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ORIGINAL ARTICLE

Optimal detection protocol of *Tetracapsuloides bryosalmonae* by environmental DNA: A comparison of qPCR and ddPCR approaches

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Abstract

Investigation of environmental DNA (eDNA) is increasingly used to precisely and non-invasively detect and monitor pathogens. Among these, *Tetracapsuloides bryosalmonae* is a myxozoan endoparasite that causes proliferative kidney disease (PKD) in salmonid fish. Although the detection of *T. bryosalmonae* DNA in water samples has been shown to be promising and successful, method comparison and cross-validation are currently lacking. This study aims to directly compare the sensitivity of different eDNA-based methods in field and laboratory applications, and to develop an easy-to-apply and sensitive protocol to monitor *T. bryosalmonae* occurrence non-invasively by its eDNA in water samples. First, we tested three existing probe-based *T. bryosalmonae*-specific detection assays in parallel by comparing the limit of detection (LOD) and limit of quantification (LOQ) using quantitative PCR (qPCR) and digital droplet PCR (ddPCR) platforms. Second, the impact of different filter types and water volumes on the detection probability was tested by sampling water directly from riverbanks with a syringe-based protocol. The most sensitive detection protocol was the combination of the probe-based assay published by Bettge et al. run via ddPCR, resulting in a LOD of 1.65 copies/µL input (6.6 copies/reaction) and a LOQ of 3.66 copies/µL input (14.67 copies/reaction). The type of filter (Sterivex™ compared to Millex®) did not significantly influence detection probability, however, the volume of water sampled (600 mL compared to 300 mL) significantly affected the probability of capturing eDNA in a sample. Based on modeled probabilities of eDNA capture and detection, we calculated that using the Bettge et al. assay via the ddPCR platform for data collection, 95% overall detection probability could be achieved with three replicates of 600 mL filtered water with Sterivex™ filters. Based on this cross-validation of assays and detection platforms, we provide a cost-effective, straightforward, and highly sensitive laboratory analysis workflow to detect DNA of *T. bryosalmonae* from water samples.

Heike Schmidt-Posthaus and Rein Brys share last authorship.

KEYWORDS
environmental DNA, method comparison, monitoring protocol, non-invasive monitoring, proliferative kidney disease, *Tetracapsuloides bryosalmonae*

1 | INTRODUCTION

Detection of environmental DNA (eDNA) is a promising approach for advancing the monitoring and surveillance of pathogens. Pathogens, which release water-borne transmission stages, are particularly appropriate target organisms for eDNA-based monitoring (Bass et al., 2023). *Tetracapsuloides bryosalmonae* is a malacosporean endoparasite and causative pathogen of proliferative kidney disease (PKD) in salmonids (Canning et al., 1999, 2002; Hedrick et al., 1993). The parasite has a complex life cycle, including its invertebrate final host (phylactolaemate bryozoans) and a fish intermediate host. Most salmonid species are susceptible to *T. bryosalmonae*, causing severe disease in many cases and highly variable mortality (Bettge, Wahli, et al., 2009; Hedrick et al., 1993; Okamura et al., 2011; Schmidt-Posthaus et al., 2015). Transmission of *T. bryosalmonae* occurs via water-borne malacospores (14–20 μm diameter), which are either released via the urine of fish (Hedrick et al., 2004) or the vestibular pore of a bryozoan host (McGurk et al., 2005; Okamura, 2013).

Monitoring the occurrence of PKD is important for understanding its emergence in wild and farmed fish stocks and to develop appropriate surveillance, management, and conservation campaigns, especially due to its status as an ecologically and economically important disease in the northern hemisphere (Feist, 2004; Hutchins et al., 2021; Sage, 2016). Its devastating effects on wild trout populations resulted in listing of PKD as a notifiable disease in Switzerland (FSVO, Federal Food Safety and Veterinary Office, 2022).

Conventional monitoring of *T. bryosalmonae* and PKD occurrence currently uses an invasive protocol, examining tissue of euthanized young brown trout (Wahli et al., 2007). For this purpose, fish are obtained via electrofishing, a procedure that is harmful to the fish populations, time-consuming, and expensive. The detection of the parasite eDNA (comprising waterborne extracellular or intracellular DNA) has the potential to solve these issues, by providing a complimentary, non-invasive monitoring tool (Beng & Corlett, 2020). Non-invasive detection protocols based on eDNA are already used in detection and monitoring campaigns of many different aquatic pathogens, for example, the oomycete crayfish plague agent – *Aphanomyces astaci* – (Strand et al., 2014, 2019), the fungal agent of chytridiomycosis – *Batrachochytrium dendrobatidis* – (Kirshtein et al., 2007; Walker et al., 2007), the bacterial agent of cold water disease – *Flavobacterium psychrophilum* – (Tenma et al., 2021), viruses such as the amphibian and reptile ranavirus (Hall et al., 2016) and also the myxozoan parasites *Ceratonova shasta* (Richey et al., 2020) and *Myxobolus cerebralis* (Barry et al., 2021). In general, the detection probability of parasite eDNA is influenced by several factors, including aggregated parasite distributions among host populations, host-condition-linked transmission stage release and complex and multi-host life cycles, resulting in water-borne parasite stages with varying resistance to degradation in the environment (Bass et al., 2023). For example, *T. bryosalmonae* spores released from fish and bryozoans may contain a different number of cells, thus affecting their relative detectability in a water sample (McGurk et al., 2005; Morris & Adams, 2008). These factors cause variation in the occurrence of parasite stages in the aquatic environment, often resulting in very low concentrations of pathogen DNA captured during sampling. Consequently, the validation of repeatability and sensitivity of applied detection methods is particularly important for large-scale pathogen monitoring and surveillance campaigns.

Several studies have successfully employed an eDNA-based detection tool for *T. bryosalmonae* under controlled conditions (Duval et al., 2021; Sieber et al., 2020), in lakes (Oredalen, Mo, et al., 2022) and in rivers with large dilution factors (Fontes et al., 2017; Hutchins et al., 2018, 2021; Sepulveda et al., 2020, 2021). Water samples have been variously filtered either in the laboratory after transport from field sites (Carraro et al., 2018; Duval et al., 2021; Hutchins et al., 2021), or on-site using a peristaltic pump (Sieber et al., 2020). Two studies relied on robotic eDNA samplers which autonomously filtered and stored eDNA samples (Sepulveda et al., 2020, 2021). However, these samplers are still costly and did not increase the detection probability of the target species. Currently, three different probe-based assays have been developed and successfully used together with quantitative PCR (qPCR) for the detection of *T. bryosalmonae* eDNA (Bettge, Segner, et al., 2009; Carraro et al., 2018; Hutchins et al., 2018). The use of one of those assays (Bettge, Segner, et al., 2009) was reported in combination with droplet digital PCR (ddPCR) by Oredalen, Saebø, and Mo (2022).

For large-scale monitoring or official surveillance programs, the selection of one simple, consistent, easy-to-use, cost-effective, and reliable eDNA-based protocol is preferable (CSA-Group, 2019). This includes appropriate collection, for example, filtering water directly in the field (i.e., in situ) and relying on single-use, sterile materials to reduce the likelihood of contamination, as proposed by Lugg et al. (2018). Further, the laboratory detection workflow needs to follow stringent and validated sensitivity and robustness criteria in the form of limit of detection (LOD) and limit of quantification (LOQ) (Klymus et al., 2020).

Despite the adoption of LOD and LOQ testing and reporting, for *T. bryosalmonae* the variation in laboratory procedures and application of standards still prevents direct comparisons of assays in most cases. For example, for the different detection assays, sensitivity has been determined either using standard dilutions of a 170 bp synthetic 18S rDNA template of the parasite (Hutchins et al., 2018), or several standard dilutions of a plasmid with insertion of parts of the *T. bryosalmonae* 18S rRNA gene (Oredalen, Mo, et al., 2022), resulting in an LOD of seven copies (Hutchins et al., 2018) and 10.33 copies/reaction (Oredalen, Mo, et al., 2022). Although the copy number variation in the LOD between both studies is relatively low, further insights on the

sensitivity of existing parasite eDNA assays may be particularly important, as for example in the case of *T. bryosalmonae*, a single spore can infect a fish and eventually lead to fatal disease (McGurk et al., 2006).

