Received: 29 June 2023 | Accepted: 30 August 2023

DOI: 10.1111/2041-210X.14217

Methods in Ecology and Evolution

RESEARCH ARTICLE

Environmental DNA-based detection of pathogens in trade and captive settings: Best practices and validation for *Batrachochytrium salamandrivorans*

Jesse L. Brunner[1] | Christian M. Yarber[1] | Robert A. I. Pearhill[1] | Caren S. Goldberg[2]

[1]School of Biological Sciences, Washington State University, Pullman, Washington, USA

[2]School of the Environment, Washington State University, Pullman, Washington, USA

Correspondence
Jesse L. Brunner
Email: jesse.brunner@wsu.edu

Funding information
Division of Environmental Biology, Grant/Award Number: 1754474; Morris Animal Foundation, Grant/Award Number: D19ZO-010

Handling Editor: Chloe Robinson

Abstract

1. Detecting pathogens in the live animal trade is critical for tracking and preventing their movement, introduction and spillover into susceptible fauna. However, the scale of the live animal trade makes individually testing animals infeasible for all but the most economically important taxa. For instance, while the fungal pathogen, *Batrachochytrium salamandrivorans* (*Bsal*), threatens amphibian, particularly caudate diversity, in Europe and the Americas, screening even a fraction of the millions of live amphibians imported into the United States, alone, is impractically laborious and expensive. A promising alternative to individual-level sampling (e.g. swabbing the skin of salamanders) is to instead collect DNA from the animals' environment (e.g. housing container or water) which allows us to screen a whole group of animals at a time.
2. We used a series of experiments with *Bsal*-spiked water and substrates and experimentally infected rough-skinned newts (*Taricha granulosa*) to determine which methods yield the most *Bsal* environmental DNA (eDNA) and evaluate the capacity of these methods to detect *Bsal*-infected animals in conditions found in captive settings and trade.
3. We found that filtering water housing infected animals for even an hour can consistently recover detectable levels of *Bsal* eDNA, that there is little evidence of *Bsal* eDNA being clumped in housing containers or swamped or inhibited by dirty housing containers, and that eDNA-based methods achieves an equivalent or higher chance of detecting *Bsal* infections in a (virtual) population of co-housed newts with fewer samples than individual swabs.
4. By sampling the genetic materials accumulated from a whole group of animals, eDNA-based methods are a powerful means of detecting pathogens, such as *Bsal*, in shipments and captive populations. These methods bring routine pathogen surveillance into reach in many more contexts and can thus be an important tool in conservation and disease control.

KEYWORDS
emerging infectious disease, live animal trade, pathogen detection, tools to prevent spillover

1 | INTRODUCTION

The international and regional trade in live animals facilitates the movement and emergence of zoonotic and enzootic pathogens around the globe (Cunningham et al., 2003; Daszak et al., 2000; Fèvre et al., 2006). These pathogens threaten the health of humans, domestic animals and wildlife, including those species that are already threatened with extinction (Cunningham et al., 2003; Daszak et al., 2000; Peeler & Feist, 2011; Smith et al., 2009). Detecting pathogens in trade, both at borders and in domestic facilities, is a critical, if challenging goal (Brunner, 2020; Kuiken et al., 2005; Smith et al., 2009).

Consider the emerging fungal pathogen (Martel et al., 2013) *Batrachochytrium salamandrivorans* (*Bsal*), which threatens salamander diversity (Martel et al., 2014; Yap et al., 2017). Like its sister species, *B. dendrobatidis* (*Bd*), which has caused declines and even extinctions in hundreds of frog species—an unprecedented loss of vertebrate diversity due to disease (Skerratt et al., 2007)—*Bsal* appears to have spread via the international trade of live amphibians (Martel et al., 2014; Nguyen et al., 2017; Yuan et al., 2018). *Bsal* was likely introduced into Northern Europe via the pet trade from Southeast Asia (Laking et al., 2017; Nguyen et al., 2017; Yuan et al., 2018), where it rapidly spread among private amphibian collections (Fitzpatrick et al., 2018; Sabino-Pinto et al., 2018) and into wild populations (Lastra González et al., 2019; Lötters et al., 2020; Martel et al., 2014). While it has not yet been detected in North America—it is so far absent from both wild and captive amphibians (Basanta et al., 2022; Klocke et al., 2017; Waddle et al., 2020)—the threat of its introduction into this hot-spot of salamander diversity led to temporary prohibitions on the importation of 201 species of salamanders into the United States (United States Fish and Wildlife Service (USFWS), 2016 and all salamanders into Canada (Canada Border Services Agency (CBSA), 2018). However, these exclusions are incomplete (several other taxa, including frogs, have since been shown to carry *Bsal*; Gray et al., 2023, Nguyen et al., 2017), can frustrate pet trade industry partners, and may have other unintended consequences such as promoting black-market trade (Eskew & Carlson, 2020; Garner et al., 2009). Moreover, such bans do not provide any data on the magnitude, routes or patterns of introduction and spread. A more holistic approach requires detecting pathogens in trade, at borders and among captive collections and facilities.

There are two key problems with detecting *Bsal* in the amphibian trade, which are common to the live animal trade more generally. First, *Bsal* infections can be difficult to detect with confidence. Infections are often asymptomatic in some species, which is especially worrying in those that are commonly traded (Gray et al., 2023; Sabino-Pinto et al., 2018), meaning sensitive diagnostic tests are required, but even these may not reliably detected infections for several weeks post-exposure (Thomas et al., 2018).

Second, and more challenging, is the massive volume of trade. Roughly 2.98 to 3.73 million live amphibians are imported annually into the United States, alone (Altmann & Kolby, 2017; Connelly et al., 2023). Screening even a fraction of these millions with traditional methods (i.e. swabbing individuals) is prohibitively costly and laborious, and if infections are rare, *large* fractions of each shipment must be screened to have an appreciable chance of detection (Brunner, 2020).

