Research Paper

Detection of three amphibian species in two city forests in Kristiansand, southern Norway, using environmental DNA analysis

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In the context of global amphibian declines, there is need to map local amphibian populations to establish baseline knowledge of potential future species losses. Even in well-studied lakes and ponds within popular recreational areas near urban centres, information on amphibian occurrence can be limited. In this study, the local distributions of *Lissotriton vulgaris*, *Rana temporaria* and *Bufo bufo* were assessed using eDNA analysis in two city forest areas of Kristiansand, Southern Norway. In the forest Baneheia, all three species were detected, although *R. temporaria* was present in only half of the lakes. In the forest Jegersberg, *L. vulgaris* was detected in nearly all lakes while *B. bufo* only in fewer than half, and *R. temporaria* was not detected. Our results support the public observations system Artsobservasjoner.no that *R. temporaria* is uncommon in the coastal area of Kristiansand. Comparison of active netting with eDNA analysis indicated that eDNA is a more reliable method for detecting amphibians. Interestingly, larvae of *L. vulgaris* were observed in one of the lakes in Baneheia inhabited by *Salmo trutta*, in a shallow bay with dense aquatic vegetation, demonstrating that newts can successfully reproduce alongside trout under certain conditions.

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INTRODUCTION

The number of amphibian species and their population sizes have declined substantially worldwide in recent decades (Halliday 2008; Daskalova *et al*. 2020). Species that were previously common, such as the common toad *(Bufo bufo)* are now experiencing serious declines in Europe (Petrovan *et al*. 2025). The two most significant drivers of this decline are habitat destruction and fragmentation, as well as the global spread of the chytrid fungus *Batrachochytrium dendrobatidis* (Becker *et al*. 2007). The chytrid fungus has also been detected in Norway (Taugbøl *et al*. 2017, 2021). Additional factors contributing to amphibian declines include environmental toxins, an increase in ultraviolet radiation, climate change, the introduction of invasive species, overexploitation (Beebee & Griffiths 2005; Halliday 2008) and pathogenic viruses (Origgi *et al*. 2017; Origgi *et al*. 2018; Origgi & Taugbøl, 2023). Given these challenges, it is increasingly important to map local amphibian populations to support potential conservation measures. Traditional amphibian mapping methods, including active netting (Dolmen 1991) and passive trapping (Dervo *et al*. 2013) are well established but may expose amphibians to varying levels of stress. Detecting sparsely populated amphibian populations using netting can be challenging as individuals may remain undetected. Moreover, passive traps can be difficult to carry around in remote and rugged terrain, and risk to be destroyed by people.

When higher organisms interact with their surroundings, they continuously release DNA into the environment through cells present in urine, faeces, hair and skin, as well as from dead individuals which decompose and release DNA into the environment (Thomsen & Willerslev 2015). This environmental DNA (eDNA) extracted

from water samples can be analysed using PCR or DNA sequencing and applied as a non-invasive method to record and monitor the presence of low-density species, especially useful for early detection of invasive species (Smart *et al.* 2015; Taugbøl *et al.* 2021). eDNA has been successfully used to detect amphibians by analysing water samples from freshwater ponds (Dejean *et al.* 2012; Ficetola *et al.* 2008; Villacorta-Rath *et al.* 2021). In several studies, eDNA methods have demonstrated higher detection success rate than traditional survey methods (Valentini *et al.* 2016; Sun *et al.* 2023). However, false negatives may occur, particularly in deeper ponds where eDNA may be more diluted or unevenly distributed (Taugbøl *et al.* 2025).