Therefore, this study aimed to (a) explore and compare the sensitivity of different field and laboratory detection workflows and (b) develop an easy-to-apply, sensitive eDNA-based protocol to monitor *T. bryosalmonae* occurrence non-invasively (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Reference tissue material for detection of *T. Bryosalmonae*

To obtain a high concentration of *T. bryosalmonae* genomic DNA for the construction of a dilution series to evaluate the LOD and LOQ for two chosen primer/probe assays (Bettge, Segner, et al., 2009; Hutchins et al., 2018), kidneys of 12 brown trout (*Salmo trutta*) severely infected with *T. bryosalmonae* were extracted and the extracted DNA samples were pooled. From this highly concentrated pool sample, measured by ddPCR (Bettge et al. assay), 18 standard dilution steps ranging from 10,000 copies/µL to 0.001 copies/µL of the target (Table S1) were setup to compare LOD and LOQ with ddPCR versus qPCR.

This procedure allows for a precise comparison of the sensitivity of different assays using a dilution series of natural DNA, containing exactly the same concentrations of the target species.

2.2 | Water sampling

Water samples were collected from six Swiss rivers (Alte Aare, Lyssbach, Urtene, Furtbach, Wigger Ron, Wigger Rot) at locations known to contain *T. bryosalmonae* infected bryozoans (Hanna Hartikainen (personal communication, 2020)) (Table 1). Each location was sampled five times (2–4 replicate water samples per site/sampling point) between the 23rd of June 2020 and the 27th of July because we expected the highest eDNA occurrence due to high *T. bryosalmonae* spore release by the final host. A sampling of negative field controls was included on the 9th of November 2020.

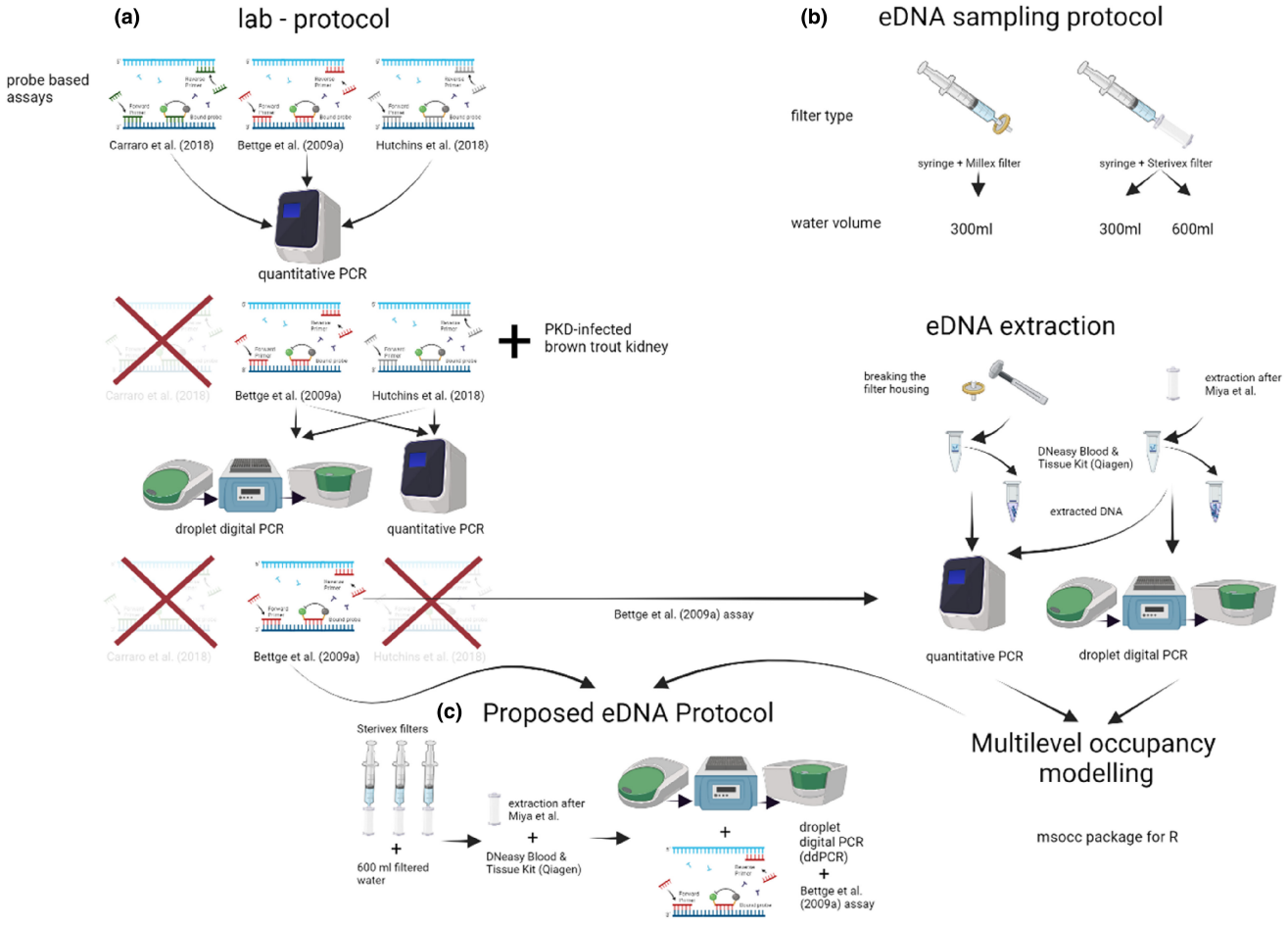

FIGURE 1 Workflow for *T. bryosalmonae* detection optimization. (a) Evaluation of the laboratory protocol with published assays that target either a region of the COI gene (Carraro et al., 2018) or a region of the small subunit 18S rRNA (Bettge, Segner, et al., 2009; Hutchins et al., 2018), as well as the two different amplification methods (quantitative PCR (qPCR) and droplet digital PCR (ddPCR)). (b) Evaluation of the eDNA field sampling protocol and extraction of eDNA samples. (c) Final proposed eDNA-based protocol to detect and monitor the presence of *T. bryosalmonae*. Figure was created with biorender.com

TABLE 1 Sampling localities, dates, and filter types used for collection of eDNA from river water.

			23, June 2020		07, July 2020		14, July 2020		21, July 2020		27, July 2020		09, November 2020	
		Coordinates	Sterivex™	Millex®	Sterivex™	Millex®	Sterivex™	Millex®	Sterivex™	Millex®	Sterivex™	Millex®	Sterivex™	Millex®
River name	Fu (Furtbach)	47.447432, 8.370180	2	0	0	2	2	2	2	2	4	2	0	0
	U (Urtene)	47.056556, 7.539222	2	0	0	2	2	2	2	2	4	2	0	0
	AA (Alte Aare)	47.111529, 7.317205	2	0	0	2	2	2	2	2	4	2	0	0
	Ly (Lyssbach)	47.049656, 7.355583	2	0	0	2	2	2	2	2	4	2	0	0
	Wi RON (Wigger Ron)	47.172951, 8.027838	2	0	0	2	2	2	2	2	4	2	0	0
	Wi ROT (Wigger Rot)	47.154506, 8.015090	2	0	0	2	2	2	2	2	4	2	0	0
	Negative controls	46.957427, 7.426536	0	0	0	0	0	0	0	0	0	0	2	2

River water was collected with a 60 mL Luer-lock BD PlastiPak™ syringe (Becton Dickinson and Company, Franklin Lakes, NJ, USA) from the riverbank, at 5–15 cm underneath the water surface, without stepping into the water. Care was taken not to stir up sediment or leaf litter. After filtering, filters were sealed with Parafilm® (Bemis Company, Neenah, WI, USA) on one end and a single-use medical screw cap (Fresenius Kabi AG, Bad Homburg, DE) was placed on the other end.

In total, 124 water samples were taken using two different models of single-use filters of 0.45 µm pore size each, comprising a Sterivex™ filter (Merck Millipore, Billerica, MA, USA) and Millex®-HV filter (Merck Millipore, Billerica, MA, USA). A subset of 60 water samples [300 mL ($n=48$) and 600 mL ($n=12$)] were filtered with Sterivex™ and another subset ($n=60$) with Millex® filters (300 mL water) (Figure 1). It was not possible to filter more than 300 mL of river water with the Millex® filters during pretrials in several locations. Four water samples from a small pond without connection to any water body nor any known history of parasite or host occurrence were included as negative controls. All materials used were single use, sterile and single packed, and medical gloves were worn during sampling to prevent contamination. Filters were transported on ice and kept at −20°C until further use.

