A seasonal follow up of *Batrachochytrium dendrobatidis* (Bd) and amphibian species detection in Norwegian watercourses using environmental DNA

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Preface

The fungus *Batrachochytrium dendrobatidis* (Bd) was first detected in Norway in 2017, and thus indicates the arrival of an invasive species in the country. The presence of Bd in Norway has previously been reported by Taugbøl *et al.*, 2017, 2018 and Ahmed *et al.*, 2019.

This report summarises data on Bd detection in Southern Norway from 2017 to 2020. Water samples from over 100 ponds were collected and described in previous reports (Taugbøl *et al.*, 2017, Taugbøl *et al.*, 2018, Ahmed *et al.*, 2019). A follow-up from the sampling sites with positive Bd results, as well as additional sites in the Viken region, are included in this report. In addition, we collected water samples in the Agder region and swab samples from captured amphibians.

This report summarises results from water samples and swabs collected from 173 ponds in Southern Norway and 126 ponds in Southern Sweden in order to assess the spread of the chytrid fungus and the risk for chytridiomycosis in Norway. In addition, the Norwegian samples were used to determine the distribution of amphibian species and their potential co-occurrence with Bd.

The project was funded by the Norwegian Environment Agency (Miljødirektoratet) and performed by eDNA solutions AB under the lead of Alexander Eiler. Johan Andersson carried out the sampling while Omneya Ahmed performed most of the method testing and DNA analyses at the labs of the Department of Biology and Environmental Sciences, University of Gothenburg. Mats Töpel and Tomas Larsson supported the data analysis and report writing.

1. Introduction

This project was initiated by the Norwegian Environment Agency with the purpose of i) developing a monitoring program for the fungus *Batrachochytrium dendrobatidis* (Bd) in Norway based on eDNA technology, ii) presenting an overall picture of the distribution of Bd in Norway (including the collection and analysis of new samples), and iii) developing cost-efficient and feasible ways to stop further spread of the fungus. In addition, information on the distribution of Bd in Sweden has also been included in this report, to assess the risk of spread of Bd between the countries.

1.1 Biodiversity loss and the need for environmental monitoring programs

Freshwater environments are threatened by loss of biodiversity, and there are over 100 documented cases of extinction in such environments during the 2000s (Dudgeon *et al.*, 2006; Tickner *et al.,* 2020). In the case of amphibians, 30% of all freshwater species are threatened by extinction. Therefore, an emergency recovery plan for freshwater biodiversity has been presented which includes protecting and restoring critical habitats and preventing and controlling introduction of non-native species (Tickner *et al.*, 2020). In order to document and evaluate biodiversity loss, a number of national monitoring programs have been introduced (e.g. the United Nations Environment Program, and the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services). For these programs to be successful, they must be designed in such a way as to minimise the main sources of error (Skalski and Robson, 1992; Thompson *et al.*, 1998; Thompson 1992). A common source of error comes from the fact that no survey method can detect all individual species in an ecosystem. A second source of error arises from the difficulty in efficiently investigating large areas, meaning that conclusions must be based on samples taken from a few locations within an area. This is compounded by the fact that many collection strategies are based on subjective assessments of how representative certain sampling locations are, or how easily accessible some premises are. Thirdly, researchers face major problems in identifying and counting species, since organisms can be difficult to detect or difficult to distinguish from one another (e.g. microbial species such as Bd).

1.2 Environmental DNA (eDNA) analysis can improve environmental monitoring

Technological advances over the past decades now allow us to overcome the third source of error. Environmental DNA (eDNA) analysis is a rapidly evolving methodology and is used for studies of current and past biodiversity (Tablerlet *et al.*, 2012; Valentini *et al.*, 2009). eDNA has broad applications in, for example, the analysis of biodiversity in microbes, plants, and animals (Eiler and Bertilsson, 2004; Valentini *et al.*, 2015; Zinger *et al.*, 2012), analysis of diet (Deagle *et al.*, 2005, Pompanon *et al.*, 2012), reconstruction of past biodiversity or environmental changes (Gigue-Covex *et al.*, 2014; Jørgensen 2012; Langenheder *et al.,* 2016; Parducci *et al.*, 2013) and environmental monitoring (Eiler *et al.*, 2013; Jerde *et al.*, 2011).

Despite the high sensitivity of eDNA methods, the research community is well aware that species detection by this approach is not free from sources of error similar to those found in conventional methods. In fact, incomplete detection is inevitable when assessing the presence/absence and number of species, regardless of the methods used (MacKenzie *et al.*, 2006; Valentini *et al.*, 2015). This can be partially addressed by the high capacity of eDNA methods that allow more samples to be processed than when conventional methods are used. In addition, adaptive sampling can increase the likelihood of detection. An example of this is sampling during the optimal season, which seems to be May/June for the fungal pathogen *Batrachochytrium dendrobatidis* (Ahmed *et al.*, 2019).

1.3 The case of an invasive fungal species threatening amphibian diversity in Norway

A focus area of environmental monitoring programs in recent years has been amphibians and, in particular, various species of salamander (order Urodela) which have decreased in number and diversity since the 1950s throughout the world. The spread of invasive species as a result of human activity is the greatest threat, but there is also a link to climate change (Beaury *et al.*, 2020). One such invasive species is the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), which was first discovered in Norway in 2017 by examining eDNA from five sites in Akershus (Taugbøl *et al.*, 2017). Bd causes the infectious disease chytridiomycosis via its zoospores, where amphibians act as hosts for the parasite. The disease itself was first described in 1998 (Berger *et al.*, 1998, Longcore *et al.*, 1999) and it was then shown that chytridiomycosis was the cause of massive declines in frog populations in mountainous regions of Australia in the 1970s, leading to a large reduction in the number of species, and in some cases to total extinction (Berger *et al.*, 1998; Laurance *et al.*, 1996; Longcore *et al.*, 1999; Mendelson *et al.*, 2006). A similar decline cannot be ruled out in Norway, which is why a monitoring program has been put in place by the Norwegian Environment Agency to follow the presence and spread of Bd.