In Norway, amphibians inhabit their northwesternmost range in mainland Europe, and the number of species is low. Out of the six known amphibian species, only three are found in Kristiansand, the largest city in southern Norway, the common toad (*Bufo bufo,* Linnaeus, 1758), the common frog (*Rana temporaria,* Linnaeus, 1758), and the smooth newt (*Lissotriton vulgaris,* Linnaeus, 1758; Artsdatabanken, 2024). No other amphibian species have been recorded in Kristiansand region in Artskart.no, nor at the Natural History Museum, University of Agder. There has been no systematic mapping of the amphibian species in this area, and existing records consist mainly of anecdotal reports from a few ponds. Fish species (*Salmo trutta, Perca fluviatilis, Anguilla anguilla*) are found in some of the lakes, and brown trout (*S. trutta*) is known to displace native amphibians through predation on tadpoles, larvae and adults, or by outcompeting amphibians for macrobenthic prey (Lowe & Bolger 2002; Manenti & Manenti 2016; Tiberti & von Hardenberg 2012). Prior to this study, the local fishing organization planned to introduce brown trout (*S. trutta*) into the ponds located north of the city centre of Kristiansand, which made it urgent to map the distribution of amphibians in the area. Given this direct management and conservation context, our study aimed to map the local distribution of *B. bufo, R. temporaria* and *L. vulgaris* using eDNA analysis in small and large water bodies in two urban forest areas of Kristiansand, southern Norway. Active netting in one of the study areas was used to compare this traditional survey method with the eDNA approach. In addition, active netting during the larvae period was used to determine whether amphibians did successfully reproduce despite the co-occurrence with brown trout in one of the lakes.

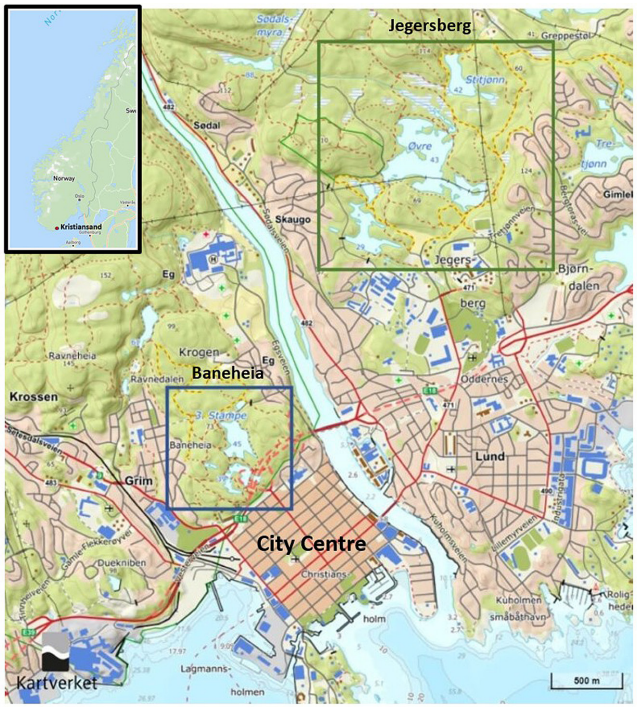


Figure 1. Map of the two study areas in Kristiansand, southern Norway. Jegersberg in the north (green square) and Baneheia in the south (blue square) The two forest areas are separated by the river Otra. (Kartverket, 2023).

MATERIALS AND METHODS

The two city forests named Baneheia and Jegersberg are situated about 40 meters above sea level north of the city centre of Kristiansand and are popular recreation spots easily accessible (Figure 1).

Baneheia

Baneheia forest area is a very popular recreational area located in direct contact with the city centre (Figure 1). There are six lakes of various sizes (Figure 2), which we divided into nine locations. The most eastern pond (A) is known to be a breeding pond for all three amphibians (*R. temporaria, B. bufo and L. vulgaris*), as several observations are made each year and some recorded by the public in Artsdatabanken (2024). We therefore hypothesize that these species are also found in the nearby lakes or are travelling through them on their way to pond A. The two largest lakes (B and D) were each split in two sampling sites (B1, B2, D1, D2), to maximize the amphibian detection probability. In addition, the largest lake (B) featured a constriction, consisting of a nutrient-rich area with aquatic vegetation, therefore, this was regarded as a third part of this pond (B3).