2.3 | DNA extractions

DNA from infected brown trout kidneys (which were used as reference tissue material) was extracted using DNeasy® Blood & Tissue Kit (QIAGEN N.V., Venlo, Netherlands) according to the manufacturer's instructions as described below for Millex®-HV filters.

DNA from Sterivex™ filters was extracted without opening the housing according to Miya et al. (2016) with minor adaptations (Appendix S1). To extract DNA from Millex®-HV filters, their housing was cracked and the membrane was removed. DNA was extracted from the membranes using the DNeasy® Blood & Tissue Kit according to the manufacturer's instructions with minor adaptations (Appendix S2). DNA concentration and quality were checked with the NanoDrop™ One UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4 | qPCR and ddPCR analysis

The comparison between primer/probe assays and amplification methods (qPCR vs. ddPCR) was conducted in two main laboratories using aliquots of the same original field samples and the standard dilutions. The samples and dilutions were prepared once and shipped on dry ice to the different laboratories. Particularly, the number of freeze–thaw cycles was kept identical between laboratories to reduce variation among low-concentration dilutions. The initial performances of the three published probe-based qPCR assays were compared on an Applied Biosystems 7500 Real-Time PCR instrument (Applied Biosystems, Waltham, MA, USA) in their original confirmation (assay details summarized in Table 2). In the initial step, the

TABLE 2 Probe-based assays, liquid volumes (& concentrations), and cycling conditions used within single steps of this study.

qPCR assay first described by	Bettge, Segner, et al. (2009)	Hutchins et al. (2018)	Carraro et al. (2018)
PCR method used in first publication	qPCR	qPCR	qPCR
Total amplicon length	73 bp	90 bp	102 bp
Forward primer	GCGAGATTTGTTGCATT TAAAAAG	CGAACGAGACTTCTTCCTT	GGTTGTTTAGTTTGGGCTCACC
Reverse Primer	GCACATGCAGTGTCCAATCG	CTTCCTACGCTTTTAAATAGCG	TCCCTGTAGGGACAGCTATTG
Originally published probe	FAM-CAAAATTGTGGAACCGT CCGACTACGA-TAMRA	FAM-CCCTTCAAT-ZEN-TAGTT GATCTAAACCCCAATT-IBFQ	FAM-CAAGATCTTATTTTATG GCTGCCAC-BHQ-1
Target gene in *T. bryosalmonae* genome	18 s	18 s	COI
1st round of comparison (qPCR only)			
qPCR machine	Applied Biosystems 7500	Applied Biosystems 7500	Applied Biosystems 7500
Number of cycles in qPCR	45	45	45
Primer/probe setup	As published previously	As published previously	As published previously
Initial denaturation	10 min; 95°C	10 min; 95°C	10 min; 95°C
Cycles	15 s at 95°C; 60s at 60°C	15 s at 95°C; 60s at 60°C	15 s at 95°C; 60s at 60°C
Total reaction volume	20 µL	20 µL	20 µL
Promega GoTaq® Probe qPCR master mix	10 µL	10 µL	10 µL
Concentration of forward/reverse primer	0.3 µM/0.3 µM	0.5 µM/0.5 µM	0.3 µM/0.9 µM
Concentration of probe	0.2 µM*	0.25 µM	0.2 µM
Sample input volume	4 µL	4 µL	4 µL
Inhibition control used	No	No	No
2nd round of comparison (qPCR vs. ddPCR) & evaluation of eDNA samples			
Probe in qPCR		FAM-CAAAATTGTGGAACCGTCCG ACTACGA-TAMRA	FAM-CCCTTCAAT-ZEN-TAGTTGATCTAAAC CCCAATT-IBFQ
Probe in ddPCR		FAM-CAAAATTGT-ZEN-GGAACCGTCCG ACTACGA-IBFQ	HEX-CCCTTCAAT-ZEN-TAGTTGATCTAAAC CCCAATT-IBFQ
qPCR/ddPCR machine		bms Mic/bioRad	bms Mic qPCR/bioRad
Number of cycles in qPCR/ddPCR		50/45	50/45
Initial denaturation (both qPCR & ddPCR)		10 min; 95°C	10 min; 95°C
Cycles in qPCR/ddPCR		15 s at 95°C; 60s at 60°C/15 s at 94°C; 60s at 60°C	15 s at 95°C; 60s at 60°C/15 s at 94°C; 60s at 60°C
Total reaction volume qPCR/ddPCR		20 µL/20 µL	20 µL/20 µL
Master Mix in qPCR: TaqMan™ Gene Expression Master Mix		10 µL	10 µL
Master Mix in ddPCR: Bio-Rad ddPCR supermix for probes (no dUTP)		10 µL	10 µL
Concentration of forward + reverse primer in qPCR/ddPCR		0.5 µM + 0.5 µM/0.75 µM + 0.75 µM	0.5 µM + 0.5 µM/0.75 µM + 0.75 µM
Concentration of probe in qPCR/ddPCR		0.2 µM/0.375 µM	0.2 µM/0.375 µM
Inhibition control in testing phase qPCR/ddPCR		No/–	No/–
Inhibition control in eDNA samples (volume) qPCR/ddPCR		1.8 µL/no	1.8 µL/no
Sample input volume (undiluted) qPCR/ddPCR		4 µL/4 µL	4 µL/4 µL

* indicates according to Strepparava et al., 2018.

three assays were compared using the same set of field-collected test samples following the conditions in Table 2. Each qPCR reaction contained 10 μL of TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), assay-specific amount of each primer and probe (Table 2), 4 μL of sample DNA and molecular grade water filling up to a total reaction volume of 20 μL.

In the second step, the LOD and LOQ of the Bettge and the Hutchins 18S assays were compared using a Mic qPCR (Bio Molecular Systems, Upper Coomera, Australia). The cycling conditions (10 min of 95°C initial denaturation, 50 cycles of 15 s at 95°C and 60 s at 60°C with fluorescence measurement after the latter step), total reaction volume and sample input were kept identical for all three assays in all qPCR reactions (see Table 2).

The ddPCR analyses were conducted only with two assays (Bettge, Segner, et al., 2009; Hutchins et al., 2018), using a QX200 ddPCR system (Bio-Rad, Hercules, CA, USA), including an Automated Droplet Generator. Each ddPCR reaction contained 10 μL of Bio-Rad ddPCR supermix for probes (Bio-Rad, Hercules, CA, USA) (no deoxyuridine triphosphate), 750 nM of each primer, 375 nM of each probe (Integrated DNA Technologies, Coralville, IA, USA) and 4 μL of template DNA, and was adjusted to the final volume of 20 μL by adding diethylpyrocarbonate (DEPC) water (Merck, St. Louis, MO, USA). After droplet generation, the ddPCR 96-well plate (Bio-Rad, Hercules, CA, USA, cat no. 12001925) was sealed with pierceable foil (Bio-Rad, Hercules, CA, USA, cat no. 181-4040) and brought into a C1000 Touch™ Thermal Cycler with a 96-well Deep Reaction Module (Bio-Rad, Hercules, CA, USA). PCR conditions were 10 min at 95°C, followed by 45 cycles of denaturation for 30 s at 94°C and extension at 60°C for 1 min, with a ramp rate of 2°C s^{-1}, followed by 10 min at 98°C and a hold at 12°C (see also Table 2). Following amplification, all samples were incorporated into a QX200 droplet reader (Bio-Rad, Hercules, CA, USA) to visualize the total amount of target-positive and target-negative droplets. Calculation of eDNA copy numbers (per reaction volume) was performed by QuantaSoft software (v.1.7.4, Bio-Rad, Hercules, CA, USA).

