Validation of an eDNA-based method for the detection of wildlife pathogens in water

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Abstract

Monitoring the occurrence and density of parasites and pathogens can identify high infection-risk areas and facilitates disease control and eradication measures. Environmental DNA (eDNA) techniques are increasingly used for pathogen detection due to their relative ease of application. Since many factors affect the reliability and efficacy of eDNA-based detection, rigorous validation and assessment of method limitations is a crucial first step. We evaluated an eDNA detection method using in-situ filtration of large volume water samples, developed to detect and quantify aquatic wildlife parasites by qPCR. We assessed method reliability using *Batrachochytrium dendrobatidis*, a pathogenic fungus of amphibians and the myxozoan *Tetracapsuloides bryosalmonae*, causative agent of salmonid proliferative kidney disease, in a controlled experimental setup. Different amounts of parasite spores were added to tanks containing either clean tap water or water from a semi-natural mesocosm community. Overall detection rates were higher than 80 %, but detection was not consistent among replicate samples. Within tank variation in detection emphasises the need for increased site-level replication when dealing with parasites and pathogens. Estimated parasite DNA concentrations in water samples were highly variable and significant increase with higher spore concentrations was observed only for *B. dendrobatidis*. Despite evidence for PCR inhibition in DNA extractions from mesocosm water samples, the type of water did not affect detection rates significantly. Direct spiking controls revealed that the filtration step reduced detection sensitivity. Our study identifies sensitive quantification and sufficient replication as major remaining challenges of eDNA-based methods for detection of parasites in water.

- 1 **Key words:** aquatic parasites, environmental DNA, *Tetracapsuloides bryosalmonae*,
- 2 *Batrachochytrium dendrobatidis*, in-situ filtration, quantitative real-time PCR

1. INTRODUCTION

Monitoring the occurrence and prevalence of parasites and pathogens (hereafter summarised as parasites) is crucial for identification of high infection-risk areas, as shown for example in intestinal protozoan parasites (Helmi et al. 2011) and viruses (Grewar et al. 2019). Knowledge of pathogens in the environment is also important for human health and for the mitigation and prevention of zoonoses (Cunningham et al. 2017). Furthermore, it eases planning of disease control, and even informs eradication measures, for example of *Schistosoma mansoni* in Kenya (Sengupta et al. 2019). Therefore, comprehensive and regularly updated monitoring campaigns and surveillance for the creation of maps of parasite prevalence (Diarra et al. 2019) are important to implement management measures effectively. However, current management of most diseases is re-active, rather than pro-active, in that disease outbreaks are often detected by chance, then triggering measures and recommendations aimed at preventing further spread of the disease, such as Ebola in West Africa (Woolhouse et al. 2015). Conventional methods of parasite monitoring are cost- and labour-intensive, further impeding regular and comprehensive monitoring campaigns for parasites in the environment.

A fungus of the Phylum Chytridiomycota, *Batrachochytrium dendrobatidis*, causative agent of chytridiomycosis, has become infamous by causing mass mortalities in amphibians worldwide (Fisher et al. 2009). It is suspected to be a major driver of extinction of frog species (Skerratt et al. 2016) and has sparked extensive monitoring programs (Seimon et al. 2017). Conventional monitoring requires capture of sometimes rare and elusive amphibian species and taking skin swabs from the animals, before releasing them again. Fish hosts of *Tetracapsuloides bryosalmonae* (Myxozoa), causative agent of proliferative kidney disease (PKD) are less fortunate:

1 after capture by electrofishing, pathological examination requires extraction of their
2 kidneys (Wahli et al. 2007). *T. bryosalmonae* causes high mortality in young-of-the-
3 year salmonid fish both in the wild and in fish farms in Europe and North America
4 (Clifton-Hadley et al. 1984, Hedrick et al. 1993). It is, therefore, of both conservational
5 and economic import to closely monitor such diseases, whilst reducing the costs and
6 conservation impacts of the surveillance itself.

7
8 As a non-invasive alternative to the described approaches, parasite detection in
9 water samples using environmental DNA (eDNA) techniques (see Bass et al. 2015
10 for a review) could alleviate the aforementioned issues. Environmental DNA is
11 defined as DNA that is not directly retrieved from the target organism, but from its
12 environment and can be in the form of dead skin cells, mucus, blood, extracellular
13 DNA and more (Thomsen & Willerslev 2015). The same or similar eDNA techniques
14 can be implemented for parasite detection, which, however, will mostly be detected
15 as whole organisms, e.g. as zoospores or eggs. Research on detection in water has
16 mostly focused on human parasites and pathogens, e.g. *Listeria* and *Salmonella*
17 (Arvanitidou et al. 1997, Lyautey et al. 2007, Papić et al. 2019), *Legionella* (Moreno
18 et al. 2019), and other potentially pathogenic microorganisms (Dalu et al. 2011).
19 Furthermore, disease agents of economically important species such as *Vibrio* spp.
20 in shellfish (Mok et al. 2019), or *Myxobolus cerebralis*, etiological agent of whirling
21 disease in salmonids (Richey et al. 2018), are also of great interest. The
22 advancement of molecular analytical methods has increased the potential of eDNA
23 techniques and water sampling for parasite and pathogen detection across a broader
24 range of systems and habitats and has been reviewed for microorganisms in drinking
25 and recreational waters (Aw & Rose 2012, Botes et al. 2013, Bonadonna et al. 2019)
26 and eukaryote parasites (Bass et al. 2015). Crayfish plague agent *Aphanomyces*

1 *astaci* spore concentrations, derived via quantitative real-time PCR (qPCR), positively
2 correlated with parasite prevalence in captured signal crayfish from three Nordic
3 lakes (Strand et al. 2014). Rusch et al. (2018) developed and successfully applied for
4 the first time an eDNA-based detection method of *Gyrodactylus salaris*, an
5 ectoparasite severely damaging Atlantic salmon populations and fisheries. These
6 studies illustrate the value that molecular, or eDNA-based, detection techniques of
7 parasites in water add to achieve deeper understanding of parasite distribution,
8 abundance and spread, and ultimately, implementation of management measures.