In Norway, Bd has previously been detected in seven locations in the region of Viken where Bd was found in watercourses of various sizes during 2017 and 2019. While Bd has been detected in watercourses, the few animals tested have so far shown no signs of infection, although it is very difficult to visually identify Bd-infected individuals. Similar studies in other Nordic countries have found Bd in various water bodies in Denmark and Sweden, and also in association with infected amphibians, in particular when using genetic assays (Rosquist 2020).

One way to identify these hosts, which requires significant resources in the form of time-consuming field work, is to visually identify Bd-infected individuals in the field. The limitations of this approach can be mitigated by DNA-based methods for detection, which provide a better opportunity to identify infected individuals. These DNA-based methods still involve invasive and time-consuming handling of individuals in the field, entail a risk that the animals are unintentionally harmed, and provide little statistical support for the number of infected animals in an area. A number of studies have also shown that the combination of multiple samples followed by species occurrence modeling can show the probability of Bd occurrence in an area and thus also give an estimate of the uncertainty in the measurement result (Chestnut *et al.*, 2014; Kerry and Schaub 2012; MacKenzie *et al.*, 2006).

2. Objectives

At present, we have little knowledge about how common the invasive fungal parasite Bd is in Norway. In addition, water bodies known to harbour amphibian species, where Bd has not been detected, need to be resampled in order to minimise false negative detection. Previous results have suggested that the best sampling time for Bd in water is during late spring (Chestnut *et al.*, 2014; Ahmed *et al.*, 2019).

After a first broad screening of over 100 water bodies throughout Southern Norway in 2019, sampling in 2020 was focused on the regions of Viken (area between Oslo and the Swedish/ Norwegian border) and Agder. In addition to water samples, swab samples from around 20 amphibian specimens were also collected in order to test for the presence of Bd.

To assess the risk of further spread of Bd in Norway, we included results of Bd distribution in Sweden. Distribution of Bd in Sweden can help to define the current invasive front and the environmental boundaries (e.g. climate) of Bd occurrences in Scandinavia.

3. Methods

3.1 Sampling design

Samples were obtained in Southern Norway, along the Swedish border, around Oslo, and in the Agder region. Based on the results from the 2019 study (Ahmed *et al.*, 2019), the end of May and beginning of June were chosen for sampling. In total, 91 bodies of water, including forest-, agricultural- and urban ponds, were sampled, and 16 swabs from various amphibian specimens were obtained in 2020.

Figure 1. Swabbing a Smooth Newt (*Lissotriton vulgaris*) at Nesodden outside Oslo.

Swabs were taken from the frogs' skin, and tadpoles were captured from a few sampling sites. Swabs and tadpoles were preserved in 70% ethanol on site and stored at 4°C until further analysis (Figure 1). Water samples were obtained by filtering between 75 and 1440 ml (median 480 ml) from each site. Filters were immediately frozen in liquid nitrogen and then stored at -80°C until further analysis. Chemical and physical parameters such as temperature, conductivity, pH and turbidity were also measured on site.

3.2 DNA extraction

For water samples, DNA extraction was performed using a Qiagen DNeasy PowerWater Sterivex Kit. The quality of the extracted DNA was estimated using NanoDrop. An internal control (genesig Advanced Kit) was added to 30 random samples and co-extracted. 4 µL of the internal control were mixed with the lysis buffer, as described in the extraction protocol. Purification and amplification of the internal control DNA was tested, as well as the presence of PCR inhibitors.

Prior to DNA extraction with the Qiagen DNeasy Blood & Tissue Kit, swabs and tadpoles were dried to remove the ethanol. The quality of the extracted DNA was estimated using NanoDrop.

3.3 qPCR assays

Laboratory workspace and equipment were sterilized by UV-light, DNase solution (5% chlorine solution) and 70% ethanol. Filter pipette tips were used in all steps of the laboratory work, and handling of positive controls was performed in a post-PCR room which is physically separated from the pre-PCR room to avoid contamination. Negative controls of DNase/RNase-free water were used in each qPCR assay.

Two real-time PCR assays were used to screen for *Batrachochytrium dendrobatidis* as follows:

The first assay was the genesig Advanced Kit (Primerdesign Ltd), containing a positive control for Bd, an internal control, and a specific primer/TaqMan probe mix targeting the 5.8S ribosomal RNA gene. Serial dilutions of the positive control ($1e^{4}$ - $1e^{-1}$ cells) were prepared. The real-time PCR assay was conducted in a BioRad qPCR machine in 10 µL reaction mixtures. The reaction mixtures contained 5 µL BioRad SsoAdvanced Universal Probes Supermix, 0.5 µL primer/probe mixture, and 1.5 µL DNAse/RNase-free water. 3 µL of positive control or unknown DNA sample was used to reach 10 µL final volume. Amplification conditions were 2 min at 95°C, followed by 10 s at 95°C and 1 min at 60°C for 50 cycles. The protocol was published on protocols.io (dx.doi.org/10.17504/protocols.io.baiticen).