The assay LOD and LOQ were experimentally evaluated for the 18S assays published by Bettge, Segner, et al. (2009) and Hutchins et al. (2018) with the same procedure for both, qPCR and ddPCR. DNA from *T. bryosalmonae*-positive brown trout kidney was used to create an 18-point dilution series, using 10 replicate reactions for each dilution. Molecular grade water was used as a technical negative control template ($n = 8$ replicates per run). All field water samples (60 Millex®, 60 Sterivex™, four field negative controls) were examined by qPCR according to Bettge, Segner, et al. (2009). 62 Sterivex™ filter samples (including two field negative controls) were investigated by ddPCR to compare assay sensitivity.

With a few exceptions, five replicates of every field water sample were examined by qPCR with a sample input volume of 4 μL per replicate. However, for better comparison with ddPCR, only three of those replicates were used within the multilevel occupancy modeling described below. On the other hand, three replicates (sample input volume of 4 μL, each) of all Sterivex™ field samples were tested by ddPCR, to evaluate assay and method sensitivity under field conditions.

Every field sample was tested for inhibition by replacing 1.8 μL of the molecular grade water with the equivalent volume of inhibition control (IC; TaqMan™ Exogenous Internal Positive Control Reagents, Thermo Fisher Scientific Inc., Waltham, MA, USA) within all qPCR reactions. A sample was considered significantly inhibited if there was a shift of >3 in the average Cq value of the IC compared to those of the positive and negative controls according to Hartman et al. (2005) and Goldberg et al. (2016). As this shift would account for an >8-fold inhibition in an ideal assay, we also checked for signals of moderate inhibition (>1 Cq value) among sample average IC Cq values.

For each qPCR run on field samples, using the Bettge assay, at least two negative control samples (molecular grade water) were used. Results with Cq values > 40 were interpreted as negative as proposed by Klymus et al. (2020). Copy number estimates were derived from Cq values via a linear model fit to Cq measurements of known standard concentrations (Cq ~ log(conc.)).

For each ddPCR run on field samples, using the Bettge assay, two technical negative control samples were included (molecular grade water).

In general, all technical replicates of a field sample as well as of a standard dilution were done on one plate within qPCR. However, various field samples were randomly repeated in another run and the setup for LOD and LOQ calibration contained always both different 18 s assays of one dilution within the same run to ensure reproducibility.

In ddPCR, the setup for the LOD/LOQ tests using standards has included one standard dilution that has been tested on all plates to ensure reproducibility and encounter for batch effects.

2.5 | Statistics

Statistical analyses were performed using R version 4.2.2 (R Foundation for Statistical Computing). Plots were generated using the ggplot2 package. All analysis scripts are available on GitHub (https://github.com/jamesord/PKD_eDNA).

2.5.1 | Estimating the LOD and LOQ

To calculate LOD and LOQ, we followed the protocol of Klymus et al. (2020) and Merkes et al. (2019). We used aliquots of *T. bryosalmonae* genomic DNA in the same dilution series for both methods, ddPCR and qPCR. The same applies to the two assays used, Bettge and Hutchins assays. Probit models from the drc package were fit to binary [positive (=1)/negative (=0)] response data from the dilution series ($n = 10$ replicates at each dilution point). For each assay-detection platform combination, the best model (based on log-likelihood, Akaike Information Criterion, lack of fit, and residual variance) was selected using the mselect() function.

LODs and their standard errors were calculated from model estimates using the predict() and approx() functions to identify the lowest concentration value at which the probability of detection was no less than 0.95.

To estimate the LOQ, coefficients of variation were calculated on obtained concentration estimates (from linear models of Cq values for qPCR and droplet numbers for ddPCR). Moreover, a 4th-degree polynomial model was fit to calculated coefficient of variation (CV) values of those estimated concentrations, and the predict() and approx() functions were used to identify the lowest concentration value at which the estimated CV did not exceed 0.35 (threshold as applied by Klymus et al., 2020). We tested polynomial models of CV as a function of starting concentration, with the maximum being a 4th-degree polynomial, reasoning that higher polynomial degrees are unlikely to represent the true relationship. The 4th-degree polynomial model did however have the lowest AIC and was therefore selected for calculating the LOQ.

2.5.2 | Comparison of ddPCR and qPCR quantification on eDNA from river water samples

The relative accuracy of quantification estimates obtained via qPCR and ddPCR was assessed by calculating a mean concentration for each sample. A linear regression model was used to estimate the correlation between qPCR and ddPCR sample concentration among the collected water samples. Only samples with non-zero estimates from both methods were included. The difference in estimated copy numbers obtained using qPCR and ddPCR was assessed using a repeated measures ANOVA, fit as a linear mixed model with Kenward-Roger approximation of degrees of freedom, using the lme4 (Bates et al., 2015) and lmerTest packages (Kuznetsova et al., 2017). The model was fit to square-root transformed data (which resulted in normally distributed residuals) and used only samples whose mean copy number estimate was above the LOQ of both qPCR and ddPCR.

2.5.3 | Multilevel occupancy models

Multilevel occupancy models were used to evaluate and compare the presence-absence data derived from qPCR and ddPCR assay replicates of environmental samples. A three-level occupancy model derives three probability parameters: probability that *T. bryosalmonae* was present at the site (site-level occupancy probability, ψ), probability that eDNA of *T. bryosalmonae* is present in the sample (herein called capture probability, θ), and probability that eDNA of *T. bryosalmonae* will be detected in a replicate (replicate-level detection probability, p). Each of these can be modeled as a function of one or more covariates.

We used the R package msocc (Stratton et al., 2020) which implements Gibbs sampling to estimate multivariate posterior distributions of the multilevel model described by Dorazio and Erickson (2018). In addition to a null model with no covariates [$\psi(1),\theta(1),p(1)$], a series of models were fit which included different combinations of covariates on θ and/or p. The covariates were sample volume, filter type (Millex® or Sterivex™), and detection platform (qPCR or ddPCR). Sample volume (either 300 mL or 600 mL for most samples) was included as a covariate on θ, reasoning that capture probability should be greater given a larger sample volume. Filter type was included as a covariate on p given that the two filters may affect DNA extraction (and therefore detection probability); as the two filters have identical pore size, we did not consider filter type as a covariate on θ. Detection platform was included as a covariate on p. Eight models were run including the null model, each with 11,000 Markov chain Monte Carlo (MCMC) samples. The first 1000 MCMC samples were excluded as 'burn-in' prior to further analyses. The widely applicable information criterion (WAIC) (Watanabe, 2010) was derived for each model to compare the quality of models based on the overfitting–underfitting trade-off. Model-based estimates of θ at different sample volumes and p for different filter types and detection platforms were calculated from the intercepts and regression slopes of α and δ – the logit-scale parameters from which θ and p are derived, respectively. These θ and p estimates were calculated from all 10,000 (non-burn-in) MCMC samples of the joint posterior distribution, such that they could be summarized as medians and 95% credible intervals.

3 | RESULTS

3.1 | Determination and evaluation of LOD and LOQ

Comparison of the three assays via the qPCR platform, examining a panel of eDNA samples known to be positive for *T. bryosalmonae*, revealed that the two assays targeting the 18S rRNA gene (Bettge, Segner, et al., 2009; Hutchins et al., 2018) detected the target one cycle earlier than the assay targeting COI (Carraro et al., 2018). Based on these findings, the LOD and LOQ analyses were further conducted on those two assays. The LOD (the lowest concentration of a target with >95% detection probability, if present) of the qPCR using the Bettge assay was 3.59 copies/µL input (14.36 copies/reaction) and 5.04 copies/µL input (20.16 copies/reaction) using the Hutchins assay (Figure 2a). The ddPCR method revealed a LOD of 1.65 copies/µL input (6.6 copies/reaction) for the Bettge assay and 3.74 copies/µL input (14.96 copies/reaction) for the Hutchins assay, respectively.