9
10 However, as eDNA-based detection methods have moved out of their infancy,
11 limitations have become more apparent. The target species might not be detected if it
12 is rare or exists in low densities, such as invasive species at the invasion front (Jerde
13 et al. 2011), or even in controlled experiments (Moyer et al. 2014). This could be the
14 case for parasites, i.e. when infection prevalence and / or intensity in the host
15 population is low or when, as for chronic infections, parasite shedding rate is low.
16 This can be alleviated by collecting larger sample volumes, which in turn, however,
17 could lead to increased accumulation of inhibitory compounds (Wintzingerode et al.
18 1997 and references therein). Coincidence of eDNA sampling timing with active
19 parasite release from hosts can be crucial to maximise detection rates and therefore
20 efficacy of the method. For parasites, this requires knowledge of periods of highest
21 transmission and proliferation, which are often seasonal, e.g. related to water
22 temperature (Mok et al. 2019), or can be tightly linked to host activities, such as the
23 mating season in North American crayfish (Wittwer et al. 2018). Furthermore, most
24 parasite DNA in environmental samples is in the form of spores, cysts or eggs, which
25 need to be physically disrupted to access the DNA, requiring rigorous extraction
26 protocols involving multiple rounds of alternate freezing and boiling of samples (Leles

et al. 2009). These aspects all affect the reliability and efficacy of eDNA-based detection methods of parasites in water. Rigorous validation is therefore crucial to assess limitations of such methods.

In this study we validated an eDNA detection method developed for aquatic parasites. The method consists of in-situ filtration of large volume samples (5 L), a DNA extraction protocol deemed efficient at removing inhibition, and quantitative real-time PCR (qPCR) analysis, which is more sensitive than endpoint PCR (Wilcox et al. 2013). The study followed four main aims: Firstly, we aimed to assess reliability of the method, by investigating in a controlled environment the detection success of two parasite species: The amphibian chytrid fungus *Batrachochytrium dendrobatidis*, and the PKD-causing *Tetracapsuloides bryosalmonae*. This was achieved by adding different estimated concentrations of parasite spores to water tanks, followed by taking water samples from these tanks and measuring detection success in the water samples. The detection results were evaluated using occupancy models that estimated detection probabilities on tank, sample and qPCR replicate levels. Occupancy models can quantify the reliability of the detection on different hierarchical levels, i.e. by quantifying the probability of detection in a single water sample, given a successful detection on the level of the sampling site from which multiple water samples were taken (Dorazio & Erickson 2017). Occupancy models furthermore help in determining factors influencing detection success, such as elevation or water quality (Schmidt et al. 2013), or in our case, spore concentrations and water source. Secondly, to test the potential effect of inhibitors and to simulate more realistic conditions, half of the tanks were filled with water originating from a large mesocosm containing a semi-natural community of aquatic organisms, and compared to tanks filled with tap water. Thirdly, to estimate accuracy of the quantification with qPCR we

1 compared estimated parasite DNA concentrations in water samples with spore
2 concentrations in the tanks from where the samples were taken. Lastly, effects of the
3 filtration process and the filter on detection variability were investigated by
4 comparisons with samples directly spiked with spores in the lab, with and without
5 filter. Validation of eDNA detection methods as conducted in this study are important
6 for comparability between methods and help towards finding a consensus of which
7 methods are most efficient and reliable for species detection in surveys.

9 2. MATERIALS & METHODS

10 2.1. Generation of parasite spore solutions

11 Zoospores of *Batrachochytrium dendrobatidis* (isolate BEW2; Farrer et al. 2013) were
12 obtained from cultures maintained according to Longcore et al. (1999) and Longcore
13 et al. (2000; see the Supplement for a detailed protocol). To generate a solution
14 containing *B. dendrobatidis* zoospores, culture medium was transferred to a 1.5 ml
15 plastic tube with a pipette, taking care not to touch the bottom of the culturing flask to
16 prevent the collection of zoosporangia. *Tetracapsuloides bryosalmonae* spores were
17 obtained from spore sacs extracted from bryozoans (*Fredericella sultana*) collected in
18 the field one day prior to the experiment (River Glane, Switzerland, September 2018).
19 Under a microscope with x8 magnification, *T. bryosalmonae* spore sacs were
20 carefully excised from the zooids using forceps and collected in a separate petri dish.
21 The spore sacs were ruptured with a needle to release the spores. The solution
22 containing spores was pipetted into a 1.5 ml plastic tube and mixed thoroughly by
23 pipetting up and down.
24 For both parasites, ten 6.6 µl subsamples of the solution were used to estimate spore
25 densities microscopically using a counting chamber (KOVA Glasstic slide 10 with
26 grids, Kova International Inc., Garden Grove, California). The mean spore

concentration of both parasites was calculated from the ten subsamples and used to further dilute the solutions to 200 spores per ml, which were then stored in glass Schott flasks on ice until further usage on the same day. For dilution, clean tap water used for culturing bryozoans and fresh MGHTL culture media was utilised for *T. bryosalmonae* and *B. dendrobatidis*, respectively.

2.2. Experimental setup

The experiment with *B. dendrobatidis* was conducted on 14.11.2018 and the experiment with *T. bryosalmonae* on 18.09.2018 in a designated outdoor mesocosm facility on the roof of a laboratory building. The experiment included either 20 (*B. dendrobatidis*) or 16 (*T. bryosalmonae*) 90 L polyethylene tanks (Eurokraft Kunststoffmulde by JOPA® Kunststofftechnik S&W GmbH, Ahlen, Germany) filled with 20 L of either tap water or water obtained from a 1000 L mesocosm that simulated a natural pond community. The pond mesocosm was set up in May 2018. It contained tap water and local leaf litter as a source of nutrients, and it was inoculated with a townet sample of phyto- and zooplankton from nearby Lake Greifensee. In both experiments, the tanks holding 20 L of water were spiked with 100 µl, 1 ml or 10 ml of spore solution for an estimated final concentration of 1, 10 or 100 spores L⁻¹ with four tanks per concentration. As a control, an additional four tanks, two for each water source, were not spiked, to test for background parasite DNA in the water sources (n = 16 tanks in total). In the experiment with *B. dendrobatidis*, an additional treatment of 1000 spores L⁻¹ in four tanks was included (n = 20 tanks in total), after the earlier experiment on *T. bryosalmonae* yielded surprisingly inconsistent detection at concentrations up to 100 spores L⁻¹. All tanks were filled with source water (mesocosm or tap) before the start of the experiment, and three 5 L subsamples were collected and filtered from each tank immediately