The second assay used a primer set of ITS1-3 Chytr (5'- CCTTGATATAATACAGTGTGCCATATGTC-3'), 5.8S Chytr (5'- AGCCAAGAGATCCGTTGTCAAA-3'), and Chytr MGB2 (FAM-5'TTCGGGACGACCC-3'-NFQ- MGB), with a final concentration of 250 nM of TaqMan probe and 900 nM of primers (Boyel *et al.*, 2004). The same amplification conditions were used as described above. Aliquots of a DNA extract of Bd from the lab of Anssi Laurila (Uppsala University) were tested with the TaqMan assay and proven to produce good

results (R2 > 0.98 and efficiency 89-100%). A serial dilution of 100, 50, 25, 10, 1, 0.1 and 0.01 genomic equivalents (GE) of the standard was tested and the first five dilutions (100, 25, 10, 1, 0.1 GE) were used in further qPCR assays. The reaction mixtures contained 10 µL BioRad SsoAdvanced Universal Probes Supermix, 1.8 µL of each forward and reverse primer, 0.5 µL of probe and 0.9 µL DNAse/RNase-free water. 5 µL of positive control or unknown DNA sample was used to reach 20 µL final volume. The same amplification conditions as described above were used. The protocol was published on protocols.io (dx.doi.org/10.17504/protocols.io.bn2z-mgf6).

In the case of the TaqMan qPCR assays, 0.5 µL of internal control DNA was added to a single reaction per sample. This included all samples with no co-extracted internal control DNA.

Two samples (no. 10 and 47; Table 2) showing high Cq values in the qPCR analysis were purified for Sanger sequencing to confirm the identity of the amplified fragment. Samples were purified by QIAquick PCR Purification Kit (Qiagen) and sequenced by Eurofins, Sweden.

3.4 Amphibian species amplification

Prior to library preparation, both workspace and equipment were cleaned as described above. The first PCR experiment was performed in a pre-PCR laboratory while the second PCR and purifications were carried out in a post-PCR environment to safeguard against contamination. We also used controls to monitor contamination, including PCR blanks for each experiment.

A mock community of six amphibian species was prepared from swabs (preserved in 95% ethanol) provided by the lab of Anssi Laurila (Uppsala University) and Nordens Ark, including DNA extracts of *Rana temporaria, Rana arvalis,* and *Bufo bufo,* and swabs of *Bufotes variabilis, Epidalea calamita,* and *Pelophylax lessonae.* The swabs were removed from ethanol and left to dry in clean Eppendorf tubes for 2 hours at 50°C to evaporate residual ethanol. DNA from the dried swabs was extracted using the Qiagen Blood & Tissue DNA extraction kit. Quantification of the DNA concentration was performed using the PicoGreen assay (ThermoFisher, US). Approximately 10 ng of DNA was used for the amphibian amplification procedure. A pool of the six species was mixed in equal amounts and a serial dilution from 1.0-0.001 ng was prepared for the sequencing.

Paired-end sequencing on the Illumina MiSeq platform required two steps of PCR. The first step amplified the 12S rRNA gene of amphibian species using primers: batra-F 5'-ACACTC TTCCCTACACGACGCTCTTCCGATCTNNNNNNACACCGCCCGTCACCCT-3' and batra-R 5'-AGACGTGTGCTCTTCCGATCTNNNNNNGTAYACTTACCATG TACGACTT-3'. Human blocking primer: TCACCCTCCTCAAGTAACTTCAAAG CA-SPC3I was used to bind to human DNA and prevent its amplification (Valentini *et al.*, 2016). This first PCR was performed in a final volume of 25 µL containing 5 µL of 5X Q5 reaction buffer, 0.2 µM batra primers, 0.2 mM dNTPs, 4 µM human blocking primer and 0.02 U/µL Q5 High-Fidelity DNA Polymerase (New England Biolabs). 1 µL of positive control or

5 µL of environmental DNA samples was used as a template for the PCR reaction. The expected size of the amplified region was 170 bp, and a 1% agarose gel was used to visualise the target bands (Figure 2). Amplification conditions were 30 s at 98˚C, followed by 35 cycles of 30 s at 98˚C, 30 s at 57˚C and 1 min at 72˚C. A final extension step at 72˚C for 7 min was then performed.

Each sample was amplified in triplicate and then pooled before the purification step. After amplification, the PCR products were purified using the AMPure magnetic beads assay (Beckman Coulter, US). A combination of indexed forward and reverse primers (0.25 µM) and 2 µL of purified PCR product was used in the second PCR. Amplification conditions were 30 s at 98˚C, followed by 15 cycles of 10 s at 98˚C, 30 s at 66˚C, and 30 sec at 72˚C, then a final extension step at 72˚C for 2 min. The second PCR was purified using the same assay as above, and quantified using the PicoGreen assay. The protocol was published on protocols.io (dx.doi.org/10.17504/protocols.io.973h9qn).

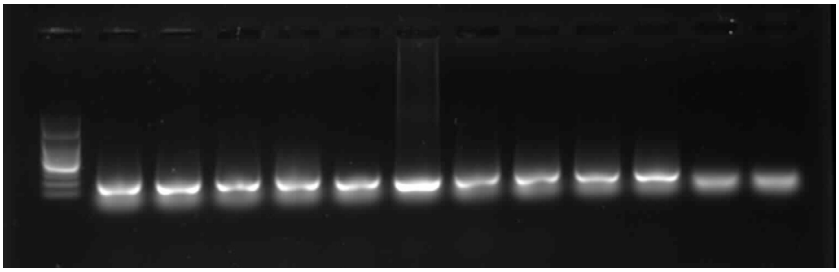

Figure 2. Agarose gel of the PCR products from the amphibian assay.

Equal amounts of DNA from each sample were mixed to prepare the sequencing library. The final pooled samples were visualised by gel electrophoresis to ensure that no extra unwanted fragments existed. The amplicon library (MiSeq Reagent Kit v2 Micro) was then sequenced on an Illumina MiSeq machine, generating paired-end reads of 150 bp length.