One promising alternative to reduce the burden of surveillance is to collect environmental samples to test for genetic material from pathogens (environmental DNA; eDNA). This approach is non-invasive, collects genetic material accumulated over the preceding hours or days, and theoretically samples from all individuals in a shipment—tens or potentially hundreds of animals—simultaneously (Brunner, 2020). Thus, by dramatically reducing the number of samples required to ensure detection, eDNA-based screening could facilitate routine *Bsal* surveillance in shipments of live animals and captive collections. However, while eDNA has been used to detect amphibian pathogens in natural settings (e.g. Hall et al., 2016; Hyman & Collins, 2012) and is being adopted rapidly, its application and study in captive, closed populations has been fairly limited (e.g. Kawato et al., 2021; Mahon et al., 2018; Trujillo-González et al., 2019).

In order for eDNA to be used appropriately to surveil for *Bsal* in captive populations three general questions need to be addressed: (1) How should samples be collected? That is, how much do the details of collection (e.g. water vs. substrates, filtering vs. centrifugation) matter to detection? With hundreds of species of amphibians in trade it is impossible to establish universal protocols, but general guidance would be helpful. (2) Is it reasonable to assume that samples are interchangeable (i.e. not masked by non-target DNA in realistic settings, not especially clumped)? The statistics of inference from eDNA in closed populations assume that sensitivity—the probability of detecting *Bsal* if present—does not change substantially as population size, and concomitant waste and non-target host and microbial DNA, increase, nor vary tremendously among samples (Brunner, 2020). These assumptions must be tested. (3) How well eDNA-based detection works at detecting infections under realistic conditions? While we caution against simple estimates of sensitivity—*Bsal* infection intensities vary by orders of magnitude over weeks if not days (Gray et al., 2023)—it is important to establish whether *Bsal* is rarely or commonly detected in eDNA from known infected animals over time, especially in comparison to traditionally collected swabs.

Here we present the results from a series of experiments in which we address each of these questions using samples spiked with *Bsal* zoospores as well as experimentally infected rough-skinned newts (*Taricha granulosa*).

2 | MATERIALS AND METHODS

2.1 | *Bsal* culturing

A culture of *Bsal* was obtained from J. Piovia-Scott (Washington State University) with permission from F. Pasmans (Ghent University) and grown on tryptone–gelatin hydrolysate–lactose agar plates at 15°C according to established methods (Martel et al., 2013; Robinson et al., 2020). Zoospores were collected by washing plates with 2 mL of water, counted using a haemocytometer, and then used for experiments or inoculation immediately.

2.2 | Experiment 1: Determining the limits of detection for two methods of collecting eDNA from water

In each of three distinct trials carbon-filtered, dechlorinated water used to house animals was spiked with *Bsal* zoospores and diluted to one of several concentrations ($1.67 \times 10^{1,2,3,\ \&\ 4}$ zoospores/mL in trial A, $10^{-2,\ -1.5,\ -1,\ -0.5,\ \&\ 0}$ zoospores/mL in trial B, and $10^{-1,\ -0.5,\ 0,\ 0.5,\ \&\ 1}$ zoospores/mL in trial C)—the range of dilutions was adjusted between trials to better establish the limit of detection—thoroughly mixed with a weighing spatula, and then eDNA immediately collected in one of two ways. First, either 250 mL (trial A) or 100 mL (trials B and C) of water was filtered through a 47 mm diameter 0.45 m cellulose nitrate analytic filter in a single-use cup (Sterlitech, Auburn, Washington, USA) using a vacuum pump, after which the filter was removed with bleach-cleaned forceps. Second, in trials A and B 50 mL of water was placed in a sterile 50 mL conical tube and the suspended material pelletized by centrifugation at 5500 rcf for 35 min at 6°C using a benchtop centrifuge (Ficetola et al., 2008), the supernatant carefully removed with a single-use serological pipette, and the pellet retained. Unspiked water samples treated similarly served as controls in this and the next two experiments to detect possible contamination. Moreover, in this and all subsequence experiments nitrile gloves were worn and changed between samples and all implements (e.g. forceps) were disinfected for ≥1 min. in a 50% commercial bleach solution to prevent contamination among samples and then rinsed thoroughly with clean water.

2.3 | Experiment 2: Determining how *Bsal* eDNA recovery changes with volume filtered

Five replicate volumes of carbon-filtered, dechlorinated water were each spiked with zoospores to achieve a 5×10^3 zoospores/mL concentration, mixed well, and then samples of 50, 125, 250, 500 and 1000 mL of water were collected from each replicate and filtered through a 0.45 m cellulose nitrate analytic filter.

2.4 | Experiment 3: Evaluating methods for collecting *Bsal* eDNA from substrates

Small (~1 cm^2) pieces of unbleached, single-fold paper towel (SK1850A, Tork, Philadelphia, PA, USA) and sphagnum moss, both common substrates for housing and shipping amphibians (JLB pers. obs.) were soaked in a solution with 5×10^5 zoospores to simulate the accumulation of zoospores on these substrates. They were then dried at room temperature for 24 h, after which five pieces of each substrate type were frozen for DNA extraction from the material itself (direct subsampling treatment) and five additional pieces were soaked in 50 mL of water for an hour, with agitation, which was then filtered through a 0.45 m cellulose nitrate analytic filter (soak then filter treatment).