The LOQ (the lowest reliable quantification with a CV of 0.35) was determined for qPCR at 4.27 copies/µL input (17.08 copies/reaction) with the Bettge assay and 5.83 copies/µL input (23.32 copies/reaction) with the Hutchins assay. The LOQ for ddPCR was 3.66 copies/µL input (14.67 copies/reaction) for the Bettge assay and 4.39 copies/µL input (17.56 copies/reaction) for the Hutchins

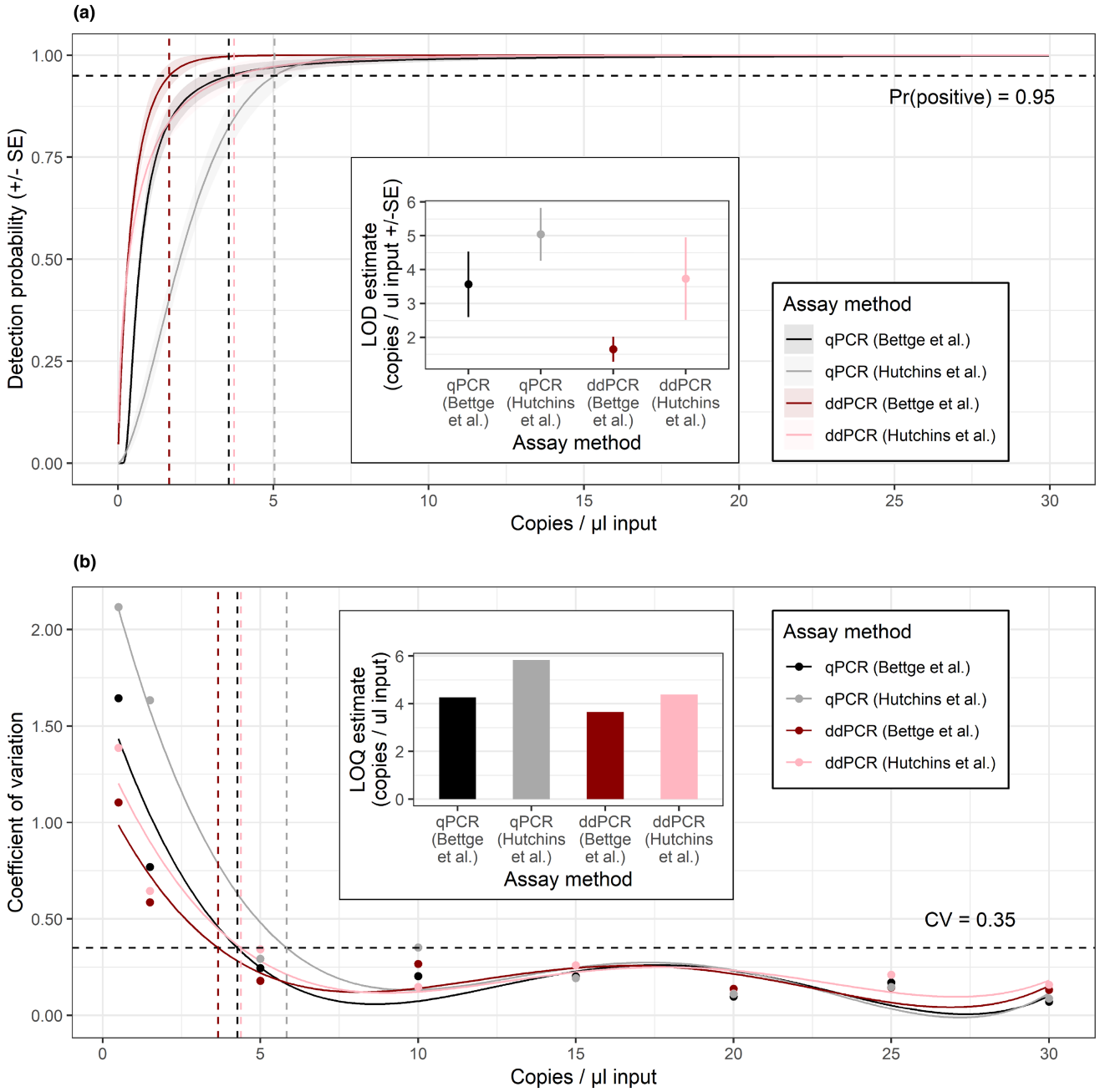

FIGURE 2 Limit of detection (LOD) and limit of quantification (LOQ) for the probe-based assays by Bettge, Segner, et al. (2009) (black and dark red) and Hutchins et al. (2018) (gray and light red) using qPCR (black/gray) and ddPCR (red). (a) LOD: The colored dashed vertical lines show the mean LOD value with a detection probability of 0.95 (black dashed horizontal line). The shadows reflect the 95% confidence interval for the LOD. Inset: Mean LOD estimates (dots) with confidence intervals (corresponding vertical lines). (b) LOQ is calculated with a coefficient of variation (cv) of 0.35 (dashed black horizontal line). The vertical lines show the LOQ also in copies/µL input. Inset: LOQ estimates of the assay/detection platform combinations.

assay, respectively (Figure 2b). The average efficiency was 0.90 for the Bettge assay and 0.91 for the Hutchins assay in qPCR.

Overall, there was a general tendency of ddPCR having lower LOD and LOQ values compared to qPCR, and the Bettge assay compared to the Hutchins assay but as the standard errors overlap, these differences were not significant. However, the LOD of the Bettge assay on the ddPCR detection platform was significantly lower than that of the other combinations.

3.2 | Assay- and method performance on natural river eDNA samples

The correlation coefficient between qPCR and ddPCR regarding total copy numbers was $r = 0.94$ (Figure 3a). Twenty-one (35%) samples showed quantifiable detections above LOQ in ddPCR, whereas only 19 (31.7%) samples showed detections above LOQ. All of those samples above LOQ had higher estimated mean copy

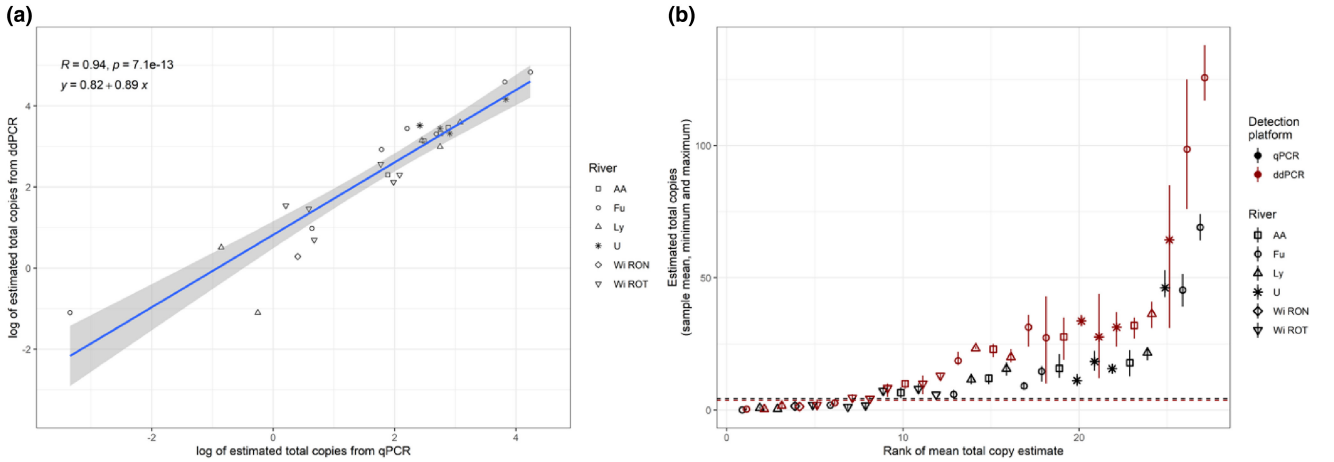

FIGURE 3 (a) Correlation between estimated total copies of the *T. bryosalmonae* 18S rRNA gene by ddPCR and qPCR, respectively. (b) Field samples ranked by mean copy number estimates of the *T. bryosalmonae* 18S rRNA gene by qPCR (black) and ddPCR (red) from low (left side) to high (right side) estimates. The dashed horizontal lines indicate the LOQ for the Bettge, Segner, et al. (2009) assay in combination with qPCR (black) or ddPCR (red). AA = Alte Aare; Fu = Furtbach; Ly = Lyssbach; U = Urtene; Wi RON = Ron; Wi ROT = Rot.

numbers in ddPCR compared to qPCR (Figure 3b), and the effect of ddPCR was significant when tested by repeated measures ANOVA ($F_{(1,122.2)} = 146.7$, $p < 0.001$).

Using the qPCR approach, 31 of 60 samples (51.7%) filtered with Sterivex™ filters were positive for *T. bryosalmonae* DNA in at least one of three replicates. Using ddPCR, 30 of these 60 samples (50.0%) were positive in at least one of three replicates. Agreement between qPCR and ddPCR was achieved in 57 of 60 samples (95%). Two samples (3.3%) were only detected by qPCR, one sample (1.7%) was only detected by ddPCR.

