland and northern Kalmar County, Sweden, were sampled for eDNA to conduct general amphibian, bird, and fish surveys from 2021 to 2023 (Figure 1). eDNA sampling occurred in May for all 31 sites, with 6 sites being sampled again in August for a total of 37 eDNA samples. May sampling was chosen to coincide with the main amphibian breeding season in Sweden, to maximize detection probabilities. Due to logistics restrictions and to maximize the number of sampling sites, a single eDNA sample was taken per survey, followed by the same sampling protocol used for the translocation experiment. For each field day, a negative field filter control was collected. Additionally, we tested each eDNA water sample for the fungus *Batrachochytrium dendrobatidis* (Bd), using the MCP gene and the qPCR protocols provided in Kamoroff and Goldberg (2017) with 12 PCR replicates per sample. Pond area for each of the sampling sites was measured using Google Earth. Water temperature for each of the sampling sites was recorded with a temperature probe.

2.3 | Negative Controls

In order to assess potential cross contamination of eDNA in the field and laboratory setting, negative controls were analyzed together with the samples. For each sampling day, negative field filter samples were processed by filtering bottled mineral water in the field. The controls were treated as the samples. In the laboratory, negative eDNA extraction controls were introduced for each extraction session, where a negative filter extraction control and negative reagent control were included in the analysis. Four replicates of non-template negative controls were added to each PCR plate. eDNA from the controls was included in all the downstream analyses and sequenced. For each PCR plate, the following controls were included: field filter negative control, extraction filter negative control, extraction reagent negative control, PCR non-template negative control, and PCR positive control (containing a mock community of known tropical fish species).

2.4 | DNA Extraction

DNA extraction followed a modified Qiagen DNAeasy blood and tissue kit (Qiagen, Germany) protocol for enclosed filters (Spens et al. 2017). Modifications to the standard kit protocol included adding Proteinase K and buffer ATL directly to the filter unit, allowing the lysis in the filter unit to be carried out overnight, and pooling the lysate to increase the DNA yield.

2.5 | Batrachochytrium Dendrobatidis qPCR

We conducted screening for Bd over the course of the survey, which was assessed at sites most suspected of possible infection due to logistics constraints. Prior to the survey, Bd was not known to be present on Öland but had been observed on the Swedish mainland. In 2021, eight sites were examined using qPCR for the presence of Bd. In spring 2021, three sites located on the mainland and seven on Öland Island were examined. In spring 2022, 22 sites were analyzed for Bd and in autumn 2022, the sites were re-examined.

The laboratory work for Bd detection was performed by NatureMetrics Ltd. UK. Shortly, detection of Bd from the eDNA sampled utilized TaqMan qPCR assay described by Boyle et al. (2004), as applied in Kamoroff and Goldberg (2017). Reactions for each sample were prepared in 12 replicates. Reaction volumes consisted of 15 μL containing 7.5 μL of 2× TaqMan Master Mix (Applied Biosystems, USA), 900 nM of