2.5 | Animal collection, housing and *Bsal* exposure

Adult rough-skinned newts (*T. granulosa*) were collected with dip nets and in minnow traps from ponds in the Virgil Phillips Farm (VPF) County Park, Moscow, ID, USA ($n = 15$; ID Dept. of Game & Fish wildlife collection permit 120303) and Capitol State Forest (CSF), Olympia, WA, USA ($n = 35$; WA Dept of Fish & Wildlife permit 19-128, WA Dept. Natural Resources licence 60-WS1038) in April 2019, returned to the laboratory, and housed individually in plastic deli containers (20.3 × 20.6 × 6.4 cm; Genpak, Charlotte, NC, USA) with moist, unbleached paper towel as substrate and a small pool of water created by holding the containers at an angle. Animals were held at 15°C with a 12-hr light/dark cycles and fed three to four crickets every 3 days when containers were changed. These laboratory studies were approved by the Washington State University Institutional Animal Care and Use Committee (ASAF #6332).

A month and a half after being brought to the laboratory roughly half of the newts from both sources began displaying inappetence and weight loss. Chytridiomycosis was confirmed with Taqman qPCR for *Bd* DNA (Hyatt et al., 2007) in swabs collected from several individuals and was suspected in many others based on shedding and inappetence. All individuals were therefore treated for chytridiomycosis with daily 5 min bath of 0.005% solution of itraconazole for 10 days following established methods (Forzán et al., 2008). Before and after treatment many individuals lost weight and were thus hand fed and, for some individuals, force fed until they began eating on their own again. In this period two individuals from CSF were euthanized for histological and diagnostic testing, an additional three from CSF were euthanized due to continued poor body condition and anorexia, and two from VPF died from bacterial septicaemia following declining body condition (histopathology report). Thus, an additional 15 newts were collected in early August 2019 and prophylactically treated for *Bd* infections with itraconazole baths. The absence of *Bd* was

confirmed with negative qPCR results skin swabs from all animals. After animals had recovered from treatment for ≥10 days and were observed regularly eating the animal experiments began.

We individually exposed 20 randomly selected newts to 5×10^5 zoospores of *Bsal*, a dose known to cause infection in *Taricha* but that is not generally lethal (Gray et al., 2023), and 20 individuals to an equivalent amount of culture media as a control in 100 mL of water in plastic cylindrical tubes (19 × 6 cm diameter) that ensured contact with the inoculum without submersion. After 24 h the animals were returned to their individual housing containers.

2.6 | Experiment 4: Evaluating how *Bsal* eDNA recovery changes with the duration of shedding

Thirty-four days post-exposure (DPE) nine infected and five control animals were transferred to individual 19 × 30 cm Whirl-pak (Madison, WI, USA) bags with 250 mL of water and air to breathe. Then 1, 3, 6, 12 and 24 h later the animals were moved to new Whirl-pak bags, or, at 24 h, to their original housing container, producing water samples in which the animals had shed eDNA for durations of 1, 2, 3, 6 and 12 h. Each sample was immediately filtered through a 0.45 m cellulose nitrate analytic filter.

2.7 | Experiment 5: Estimating the distribution of *Bsal* eDNA among samples, determining whether physical homogenization reduced the variation and establishing whether debris from group housing interferes with eDNA recovery

Each trial consisted of two parts. In the first part a *Bsal*-infected newt (previously confirmed with qPCR) was placed in a large, shallow plastic tub (31 × 77 cm) filled with 10 L of dechlorinated water (~4 cm deep) and housed for 24 h. The animal was removed and then 20 eDNA samples were collected at haphazardly selected points in the tub (control treatment). The remaining water was then homogenized with an immersion blender for 1 min and an additional 20 eDNA samples taken (homogenized treatment). In the second part, the same animal was placed in a similar container with 10 L of water that had just housed 20 uninfected newts for the prior 72 h to create conditions that might be expected in group-housing settings (dirty water treatment). Then, after 24 h, 20 eDNA samples were collected. This was repeated for each of five *Bsal*-infected newt, though the order of treatments was randomized (i.e. clean water then dirty water, or vice versa) to avoid priority effects.

2.8 | Experiment 6: Establishing how *Bsal* detection varies over time in swabs and eDNA

Beginning 10 DPE the newts were housed for 3 days on either moistened paper towel or sphagnum moss, changing from one type of substrate to the other during each water and container change, for a total of two periods on each substrate. During each water change the animal was removed, transferred to Whirl-pak bags containing 250 mL of water for 1 h, then swabbed with a sterile rayon-tipped swab (25-806 1PR; Puritan Medical Products; Guilford, ME, USA)—five strokes on each of the salamander's feet, ventral surface and dorsal surface—and moved to a new, clean container with crickets and the appropriate substrate. The used substrates were collected from the old container and the water from the Whirl-pak filtered.

An additional eDNA filter and swab sample were collected from each animal 44 DPE and then at 110 DPE two water samples and two swab samples were collected. The newts were then euthanized with an overdose of MS-222 buffered with sodium bicarbonate.

2.9 | DNA extraction and quantitative Taqman real-time PCR (qPCR) methods

All samples were frozen at −80°C until DNA extraction. DNA was extracted from one half of each cellulose nitrate filter and from the substrate samples using the Qiashredder + DNeasy (Qiagen; Redwood City, CA, USA) protocol of Goldberg et al. (2011) in a laboratory dedicated to extracting low-copy, low-quality DNA samples. Swabs were extracted using the DNeasy Blood and Tissue kit with buffer ATL, as in Goldberg et al. (2011), but without the Qiashredder step.