One of the two negative field controls showed one positive droplet in one of the three ddPCR replicates, whereas, for the qPCR analyses, none of the field negative controls showed any fluorescence above the threshold.

Neither significant inhibition was detected on a field replicate level (IPC amplified consistently within three cycles of expected Cq value), nor did the amplitudes or curves show any difference in comparison to negative and positive controls. However, one sample showed signs of moderate inhibition (sample average IC Cq value shift of 2.82) (Figure S1).

3.3 | Multilevel occupancy models

Sample volume had a large and consistent effect on model fit; the four top-scoring models all had sample volume as a sample-level covariate (Table S2). Meanwhile, the replicate-level covariates had negligible effects: although the model with sample volume and filter type as covariates [$\psi(1)$,θ(volume),p(filter)] performed best (lowest WAIC), a model with no replicate-level covariates [$\psi(1)$,θ(volume),$p(1)$] differed by less than one WAIC unit. Nevertheless, we used the model with all covariates included [$\psi(1)$,θ(volume),p(filter + detection_platform)] to obtain separate estimates of θ for two sample volumes and separate estimates of p for three filter type/detection platform combinations.

The distributions of θ values indicated better capture probability with the larger (600 mL) filtered water volume. The median value of θ was 0.49 for 300 mL and 0.91 for 600 mL (Figure 4a).

The filter type and detection platform did not alter the detection probability p (Figure 4b). Estimates of p were high regardless of filter type/assay combination, with median values of 0.92, 0.88, and 0.85 for Millex®/qPCR, Sterivex™/qPCR, and Sterivex™/ddPCR, respectively. Although all combinations had overlapping credible intervals, the estimates for Sterivex™/ddPCR were slightly lower, especially compared to the Millex®/qPCR combination.

To test whether the slightly lower p estimates for Sterivex™/ddPCR were driven by site-specific influences on detection probability of qPCR versus ddPCR, we fit an additional multilevel occupancy model that included filter type, detection platform, and site as covariates at the replicate-level [$\psi(1)$,$\theta(1)$, p(filter_type+detection_platform*site)]. In this case, the site was an interacting covariate which was allowed to influence the effect of detection platform on p. Estimates of p varied across sites, being high in the Alte Aare, Lyssbach, Furtbach, and Wigger Rot, and medium to low in Urtene and Wigger Ron (Figure S2). In the locations Lyssbach and Furtbach, the ddPCR showed a trend for lower detection probability than the qPCR, suggesting that conditions at these specific sites may have driven the slightly lower estimates of p for the Sterivex™/ddPCR assay combination, although credible intervals overlapped.

Model estimates of θ and p were used to calculate the cumulative probability of obtaining a 'positive' field sample (assuming eDNA presence at the site) considering different numbers of collected samples. The cumulative probability of a positive sample of more than 95% detection probability of *T. bryosalmonae* was reached with three field replicates of 600 mL sampled water if the decision criterion to consider a field sample "positive" was "one positive out of 3 technical replicates". When the sample volume was lower (300 mL), six field replicates were needed to reach a 95% detection probability with the same decision criterion. In all cases, the respective minimum number of samples (depending on the decision criterion) is deemed

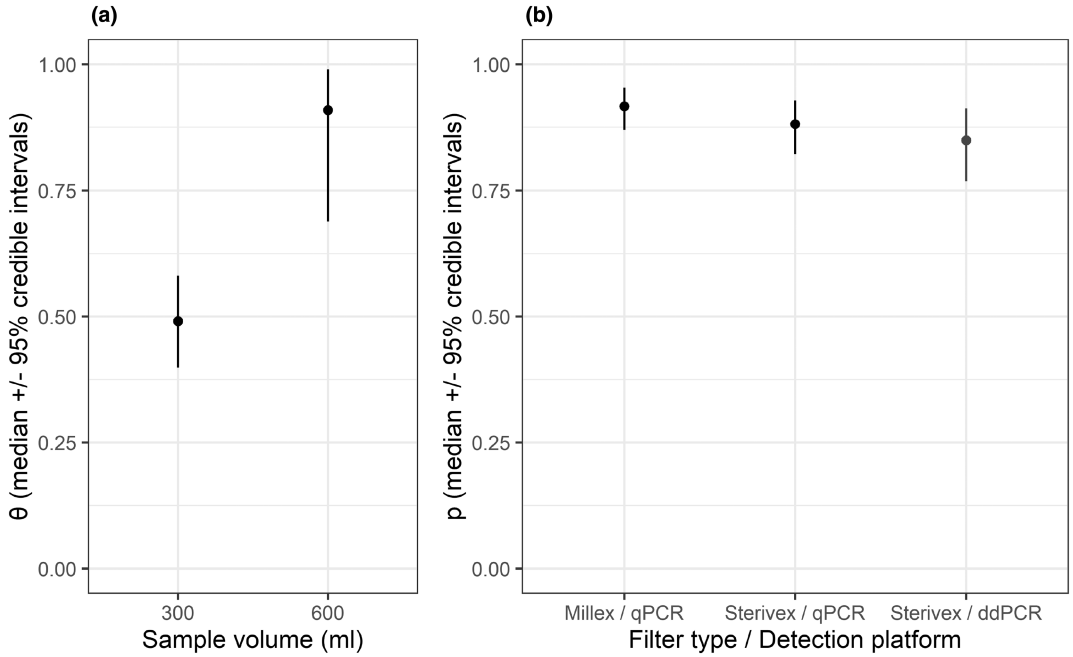

FIGURE 4 MCMC estimates of multilevel occupancy model parameters, summarized as median and 95% credible intervals of estimated values. (a) Estimates of θ (capture probability) for 300 and 600 mL filtered water volume. (b) Estimates of p (replicate-level detection probability) under different combinations of filter type and detection platform.

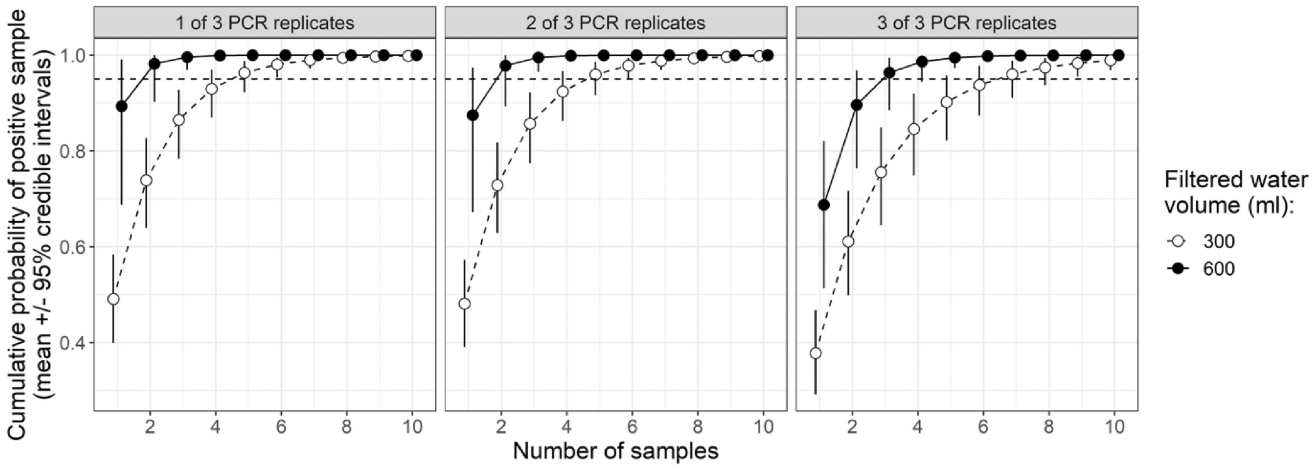

FIGURE 5 Cumulative probability of obtaining a positive sample if the parasite DNA is present, calculated for up to 10 samples collected using either a 300 mL or 600 mL sample volume. Three possible criteria for a positive sample are considered: one out of three, two out of three, and all three technical replicates being positive for parasite DNA, respectively. Cumulative probability calculations were derived from estimates of capture probability and replicate-level detection probability from a multilevel occupancy model which considered only filtered water volume as a sample-level covariate (and therefore did not consider effects of filter type or detection platform on detection probability). The dashed horizontal line indicates a 95% detection probability threshold that should be passed by the mean and the credible interval of the field replicate.

acceptable if at least 95% of the cumulative detection probability estimates exceed the 0.95 detection probability threshold. The number of field replicates increased if the more conservative decision criterion "3 positive out of 3 technical replicates" was applied (Figure 5). Due to the model estimates of p already being high, the probability of detection in at least one sample was only negligibly higher than detection in at least two samples.