3.5 Sequence data analysis

Raw sequences were first processed with cutadapt v1.18 (Martin, 2011) to remove PCR primers, and then analysed with the R package dada2 v1.14.1 (Callahan *et al.*, 2016) for denoising and sequence-pair assembly. After manual inspection of quality score plots, forward and reverse reads of the amplicon sequencing run were trimmed to 50 bp length. Additional quality filtering removed any sequences with unassigned base pairs and reads with a single Phred score below 20. After reads were dereplicated, forward and reverse error models were created in dada2 with a subset of the sequences (10^8 nucleotides). Amphibian 12S rRNA gene amplicons were assembled by merging the read pairs. Chimeras were removed using *removeBimeraDenovo* from the dada2 package, which resulted in the final amplicon sequence variant table. Taxonomy (to species level) was assigned using blastn v2.8.1 (Altschul *et al.*, 1990) and an in-house 12S rRNA gene database. Sequences were assigned to specific species using cutoff criteria of identity >96% (which corresponds to approximately two mismatches) and alignment length >45 bp.

4. Results and Discussion

4.1 Molecular assays for Bd detection

The two tested assays amplify different regions of the 5.8S rRNA gene and intergenic spacer region of the rRNA operon located in the mitochondrial genome of Bd. qPCR efficiency of the genesig Advanced Kit and the TaqMan assay was on average 98.82% and 99.34%, respectively. The limit of quantification of the genesig kit was 1 and 0.5 genome equivalents for the TaqMan assay. The limit of detection of the genesig kit was 0.1 and 0.05 genome equivalents for the TaqMan assay (Figure 3). Thus, it can be speculated that the TaqMan assay is slightly more sensitive than the genesig kit assay.

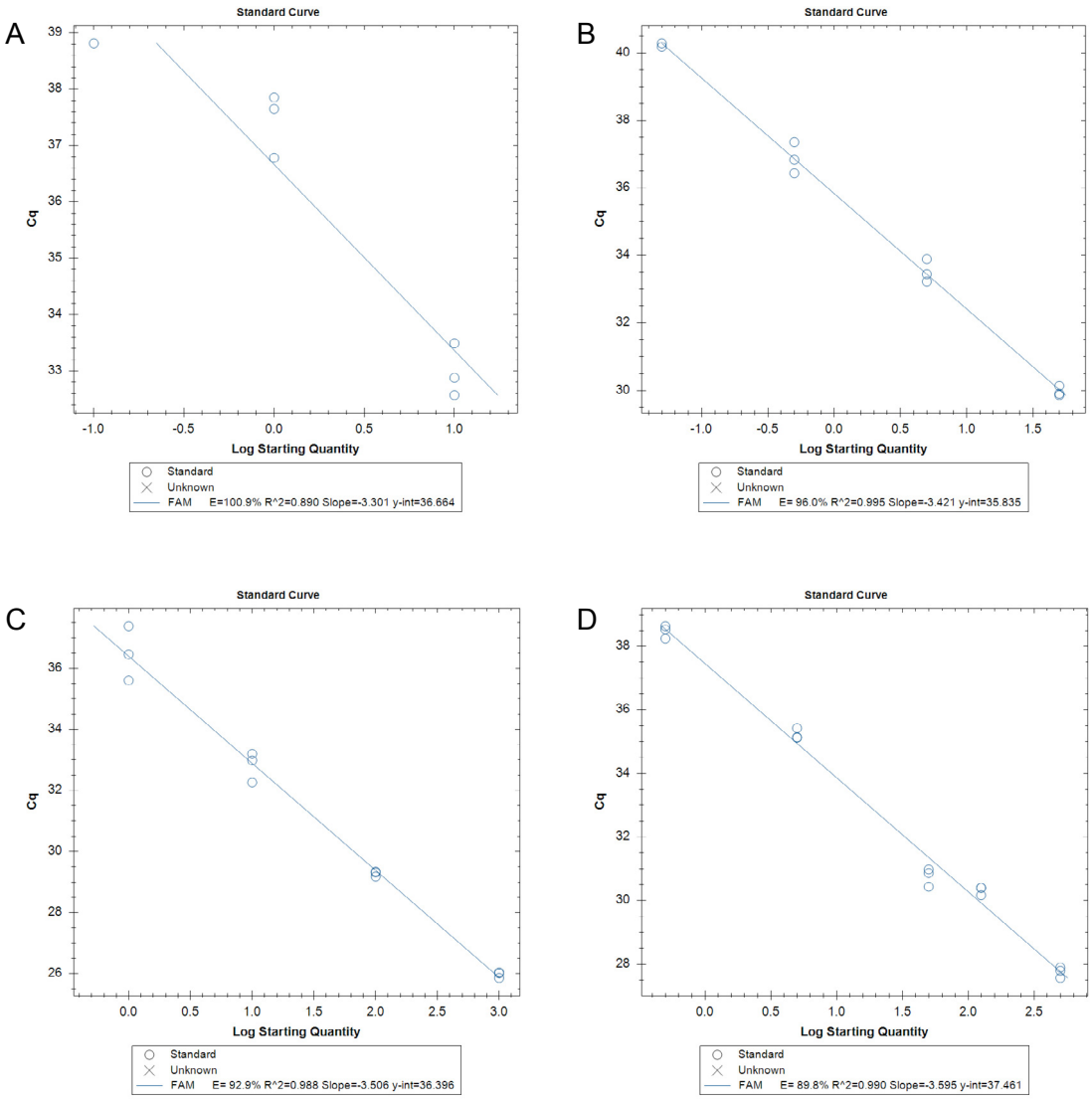

Figure 3. Standard curves visualizing the limit of detection and quantification of genesig (A) and TaqMan (B) assays and limit of quantification for the genesig (C) and TaqMan (D) assays.

Both assays generated positive Bd signals in all replicates from the three locations Østre Støken, Ringveien 52, and Odden, and from one swab from the location Værmyr (Table 2). Two additional locations, Butjenna and Rokkevannet, generated a positive Bd signal when using the TaqMan assay. However, as a high Cq value (as in the case of Butjenna and Rokkevannet) can represent unspecific amplification and thus false positive Bd results, both PCR products were purified and Sanger-sequenced for validation. The resulting sequences were used to query the NCBI nt-database using blastn, which resulted in 100 % matches with sequences from Bd. These results validate Bd detection and indicate that the TaqMan assay can potentially detect Bd at lower concentrations than the genesig Advanced Kit, with similar sensitivity.