We quantified the amount of *Bsal* DNA using the TaqMan real-time PCR assay of Blooi et al. (2013) that targets the 5.8S rRNA gene of *Bsal* run in triplicate 20 L reactions with iQ Supermix (Bio-Rad; Benicia, CA, USA) for 50 cycles on a CFX96 Touch Real-Time PCR Detection System. Standard curves of gBlock oligonucleotides (Integrated DNA Technologies; Skokie, IL, USA) with known numbers of copies of the target sequence ($5 \times 10^{0-3}$ copies) were used to quantify *Bsal* gene copy number. One microlitre of exogenous internal positive control (ExoIPC) assay and target (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was added to the third well of each sample to look for evidence of PCR inhibition. PCR inhibition causes notably reduced amplification of the ExoIPC assay, relative to that seen in the no-template control wells, even when the *Bsal* target did not amplify. Extracted DNA from inhibited samples were cleaned using a OneStep PCR Inhibitor Removal kit (Zymo Research; Irvine, CA, USA) and retested; if necessary, samples were diluted 1:10 until there was no evidence of inhibition. Concentrations were adjusted to account for dilution (multiplied by the dilution factor) or loss in the column (multiplied by two, which represents the worst-case recovery per manufacturer's materials, though on the scale of analysis the size of this adjustment has little effect; JLB pers. obs.). Samples in which just one of three wells showed clear signs of amplification, or were ambiguous for any other reason, were re-run.

2.10 | Analyses of *Bsal* quantities

We modelled the quantity of *Bsal* gene copies (Q) estimated from each qPCR reaction (well) in a hierarchical, Bayesian framework to account for the structure of the data (e.g. multiple samples from the same individual animal or replicate, multiple replicate experiments following the same design), uneven sample sizes, and reasonable values for parameters (e.g. the ratio of gene copies per zoospore must be ≥0 but is unlikely to be larger than an order of magnitude). We also needed to account for zeros, or non-detections, in our data, which do not necessarily correspond to the true absence of *Bsal* genes (e.g. samples taken from water with low concentrations of zoospores in experiment 1). At low copy number, the chances that a copy of the DNA target ends up in a qPCR reaction, and thus that the sample has any amplification, can be modelled as a Poisson distribution (Lesperance et al., 2021), but when the copy numbers in samples are higher and detection is virtually assured the logarithm of the quantity is reasonably modelled with a normal distribution. We combine these approaches as follows.

The likelihood of observing quantity $\log(Q_{i,j,k})$ in well i from sample j in treatment k is,

$$\log(Q_{i,j,k}) \sim \begin{cases} \text{Poisson}(0|\,\lambda_{j,k}). & \text{if } Q_{i,j,k}=0 \\ [1-\text{Poisson}(0|\,\lambda_{j,k})]\times\text{Normal}[\log(\lambda_{j,k}).\sigma_{qpcr}]. & \text{if } Q_{i,j,k}>0 \end{cases}$$

where λ is the expected number of copies in the qPCR reaction. The second term in the likelihood accounts for the probability of not being negative *and* having an estimate of $\log(Q_{i,j,k})$ given a mean of $\log(\lambda_{j,k})$, and a standard deviation among wells of a qPCR reaction of σ_{qpcr}. In the cases where there were no zeros (e.g. experiment 2) this collapses to a typical likelihood with a normal distribution of observations.

In experiment 1 with various concentrations of zoospore-spiked water we assumed the expected number of copies in a reaction was a multiple of the number of zoospores processed,

$$\lambda_{j,k} = \exp(\varphi_{j,k}) \times \text{processed}_{j,k},$$

where $\exp(\varphi_{j,k})$ is the recovery efficiency of the entire process, from sample collection to DNA extraction, for sample j in treatment k. The principal goal was estimating this recovery efficiency for each method of collection, as well as the sample-to-sample variability, but we also used these estimates to infer the limits of detection with each method given the various sources of uncertainty.

For experiment 2 the goal was to determine whether the expected recovery efficiency changed with the volume of water filtered. We assumed that recovery efficiency, $R_{i,j} = \log(Q_{i,j}) - \log(\text{processed}_{i,j})$, was normally distributed:

$$R_{i,j} \sim \text{Normal}(\varphi_{i,j}, \sigma_{qpcr}).$$

The expected recovery efficiency was modelled as a linear function of the volume sampled:

$$\varphi_{i,j} = \alpha_j + \beta_j \times \text{Volume}_{i,j}.$$

If increasing volumes did not reduce recovery efficiency then $\beta_j \approx 0$ and the expected recovery efficiency (per zoospore processed) would be constant.

In experiment 4 we expected eDNA to accumulate at a rate that was allowed to increase or decrease with the duration animals were held in the water:

$$\lambda_{i,j} = \exp(\alpha_j + \sigma_{\varphi_1}) \times \text{duration}_i^{\beta_j}.$$

If $\beta_j = 1$ this would revert to a simple time-invariant model with a constant rate of accumulation. The first term reflects the animal-specific shedding rate and average recovery, α_j, plus the random deviations in recovery from sample to sample, σ_{φ_1}.

Similarly, in experiment 5 we modelled the expected number of *Bsal* gene copies in a reaction from animal j in treatment k as

$$\lambda_{i,j} = \exp(\alpha_j + \sigma_{\varphi_1,k}) \times \exp(\beta_k).$$

The treatment (control vs. homogenized water vs. dirty water) was allowed to influence both the average amount of copies shed by an animal, $\exp(\beta_k)$ and the degree of sample-to-sample variation, $\sigma_{\varphi_1,k}$.

Finally, we considered the probability of detecting *Bsal* in a hypothetical population based on our swab and eDNA filter samples collected over the course of the experiment. In essence, we imagined a population comprised of our 20 *Bsal*-exposed animals with the individual-level infections status we observed. We then estimated the probability of detecting *Bsal* in this population at each time point as the probability that at least one of the imagined 1, 3, 5 or 10 samples collected tested positive. For swab samples this amounted to the probability that at least one of the positive-testing animals at the time point would be included in the group of samples. For filter eDNA samples we calculated the concentration of *Bsal* eDNA expected in a 20 L volume housing this imagined population as the sum of the *Bsal* eDNA shed by each animal divided by the volume. We repeated this exercise for each time point independently, which ignores the possible accumulation of *Bsal* eDNA over time, because we are uncertain of the rate at which *Bsal* eDNA degrades under these or other conditions. We then simulated eDNA samples from this volume of water given the upper range of the sample-to-sample variation estimated in experiment 5 (the upper 95th percentile of the posterior for this parameter). Note that these calculations ignore transmission and other interactions that might increase the prevalence or intensity of infections and so they are likely conservative estimates for both types of samples.