after addition of the parasite spore spike to the tank. Prior to filtering, the spike was mixed into the tank water thoroughly by hand with clean gloves. The master spore solution was kept on ice between filtration of different tanks. In order to accomplish each of the experiments in one day, spiking and filtration were conducted for both water types in two blocks, resulting in two replicates of each water quality treatment ("mesocosm water" and "tap water") per spore concentration. Within blocks, the order of filtration was randomised. Water was filtered from each tank as it would be in the field (Fig. 1), including a negative control at the beginning to test for cleanliness of the equipment. Sampling of one tank, including filtration of the negative control at the beginning, lasted around 45 minutes. The filters were stored on ice for a maximum of 4 h and frozen at -80°C until DNA extraction.

2.3. Sampling method

Environmental DNA samples were collected using an in-situ filtration system with a peristaltic pump (Alexis peristaltic pump, Proactive Environmental Products LLC, Bradenton FL, USA). Figure 1 shows a sketch of the assembled filtration system. A PVC tube (length = 2 m, $\varnothing$ (outer / inner) = 13 / 10 mm) is attached to a polypropylene plastic funnel ($\varnothing$ (outer / inner) = 100 / 95 mm) with its opening covered with a synthetic polyamide mesh with particle retention size of 100 μ m (Sefar AG, Heiden, Switzerland), to serve as an inlet for the unfiltered water. To ensure submersion, a 1 kg coated lead diving weight was attached to the funnel with a 20 cm long rope and a longer piece of rope was fastened to the weight to facilitate retrieval from the water. The PVC tube was connected to a silicone tube (75 cm long, $\varnothing$ (outer / inner) = 10 / 5 mm) with a plastic reducer (PP $\varnothing$ 8-12/4-8 mm). A filter holder (Swinnex, 47 mm, Merck Millipore, Schaffhausen, Switzerland) was attached to the other end of the silicone tube using two connectors (double nipple PVC 1/4" x

1 ¼" inner thread and screw-in connector GES 6 R1/4", straight 6 mm / R1/4"). A 75
2 cm long PVC tube ($\varnothing$ (outer / inner) = 10 / 6 mm) was fastened to the outlet of the
3 filter holder to direct the filtered water to a measuring bucket (Fig. 1).

4 A "site kit" was assembled and sealed under clean conditions in the lab (see Fig. S1
5 in the Supplement for a picture of its components). It consisted of a plastic bag
6 containing an assembled filtering apparatus with a ready to use glass fibre filter with
7 1 μ m pore size and 47 mm diameter (Grade GF/B, Whatman, VWR, Dietikon,
8 Switzerland) in the filter holder, a pair of plastic forceps, three glass fibre filters, four 5
9 ml tubes (PowerWater DNA bead tubes, Qiagen AG, Hombrechtikon, Switzerland)
10 and a pair of nitrile lab gloves. A new "site kit" was used for each experimental water
11 tank. Parts of the equipment were cleaned and reused for the experiments since they
12 had been used in the field before. Reused parts were the filter holder and its two O-
13 rings, the silicone tube, all the connectors, the forceps, the funnel and the weight
14 including the ropes. The cleaning procedure for all parts included a minimum of 10
15 min soak in 2.5 % diluted technical bleach (Sodium hypochlorite (14 % Cl_2), VWR,
16 Dietikon, Switzerland), followed by a 10 min soak in de-ionised water. De-ionisation
17 was achieved with reverse osmosis. Equipment was rinsed in MilliQ water and air-
18 dried on a lab bench, covered with household paper. To control for the cleanliness of
19 the filtration equipment, a negative control was run through the system prior to
20 sampling each tank. The negative control consisted of 5 L of MilliQ water in a flexible
21 plastic container (Brainypack, Bottleshop, Menshen Schweiz GmbH, Obernau,
22 Switzerland).

23 Before sampling, wearing the gloves included in the site kit, the silicone tube was
24 inserted into the pump head and the short PVC tube end was put into the bucket and
25 secured in place with a clamp. The negative control was then filtered, with a helper
26 pouring the water into the funnel held by the person with the gloves. After filtration

the filter holder was opened, and the filter carefully folded with the feed side inwards using forceps and put into a 5 ml tube. A new filter was inserted into the holder and the funnel was lowered into the experimental tank by the rope attached to the weight. After 5 L had been filtered the filter was exchanged as before. This procedure was repeated until three 5 L water samples were filtered, in addition to the negative control. The tubes containing the samples were stored on ice for a maximum of four hours and then put to -80°C until extraction.

2.4. Validation of the *T. bryosalmonae* spike consistency

In a validation experiment, the spore solution of *T. bryosalmonae* was added to pre-wetted filter papers directly, omitting the filtration step. This experiment could not be conducted with *B. dendrobatidis* due to lack of remaining spore solution. The filters were spiked with the number of spores they would theoretically have captured in the experiment according to the spore concentrations in the tank, i.e. for 5 L from a 1 spore L⁻¹ tank = 5 spores and then 50 and 500 spores, respectively, for the 10 and 100 spores L⁻¹ concentrations. Filters were placed onto petri dishes and the adequate amount of spore solution was pipetted onto the filter. Before spiking with spores, to simulate the wetness of a filter during and after the filtration process, Sigma water was added to the filter up to a total liquid (spore solution + H₂O) of 1.5 ml per filter, which was absorbed completely by the filter. Three filters per concentration were tested. To test the effect of filter presence on detection and concentrations of spiked parasite, the same number of spores were added to the DNA extractions without a filter. Due to a dwindling stock of *T. bryosalmonae* spore solution only three samples per 5 and 50 spores were obtained without filter.