4 | DISCUSSION

4.1 | Assay choice

Here we discuss the comparison of most recently established eDNA-based detection assays for *T. bryosalmonae* and provide recommendations for best practice.

We found, that out of the three published *T. bryosalmonae* assays used previously in eDNA studies, the two assays targeting regions of the nuclear 18S rRNA gene (Bettge, Segner, et al., 2009; Hutchins et al., 2018) were twice as sensitive (one cycle difference in a standardized qPCR test) compared to an assay targeting the mitochondrial COI gene (Carraro et al., 2018). Furthermore, our analyses revealed that the detection platform in combination with assay selection can affect detection sensitivity, with ddPCR performing more sensitive than qPCR, and the Bettge assay more sensitive than the Hutchins assay. However, it needs to be mentioned that, since the standard errors overlapped, the differences between the two 18S rRNA gene assays can only be interpreted as tendencies.

Differences in the sensitivity of the probe-based quantification assays can arise from differential PCR efficiency, primer/probe compatibility, and the general design and analysis settings used. In this study, all three assays used were previously published, with PCR efficiency determined from standard curves. In all cases, the efficiency was deemed to be within the desired range of 90%–110% according to Vandesompele and Hellemans (2010) and Svec et al. (2015) and in our initial qPCR comparisons in natural river water eDNA samples, the quantification reactions were set up using the published, optimized assay-specific parameters. Therefore, the lower Cq values obtained from the COI assay for the same samples as processed with the 18S assays more likely reflect lower COI copy numbers in the starting material than lower PCR efficiency.

Mitochondrial genes are commonly used as targets for species-specific, sensitive detection as mitochondrial genomes are present in 10 to 1000 s of copies in most eukaryotic cells (Robin & Wong, 1988), and in *T. bryosalmonae* both the sporoplasmogenic and capsulogenic cells, which together give rise to the mature spores, contain abundant mitochondria (Canning et al., 2000). The abundance of mitochondria in mature *T. bryosalmonae* spores shed from the host has not been quantified but might be expected to be lower than in developing sporogenic cells (Hartmann et al., 2011). Therefore, tandemly repeated multicopy nuclear gene regions may represent a higher copy number target (Dysthe et al., 2018; Minamoto et al., 2017), with a higher detectability and yield in eDNA samples (Jo et al., 2022). The 18S rRNA gene copy number is not known for *T. bryosalmonae* and further particularly hard to estimate within eDNA samples due to its origin from different spore stages, including their difference in cell number and ploidy (Fontes et al., 2017). However, in a related myxozoan (*Ceratonova shasta*), each parasite spore was shown to contain at minimum 2000–4000 gene copies (Hallett & Bartholomew, 2006), suggesting the 18S rRNA gene to be a potentially higher copy number target than the COI gene.

The LOD and LOQ values of the two 18S rRNA gene assays were relatively similar in both ddPCR and qPCR experiments. Generally, the Bettge assay performed better, resulting in lower LOD values compared to the Hutchins assay. Although both assays differed in size, with the Bettge assay amplifying a slightly shorter 73 bp long sequence compared to the Hutchins assay (amplifying a 90 bp target amplicon), it seems rather unlikely that this size difference of the target amplicon may have caused a difference in LOD values. In this context, Brys et al. (2023) recently documented that different mitochondrial target amplicons, that were relatively similar in size (ranging between 120 and 84 bp, and 70 and 119 bp for the detection of American bullfrog and Weather loach respectively), showed very similar detection rates when analyzed by ddPCR. Also, efficiency values of the assays in qPCR (Bettge assay = 0.90; Hutchins assay = 0.91) were very similar in our study. To date, there are only two published studies documenting a LOD threshold for the eDNA assays developed for *T. bryosalmonae* detection. For the Hutchins assay, the LOD threshold was reported at seven copies by Hutchins et al. (2018) and for the Bettge assay, the LOD was reported at 10.33 copies/reaction by Oredalen, Mo, et al. (2022). This does not completely match our results of 20.16 copies/reaction (Hutchins assay) and 14.36 copies/reaction (Bettge assay) on qPCR. The LOQ, on the other hand, was reported at 100 copies (Hutchins et al., 2018) and not reported by Oredalen, Mo, et al. (2022). Our LOQ thresholds were determined at 23.32 copies/reaction for the Hutchins assay and 17.08 copies/reaction for the Bettge assay respectively on qPCR within this study. Both previous studies (Hutchins et al., 2018; Oredalen, Mo, et al., 2022) have used qPCR, and differences in study-specific LOD/LOQs may arise from technical differences (e.g., pipetting and quantification) or the origin of the positive reference samples used for the dilution series (i.e., synthetic templates like GBlock fragments and plasmids or natural reference template). In our study, we used natural *T. bryosalmonae* DNA template from infected and extracted kidneys for the comparison of LOD and LOQ between both assays, as well as both detection platforms. Before starting, the initial concentration of *T. bryosalmonae* DNA template was quantified via ddPCR and diluted to the different standard concentrations. This dilution series was aliquoted and sent to the other collaborating laboratories. Since an accurate and direct comparison of assays and detection platforms is only possible with this approach and resulted in better performance of the Bettge assay (lower LOD and LOQ estimates for both qPCR and ddPCR), we decided to use this assay for the second step of the platform comparison which was based on eDNA samples.

The theoretical LODs described above enable a direct comparison of the assay performance and reflect a threshold at which the target is detected in 95% of the cases, if present. In our study 25% of river eDNA samples were determined to be positive according to this criterion (16.7% by qPCR and 33.3% by ddPCR), using the Bettge assay. Since values below LOD could mean a valid and true detection and should be still reported (Klymus et al., 2020) and due to a lack of any signal during amplifications in water negative reactions as well as a negligible signal in negative field controls (one of three replicates showed one positive droplet in ddPCR, no fluorescence signal in qPCR), we considered detections below the LOD as positive detections within the multilevel occupancy models. Thus, an additional 25.9% of samples showing detection signal below LOD (35.0% for qPCR and 16.7% for ddPCR, both using the Bettge assay) were included as "positive" reactions.

4.2 | Detection platform choice

Our findings suggest that ddPCR is more sensitive than qPCR, resulting in lower LOD and LOQ thresholds for both assays. ddPCR is considered a very sensitive and robust detection method especially in samples with low target copy numbers and when PCR inhibition is present (Brys et al., 2021, 2023; Doi et al., 2015). ddPCR may be particularly suited for eDNA samples, as the target DNA concentrations are typically low, and the diverse "background" non-target DNA, as well as compounds with PCR inhibiting properties, can be high and variable. On the other hand, qPCR inhibition is often reported for environmental samples (Harper et al., 2019; Jane et al., 2015) and can bias the detection of target species compared with ddPCR (Brys et al., 2021, 2023).

However, none of the eDNA samples analyzed in this work showed significant inhibition when tested with the internal positive control approach as recommended for example by Goldberg et al. (2016), Klymus et al. (2020), and Brys et al. (2021).

The comparison of ddPCR and qPCR-based quantification, using both, the Hutchins assay and the Bettge assay and identically treated and prepared dilutions, highlighted a trend of lower quantifications by ddPCR (see Figure 3a). The amplification from the tandemly repeated, multicopy, small subunit 18S rRNA gene target region may result in a biased quantification in ddPCR reactions, particularly when starting DNA is of high molecular weight (Devonshire et al., 2015). This could have been the case with the dilution series used for assay and amplification comparison in this study, where DNA from intact parasite stages was used as a template.