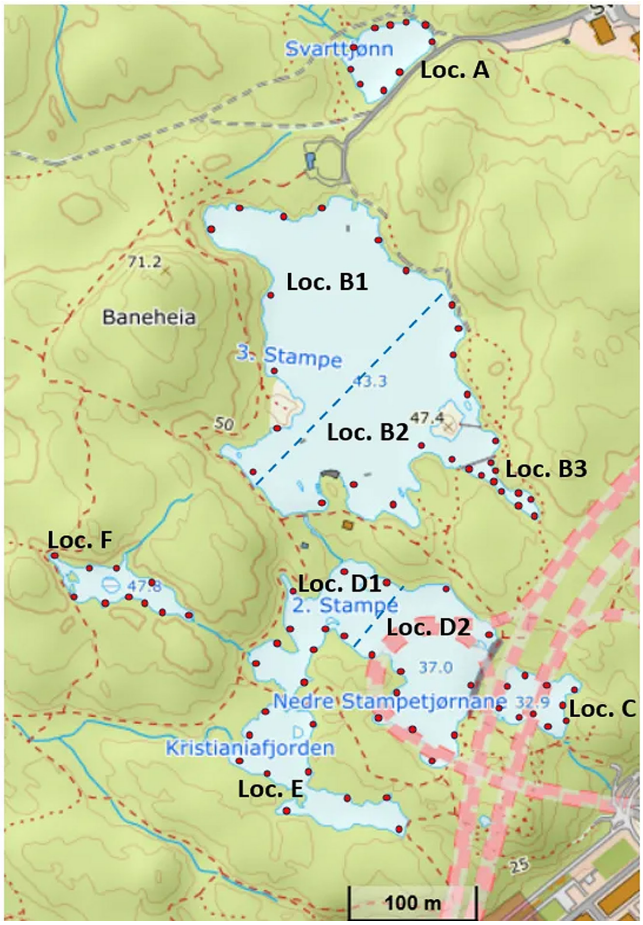


Figure 2. Map of Baneheia in Kristiansand 2019 with locations for sampling (Loc.A – Loc. F). Round symbols marking the water sampling and active netting. Map from NVE, (2020).

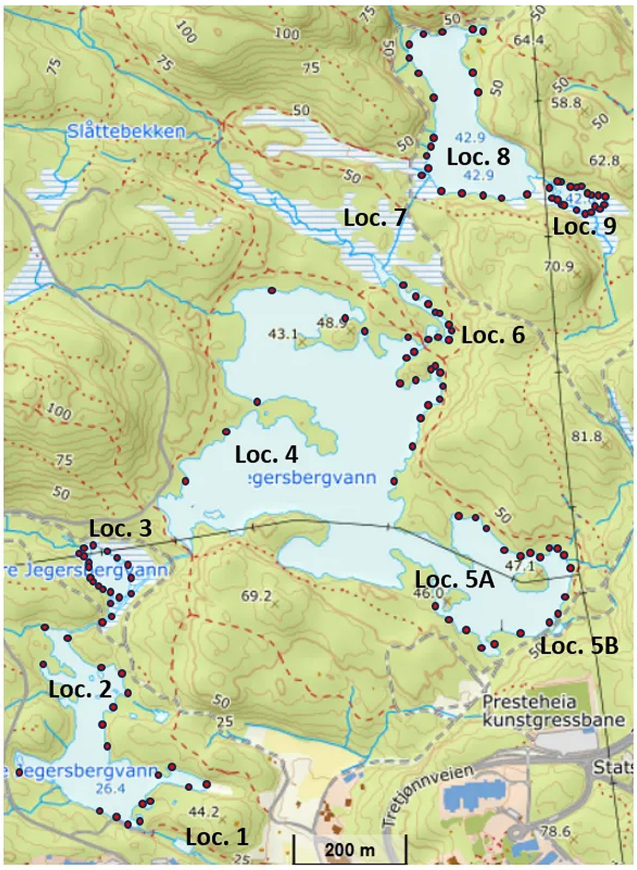


Figure 3. Map of Jegersberg in Kristiansand 2020 with locations for sampling (Loc. 1 – Loc. 9). Round symbols marking sampling spots. Map from Google Earth Pro (Google, 2023) with map overlay from Norgeskart (Kartverket, 2023).