2.5. DNA Extraction

All extraction work was conducted in a dedicated lab only used for processing sensitive samples with low DNA content and for pre-PCR work. The filters were extracted following the DNEasy PowerWater Kit protocol (Qiagen AG, Hombrechtikon, Switzerland) with the following adjustments: after adding 1 ml of solution PW 1 and shredding of the filter into small pieces using a pipette tip, the tubes were incubated at 65°C for 10 minutes in an oven (VWR Peqlab, Dietikon, Switzerland). After vortexing for 5 minutes at full speed on a Vortex-Genie 2 (VWR, Dietikon, Switzerland) with a 5 ml tube adapter (QIAGEN AG, Hombrechtikon, Switzerland) samples were subjected to an additional incubation period of 10 minutes at 65°C. Samples were then centrifuged at 8000 x g with an Eppendorf 5427 R centrifuge and rotor FA-45-12-17 for 5 ml tubes (VWR, Dietikon, Switzerland). The extraction protocol used is described in detail in the Supplement (Supplementary data 2). A no-template extraction control containing only a clean filter paper was included in all extraction runs. The processed samples were stored at -20°C until further analysis.

2.6. Real-time quantitative qPCR

2.6.1. Reaction setup & thermal cycling

The samples were analysed with real-time quantitative PCR (qPCR) on a LightCycler 480 (Roche, Basel, Switzerland) for both parasite species. A QIAgility pipetting robot (Qiagen AG, Hombrechtikon, Switzerland) was used for setting up the reactions. Reactions were run in triplicate. Each qPCR run included a five-fold dilution series of eight standards of a Gblocks fragment (Integrated DNA Technologies, see Supplementary data 3) with DNA concentrations ranging from 69'335 copies μl^{-1} to 0.9 copies μl^{-1} to generate a standard curve, and a negative PCR control. To detect and quantify *B. dendrobatidis* DNA in water samples we used the TaqMan assay

1 developed by Boyle et al. (2004), which was applied to water samples before (Walker
2 et al. 2007), using specific forward primer ITS1-3 Chytr (5'-
3 CCTTGATATAATACAGTGTGCCATATGTC-3'), reverse primer 5.8S Chytr (5'-
4 AGCCAAGAGATCCGTTGTCAAA-3') and minor groove binding probe Chytr MGB2
5 (5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3'). Reactions contained 5 µl of
6 LightCycler 480 Probes Master buffer (Roche, Basel, Switzerland), both primers at
7 concentrations of 900 nM, the MGB probe at 200 nM and 2.5 µl of DNA template for
8 a total volume of 10 µl per reaction. To detect and quantify *T. bryosalmonae* DNA in
9 water samples we used the TaqMan assay developed by Carraro et al. (2018), which
10 has already been used to detect *T. bryosalmonae* in water samples, with forward
11 primer Tb_COI_F1q (5'-GGTTGTTTAGTTTGGGCTCACC-3'), reverse primer
12 Tb_COI_R1q (5'-TCCCTGTAGGGACAGCTATTG-3') and TaqMan probe
13 Tb_probe_COI_1 (5'-6FAM CAAGATCTTATTTTATGGCTGCCAC BHQ-1 NFQ-3').
14 Ten microliter reactions for *T. bryosalmonae* contained 5 µl of LightCycler 480
15 Probes Master buffer (Roche, Basel, Switzerland), forward primer at concentration of
16 300 nM, reverse primer at 900 nM and 250 nM for the probe and 2.5 µl of template
17 DNA. To control for inhibition in water samples, a synthetic template (Carraro et al.
18 2017) was used (5'-
19 GTATTCCTGGTTCTGTAGGTTGAGCGTAAACGACGGCCAGTGAATTGTAATAC
20 GACATGGTCATAGCTGTTTCCCGATACGGAAGTCCAGTCACAT -3'). This internal
21 positive control (IPC) does not match any published sequence data. The IPC reaction
22 setup was as follows: 5 µl of LightCycler 480 Probes Master buffer, forward primer
23 MIMf (5'- GTATTCCTGGTTCTGTAGGTTGAGC -3') at concentration of 50 nM,
24 reverse primer MIMr (5'- ATGTGACTGGACTTCCGTATCG -3') at 900 nM, the IPC
25 hydrolysis probe (5'- Cy3 CGACGGCCAGTGAATTGTAATACGA BHQ-1-3') at 250
26 nM, 2.5 µl of water sample DNA and the IPC at a final concentration of 8.33E-19 mol

L⁻¹, or 5.01833 copies reaction⁻¹. The IPC was run separately from the parasite assays in triplicate for each sample.

Thermal cycling for all assays started with an initial ten minutes at 95°C to activate the DNA polymerase and denature template DNA, followed by 50 cycles of 15 seconds at 95°C and one minute at 60°C. A short cooling step of ten seconds at 40°C at the end of the cycling is suggested by the manufacturer of the machine and was implemented here.

2.6.2. Standard curve

A double-stranded Gblocks fragment (Integrated DNA Technologies) consisting of target sequences of all assays used in this study was used to create a five-fold dilution series with 15 dilutions from concentrations of 21.67*10⁷ to 0.035 copies µl⁻¹. The Gblocks sequence information is listed in the Supplement (Supplementary data 3). For each parasite assay a qPCR run was conducted with 30 replicates each from the fifth to eleventh dilution of the initial dilution series, and 40 replicates each of dilutions 12 to 15 (see Table S1 in the Supplement for detailed results). All replicates in each dilution were positive up to dilution 12 (100% detection). In dilution 13, 50% of the replicates were positive for both *T. bryosalmonae* and *B. dendrobatidis* assays. Therefore, the mean cycle value (Cq-value) of positive replicates of the dilution with 50 % detection (dilution 13 = 2.22 copies reaction⁻¹) was defined as limit of detection (LOD) for each parasite assay, respectively (*T. bryosalmonae* Cq = 35.646, *B. dendrobatidis* Cq = 37.706). We are aware that this limit is less stringent than the frequently used 95% detection (Bustin et al. 2009), but we deem it more appropriate when dealing with low content eDNA samples of potentially dangerous pathogens. For the sake of comparability, we applied the LOD calculator method developed by Klymus et al. (2019), which determines the LOD concentration at 95 % detection, to

the assays used in this study, resulting in LOD concentrations of 10.27 copies reaction⁻¹ for *B. dendrobatidis* and 9.64 copies reaction⁻¹ for *T. bryosalmonae*. The limit of quantification (LOQ) was defined as the concentration of the last dilution of the linear range of the standard curve, which was at 22.2 copies µl⁻¹ for both parasite assays. Parameters for the standard curve were R² = 0.9995, slope = -3.467 and efficiency = 94.30 % for *B. dendrobatidis* and R² = 0.9999, slope = -3.410 and efficiency = 96.46 % for *T. bryosalmonae*. The standard curves used to define the LOD and LOQ are visualised in Fig. S2 in the Supplement for both parasites. We included dilutions six (69.33*10³ copies µl⁻¹) to 13 (0.89 copies µl⁻¹) on each run for absolute quantification of the DNA samples of the same run.