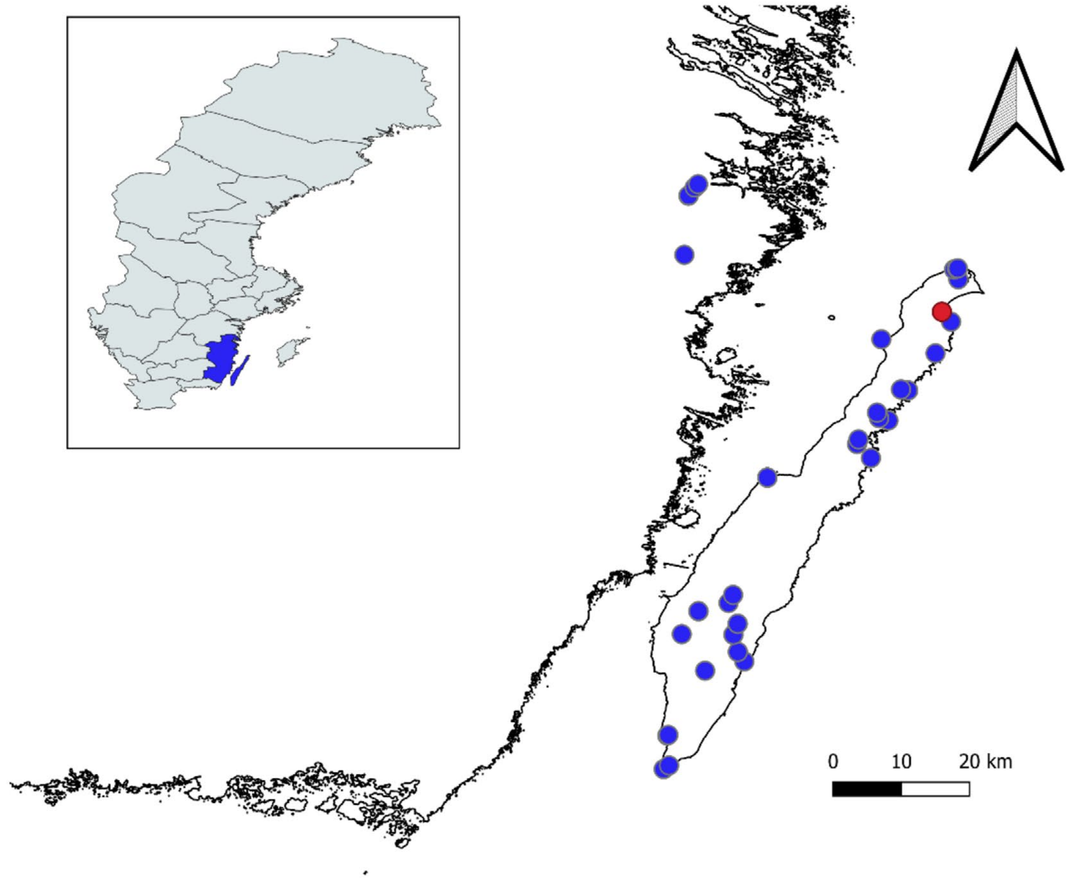

FIGURE 1 | Site locations for *B. viridis* translocation experiment (red) and the general eDNA survey (blue). The map insert shows the geographic position of Öland Island and northern Kalmar county (blue) within Sweden.

each primer (Forward Primer: ITS1-3 Chytr, 5′-CCTTGATATAATACAGTGTGCCATATGTC-3′; Reverse Primer: 5.8S Chytr, 5′-AGCCAAGAGATCCGTTGTCAAA-3′), 250 nM of the MGB probe (Chytr MGB2, 5′-CGAGTCGAACAAAAT-3′), and 2 μL of diluted DNA (10–1 in Tris pH 8). PCR conditions included an initial step of 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. A standard curve was constructed using control reactions containing 100, 10, 1, and 0.1 Bd zoospore equivalents, and no-template controls were included to ensure accuracy. The Ct values were determined using a ΔRn threshold of 0.10, and the concentration of Bd in test samples was expressed as zoospore equivalents.

2.6 | Metabarcoding

Library preparation and sequencing followed established 12S metabarcoding protocols described in Griffiths et al. (2023). Briefly, nested metabarcoding was carried out following a two-stage PCR approach (Bohmann et al. 2022) following the protocols described in Kitson et al. (2019). A positive control (quantified at 0.05 ng/μL) from the non-native cichlid (*Maylandia zebra*) and a negative control of molecular grade water were used for each library. The first PCR was performed in triplicate to amplify a 106 bp fragment using published 12S ribosomal RNA primers 12S-V5-F (5′-ACTGGGATTAGATACCCC-3′) and 12S-V5-R (5′-TAGAACAGGCTCCTCTAG-3′) (Kelly et al. 2014; Riaz et al. 2011).

Following the first-stage PCR, samples were visualized via gel electrophoresis to confirm successful amplification. Once successful amplification was confirmed, samples were pooled within sub-libraries based on band strength (Alberdi et al. 2018). Subsequently, to remove non-specific amplification, libraries underwent a double size selection clean-up (Bronner et al. 2013) using MagBIND RxnPure Plus magnetic beads (Omega Biotek Inc., Norcross, GA, USA). Second-stage PCR was carried out in duplicate to bind pre-adapters, indices, and Illumina adapters to each sub-library, which was followed by a second double size selection.

Cleaned sub-libraries were quantified using the Qubit 3.0 fluorometer high-sensitivity (HS) dsDNA assay (Thermo Fisher Scientific, USA) and normalized proportionally according to sample number and concentration. The normalized library underwent a final double size selection clean-up. The library was then diluted to 4 nM before being quantified via qPCR using the NEBNext Library Quant Kit for Illumina (New England Biolabs, USA). Once the desired quantification was confirmed, the final library was denatured and sequenced at 13 pM with 10% PhiX Control on an Illumina MiSeq using a MiSeq Reagent Kit v3 (600 cycle) (Illumina, USA). Due to project logistic constraints and timing, sequencing for the translocation samples was carried out at the University of Hull and the survey samples were sequenced via Nature Metrics. Subsequently, the bioinformatics for the two parts of the study were carried out at their respective sequencing locations.

2.7 | Bioinformatics

Sequencing data were demultiplexed using the Illumina MiSeq Reporter software post sequencing. Quality trimming was performed using fastp (Chen et al. 2018). Paired-end reads were merged into single sequences with an overlap of 20 bp, no more than 5% mismatches, and no more than 5 mismatched bases. Redundant sequences were removed by clustering at 100% identity and length using VSEARCH (Rognes et al. 2016). Sequences were globally denoised using the unoise algorithm (Edgar 2016) to remove sequencing errors and clustered at 99% identity to define amplicon sequence variants (ASVs). Chimeric sequences were identified and removed using VSEARCH (–uchime3_denovo). An ASV table was generated by mapping dereplicated reads to the denoised sequences at a 97% identity threshold. False positives were filtered by removing ASVs representing less than 0.05% of the sequences in each sample. Taxonomic identities were assigned by comparing ASV sequences against curated reference databases, including the UK vertebrate reference database for the translocated samples (Harper et al. 2020) and the NatureMetrics 12S database for the field survey samples, using BLAST (Camacho et al. 2009). Taxonomic assignment was based on the highest percentage identity (98%–100%), an e-score of 1e-20, and a hit length covering at least 80% of the query sequence. A majority, lowest common ancestor (MLCA) approach was applied to resolve ambiguous matches, requiring at least 90% query coverage and agreement among 80% of unique taxonomic lineages at descending ranks. Only species- or genus-level identifications were retained in the final results.

2.8 | Statistics

Probability of detection was calculated as the number of positive detections divided by the number of replicate samples for each amphibian species before and after the translocation of *B. viridis* to each of the three ponds. Changes in probability of detection were statistically assessed for each amphibian species using a generalized linear model with a binomial error distribution. Probability of detection was used as the response, and pond identity and time (i.e., before and after translocation of *B. viridis*) were used as the explanatory variables.