See the supporting information for the details of analyses, including model structure accounting for the hierarchical nature of our data, our choices of prior distributions, and code for fitting the models to data using Stan via the Rstan package (Stan Development Team, 2023a, 2023b).

3 | RESULTS

3.1 | Experiment 1

Average recovery, defined as the estimated number of *Bsal* gene targets per zoospore processed, was usually between 0.1 and 0.5 across sample collection methods and trials, with the exception of the filtering in the first trial, which, for unknown reasons, had a much higher and less variable recovery of ~1.5 copies per zoospore (Figure 1). Outside of filter samples in this first trial there was substantial variation—up to two orders of magnitude—in the recovery rate among replicate samples (note the width of the distributions of estimated recovery efficiency in Figure 1b). This might stem from zoospores occurring in aggregations or variation in the collection and extraction procedures. In contrast, the variation in estimated quantities among qPCR reactions for individual samples (across wells and plates) was just $\sigma_{qPCR} = 0.18$ on the $\log_{10}$-scale.

Based on these recovery efficiencies, the limit of detection (LOD), operationally defined as the minimum number of zoospores processed to ensure a ≥95% probability of being scored as positive, *per sample*, were quite low (Figure 2), but varied a great deal among experiments, sample collection method, and the criterion for scoring a sample as positive. The *median* LOD in a single well was less than ~25 zoospores with a 90% chance of being ≲ 130 zoospores. The large uncertainty in LOD (shaded areas in Figure 2) stems from the variability in recovery observed among samples.

If samples were run in triplicate and scored with the often-used 'majority rules' criterion—positive if two of three wells are positive, negative if none are positive and otherwise re-run and then scored as positive if at least one of the three wells is positive—the LOD was reduced to a median of <10 zoospores with a 90% chance of being ≲ 47 zoospores across trials and sample collection method. Note that the uncertainty in the LOD is not reduced because the variability in recovery is among *samples*, not among *wells* for a given sample.

Pelletizing and filtering materials from water were broadly equivalent in their recovery and LOD. However, filtering enabled us to process much larger volumes, and thus more zoospores, and was easier, so we used filtering for the remaining experiments.

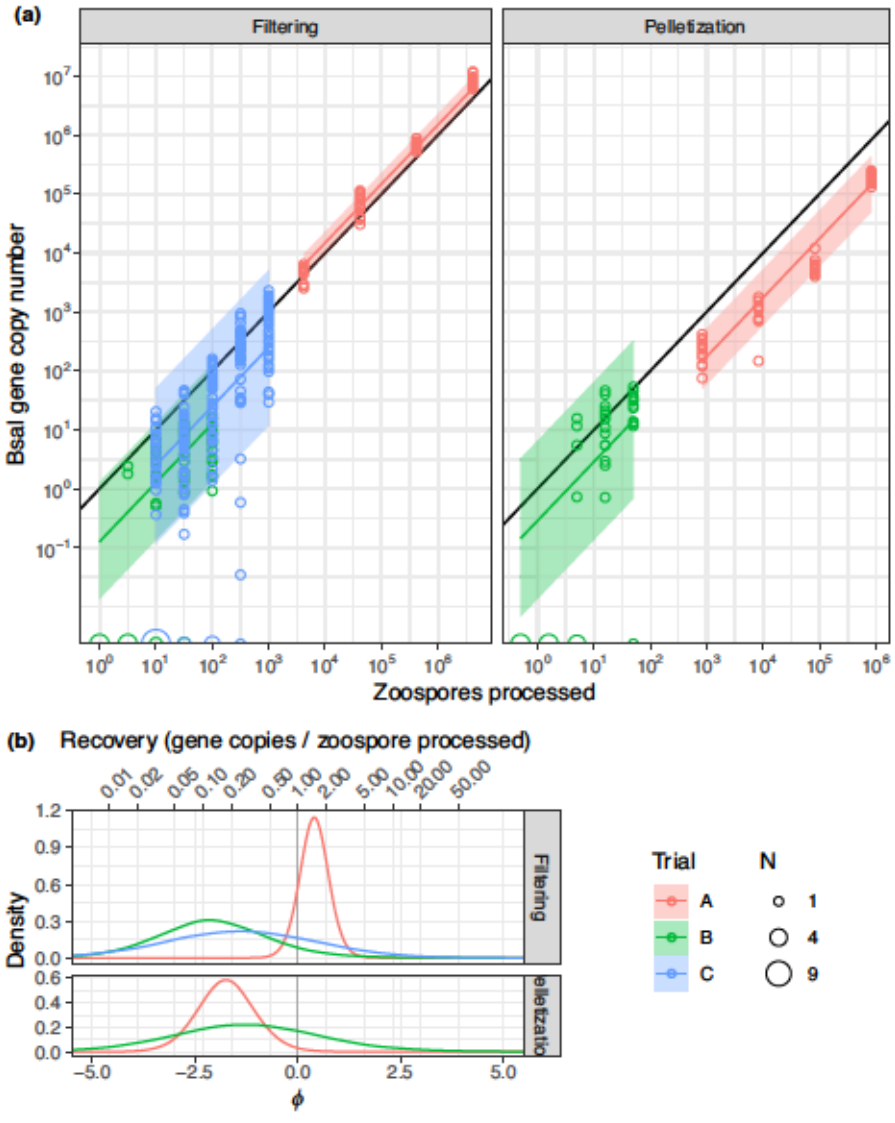

FIGURE 1 (a) Estimated copies of the *Bsal* gene target in each qPCR reaction (circles) against the number of zoospores that were processed (volume filtered × concentration) by filtering or pelletization (facets) in each of three experimental trials (colours) in experiment 1. Lines and shaded areas are model-derived mean and 90% CIs. The black lines highlight the 1:1 relationship. The non-detections at each level of zoospores processed have been aggregated so that they are more visible along the abscissa (zero is undefined on the log-scale). (b) Estimated recovery efficiency ϕ for each method of concentrating eDNA (facets). The distributions of recovery rates do not reflect parameter uncertainty as much as the variability in recovery efficiency among samples.