2.7. Data analysis

The qPCR raw data was first prepared using the LightCycler 480 Software version 1.5.1 (Roche, Basel, Switzerland). Cq-values were determined with the Absolute Quantification – Second Derivative Maximum method and the high confidence algorithm (LightCycler Software version 1.5.1). A water sample was considered positive if the DNA concentration of the target parasite exceeded the LOD in at least one of the three replicate qPCR reactions. Detection of the target parasite in a water tank was considered successful if at least one sample from the tank was positive for parasite DNA. Non-detections were considered as “no answer” (N.A.) and excluded from calculations of the mean. Mean Cq-values or DNA concentrations of water samples were calculated with values from positive qPCR replicates and mean values of tanks from mean values of water samples. Statistics were conducted in R version 3.6.1. (R Core Team 2019). The R package “eDNAOccupancy” (Dorazio & Erickson 2017) was used for running hierarchical occupancy models and model selection to test if spore concentrations, water source, order of sampling, block and inhibition had

an effect on detection probability. All occupancy models were run with 11'000 iterations of the MCMC algorithm. Model selection was conducted using the posterior Predictive Loss (PPLC, Gelfand & Ghosh 1998) and Watanabe-Akaike Information criteria (WAIC, Watanabe 2010). If the addition of a covariate or factor did not improve model fit, it was considered not to influence parasite DNA detection. The equation $1 - (1-\theta)^n \geq 0.95$ was used to determine the number of water samples (n) required for successful detection probability of 95 %, with θ being the probability of detection of parasite DNA in a water sample.

Linear mixed effects models were used to test the effect of spore concentrations, water source, order of sampling and block on estimated parasite DNA concentrations, including tank and sample ID as nested random factors. Block was considered a fixed factor because it contained only two levels. After testing for model fit using AIC, order of sampling and block effects were pooled into the residual term since they did not improve the model fit and were not significant for either *B. dendrobatidis* ($p = 0.845$ and $p = 0.316$, respectively) or *T. bryosalmonae* ($p = 0.692$, $p = 0.728$, respectively).

Detection success between pipette-spiked filter and experimental samples containing the same number of spores was compared using Fisher's exact test for count data.

Comparisons of parasite DNA concentrations between pipette-spiked samples with and without filter, and between pipette-spiked filter samples and experimental samples were conducted with linear models with spiked number of spores as a covariate. Significance scores of all linear models were extracted with type III analysis of variance with Satterthwaite's method.

Inhibition of water samples was quantified by calculating Cq-value differences between water samples spiked with IPC DNA and control samples containing MiliQ water spiked with IPC DNA. The larger the Cq-value differences, the more inhibited

the sample, with ΔCq -values ≥ 3 signifying substantial inhibition. We tested the effect of water source on inhibition using a Wilcoxon Rank Sum test.

3. RESULTS

3.1. Detection consistency

Batrachochytrium dendrobatidis DNA was successfully detected in 14 out of 16 spore-spiked tanks (87.5 %) and *Tetracapsuloides bryosalmonae* DNA in 10 out of 12 tanks (83 %). The two tanks where detection failed for *B. dendrobatidis* were spiked at concentrations of 1 and 100 spores L⁻¹, for *T. bryosalmonae* at 10 and 100 spores L⁻¹. Detection of parasite DNA was not consistent among samples of the same tanks. DNA of *B. dendrobatidis* was successfully detected in all three samples of only two tanks, while five tanks had two, and the rest had one DNA-positive sample (n = 7). For *T. bryosalmonae* two tanks had two positive samples out of three, while the remaining eight had only one positive sample. The mean Cq-values and concentrations are reported in full in Table S2 in the Supplement. All negative controls filtering 5 L of MilliQ water to test the cleanliness of sampling equipment (n = 36) and all samples from tanks without parasite spores (n = 24) were consistently negative.

Hierarchical occupancy models with constant parameters estimated occupancy probability in a tank to be $\Psi(.) = 0.916$ for *B. dendrobatidis* and $\Psi(.) = 0.910$ for *T. bryosalmonae*. The estimates of DNA detection per sample were $\theta(.) = 0.53$ and $\theta(.) = 0.356$, respectively. Thus, to reach detection rates per tank of 95 % or above, four and seven water samples per tank need to be taken for *B. dendrobatidis* and *T. bryosalmonae*, respectively. Detection probability in a qPCR replicate, given successful parasite detection in the sample, was estimated to be $p(.) = 0.665$ and $p(.)$

= 0.955, respectively. *Batrachochytrium dendrobatidis* model fit improved when spore concentration and water source were added as covariates at the qPCR replicate level. This result suggests that *B. dendrobatidis* DNA detection in positive samples at the level of qPCR replicates was more likely in tap water samples ($p(\text{tap water}) = 0.813$ versus $p(\text{mesocosm water}) = 0.553$) and in tanks with higher spore concentrations ($p(1 \text{ spore L}^{-1}) = 0.576$, $p(10 \text{ spores L}^{-1}) = 0.579$, $p(100 \text{ spores L}^{-1}) = 0.608$, $p(1000 \text{ spores L}^{-1}) = 0.843$). Since none of the covariates improved *T. bryosalmonae* model fit, detection success did not depend on water source or spore concentrations per tank. All tested models are listed in Table S3 in the Supplement.

3.2. Real-time qPCR inhibition

Most samples from the *B. dendrobatidis* experiment with water originating from the mesocosm (19 of 24) showed signs of inhibition according to IPC Cq-value shifts, while none of the tap water samples seemed to be affected ($p < 0.001$; Fig. 2). In contrast, only three mesocosm water samples were affected by inhibition (Cq-value shift >3 cycles) in the *T. bryosalmonae* experiment (conducted at a different time point) and therefore, no significant effect of water source on inhibition was detected ($p = 0.064$).