For the sites surveyed, we assessed the potential effect of Bd occurrence, pond area, and water temperature on amphibian detectability using a generalized linear model with a binomial error distribution. Amphibian presence/absence was used as the response variable, with Bd presence/absence, pond area, and water temperature used as the explanatory variables.

Co-occurrence analysis was used to infer associations between all species detected, based on presence-absence data, using the R package co-occur (Griffith et al. 2016). The co-occur package assesses the co-occurrence probability between species pairs compared to the null hypothesis of random species distribution across the sampling sites. We excluded species pairs with less than one co-occurrence from the analysis, using the combinatorics approach parameter option.

To prioritize ponds for potential translocation based on biotic interaction information, a systematic approach was employed to evaluate each pond using specific biotic indicators. First, data on the four key factors identified from the co-occurrence analysis, including cattle presence (negative influence), heron presence (negative influence), ninespine stickleback presence (negative influence) and herbivorous bird presence (mallard or crane) (positive influence), were extracted and normalized for each of the surveyed ponds. The effects of temperature, Bd, and area were found to be non-significant, so they were not included in the model. A Multi-Criteria Decision Analysis (MCDA) framework (Belton and Stewart 2012) was then constructed, assigning weights ranging from 0.1 to 1 to each factor to explore their relative influence on pond prioritization. The MCDA model computed a composite score for each pond by combining the weighted values of the four factors. To assess the robustness of the prioritization, a sensitivity analysis was conducted by systematically varying the weights of each factor across the full range of 10,000 possible value combinations (0.1–1 in increments of 0.1). This allowed for the evaluation of how changes in the importance assigned to each factor affected the final rankings. The results of the sensitivity analysis were aggregated, and the mean rank for each pond was calculated to provide a comprehensive overview of its priority across all weighted combinations.

3 | Results

3.1 | Sequence Data Summary

The translocated sequences generated 3,140,171 pair end reads across 18 eDNA samples (mean 16,3342 paired end reads/sample). Survey sequences generated 3,029,794 paired end reads across 37 eDNA samples (mean 84,161 paired end reads/sample). PCR negative controls were all negative and positive controls were positive for the *M. zebra*. Field negative controls were essentially negative with relatively low levels of human contamination detected compared to the eDNA samples, but this did not impact the downstream analysis.

3.2 | Translocation Experiment

Overall, we recorded 4 amphibian species from the sequence data, including *Triturus crisatus*, *Lissotriton vulgaris*, *Bufo bufo*, and *Bufotes viridis* (Figure 2). We also observed occurrences of fishes (8 species and 1 genus level assignments), birds (8 species and 5 genus level assignments) and mammals (8 species and 1 genus level assignments) (Figure 2). The amphibian biodiversity across the ponds varied prior to the translocation, whereby pond 1 and pond 3 harbored 2 species of amphibians and 4 and 3 fish species, respectively. Pond 2 was devoid of amphibians or fish species, with limited bird and cattle eDNA detected (Figure 2). The probability of detection was significantly greater ($p < 0.01$) after the translocation of the tadpoles but did not differ

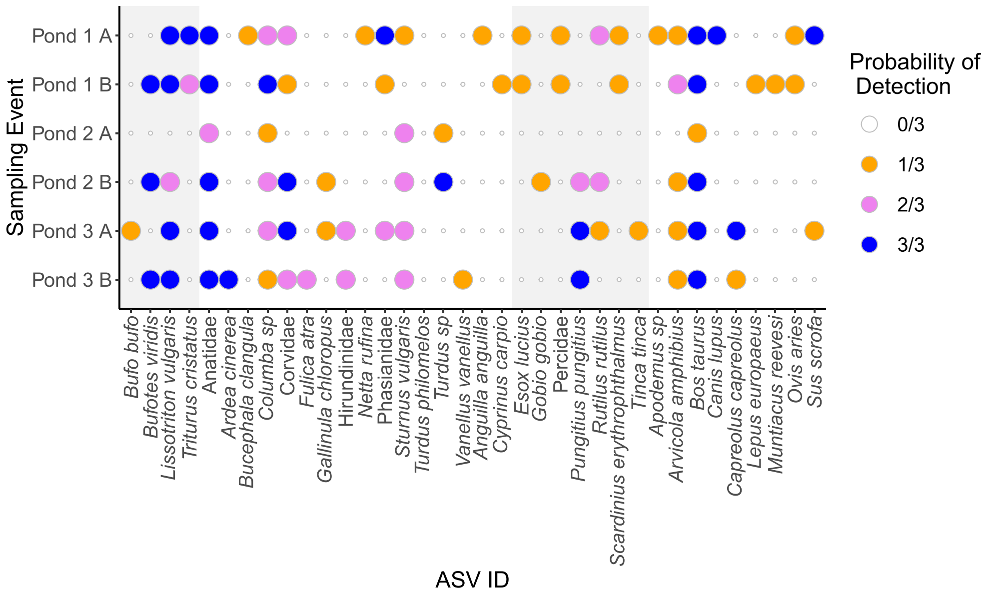

FIGURE 2 | Community dynamics across the translocation sites, including before (A) and after (B). Showing unique ASV identities as their taxonomic assignments on the x-axis. Groups are ordered by main groups and vertical lines, including amphibians, birds, fishes and mammals from left to right. Along the y-axis are the individual ponds with the number indicating the pond identity and (A) indicating before translocation and (B) referring to post- translocation. Color corresponds to the probability of detection for the given ASV identity for the given sample event with white being zero and blue being 3 out of 3 (100%) as indicated by the legend.