In addition, standards and samples were spiked with an internal control (IC), using the VIC fluorophore channel, multiplexed with the TaqMan assay. The Cq value of the IC is usually close to 27 (genesig Advanced Kit). While most of the samples amplified in the same range, two samples (samples 22 and 67) showed a delayed Cq value of around 38 (Figure 4). Three samples, 12, 24 and 26 which were co-extracted with the IC gave no positive signal for the IC. Only sample 26 generated a positive IC signal after 1:50 dilution, while 12 and 24 generated no signals after dilution. Furthermore, sample 72 was not co-extracted with IC but had shown normal positive IC signal after a 1:50 dilution factor. This variability in Cq results strongly suggests the presence of inhibitory factors in a minor fraction of samples, which needs to be taken into account when interpreting negative results. For samples 12 and 24, the IC DNA might have been bound to inhibitory compounds, or the PCR polymerase might have been inhibited, either of which would have interfered with amplification.

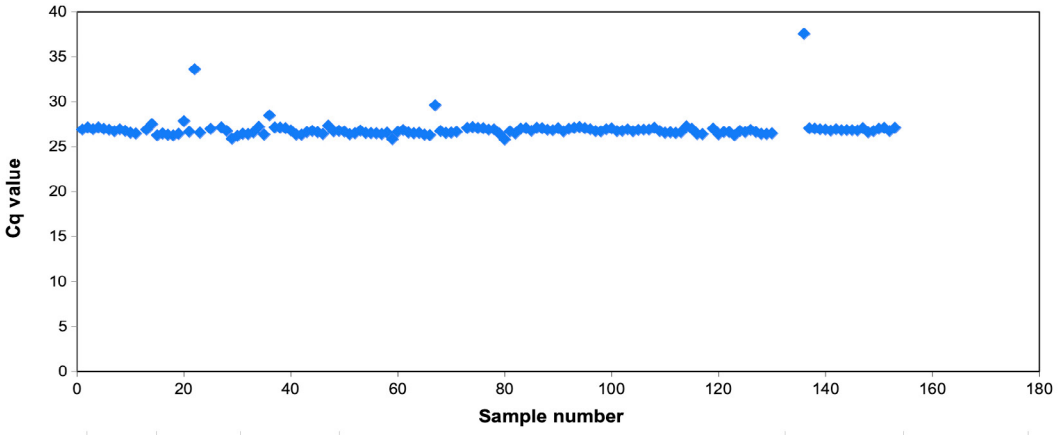

Figure 4. Scatter plot of Cq values of standard and samples multiplexed with the positive internal control.

Table 1. Location of samples which generated Cq values greater than 27 Cq and thus were likely compromised by PCR inhibition.

ID	Cq	Date	Type	Locality	Lattitude	Longtitude
22	34	26-May	forest	Svarttjønn, Eg, Baneheia, Kristiansand, Ag	58.155545	7.987209
67	30	1-June	forest	Bogslunden	59.40177	10.66751
26	38	26-May	forest	Myanstjønna, Lindesnes, Ag	58.037285	7.613671
12	> 50	25-May	agriculture	Harveland, Arendal, Ag	58.538413	8.827017
24	> 50	26-May	urban	Kolekniben, Brennåsen, Kolekniben, Kristiansand, Ag	58.130066	7.866646

Table 2A. Metadata of samples with positive Bd-qPCR signals. Sample 38B represents a swab sample. Vol: volume, Temp: temperature and Turb: turbidity.

ID	Date	Vol	Temp	Turb	pH	Type	Locality	Municipality	Region	Latittude	Longitude
69	01-jun	600	24	2.16	7.9	Agriculture	Østre Støkken	Ås	Viken	59.636339	10.74289
75	01-jun	360	23.4	4.47	7.09	Suburb	Ringveien 52	Nesodden	Viken	59.8396	10.692585
74	01-jun	NA	24.6	0.81	7.7	Suburb	Odden	Nesodden	Viken	59.86984	10.660407
38 B	30-maj	540	21.9	6.33	7.8	Agriculture	Værmyr, Enningdalen	Halden	Viken	58.926428	11.493064
10	25-maj	480	18	2.12	5.96	Forest	Butjenna	Tvedestrand	Agder	58.600471	8.888018
47	30-maj	450	16	1.75	7.4	Agriculture	Rokkevannet. Vi	Halden	Viken	59.200981	11.342246

Table 2B. qPCR results of genesig and TaqMan assays from samples with positive Bd-qPCR signals. Sample 38B represents a swab sample. The positive signals for samples 10 and 47 (as indicated by *) were validated by 100 % sequence similarity of the PCR product with Bd. Ct: cycle threshold and STDEV: standard deviation.

ID	Genesig advanced assay		TaqMan assay	
	Average Ct	STDEV	Average Ct	STDEV
69	35.72	0.68	41.62	5.66
75	36.26	0.82	39.37	2.08
74	36.67	0.49	37.36	0.35
38 B	33.76	0.32	34.74	0.47
10*	-	-	45.92	0.79 (two replicates)
47*	-	-	39.3	0.26 (two replicates)

4.2 Distribution of Bd in Norway

During 2019, 110 water samples were collected for eDNA analyses in Norway, and in 2020 an additional 91 samples were collected. DNA from 16 wild amphibian individuals was also collected in 2020. While three water samples were positive for Bd in 2019 (Ahmed *et al.*, 2019), five water samples and one swab sample were positive in 2020 (Table 2A and 2B). The positive swab, from Norway, came from a *Bufo bufo* (Common toad, Nordpadde) captured from a pond close to the Swedish border. The establishment of the chytrid fungus in the region of Viken is now well documented (Taugbøl *et al.*, 2017). Still, the distribution of the fungus seems to be restricted to a few bodies of water in the region.