3.3. Quantification of parasite eDNA

Parasite DNA concentrations in water samples increased significantly with higher spore concentrations in tanks for *B. dendrobatidis* ($F = 4.787$, $p = 0.041$; Fig. 3a), but not *T. bryosalmonae* ($F = 1.239$, $p = 0.29$; Fig. 3b). Water source did not influence DNA concentration estimates of either parasite ($F = 0.115$, $p = 0.738$, and $F = 0.09$, p

= 0.771, respectively). Parasite DNA concentrations on qPCR replicate level versus tank spore concentrations are visualised in Fig. S3 in the Supplement.

3.4. Effect of filtration and extraction on *Tetracapsuloides bryosalmonae* detection and quantification

Tetracapsuloides bryosalmonae DNA was successfully detected in all replicates of samples where no filter paper was introduced. No significant differences in *T. bryosalmonae* DNA detection success between pipette-spiked filters and filters from experimental samples were observed ($p = 0.119$). No-filter samples of *T. bryosalmonae* yielded higher DNA concentration estimates than pipette-spiked filters ($F_{1,9} = 52.281$, $p < 0.001$; Fig. 4) with significant differences in concentrations depending on the number of spores per sample ($F_{1,9} = 53.785$, $p < 0.001$). The increase of *T. bryosalmonae* DNA concentration in samples with 50 spores compared to samples with five spores, was significantly different between no-filter and pipette-spiked filter samples (interaction: $F_{1,9} = 11.469$, $p = 0.008$). Pipette-spiked filter samples did not have higher *T. bryosalmonae* DNA concentrations than experimental filter samples ($F_{1,21} = 0.119$, $p = 0.733$; Fig. 4). *T. bryosalmonae* DNA concentrations of pipette-spiked filter samples significantly increased with increasing spores filter⁻¹ ($F_{1,8} = 27.675$, $p < 0.001$; Fig. 4). The mean Cq-values and concentrations recorded from no-filter and pipette-spiked filter samples are found in Table S4 in the Supplement.

4. DISCUSSION

4.1. Parasite detection and occupancy

The detection success of parasite spores in water using eDNA techniques was assessed in a controlled environment for a filtration method capturing 5 L water

1 samples, developed for application in the field. We detected DNA of
2 *Batrachochytrium dendrobatidis* in 14 out of 16, and of *T. bryosalmonae* in 10 out of
3 12 tanks spiked with the target parasite spores. Therefore, the overall detection rate
4 was similar for both tested parasites, i.e. 87.5 % for *Batrachochytrium dendrobatidis*
5 and 83 % for *Tetracapsuloides bryosalmonae*. However, per sample detection was
6 far from perfect even under these controlled conditions. Sample level non-detections
7 were observed at all spore densities of both target organisms. *B. dendrobatidis* DNA
8 was detected in all three samples from two tanks only (2×100 spores L^{-1}), while
9 most tanks yielded only one positive sample out of three ($n = 7$). *T. bryosalmonae*
10 DNA was never detected in all three samples in any of the experimental tanks. Non-
11 detections were not restricted to low concentration treatments, nor to mesocosm
12 water where PCR inhibition was predicted to be higher (Fig. 2).

13
14 Inconsistency of detection in water samples can arise when the amount of DNA
15 captured in a sample is low, i.e. around the limit of detection (LOD). We deliberately
16 adopted a relatively permissive LOD for reporting of positive detections, in line with
17 recommendations for low-DNA samples (Hunter et al. 2017). Since we expect most
18 of the parasite DNA in water to originate from intact spores and not from free
19 extracellular DNA, encounter rate with parasite spores might not be high enough for
20 successful detection unless they occur in sufficient densities. In our experiments, the
21 density of spores could have been reduced through adhesion of spores to plastic or
22 glass surfaces (tank sides, bottle lid, pipette tips etc.), which would render them
23 unavailable for capture via water filtration. However, *T. bryosalmonae* DNA
24 concentrations of experimental water samples were not lower than pipette-spiked
25 filter samples (Fig. 4), indicating that loss by adhesion in the tanks and by the filtering
26 equipment was negligible. We note that detection rate did not increase with higher

1 spore densities in our experiments, and even the lowest spore concentrations led to
2 successful detections. This suggests that detection success may be compounded by
3 other factors than low DNA concentrations, for example due to heterogeneous
4 distribution of parasite spores in water.

5
6 Heterogeneity in the *T. bryosalmonae* experiment could have arisen by the presence
7 of spore / sac wall clusters or by spores sticking together. Presence of spore sac
8 fragments was not observed during counting, but cannot be completely excluded.
9 Clumping of spores in dissected material is possible, as *T. bryosalmonae* spores
10 contain four polar capsules, each with a polar filament used for attachment to the fish
11 host (McGurk et al. 2005, Morris & Adams 2007) and we cannot exclude the
12 possibility that the spores could have formed clusters post-release. Further, parasite
13 sacs likely contained spores in different stages of maturity, whereas spores released
14 in natural conditions from live bryozoan hosts are likely to be a more homogenous
15 population of mature spores (the parasite sacs generally burst inside the host and
16 only spores are ejected into the water column; Hartikainen & Okamura 2015). To
17 further evaluate experimentally the consistency of *T. bryosalmonae* detection,
18 inoculation with naturally released spores would be an informative addition to the
19 quantitative results obtained here. *B. dendrobatidis* zoospores are motile and less
20 likely to form aggregations (Berger et al. 1998). However, most spores encyst in
21 under 24 h after their release from the zoosporangium (Berger et al. 2005) and move
22 only short distances during that time (Piotrowski et al. 2004). The spores spiked into
23 the tanks were of different ages. Therefore, we cannot exclude heterogeneous
24 distribution of *B. dendrobatidis* due to formation of immobile cysts. Finally, insufficient
25 mixing of the water could be a possible reason for heterogeneous distribution of
26 spores in the tanks, since we only mixed the water before filtering the first sample of

1 each tank, and the filtration of three 5 L samples took about 30 minutes. This might
2 be enough time for spores to settle or otherwise distribute unevenly in the tank,
3 although there was no pattern in the results suggesting this (e.g. no higher probability
4 of detection in the first samples taken from a tank).