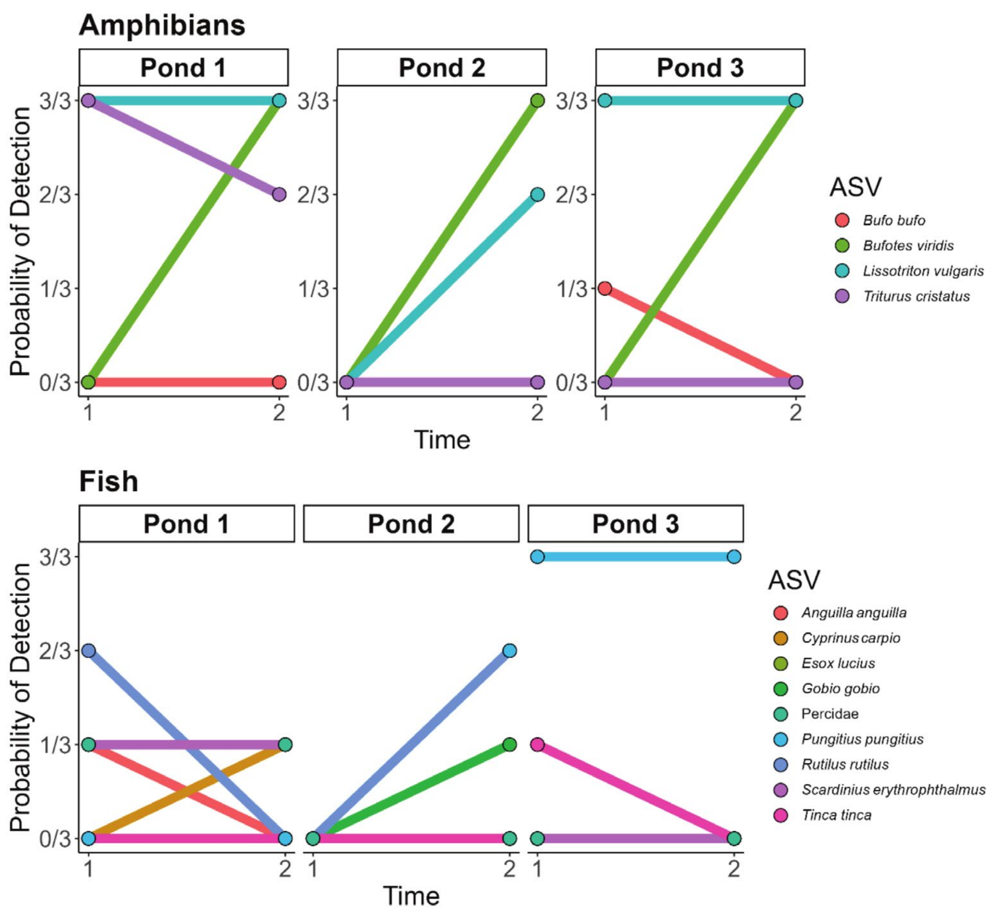

FIGURE 3 | Changes in detection probabilities for aquatic species detected across the translocation ponds. *B. virdis* is the only one to consistently increase after the introduction event. Each panel is a unique pond, with the upper panels showing amphibians and the lower panels showing fishes. Each color is a unique ASV (e.g., species) as indicated in the corresponding legend.

TABLE 1 | Probability of detection versus time (before/after *B. virids* translocation) and pond identity. Shown are the degrees of freedom (DF), deviance, and *p*-value for each of the generalized linear models using a binomial error distribution.

Explanatory	DF	Deviance	*p*
Bufotes viridis			
Time	1	9.56	**< 0.01**
Pond	3	0	1
Bufo bufo			
Time	1	0.88	0.83
Pond	3	0.53	0.47
Lissotriton vulgaris			
Time	1	4.56	0.21
Pond	3	1.27	0.26
Triturus cristatus			
Time	1	5.3	0.15
Pond	3	0.53	0.47

Note: Boldface indicates a significant effect.

significantly between the ponds ($p=1$) (Figure 3). All other amphibian probabilities of detection were non-significant with regard to the introduction of *B. viridis* and with regard to pond identity (Table 1 and Figure 3).

3.3 | Survey Study

Seven amphibian species and one genus were detected across 22 of 37 surveys, including *Bufo bufo* (17 surveys), *Bufotes viridis* (1 survey), *Lissotriton vulgaris* (7 survey), *Rana arvalis* (4 survey), *Rana dalmatina* (1 survey), *Rana temporaria* (1 survey in Kalmar) and *Triturus cristatus* (4 survey). We also detected instances of *Lissotriton* sp. across 5 of the surveys, which is most likely a sequence variant of *Lissotriton vulgaris* that was unable to be taxonomically assigned to species (Figure 4).

Comparatively, bird species were found across 31 of the 37 surveys, with richness ranging from 0 to 12 (mean = 3.32, SD = 3.10). Fish were detected in 29 surveys with richness ranging from 0 to 7 (mean = 2.30, SD = 1.66). Mammals were detected in 24 surveys with richness ranging from 0 to 5 (mean = 1.41, SD = 1.46) (Figure 4).