Prior to ddPCR analyses, the target DNA can be additionally fragmented by applying restriction enzymes, in order to digest long DNA fragments into smaller, linear DNA fragments, which may equalize for template differences (Devonshire et al., 2015). This could be used to increase the accessibility of the target DNA for amplification, hence increasing copy number estimates. Which, in turn, could improve precise quantification in ddPCR (Devonshire et al., 2015; dMIQE Group & Huggett, 2020).

eDNA of *T. bryosalmonae* may, depending on the site and proximity to infected hosts, consist of a mixture of DNA deriving from entire spore stages or from degraded, fragmented DNA. Therefore, the use of restriction enzymes could further reduce between-site variation in detectability.

On the other hand, restriction enzymes can cause decrease in sensitivity of ddPCR reactions with well-optimized PCR assays by digesting within a target sequence and thus reducing the number of amplifiable targets (Devonshire et al., 2015; Kline et al., 2016). To avoid target fragmentation, an analysis of restriction sites within a whole genomic ribosomal gene unit should be performed. This can be difficult in species like *T. bryosalmonae*, where no reference genomes are available or where a high level of intragenomic variation in ribosomal gene copies persists.

In this study, we focused mainly on the sensitivity and detection probability of the already used and optimized probe-based assays and wanted to compare both PCR platforms in a similar way on both types of DNA samples, the infected tissue material and the natural eDNA river samples, to keep our findings also comparable with previous studies applied on the same topic (Brys et al., 2021). Therefore, we aimed to avoid the possible negative influence of restriction enzymes on assay sensitivity.

However, it is important to pronounce that the ddPCR results obtained in this work can be partly biased by erroneously low copy numbers, particularly when intracellular genomic DNA was amplified. In any case, this would be the same for both assays in ddPCR but could have caused a reduced sensitivity in comparison to the outcome of the qPCR analyses. Since ddPCR appeared already more sensitive than the qPCR platform, it might be possible that inclusion of a restriction enzyme treatment would have even increased the sensitivity of the ddPCR analyses for the primer/probe assays used.

Nevertheless, and despite all this, the ddPCR showed lower LOD and LOQ than qPCR for both assays and consistently detected higher mean copy numbers than qPCR in all examined eDNA samples whose estimated copy numbers exceeded the LOQ. This could possibly indicate less inhibition on the ddPCR platform. Likewise, the possibility of additional detection or quantification improvement via analysis of restriction sites should be evaluated experimentally.

4.3 | Filter type and water volume choice

The sampled water volume had a significant influence on *T. bryosalmonae* detection probability, with 600 mL showing significantly higher detection rates compared to 300 mL of water. The lowest number of field replicates with a cumulative detection probability (a function of capture probability, replicate-level detection probability, and N samples) of over 95% positive parasite DNA detection, was reached with the combination of 600 mL filtered water through Sterivex™ filters in combination with either qPCR or ddPCR at 3 field replicates. Increase in detection probability with higher sample volumes have been described previously (Sakata et al., 2021; Schabacker et al., 2020), and for consistent detection (detection in 100% of water sample level replicates within a site), even higher water volumes may be beneficial (Sieber et al., 2023). Such approaches might be of particular importance when targeting parasite spores in the water (Alama-Bermejo et al., 2013; Bass et al., 2023; Fontes et al., 2017). However, other studies report no significant correlation between higher filtered water volumes and detection success for free- or membrane-bound eDNA (Mächler et al., 2016).

No significant difference was found in detection probability between Millex® and Sterivex™ filters. Sterivex™ filters were, however, less susceptible to clogging, and filtering high amounts of water (>300 mL) was not possible using Millex® filters. By comparison, Sterivex™ filters consistently processed 600 mL of water. The multilevel occupancy models showed that the amount of field replicates can be reduced using Sterivex™ filters in combination with higher water volumes filtered. The Sterivex™ cartridges are easier to handle

compared to Millex® filters during field work and extraction, less prone to contamination, and easier accessible to a broader range of users, such as fish farmers or wardens.

It is important to point out that if the number of field replicates is increased, >95% cumulative probability of positive detection can be reached with all combinations of the filters, filtered water volumes, and the detection platform used. The number of field replicates is thus dependent on the decision criterion of how many technical replicates must be positive to consider a field replicate and thus a sampling site "positive". Our method of testing allows selecting theoretically any combination of filter type, filtered water volume and detection platform to be applied. *T. bryosalmonae* detection is therefore possible in many situations where favorable materials or methods (Sterivex™ filters and ddPCR) are not available, but should be compensated for by higher sampling effort and costs.

4.4 | Large-scale implementation and outlook of eDNA PKD monitoring

The currently established PKD monitoring uses conventional methods involving electrofishing and culling of salmonids for pathological, histological, or molecular investigations for the presence of pathogens and pathology which reduces their population on top of disease-induced mortality. This invasive monitoring is conducted without a priori knowledge of whether the hosts are infected or not. The application of a non-invasive eDNA detection and quantification to evaluate the parasite's presence in a locality, combined with follow-up monitoring via conventional methods may thus allow more targeted and informative surveillance of PKD in fish populations.

As the development of clinical signs of PKD in an infected host is temperature-dependent (Bettge, Wahli, et al., 2009; Waldner et al., 2021), the detection of *T. bryosalmonae* in water cannot be used to infer the presence or severity of disease without further modeling and calibration work. Therefore, a direct comparison of parasite eDNA concentrations in the environment and PKD prevalence in fish populations is required to develop predictive models of PKD epidemiology and disease risk.

Although there may be seasonal differences in the timing and quantity of spore release from bryozoan and fish hosts (Carraro et al., 2018; Tops et al., 2006), the actual seasonal presence and concentration of spores, and thus also of eDNA from *T. bryosalmonae* within flowing waters, have to be investigated in further studies.

Further, the non-invasive, eDNA-based detection protocol that we describe within this study reduces sampling efforts and costs drastically compared to the currently applied invasive monitoring protocol. We calculated costs based on laboratory setups and protocols within the participating laboratories of this study and used current prices of materials and salaries based on the Swiss market. Whereas we identified a big difference in costs (mainly based on additional sampling effort) between the invasive monitoring protocol and the non-invasive protocols combining the used materials and methods in four different combinations (fulfilling the requirement for a > 95% cumulative detection probability), those different combinations did not vary much between themselves (Table S3). However, those numbers have to be considered cautiously. They should only be used as rough reference since, for example, product prices (including discounts), salaries, laboratory infrastructure, processes and protocols are often subject to fast and massive changes and can vary tremendously between different laboratories and countries.

Finally, species-specific non-invasive pathogen detection protocols can, with further research and development, significantly reduce the need to sample fish for pathogen monitoring purposes. This would reduce the culling of already fragile fish populations and disturbance of their habitat for surveillance campaigns. Other situations that might particularly benefit from non-invasive surveillance are cases where restocking or resettling of salmonid fry is considered, and a risk assessment of the habitat regarding parasite presence might be decisive.

5 | CONCLUSION

Our calibration and comparison studies showed that a combination of Sterivex™ filters, 600 mL water, three field replicates and the probe-based assay published by Bettge, Segner, et al. (2009) tested via ddPCR analyses was the most sensitive and easy-to-apply *T. bryosalmonae* eDNA detection protocol. This non-invasive protocol combines an easy and cost-effective sampling method and a highly sensitive laboratory analysis to detect environmental DNA of *T. bryosalmonae* from water samples. We conclude that, with further field calibration (e.g., test for the seasonal presence of the parasite, comparison of non-invasive eDNA-based protocol with the established invasive monitoring protocol), this eDNA-based protocol has the potential to complement and partially replace the current invasive monitoring protocol.

AUTHOR CONTRIBUTIONS

Conception and design of study: Heike Schmidt-Posthaus, Rein Brys, Hanna Hartikainen, and Moritz Stelzer. Data acquisition: Moritz Stelzer, Hanna Hartikainen, Sabrina Neyrinck, Jonas Steiner, and James Ord. Data analysis: Moritz Stelzer, James Ord, Rein Brys, Jonas Steiner, Hanna Hartikainen, and Heike-Schmidt-Posthaus. Data interpretation: James Ord, Rein Brys, Moritz Stelzer, Heike Schmidt-Posthaus, Hanna Hartikainen, and Jonas Steiner. Writing of the manuscript, text editing, and final approval by all authors.

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The R script code and the data used in this study can be found at https://github.com/jamesord/PKD_eDNA.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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