An alarming finding was a positive water sample (sample 10 from Butjenna) in the region of Agder which was validated by sequencing the qPCR amplicon fragment. This first potential detection of Bd in the Agder region highlights the need for further monitoring of Bd in the area. The arrival of Bd in Agder may pose a threat to the populations of *Pelophylax lessonae* (Pool frog, Damfrosk) in the surrounding area (Figure 5).

A comparison with results in other Nordic countries was made possible by the establishment of a Nordic network for detecting amphibian diseases (Bd and *Batrachochytrium salamandrivorans* [Bsal]) (Rosquist 2020). First detections of Bd were made in Denmark in 2007 (Scalera *et al.*, 2008), in Sweden in 2010 (Kärvemo *et al.*, 2015) and in Norway in 2017 (Taugbøl *et al.*, 2017). To our knowledge, Bd has so far not been detected in Finland. Analysis of a Swedish data set that includes a total of 145 water samples and 114 swab samples taken during the years 2017 to 2019 from 126 water bodies, showed that 17 water bodies contain Bd. These infected water bodies were located in Skåne, Blekinge and Västra Götaland (Rosquist *et al.*, 2019). This presence and wide distribution of Bd in Scandinavia thus necessitates coordinated management action and public awareness in the respective infected areas, in order to minimise further spread of Bd.

4.3 Swabs vs. water samples

Three types of samples were used to detect Bd in this project; swabs from adult animals, tadpoles, and filtered water samples. The advantage of swabbing animals is that the result shows both the number of infected animals and which species are infected, but the method is time-consuming as a large number of animals must be sampled to enable an infection in the population to be detected. Water samples are easy to collect but do not provide information on the number of infected individuals or which species are infected. Furthermore, eDNA samples can be taken during the day, while swabbing is usually done at night when the animals are more active and easier to capture.

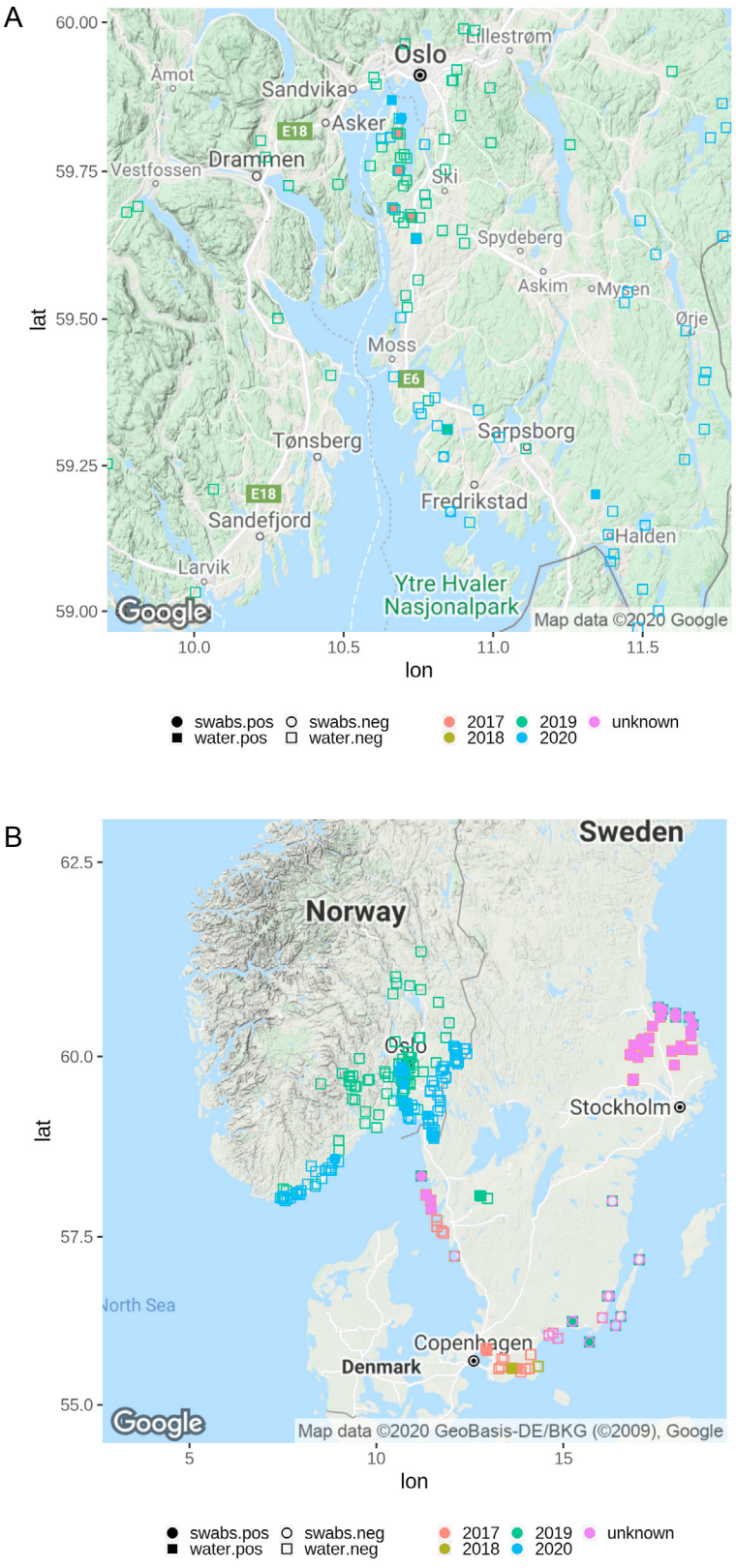

Figure 5. Distribution of Bd as assessed by the analysis of water samples, swabs, and tadpole DNA around Oslo (A) and Southern Norway and Central Sweden (B). Full symbols indicate detection of Bd by the TaqMan assays while open symbols indicate negative results.