5
6 Many previous studies using vertebrates, e.g. fish (Klymus et al. 2015) and
7 amphibians (Pilliod et al. 2013), or macrophytes (Gantz et al. 2018) have reported
8 100 % detection rates in mesocosm studies. Such promising results may arise
9 because of the continuous and more disperse release of DNA via dead cells, mucus,
10 etc. from these organisms. Previous studies with invertebrates and parasites in
11 mesocosms have reported more variable detection success, in accordance with our
12 findings (but see Sengupta et al. 2019 for 100 % detection efficiency of *Schistosoma*
13 *mansonii*). Mauvisseau et al. (2019) sampled water from mesocosms containing
14 Freshwater Pearl Mussels (*Margaritifera margaritifera*) and were able to detect their
15 DNA in all mesocosms with only one of two assays used, with evidence for
16 inconsistent detection in biological and technical replicates. Tapeworm *Echinococcus*
17 *multilocularis* DNA was detected in all 10 L water samples spiked with 100 or 1000 *E.*
18 *multilocularis* eggs, respectively, but only in two-thirds of samples spiked with 10
19 eggs (Lass et al. 2019). Furthermore, while detection rate from 1 L lake water
20 samples spiked with 10 or 1000 spores of agent of the crayfish plague,
21 *Aphanomyces astaci*, reached almost a 100 %, single spores were detected in only
22 73 % of the filter samples (Strand et al. 2011).

23
24 The inconsistency of detection at water sample level is reflected in the detection
25 probabilities estimated with occupancy models, i.e. $\theta(.) = 0.53$ and $\theta(.) = 0.356$, for *B.*
26 *dendrobatidis* and *T. bryosalmonae*, respectively. However, detection consistency

was higher on tank level, i.e. $\Psi(.) = 0.916$ for *B. dendrobatidis* and $\Psi(.) = 0.910$ for *T. bryosalmonae*, and qPCR replicate level, i.e. $p(.) = 0.665$ for *B. dendrobatidis* and $p(.) = 0.955$ for *T. bryosalmonae*. The former being probabilities of detecting the parasite when it is present in a tank, and the latter being probabilities of a positive detection in a replicate of a sample, given that the sample is positive. These results suggest that the highest inconsistencies in detection success occur at the sample level. According to per sample detection probabilities, a theoretical cumulative detection rate of 95 % or above can only be reached if four water samples per tank for *B. dendrobatidis* and seven samples per tank for *T. bryosalmonae*, are taken. This is despite our liberal approach of accepting a single qPCR replicate above LOD as successful detection, which we consider appropriate for low-quantity eDNA samples. Taking replicate samples per site thus seems to be a requirement for reliable detection. However, increasing the number of samples leads to higher processing effort and costs, requiring careful consideration of cost and benefit when planning surveys for parasites in water.

4.2. Inhibition in water samples

We observed inhibition of the IPC amplification in water originating from the mesocosm during the *B. dendrobatidis* experiment, but not during the *T. bryosalmonae* experiment (Fig. 2). In both experiments, the water came from the same mesocosm, but the experiments were separated by two months. Natural variation in the mesocosm communities (e.g. different phytoplankton densities) may thus explain the different levels of inhibition in DNA extractions between the two experiments. Larger water volumes increase the number of spores captured in a sample, but the downside of increasing volume is that it will potentially lead to higher inhibition of the samples if more inhibitory compounds accumulate. This can

decrease the efficacy of eDNA-based monitoring methods (Harper et al. 2019). In our study, water source, and therefore inhibition, did not influence detection success on tank level of either parasite in our tank experiment (Fig. 3). However, *B. dendrobatidis* mesocosm water samples did have lower detection consistency on the qPCR replicate level than tap water samples ($p(\text{mesocosm}) = 0.553$, $p(\text{tap}) = 0.813$), indicating a potential inhibition effect. This was not the case for *T. bryosalmonae* samples, which might explain the higher overall qPCR replicate detection probability of *T. bryosalmonae* ($p(.) = 0.955$) compared to *B. dendrobatidis* ($p(.) = 0.665$). No direct effect of the magnitude of inhibition (measured shift in IPC Cq-value between a clean water and experimental DNA extract) on detection success was indicated by the statistical analysis. It could be that the low amount of IPC template added to qPCR reactions (5 copies reaction⁻¹) renders the IPC inhibition control more sensitive to inhibition than the parasite detection assays.

4.3. Accuracy of quantification

Parasite DNA concentration estimates in water samples increased with spore concentrations in tanks (Fig. 3). This relationship was statistically significant for *B. dendrobatidis* (Fig. 3a) and a similar trend is visible for *T. bryosalmonae* (Fig. 3b), even though it is not statistically significant. The difference likely resulted from the fact that the *B. dendrobatidis* experiment included tank concentrations of 1000 spores L⁻¹, while the maximum concentration in the *T. bryosalmonae* experiment was 100 spores L⁻¹. Even though one correlation was statistically significant, the large variance in the data, especially in samples from tanks with high parasite concentrations, urges caution in interpreting the results quantitatively. An accurate quantification of parasite DNA concentrations is not fulfilled by the method used in this study, according to the data. A qualitative comparison of parasite spore

abundance might be possible between sites, if enough water samples are taken. Correlations between species densities and eDNA quantities has been previously found for amphibians (Thomsen et al. 2012, Pilliod et al. 2013), crayfish (Harper et al. 2018) and freshwater snails (Mauvisseau et al. 2019) in mesocosms. Infection prevalence has been shown to positively correlate or temporally coincide with DNA concentrations in water of amphibian (Huver et al. 2015), crayfish (Strand et al. 2014), fish (Hallett et al. 2012) and human parasites (Wade et al. 2010). However, a precise quantitative relationship of eDNA concentrations in water and parasite densities is yet to be described.