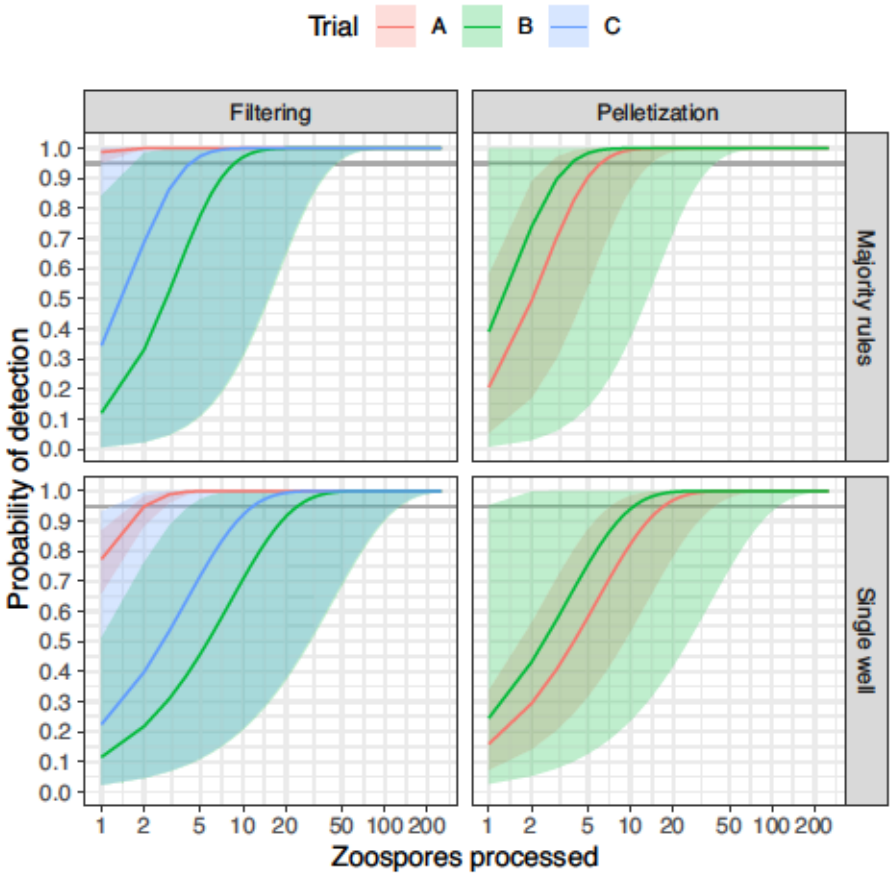

FIGURE 2 Inferred probability of detecting *Bsal* DNA in a single qPCR reaction (= well) or across triplicate wells using the 'majority rules' scoring system (see text for explanation; facet rows) for eDNA concentrated by filtering or pelletization (facet columns) as a function of the number of zoospores processed. Lines represent the median expectation and the shaded areas the 90% CI.

3.2 | Experiment 2

There was little evidence that the recovery efficiency of *Bsal* eDNA decreased with increasing volumes of water filtered (average change in per litre ± one standard deviation = −0.088 ± 0.252), although there were slight, but clearly positive and negative trends in some replicates (Figure 3). This suggests that, barring filter clogging, filtering larger volumes simply captures more *Bsal* eDNA and does not appreciably reduce recovery efficiency.

3.3 | Experiment 3

Six of 15 samples collected directly from either *Bsal*-spiked paper towel or sphagnum moss exhibited clear signs of PCR inhibition—notable reductions in the amplification of the exogenous internal positive control—although all 15 of the paper towel samples and 12 of 15 sphagnum moss samples tested positive (Table 1). Soaking the substrates in water first and then filtering the water for *Bsal* eDNA removed issues with inhibition in the paper towel samples and increased the estimated copy numbers (not shown), but led to greater inhibition and no detection among all of the 15 sphagnum moss samples (Table 1).

3.4 | Experiment 4

There was strong evidence that *Bsal* eDNA accumulates less than linearly with the duration animals are held in water; with longer soak times the amount of *Bsal* eDNA accumulated increased at a slower and slower rate (Figure 4). However, we observed much more variation in the shedding among individual animals than among durations. Collectively this suggests that increasing the time that animals are held in water to collect *Bsal* eDNA yields small gains; short durations are likely sufficient. Variation in the amount of *Bsal* eDNA shed by and collected from individuals, however, may be substantial.