Sampling using both these methods was conducted at six sites during this project. In addition, we have included the results from investigations using both methods at 16 sites in Sweden, Denmark, and Finland. Our results show cases of positive Bd detection in water samples and negative swabs (Ringveien 52) as well as positive swabs and negative water samples (Værmyr). In the case of a previous analysis (Rosquist 2020), it was argued that swabs provided a higher likelihood of Bd detection. However, the comparison of detection probability between the two methods in the Rosquist (2020) study was highly biased, as only a single water sample was taken from an individual system, while swabs were taken from multiple specimens (up to 97) during multiple sampling occasions spread over several years from a single system.

A suggested next step is therefore to assess detection probabilities of the two methods using comparable sampling efforts, i.e. analysing the same number of water samples and swabs over a single season.

Confirming results with diverse methods such as swab- and water-based detection, as well as multiple molecular assays, increases confidence in positive detection. In the case of the Butjenna and Rokkevannet sampling sites, a positive signal was only produced by the TaqMan assay. Although these results were confirmed by Sanger sequencing, further sampling is also required to increase confidence, and assess the spread of Bd in the Agder region. This may include additional sampling in the area in 2021. As the qPCR results and Sanger sequencing were finalised in August (i.e. after the optimal sampling season for Bd), additional sample collection was not performed in 2020.

4.4 Disease management

The chytrid fungus Bd spreads naturally between bodies of water via migrating animals such as amphibians, birds, and insects (Lips 2016). It can also be spread by human activities such as transfer of boats and equipment, or by capture and release of infected amphibians. Countering chytridiomycosis-driven amphibian declines should consist of a multifaceted approach adapted to the stages of pathogen emergence (pre-arrival, invasion front, epidemic, and established) (Langwig *et al.*, 2015a). The identification of pathogen invasion stages through monitoring programs is important for disease management, where pathogen abundance and distribution will determine the invasion stage. Current disease management approaches include prevention and short-term solutions (e.g. *ex situ* breeding programs, antifungals), but long-term, *in situ*, sustainable solutions are required if the goal of amphibian conservation is to be attained. This implies neutralising the disease threat in wild populations.

(1) Early detection of invading pathogens, facilitated by monitoring programs in combination with good communication between the public and government agencies, represents a very important first step. With the arrival of Bd and other invasive animal diseases, there is clearly a need to educate the public to avoid rapid spread and subsequent extinction of native species in Norway.

(2) The detection of invading pathogens in an isolated area requires fast disease management, such as application of antifungal chemicals, to eliminate pathogen establishment (Lachish *et al.*, 2010). This strategy was successfully applied to eliminate the black-striped mussel (*Mytilopsis sallei*) by using a large quantity of chlorine and copper sulfate in three Australian bays (Ferguson 2000). However, in the absence of long-term disease management *in situ*, any short-term measure is unlikely to result in significant conservation success (Hudson *et al.*, 2016).

(3) Factors involved in Bd spread such as host factors (e.g host densities, contact rates) and natural geographical features can help in disease management (Smith *et al.*, 2005; Kilpatrick *et al.*, 2006a). Augmenting host demographic rates by reducing other stressors such as predation can facilitate the evolution of host resistance or tolerance (Kilpatrick 2006).

(4) Translocations/reintroductions often have strong appeal but such approaches have little probability of success, as shown in multiple trials (Garner *et al.*, 2016).

(5) Altering biotic communities to reduce transmission and pathogen population growth (Langwig *et al.*, 2012), as well as to decrease pathogen viability in the environment (Langwig *et al.*, 2015b), may be another option. The probiotic, antifungal bacterial species *Janthinobacterium lividum,* isolated from salamander skin, has been used to reduce chytridiomycosis infection of mountain yellow-legged frogs both in the laboratory and in the field (Harris *et al.*, 2009; Woodhams *et al.*, 2019). Another potential route is the manipulation of the food web in amphibian ponds, with the aim of exploiting zooplankton grazing in order to decrease survival and dispersal of chytrid zoospores (Frenken *et al.*, 2020). However, several general issues need to be overcome before probiotics and predators can be considered viable mitigation strategies. In particular, the potential risk that biotic community changes pose to ecosystems and public health requires assessment, as well as the practicalities of such biotic management practices.

Integrating multiple actions and performing interventions in collaboration with researchers will facilitate the use of an adaptive approach, and will maximise the probability of success.

Further research combining empirical, observational, and experimental data with statistical and mechanistic models is an effective way to determine the key factors driving transmission and disease dynamics (McDermott 2019), and thus identify sustainable management practices for disease suppression.

4.5 Amphibian distribution

Amphibian diversity was assessed using a metabarcoding approach based on amplification and sequencing of the mitochondrial 12S rRNA gene of amphibian species. Most of the samples were well amplified with a clear target band corresponding to the 12S rRNA gene, but amphibians could only be detected in 41 out of the 91 samples analysed (Figure 6).

The four amphibian species *Bufo bufo* (Nordpadde), *Lissotriton vulgaris* (Småsalamander), *Rana arvalis* (Spissnutefrosk) and *Rana temporaria* (Buttsnutefrosk / Vanlig frosk) were detected in site 59 (Dyvlene) while three amphibian species were detected in Åkebergmosen, Nestangen, Dyvlene, Gylterud, and Flyginnsjøpytten. Furthermore, two species were detected in 17 sites and only one species was detected in the remaining sites. *Bufo bufo* was overall the most commonly detected species. *Lissotriton vulgaris* was identified in Østre Støkken and Ringveien 52 which are sites where Bd has been detected.