Jegersberg

Jegersberg, is a forest area north of the city centre, on the northern side of the river Otra approximately 2 km from the seaside (Figure 1). The area includes three lakes (Stitjønn, Øvre Jegersbergvann and Nedre Jegersbergvann, Figure 3) and the stream Prestebekken, which flows into the Kristiansand fjord. Recent pH measurements (Miljødirektoratets vannmiljø-database 2021) indicate values that are favourable for amphibians (Dolmen *et al*. 2018). Brown trout (*S. trutta*) has historically been released into the Jegersberg lakes (KJFF 2020). Furthermore, Jegersberg supports European perch (*P. fluviatilis*) and eel (*A. anguilla*). To obtain more detailed information on amphibian distribution within this water system, we subdivided the large lakes into individually numbered locations evenly distributed around the lakes. These locations were numbered from the stream in the south named location number 1 (Prestebekken), to the last location number 9 (Stitjønn Øst) in the north. All locations are interconnected through small streams.

Collection of water samples

Water sampling for eDNA isolation took place in late spring in Baneheia (2019) and Jegersberg (2020). 100 ml water was collected from 50 cm depth at each of ten evenly distributed spots and pooled into a 1 litre container. Sampling spots were selected in the field, to optimize mapping of amphibian distribution. Priority was given to sunny spots with aquatic vegetation and were not too steep. Equipment was disinfected with Virkon-S between ponds, to reduce the risk of spreading potential water-borne pathogens. Between individual sampling locations, the equipment was treated with a 10% chlorine solution and rinsed with water from the pond. Samples were stored on ice and transported to the laboratory. As a negative field control, a bottle holding 1 litre of distilled water was carried in the field and subsequently treated identical to the water samples, to detect any potential inter sample contamination. At the laboratory, the water samples (1 litre from each location) were filtered by using an ILMVAC vacuum pump (GmbH), through a sterile cellulose-nitrate-filter (0.45 µm pore size, Thermo Scientific Nalgene). The filters were folded, put into an Eppendorf-tube, and stored at -20°C.

Manual capture of amphibians

Active netting (sweep sampling) was done along the shoreline of all six ponds in Baneheia during spring 2019, a few metres from water collection sites for eDNA. A long-handled, fine-meshed net with a frame of approximately 25 cm by 25 cm was used. Ten horizontal sweeps were made at each sampling spot along the edge of the ponds, following the method described by Dolmen (1991). Mid-summer 2021 sweep sampling was done in two of the ponds in Baneheia (A and B), searching for larvae and adult amphibians that could confirm reproduction. Sweep sampling was not performed in Jegersberg.

Isolation of eDNA and amphibian DNA

Filters from individual ponds, and negative field control, were cut into thin strips and placed in 2 mL tubes with 0.3 g zirconia-silica beads (diameter, 0.5 mm; Biospec Products). eDNA was extracted using DNEasy Blood and Tissue-Kit (Qiagen) as described in (Halvorsen *et al*. 2020). The concentration and purity of the extracted eDNA was measured with a spectrophotometer (NanoDrop™ One, Thermo Scientific). Locally collected dead individuals of the target amphibian species were used as positive tissue controls. Genomic DNA from these was extracted with the same procedure as given above. A positive control was made by keeping a smooth newt in a 50 mL plastic tube with water for 1 minute followed by isolation of DNA from the water sample.

Recording amphibian DNA by qPCR

TaqMan qPCR assays with species-specific primers and probes were used to detect the target amphibians. Primers and probe for *L. vulgaris* were found in Tingley *et al*. (2015) whereas primers and probes for *B. bufo* and *R. temporaria* were provided by Niclas Gyllenstrand (pers. com). DNA sequence of primers and probes is shown in Table 1. The reaction volume of the PCR-mix was 20 µL and included 1x TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems), 0.9 µM of each primer, 0.55 µM fluorescent labelled probe, and 3-4 µL of eDNA template. qPCR was conducted on a StepOnePlus real-time PCR instrument (Applied Biosystems) with the temperature profile: 50 °C for 2 min, 95 °C for 10 min, followed by 60 cycles of 96 °C for 15 s and 60 °C for 60 s, each cycle followed by detection of fluorescence. Every analysis was done in triplicate. A PCR run giving a CT value below 41 was scored as positive, and a sample is scored as positive when at least one of the PCR triplicates was positive. The same reaction mix and temperature profile were used for analysis of blank, negative field control and positive controls. Positive and negative controls were analysed in all PCR runs. The qPCR assays were previously evaluated for species specificity (Kastrud & Lindbo 2020).