Regarding the Bd survey, all negative field and PCR controls were negative, and the positive controls were positive for Chytrid fungus, B. dendrobatidis (Bd). Bd was detected in five ponds. In spring 2021, one out of eight sites examined was positive for Bd, with the site located on the mainland. In spring 2022, five sites tested positive for Bd, including 2 sites on the mainland and 3 constructed ponds Öland. In summer 2022, all the tested sites were negative for Bd.

3.4 | Statistics

We found a non-significant relationship between amphibian occurrence and Bd ($p=0.85$), water temperature ($p=0.28$) and area ($p=0.56$).

For the co-occurrence analysis, of the possible 3003 species pair combinations, 2745 pairs (91.41%) were removed because the expected co-occurrence was less than 1 site, with 258 pairs analyzed. The 258 pairs included 78 species across 37 sites with 19 positive and 8 negative associations, with the remaining 231 deemed to be randomly associated. Among amphibians, *Lissotriton vulgaris*, *Bufo bufo*, and *Rana arvalis* showed negative associations with *Bos taurus* (cattle) or *Ardea cinerea* (gray heron). *Anas platyrhynchos* (mallard) and *Grus grus* (common crane) were found to have positive associations with *Triturus cristatus* (Figure 5).

MCDA site ranks were mostly consistent for each site when considering model sensitivity (Figure 6), with a few sites showing high variability. Here lower values (e.g., rank 1) indicate possible preferred habitat for amphibian translocation, whereas greater rank values indicate less favorable. Of these, 6 ponds were consistently low-ranking (lower 25%), suggesting more favorable habitat for amphibians compared to the other ponds. Seven sites had consistently high-ranking values (upper 25%) due to high cattle or stickleback influence.

4 | Discussion

We successfully showed that eDNA metabarcoding is effective for detecting *B. viridis*, as well as distinguishing them from other amphibian species, using established 12S primers. Beyond target species detection, the use of eDNA metabarcoding allowed for an in-depth biodiversity assessment across multiple vertebrate groups, including amphibians, birds, fish, and mammals using a single genetic marker. This approach proved valuable not only for tracking the presence of *B. viridis* over time to assess translocation success but also for generating fine-scale biodiversity information across Öland and neighboring northern Kalmar County. Furthermore, co-occurrence analysis revealed patterns of biotic interactions, which combined with MCDA analysis provides clear potential management strategies for translocation efforts of *B. viridis* and other species of conservation concern.

The successful detection of *B. viridis* before and after tadpole translocation in Öland's ponds underscores the utility of eDNA metabarcoding in conservation management. Traditional monitoring methods for translocated populations often pose risks of disturbance to fragile ecosystems or newly established species. In contrast, eDNA metabarcoding offers a non-invasive, efficient, and sensitive approach to assessing species presence and population dynamics over time, as demonstrated by its ability to detect the significant increase in *B. viridis* post-translocation. This finding aligns with a growing body of evidence supporting eDNA technologies for freshwater monitoring (Seymour 2022) of elusive or low-density species, including the great crested newt (*Triturus cristatus*) (Biggs et al. 2015) the North American beaver (*Castor canadensis*) (Burgher et al. 2024) and the invasive Asian carp