4.4. Influence of filtration methodology on detection success

All qPCR replicates of *T. bryosalmonae* spore samples extracted without a glass fibre filter detected parasite DNA and yielded higher DNA concentration estimates compared to the same number of spores extracted from filters (Fig. 4). *T. bryosalmonae* detection success of pipette-spiked filter samples did not differ from experimental samples employing the same filter type. Thus, glass fibre filters, rather than the filtration process, seem to have decreased DNA yield. Filtering has been previously shown to result in weaker qPCR signals during parasite detection (Hallett & Bartholomew 2006). While glass fibre filters have been shown to work well for eDNA studies (Eichmiller et al. 2016, Spens et al. 2017), in general the optimal filter paper choice may vary according to the study objectives (Djurhuus et al. 2017, Deiner et al. 2018). Glass fibre filters are absorbent and retain more of the lysate containing DNA than some other filter types, potentially decreasing yield of target parasite DNA. Glass fibre filters (GF/B) employed in this study allow a large volume of water to be filtered, which is potentially important for detection of relatively rare parasite spores in the environment. We did not test the effect of sample volume on

detection efficiency, but it seems unlikely that detection of one spore L⁻¹ could be consistently achieved with smaller sample sizes. Thus, the choice of filter type and volume of water sampled presents a trade-off that is perhaps of more importance in parasite detection than for eDNA studies in general.

4.5. Implications for parasite detection in the field

The inconsistent detection we observed when applying the method in a very simplified environment is rather sobering and contains an important message for real surveys: patchiness in detection is likely to be inherent to any experimental setting where parasites are assayed in subsamples taken from rivers or lakes. Knowledge of *B. dendrobatidis* and *T. bryosalmonae* densities in natural systems, or parasite release dynamics from host individuals, is sparse and difficult to estimate outside the laboratory settings (Maguire et al. 2016, Fontes et al. 2017). The range of spore concentrations tested in this experiment likely encompasses at least some of the concentrations encountered in the field. However, the influence of spatial and temporal fluctuations on spore densities is difficult to capture. Natural aquatic systems are complex in their hydrology and structure and thus, heterogeneous distribution and density of parasite spores and DNA, even on small scales, are expected (Shogren et al. 2017). This will particularly be the case in systems with low host abundance or parasite prevalence in the host, or where the exact sampling location and timing of sampling determines occurrence of parasite spores in a water sample. In this context, the overall detection rates obtained in this experiment were still high (87.5 % and 83 % for *B. dendrobatidis* and *T. bryosalmonae*, respectively), thanks to replicated sampling and technical replication within samples. Notably, low spore densities down to one spore L⁻¹ could still be detected, while no false positives were found in our study. For assessment of disease risk, the distinction between

viable and non-viable parasites is important and poses additional challenges for interpretation and application of monitoring results in management.

5. CONCLUSIONS

This study presents a validation of an eDNA-based method to detect parasite DNA in water. An in-situ filtration method was applied to water spiked with known amounts of spores in a controlled environment. This validation identified limits and remaining challenges of such monitoring methods, which can therefore be addressed and improved. Specifically, it showed that despite the high sensitivity of qPCR assays, detection can be very inconsistent even at high spore concentrations, presumably due to non-homogeneous spore distributions and variation introduced at the different processing steps (i.e. filtration and DNA extraction from filters). Being aware of the limitations of the method helps interpret the results from field surveys more adequately and to quantify uncertainties. In the present case, it highlights the need for sufficient replication of samples from the same collection site to maximise detection success. While we did observe a positive correlation between estimated DNA concentrations and the manipulated spore concentrations, the large variance of the data compromises the reliability of quantitative comparisons. In general, we advocate further studies that evaluate the feasibility of quantifying parasite loads in water at accuracy that is relevant for risk assessments and early warning systems of disease outbreaks. Our 5 L filtration method was generally as efficient at detecting 1 spore L⁻¹ than 100 spores L⁻¹, suggesting high sensitivity, albeit the patchy occurrence of false negatives. Studies such as ours can help practitioners decide which detection method to use for monitoring campaigns. Therefore, the development of standardised validation practices for eDNA-based methods used in species detection is an important step still to be undertaken by researchers to

facilitate their widespread implementation. As a step further, field-based evaluations of detection success are recommended to obtain the most relevant guidance for monitoring campaign designs.

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1 FIGURES

Fig.1: Sketch of the eDNA collection method developed in this study. A battery-driven peristaltic pump is used to pump water through a submerged funnel covered in a 100 µm pore-sized mesh and filtered through a glass fibre filter contained within a re-usable filter holder. Silicone tubing section compatible with the pump is extended using disposable PVC tubing to minimise contamination and requirement for cleaning.

Fig. 2: Internal positive control qPCR cycle differences (Δ Cq-values) between mesocosm and tap water samples of the *B. dendrobatidis* and *T. bryosalmonae* experiments. Points are jittered for better visibility. Δ Cq-values ≥ 3 indicate presence of inhibitors. The samples with Δ Cq-values > 30 had no IPC DNA amplification and therefore, Δ Cq-values were set to the Cq-value of the IPC control sample (Cq = 32.22).

Fig. 3: Mean estimated parasite DNA concentrations in copies μl^{-1} per spore concentration and water source (grey bars: mesocosm water, white bars: tap water) of a) *Batrachochytrium dendrobatidis* and b) *Tetracapsuloides bryosalmonae*. To account for the large variance, concentrations are presented on a logarithmic scale. Successful detections only were used for calculating the means. The data points show mean parasite DNA concentrations of qPCR replicates per 5 L water samples ($n = 6$ per bar), with closed and open circles distinguishing samples from different blocks (two tanks, one in each block, per water origin and spore concentration). Points are jittered to visualise non-detections (n.d.). Parasite DNA concentrations on qPCR replicate level versus tank spore concentrations are visualised in Fig. S3 in the Supplement.

Fig. 4: Mean concentrations of *T. bryosalmonae* DNA in copies μl^{-1} of no filter samples ($n = 3$ for each spores sample⁻¹ treatment), pipette-spiked samples ($n = 6$ each for treatments of five and 50 spores filter⁻¹ and $n = 3$ for the 500 spores filter⁻¹ treatment) and water samples of the experiment ($n = 12$ for each spore concentration [spores L⁻¹]). Successful detections only were used for calculating the means. Points show means of qPCR replicates for each sample and are jittered to visualise non-detections (n.d.). The data that was used to create this figure is found in Table S5 in the Supplement. *T. bryosalmonae* DNA concentrations on qPCR replicate level are visualised in Fig. S4 in the Supplement.