Table 1. Sequence of the primers and probes used for the studied amphibians during qPCR (Tingley *et al*. 2015; Niclas Gyllenstrand pers. com.). Probes are 5' labelled with FAM and MGB-NFQ is attached to 3' end.

	DNA sequences
	Lissotriton vulgaris
Forward primer	5'– CCTACTTCTCCTACAAAGACATGCT–3'
Reverse Primer	5'–TTTCTGGGTCTCCTAAAAGGTTTGG–3'
Probe	5'– AAGGAGCATAAGTAAGAAACC–3'
	Bufo bufo
Forward primer	5'-CGGAACCGAACTTGTTCAG-3'
Reverse Primer	5'-ATGAAGAAAAAGAAGGTGGAGT-3'
Probe	5'-ATGAAGAAAAAGAAGGTGGAGT-3'
	Rana temporaria
Forward primer	5'-CGGAGTAATCCTCTTATTCTTAGTG-3'
Reverse Primer	5'-CGAGGTCGTAGCCAATGTAG-3'
Probe	5'-CTTCCGTGAGGGAAATGTCATTTTGA-3'

RESULTS

Recording of amphibians in Baneheia

DNA from *L. vulgaris* and *B. bufo* was detected in all six ponds and lakes within the Baneheia study area. Additionally, DNA from *R. temporaria* DNA was detected in three of the six ponds (pond A, B, D), whereas the ponds C, E, F were all negative (Table 2).

Using Z-swipes during all visits to Baneheia in 2019, we captured only two adults of *L. vulgaris* across the six ponds. The first adult was captured in pond A on April 29th, 2019, and the second in pond C on May 29th, 2019. To find out if *L. vulgaris* were only in transit during spring, or stayed in the pond and produced larvae, we did additionally z-swipes in two ponds in 2021. In total, eight larvae of *L. vulgaris* were captured: four larvae in pond A (Svarttjønn) on July 12th, 2021, and four larvae in pond B (3. Stampe) on July 14th of the same year (Table 3). Pond B is a lake with brown trout, and the larvae were detected in a shallow bay with dense aquatic vegetation.

Recording of amphibians in Jegersberg

All studied locations in Jegersberg, except for Loc. 1 (Prestebekken) and Loc. 9 (Eastern Stitjønn) tested positive for *L. vulgaris*. Only four of the studied locations were positive for *B. bufo*: Loc. 2 (Southern Nedre Jegersbergvann), Loc. 4 (Western Øvre Jegersbergvann), Loc. 5A (Eastern Øvre Jegersbergvann) and Loc. 8 (Western Stitjønn). None of the studied locations tested positive for *R. temporaria* during qPCR analyzation (Table 3).

All the negative and positive control samples got the expected values in qPCR, and the isolated DNA had acceptable concentrations and purity.

Table 2. Results from the qPCR analyses conducted to map the distribution of the three target amphibian species (*Lissotriton vulgaris*, *Bufo bufo* and *Rana temporaria*) in Baneheia and Jegersberg. All samples were analyzed in triplicate, with each + (positive qPCR result) or – (negative qPCR result) representing one replicate in the assessment of eDNA detection.