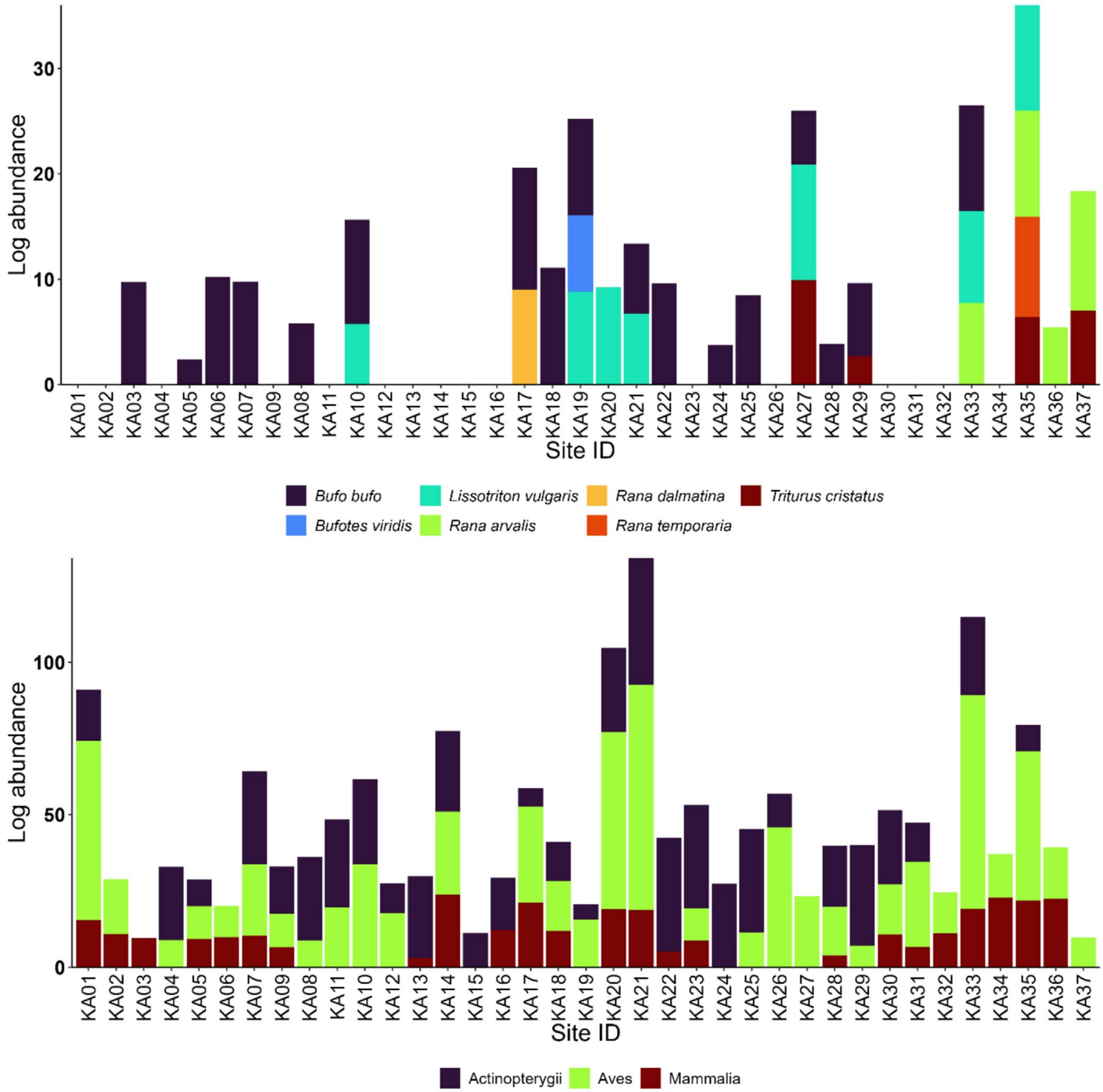

FIGURE 4 | Biodiversity as log-reads (y-axis) across the survey sites (x-axis) showing amphibians (top panel) and non-amphibian (bottom panel). Each color corresponds to a unique taxonomic group (e.g., species assignment). The size of the bar (unique sample site) corresponds to the log number of reads per the corresponding taxonomic group (y-axis). Site are arranged along the x-axis from east (left) to west (right) based on their geographic location.

(*Hypophthalmichthys* spp.) (Jerde et al. 2011). Recent applications further highlight its versatility, including disentangling population dynamics (Seymour et al. 2025; Si et al. 2025), evaluating seasonal shifts in riverine ecosystems (Perry et al. 2024; Seymour et al. 2021), and monitoring tropical mammals (Mena et al. 2021). Here, the consistent distribution of *B. viridis* tadpoles across the study area underscores the importance of incorporating eDNA metabarcoding into post-translocation monitoring programs to improve conservation outcomes and support the long-term management of vulnerable species like *B. viridis* (Burton et al. 2009). By continuing to integrate eDNA metabarcoding into post-translocation monitoring programs, conservationists can enhance detection sensitivity, optimize resource allocation, and gain critical insights into the ecological dynamics of translocated populations.

The use of eDNA metabarcoding in this study has proven to be a highly effective tool for assessing the total biodiversity of Swedish ponds, particularly regarding amphibian species. Specifically, we detected seven distinct amphibian species across 22 of the 37 surveyed sites using non-invasive and rapid assessment methods. Traditional survey methods for amphibians often involve time-consuming fieldwork, including visual encounter surveys, trapping, and netting, which can be invasive

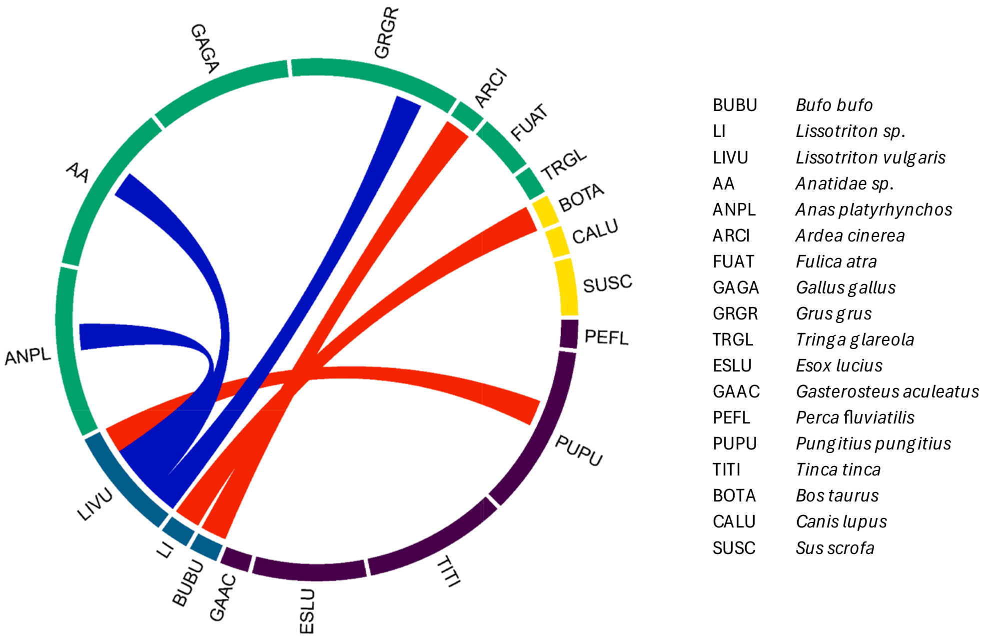

FIGURE 5 | Co-occurrence results for the amphibian specific interactions. Blue lines indicate significant positive associations, and red lines indicate negative positive associations. Each label is a unique identifier (genus or species level). Each outer ring color is a unique class color to help differentiate taxonomic subgroups.