3.5 | Experiment 5

Replicate samples collected from clean water housing a single *Bsal*-infected animal ('control' treatment in experiment 5) varied substantially, by one or even two orders of magnitude (Figure 5). We had hypothesized that physically homogenizing the water prior to sampling would reduce the heterogeneity in estimates of *Bsal* copy number among samples, but that this might come at the cost of degrading the DNA and reducing recovery. Instead, we found that, on average, homogenization not only increased recovery, perhaps by resuspending particles adsorbed to the container walls, but also increased the variation among samples, although both effects were small (Figure 5). We had also hypothesized that when animals were shedding *Bsal* eDNA into dirty water—created by housing 20 uninfected animals in the water for 3 days prior to adding the infected animal—recovery would be substantially reduced due to the eDNA being degraded by microbes, swamped by non-target DNA (e.g. from hosts or microbes), or issues with inhibition. In fact, we found that

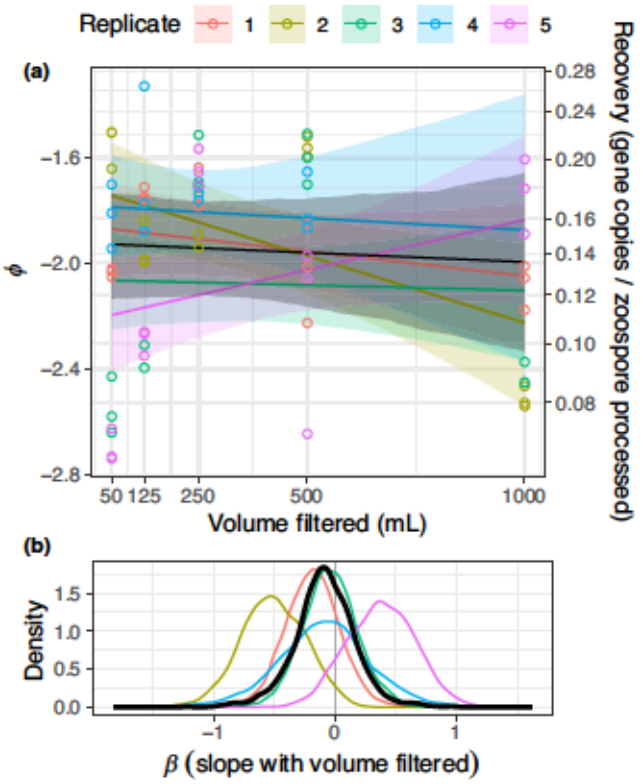

FIGURE 3 (a) Estimated recovery efficiency $\bullet\bullet$ $\exp(\varphi)$, defined as the number of *Bsal* gene copies per zoospore processed, as a function of increasing volumes filtered in experiment 2. Lines and shaded areas are model-derived mean and 90% CIs. (b) Posterior estimates of the slope of φ (=log[recovery]) with volume for each replicate (colour) and overall (black line).

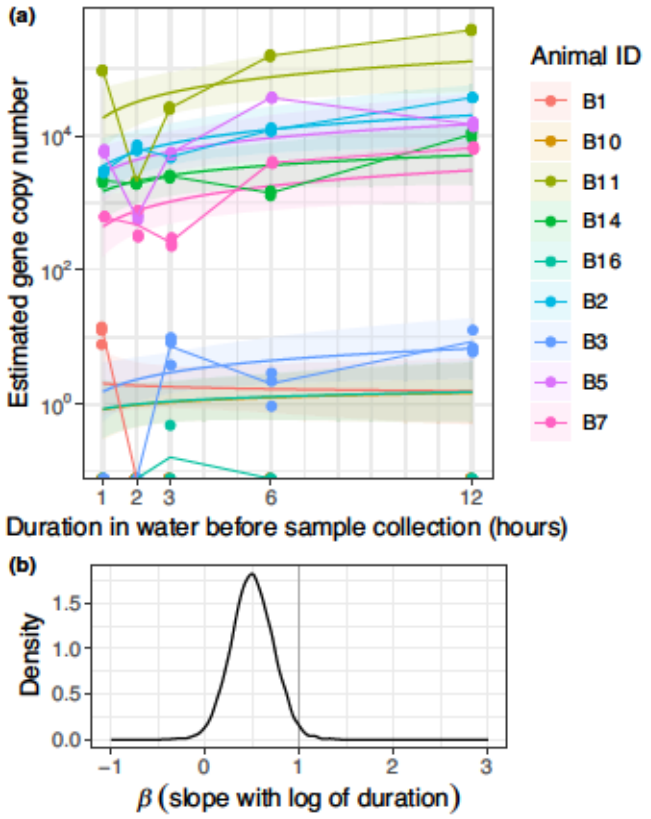

FIGURE 4 (a) Estimated *Bsal* gene copy number recovered from filter eDNA samples collected from each of 10 infected animals (colours) in experiment 4 with increasing durations of time in water. Lines and shaded areas are model-derived mean and 90% CIs. (b) Posterior estimate of the slope of *Bsal* gene copy number with log(duration). A slope of one (vertical grey line) would imply a constant rate of accumulation; this posterior estimate is consistent with *Bsal* eDNA accumulating with the square root of duration.

TABLE 1 The number of samples from experiment 3 that exhibited PCR inhibition, that were positive, and that were screened overall.

Substrate	Sampling method	Number inhibited	Number positive	Number samples
Paper towel	Direct subsampling	6	15	15
	Soak then filter	0	15	15
Sphagnum moss	Direct subsampling	0	12	15
	Soak then filter	15	0	15

recovery was slightly increased in the dirty water treatment and, surprisingly, the estimated sample-to-sample variation was substantially lower than in the other two treatments (Figure 5).

3.6 | Experiment 6

None of the 145 samples from control animals tested positive. Moreover, 10 of the 20 *Bsal*-exposed animals never tested positive in any of the $n=7$ swabs, $n\geq 7$ eDNA filter or $n=2$ paper towel and $n=2$ sphagnum moss substrate samples collected over the duration of the study, strongly suggesting that these animals escaped uninfected even after exposure to 5×10^5 zoospores of *Bsal*. Among the 10 animals with at least one positive sample, the intensity of infections, as reflected in the swab and eDNA filter samples varied a great deal through time, with generally low-intensity or inapparent infections for the first 2 or 3 weeks post-exposure and then increasing in the latter half of the study for six of the individuals and decreasing to undetectable levels in three (Figure 6).