Pond no.	Date	Location (lake/pond)	*Lissotriton vulgaris*	*Bufo bufo*	*Rana temporaria*	Coordinates (Lat/Lon)
Baneheia						
1.	29.04.2019	Loc. C (1. Stampe)	+ + +	+ + +	- - -	58.1508, 7.9895
2.	29.04.2019	Loc. D (2. Stampe)	+ + +	- + +	- + -	58.1510, 7. 9881
3.	29.04.2019	Loc. B (3. Stampe)	+ + +	+ - +	+ + +	58.1533, 7.9864
4.	29.04.2019	Loc. B (3. Stampe)	+ + +	- + +	+ + +	
5.	29.05.2019	Loc. B (3. Stampe entire pond)	+ + +	+ + +	- + -	
6.	29.04.2019	Loc. F (4. Stampe)	+ + +	+ - -	- - -	58.1513, 7.9840
7.	03.06.2019	Loc. F (4.Stampe)	+ + +	+ + +	- - -	
8.	29.04.2019	Loc. E (Kristianiafjorden)	+ + +	+ + +	- - -	58.1502, 7.9863
9.	29.04.2019	Loc. A (Svarttjønn)	+ + +	+ + +	+ + +	58.1553, 7.9867
Jegersberg						
1.	09.06.2020	Loc. 1 (Prestebekken)	- - -	- - -	- - -	58.16529, 8.00228
2.	09.06.2020	Loc. 2 (Nedre Jegersbergvann South)	+ + +	+ + +	- - -	58.16614, 7.99962
3.	09.06.2020	Loc. 3 (Nedre Jegersbergvann North)	- + -	- - -	- - -	58.16941,7.99916
4.	08.06.2020	Loc. 4 (Øvre Jegersbergvann West)	+ + +	+ + +	- - -	58.17135, 8.00382
5A.	08.06.2020	Loc. 5A (Øvre Jegersbergsvann East)	+ + +	+ + +	- - -	58.16976, 8.01051
5B.	08.06.2020	Loc. 5B (Small pond next to loc 5A)	- - +	- - -	- - -	58.16927, 8.01271
6.	08.06.2020	Loc. 6 (Bay north of Øvre Jegersbergvann)	- + -	- - -	- - -	58.17409, 8.00777
7.	08.06.2020	Loc. 7 (Stream from Stitjønn)	- + -	- - -	- - -	58.17564, 8.00701
8.	09.06.2020	Loc. 8 (Stitjønn West)	+ + -	+ - +	- - -	58.17655, 8.00874
9.	10.06.2020	Loc. 9 (Stitjønn East)	- - -	- - -	- - -	58.17606, 8.01195

Table 3. Number of *Lissotriton vulgaris* specimens captured in Svarttjønn (A), 1. Stampe (C) and 3. Stampe (B) in Baneheia by performing ten Z-swipes with nets around the edge of the water.

Lissotriton vulgaris specimens found by active netting			
Date	Location	Swipes	Captured
29.04.2019	Svarttjønn (A)	10	1 adult
29.05.2019	1. Stampe (C)	10	1 adult
12.07.2021	Svarttjønn (A)	10	4 larvae
14.07.2021	3. Stampe (B)	10	4 larvae

DISCUSSION

In Baneheia, sweeping and eDNA analysis was performed in all six ponds, with very different result. eDNA from both *L. vulgaris* and *B. bufo* was detected in all six ponds included in our study, while the common frog eDNA was detected in five ponds (Table 2). However, by sweeping, *L. vulgaris* was found in two ponds, an adult smooth newt male in pond A and an adult female smooth newt in pond C. Our study therefore supports an earlier study from southern Norway (Strand *et al.* 2022) where *B. bufo* and *R. temporaria* were easily detected by eDNA while there were few records from traps or nets, and *L. vulgaris* were detected in both nets and eDNA. Also, other studies highlight that eDNA is a more sensitive method than traditional methods (e.g. Smart *et al.* 2015; Sun *et al.* 2023). In 2021, sweeping was repeated in two ponds (A and B) in mid-July, during the period with the highest probability of finding larvae of *L. vulgaris* and larvae were detected in both. This finding proves a successful breeding of *L. vulgaris* in Baneheia. Pond A (Svarttjønn) is a small pond without fish and well known for breeding populations of all three amphibian species, thus performed as a control pond. We were surprised to detect smooth newt larvae in the large waterbody of pond B (Third Stampe) where brown trout (*S. trutta*) are known to occur. Larvae were captured by sweeping in a shallow, vegetated bay with a high density of aquatic plants, which may provide refuge from fish predation. In Jegersberg, eDNA from *L. vulgaris* was detected at 8 out of 10 locations (Table 2), *B. bufo* eDNA was detected at four locations, while eDNA from *R. temporaria* was not detected in any of the ponds. This supports our general impression from field observations in the local area, as well as from public reports (eg. Artsobservasjoner.no), that *R. temporaria* is not a common amphibian species near the coast in Kristiansand, while *B. bufo* and *L. vulgaris* are observed more frequently.