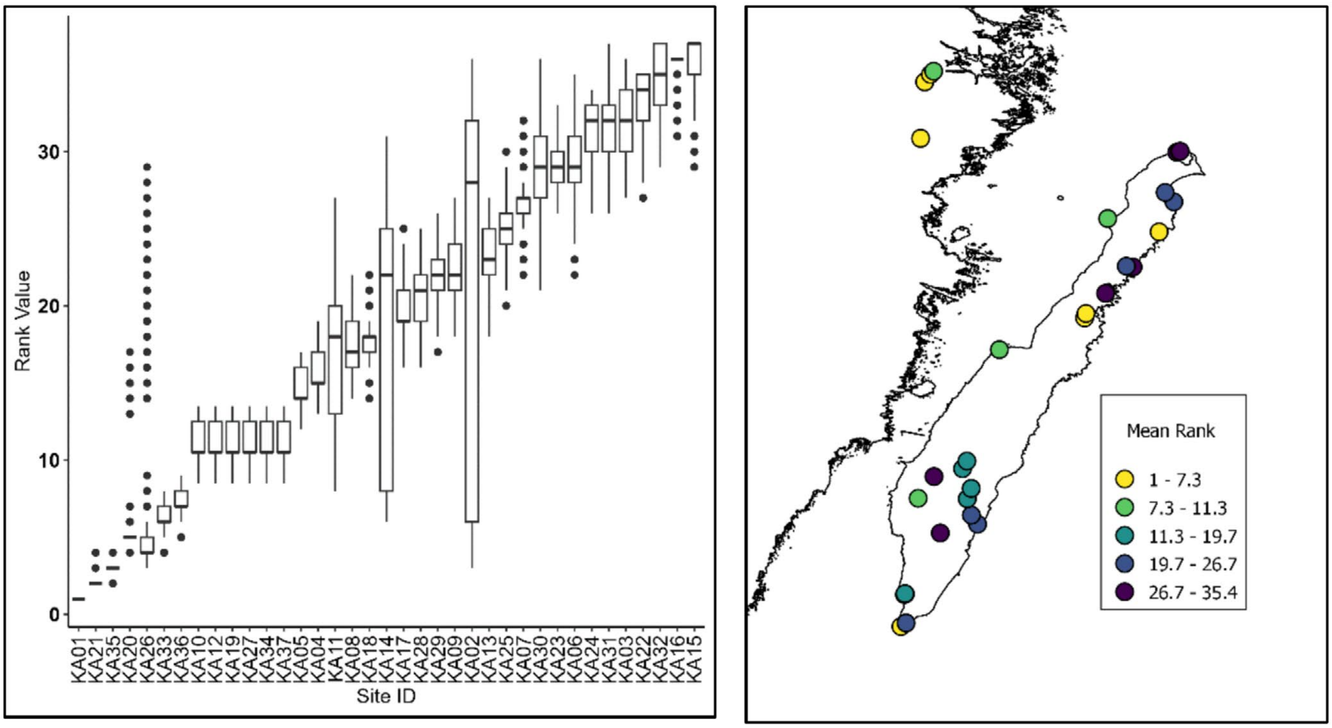

FIGURE 6 | Results of the Multi-Criteria Decision Analysis (MCDA) to rank potential pond suitability for amphibian translocation. The left panel shows each pond (x-axis) and the associated mean and standard deviation rank score across all possible MCDA models covering the sensitivity range. Lower values indicate ponds with potentially more favorable conditions for translocation. The boxes indicate the interquartile range (IQR) of the MCDA model results for each site, with the whiskers extending to 1.5 times the IQR range. Dots outside the whiskers denote outliers outside the whisker range, highlighting variability in site suitability assessments. The right panel depicts the mean values to their geographic location with the corresponding color to help illustrate the mean value distribution across the Öland and northern Kalmar county sampling sites.

and potentially harmful to the species and their habitats (Biggs et al. 2015). In contrast, our use of eDNA metabarcoding minimizes disturbance to local animal populations and their habitats while enabling simultaneous surveying of multiple species (Goldberg et al. 2016; Seymour and Smith 2023). We do note the absence, or at least the non-detection, of amphibians from several of the sites surveyed. Though we did not find indications of environmental factors attributed to amphibian presence or absence, we did find some key positive and negative biotic interactions associated with the presence or absence of amphibian species across the sampling sites.

Our co-occurrence analysis noted a negative association between amphibians and cattle, suggesting that sites with cattle usage may have detrimental effects on amphibian persistence. This has been suggested prior for other amphibian species, whereby grazing land use was found to be associated with less stable populations of amphibians compared to protected wetland sites (Burton et al. 2009), which may be related to the negative effects of either physical disturbance or alteration of vegetation (Perl et al. 2022). The negative interaction with ninespine sticklebacks is likely a case of competition. Ninespine sticklebacks prefer reedy environments, which overlap with amphibian habitat preference in most of these ponds (Hart 2003). Both are also likely to compete for similar food resources, such as small invertebrates, zooplankton, and insect larvae, particularly in ponds where resources are limited (Crnobrnja-Isailović et al. 2012). Additionally, amphibians, particularly eggs and tadpoles, can be prey for ninespine sticklebacks (Laurila and Aho 1997).