The amphibian diversity data obtained by metabarcoding supports traditional observations on the distribution of amphibian species in Norway. As such, the amphibian species *Bufo bufo, Lissotriton vulgaris, Rana arvalis, Rana temporaria,* and *Triturus cristatus,* seem to be prevalent in Norwegian waterbodies, and thus currently not threatened.

While we aim to identify potential association or dissociation of Bd with specific amphibian species, the limited number of Bd positives still preclude such a co-occurrence analysis. With more data we may be able to identify amphibian species that have a higher degree of association or dissociation with Bd than others, thus representing susceptible or resistant species, respectively.

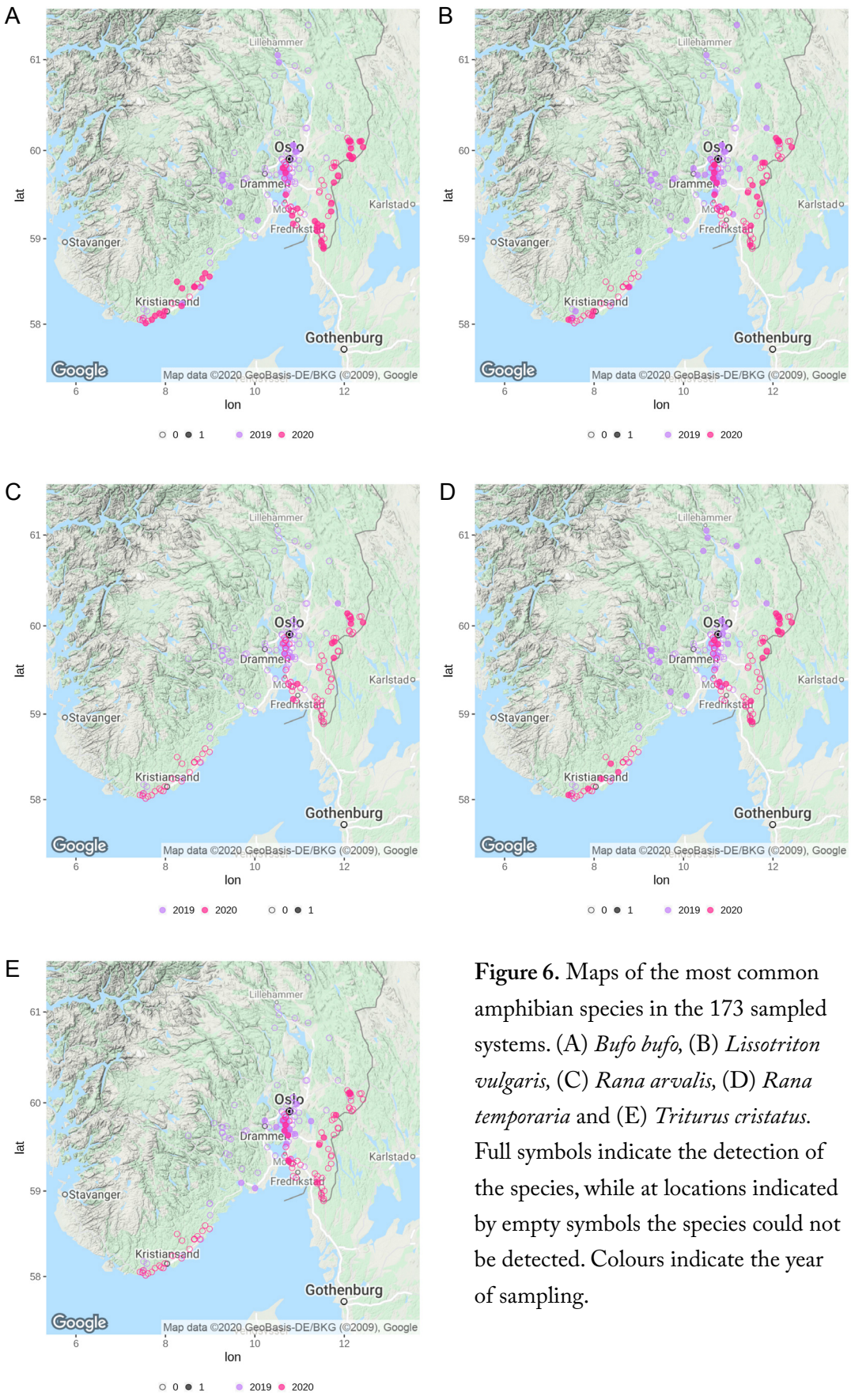

Figure 6. Maps of the most common amphibian species in the 173 sampled systems. (A) *Bufo bufo*, (B) *Lissotriton vulgaris*, (C) *Rana arvalis*, (D) *Rana temporaria* and (E) *Triturus cristatus*. Full symbols indicate the detection of the species, while at locations indicated by empty symbols the species could not be detected. Colours indicate the year of sampling.

5. Summary and future suggestions

Our results confirmed detection of Bd in five sites in south-eastern Norway. We therefore conclude that the risk of a Bd outbreak and amphibian decline caused by chytridiomycosis may be low at the moment. Still, the first detection of Bd in Agder calls for further monitoring efforts to be put in place. Observations of Bd made in this project have been reported to the citizen science database Artsportalen (www.artsobservasjoner.no), except for the novel finding at the site in Agder which requires confirmation during the upcoming sampling season.

Skin microbes living in a symbiotic relationship with frogs' skin may have a role in protecting their host from fungal infection (see disease management section). In addition, planktonic grazers can control survival and dispersal of chytrid zoospores. Therefore, we suggest that research efforts should be directed towards screening amphibian skin microbiota and fungal predators (i.e. planktonic grazers) to identify their potential as mitigators in disease management.

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