In our study, *L. vulgaris* was the only amphibian species detected by sweeping and was found only in a few ponds. In contrast, eDNA analysis yielded positive detections in nearly all the ponds. eDNA analysis is a sensitive method for surveying aquatic organisms, particularly where species occur at low abundance, largely due to the high sensitivity of qPCR (Wilcox *et al.* 2013). Some positive detections may reflect individuals passing through the area, as many sampling sites were close together and several were connected by streams.

Even though eDNA analysis is a relatively reliable method, false negatives may occur. Several factors influence eDNA detection in water, including the time an organism has been present, its distance from the sampling point, and environmental conditions such as sunlight which degrades DNA over time (Pilliod *et al.* 2014). Enzymes and microorganisms in the water can further accelerate DNA degradation (Dejean *et al.* 2011). To reduce false negatives, three PCR replicates were included in the eDNA analysis. Use of replicates increases the likelihood of detecting target DNA when eDNA concentrations are low. Ficetola *et al.* (2015) shows in their study that the number of PCR replicates needed to detect species in the samples varies between different organisms (Ficetola *et al.* 2015). When detection probability is low additional replicates are recommended. Similarly, collecting multiple samples from the same locality can increase the probability of detecting species in the environment. Defining a sample as positive when only one of three replicates yields a qPCR product, as applied in the present study, increases the probability of false positives. However, when a species is rare in an area and consequently give very low eDNA concentrations, this level of sensitivity is considered acceptable, as demonstrated in several studies (e.g., Smart *et al.* 2016; Harper *et al.* 2018; Ongulee *et al.* 2025).

To obtain optimal results when surveying amphibians in natural habitats, eDNA analysis should be combined with conventional methods such as netting and sweeping (Smart *et al.* 2015), see review by Sun *et al.* (2023). eDNA alone does not provide information on life stage or absolute abundance (Rees *et al.* 2014), however recent work indicates that eRNA can distinguish larvae from adult amphibians (Parsley & Goldberg 2024).

Negative results from the sweep samples may reflect low species density, which reduce capture probability in large ponds with abundant hiding areas. In Baneheia we detected *L. vulgaris by sweeping* in three of six ponds, whereas eDNA indicated the species was present in all six ponds. Thus, in larger freshwater ponds with limited accessibility and low amphibian density – as in our study sites – eDNA appears more sensitive for presence/absence surveys. Sternhagen *et al.* (2024) compared the costs of conventional capture-based sampling (e.g. traps and sweep sampling) with eDNA analysis and found that eDNA had a "detection rate (per site) 5.55 times higher than conventional trapping and was 18.7% less expensive." "With eDNA analysis, additional resources can be invested toward expanding survey coverage and increasing sampling frequency, allowing managers to more effectively target subsequent intensive monitoring efforts".

Our study improves knowledge of three amphibian species in Kristiansand and indicates that eDNA based surveys outperform conventional capture-based methods. If eRNA can be applied reliably during the larvae period, it could reduce time and effort required for trapping and sweep sampling and provide more informative data on breeding populations in each pond, which is critical for ecological assessment and conservation management (Parsley & Goldberg 2024). Given ongoing climate change, which is producing longer, warmer and drier spring periods in southern Norway which leaves amphibian eggs on dry land as water level sinks in the ponds, as well as threats from fish introduction and emerging diseases, we are concerned about the future viability of local amphibian populations. Although *L. vulgaris* were detected in a lake containing brown trout, successful reproduction in this system was likely facilitated by the presence of dense aquatic vegetation in a shallow bay, which may provide refuge from fish predation. Consequently, we recommend avoiding the introduction of fish species into ponds and small lakes that support amphibian populations, as the negative ecological consequences of fish introduction for amphibians are well documented. Limited knowledge of amphibian distributions in Norway may result in the loss of undocumented populations. This study contributes to addressing this knowledge gap.

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