Birds, both piscivorous (fish-eating) and herbivorous (plant-eating), can have significant effects on amphibian populations, albeit in different ways, though our current understanding of direct bird-amphibian interactions is currently limited. Piscivorous birds, such as herons and kingfishers, may indirectly impact amphibians by preying on fish that share the same aquatic habitats (Hossack et al. 2022). Predation of fish can lead to reduced competition for food and space, potentially benefiting amphibian larvae and adults. Conversely, birds might also prey directly on amphibian larvae or adults, thereby reducing their populations. Younger birds have a much wider diet, consuming smaller prey at younger ages, including amphibians, which may explain the negative association found in this study (Molnár 1990). Though adult birds of several heron species are also well known to consume amphibians as well (Oscar 1912). As many bird species breed in Sweden during the summer months, including gray heron, the need to feed growing chicks may put additional pressure on amphibian populations at sites where predatory water birds have a greater presence (Fasola et al. 1993). Herbivorous birds, on the other hand, can influence amphibian populations through their effects on vegetation. By consuming aquatic and terrestrial plants, these birds can maintain habitat structure and the availability of shelter and breeding sites for amphibians (Kloskowski et al. 2010). Additionally, as birds and amphibians may prefer different vegetation, changes in plant composition by feeding birds could improve the abundance and types of invertebrates that amphibians feed on (Semlitsch et al. 2015).

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), is a well-known pathogen linked to the global decline of amphibian populations (Skerratt et al. 2007). Here we provide the first detection of Bd in Kalmar County, with all the positive detections occurring during spring (May) and no detections in early autumn (August). This is in accordance with other studies that have shown that the optimal months for detecting the pathogens are in spring (Talbott et al. 2018). The ponds that tested positive were also man-made, which may suggest that the equipment used for digging the ponds was contaminated and made the spread possible to Kalmar County. Specifically, in spring 2021, Bd was not detected on Öland Island, but two detections were registered in constructed ponds on the mainland. In 2022, three constructed ponds on Öland Island were positive, indicating an early invasion stage.

We did not find a significant association between the presence of Bd and amphibian occurrence in this study. This could be due to the heterogeneous and seasonal nature of Bd strains, with some being less harmful or even potentially resisted by certain amphibian populations (van Rooij et al. 2015). The Bd detection here emphasizes the need for a more nuanced understanding of the Bd pathogen, particularly its strain diversity. Furthermore, the role of environmental factors, such as climate and habitat characteristics, may also influence the prevalence and impact of Bd on amphibian populations (Rödder et al. 2009). Future research should delve deeper into these potential interactions and the role of the Bd strain variation to provide a more comprehensive understanding of the dynamics between Bd and amphibian populations in Sweden.

To provide suggestions for future translocation we utilized a multi-criteria decision analysis (MCDA) to evaluate pond suitability for amphibians based on our cooccurrence results. Six ponds consistently exhibited low rank values, suggesting they are highly suitable for amphibians. These sites were characterized by elevated crane and duck detection, and limited cattle or heron occurrence. Conversely, seven ponds had consistently high rank values, reflecting increasingly less favorable conditions driven by cattle and stickleback presence. Notably, the three ponds with mean ranks greater than 34, out of 37, were associated with either high stickleback or cattle detection, highlighting their likely high unsuitability for amphibian translocation. Some sites lacked occurrence of cattle, heron, stickleback, crane, or duck, which may require further data collection to fully assess their suitability for amphibians. While not definitive, these findings show the potential of integrating eDNA metabarcoding with methods such as MCDA to identify and prioritize habitats for future conservation efforts. Given the effectiveness of eDNA metabarcoding and the insights gained from MCDA, this integrated approach could prove integral to future conservation strategies, offering a framework for tracking amphibian populations and ensuring the protection of these vital ecosystems.

Our study underscores the transformative potential of eDNA metabarcoding as a cornerstone tool for modern conservation science, offering a non-invasive, scalable, and highly sensitive approach to monitoring species and ecosystems. By successfully tracking the translocation of *Bufotes viridis* and simultaneously assessing broader biodiversity patterns, we demonstrate how eDNA can bridge the gap between species-specific monitoring and holistic ecosystem assessments. The integration of co-occurrence analysis and MCDA frameworks further illustrates how eDNA data can inform targeted conservation strategies, optimizing habitat suitability evaluations and resource allocation. As global biodiversity faces unprecedented threats, the adoption of eDNA metabarcoding represents a critical step forward, enabling researchers to unravel complex ecological interactions, monitor vulnerable populations, and guide evidence-based management decisions. This approach not only enhances our capacity to conserve biodiversity but also paves the way for more adaptive and sustainable conservation practices in an era of rapid environmental change.

Author Contributions

M.S. wrote the manuscript. M.H., M.S., C.H., R.B., B.H., D.H., J.M., S.N., G.S.S., M.N., D.S., A.F., B.L. and K.F. contributed to the acquisition, analysis, or interpretation of the data. M.S., M.H. and C.H. conceived and designed the study. All authors provided comments on the final version.

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Conflicts of Interest

Authors Micaela Hellström is the CEO of MIX Research Sweden AB.

Data Availability Statement

Working data for the translocation and survey and the site meta data are available at https://github.com/MatSeymour/Seymour_etal_Toads_2025. Raws sequence data are deposited at the European Nucleotide Archive (project PRJEB96860).

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