If we assume all 10 animals that tested positive were infected with *Bsal* for the duration of our study (110 days post-exposure; DPE)—a dubious assumption, given the evidence that individuals can recover from *Bsal* infections (Gray et al., 2023), but necessary without an independent indicator of infection status—we can estimate the diagnostic sensitivity of swabs and eDNA filters for each animal as the proportion of samples that (correctly) tested positive. We see substantial variation in the diagnostic sensitivity of both sample types among individual animals (Figure 7), but eDNA filter samples were generally equivalent in performance to swabs.

When we constructed virtual populations with these 10 infected and 10 uninfected newts and estimated the probability that at least one swab or eDNA filter sample would test positive, we observed substantial variation in the power to detect *Bsal* over time, with little

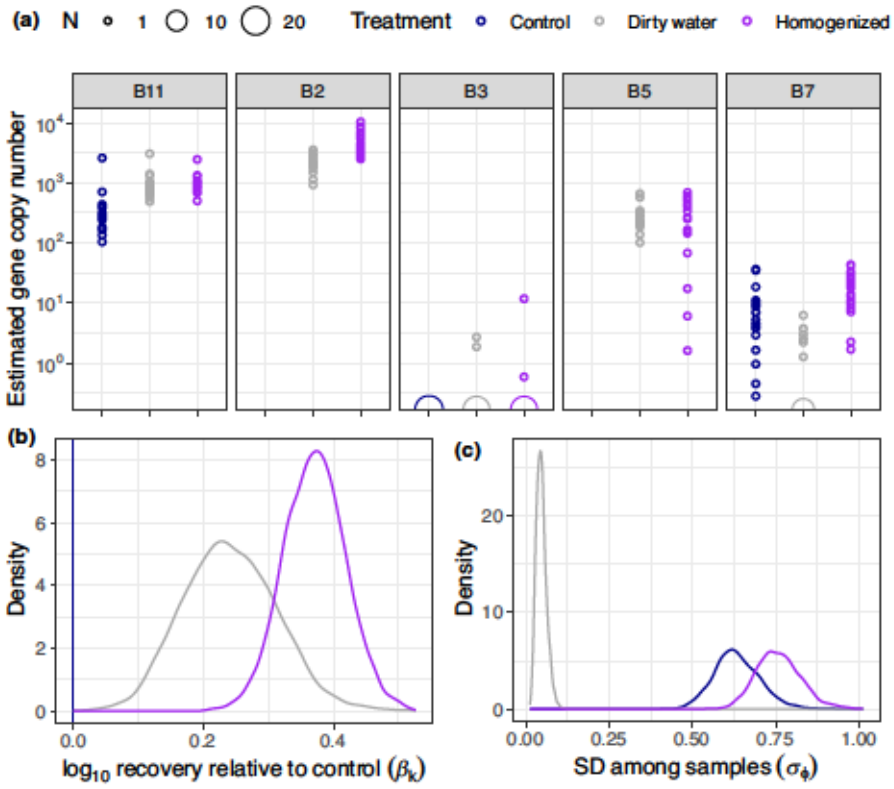

FIGURE 5 (a) Estimated *Bsal* gene copy number in 20 eDNA filter samples collected from the water housing each of five animals (panels) in experiment 5. Water was unmanipulated (control treatment) or physically homogenized with an immersion blender (homogenized treatment) prior to collection, or had housed 20 uninfected animals for the prior 72 h prior to housing the infected animal (dirty water treatment). Note that the control samples from three animals were lost. (b) Posterior estimates of the recovery in the homogenized and dirty water treatments relative to the controls (blue vertical line at zero). (c) Posterior estimates of the sample-to-sample standard deviation in each treatment.

capacity to detect *Bsal* in the first 2 or 3 weeks after exposure (Figure 8). However, eDNA filter samples had an equivalent or greater chance of detecting *Bsal* than swabs at all time points. Five eDNA filter samples would have provided a ≥95% chance of detection, outside of days 9 and 15 DPE when the number of infected animals and the overall amount of *Bsal* eDNA shed into the water were quite low, but 10 swab samples were needed to achieve the same power.

4 | DISCUSSION

Detecting pathogens such as *Bsal* in captive settings and trade is essential for preventing and mitigating the risk of pathogen emergence from the live animal trade. However, novel approaches to surveillance are necessary in the face of the enormous magnitude of animals moved in trade, even for amphibians, which make up a small portion of the overall trade of live vertebrate animals. Screening entire groups of animals in a housing container in a facility or a consignment in trade using eDNA has been proposed as a strategy to dramatically reduce the number of samples required to detect even rare infections (Brunner, 2020), but one that needed further evaluation. In addition to simply establishing best methods to collect eDNA samples, a key question was whether the capacity to detect pathogens in eDNA samples would scale to larger volumes of water and larger numbers of animals. Our studies thus represent an important step in establishing the conditions in which eDNA-based pathogen detection can work for at least this one important, emerging pathogen.

We found that analytic sensitivity of eDNA samples could be quite high, with 95% limits of detection on the order of tens and always less than 150 zoospores processed (Figure 2). But our results also demonstrate the large amount of variation in the recovery of *Bsal* DNA among replicate samples, even under ideal conditions (i.e. *Bsal*-spiked water; Figure 1), a result consistent with prior work on eDNA-based detection of *Bd* and a myxozoan parasite (Sieber et al., 2020). This variation is rarely accounted for in studies of diagnostic sensitivity but has the potential to increase false negatives when samples are near or below the limit of detection (Sieber et al., 2020). For instance, a substantial fraction of the 20 replicate samples collected from animals B3 and B7 in experiment 5 were negative, even though the animals were infected and shedding *Bsal* eDNA (Figure 5a). However, this variability is not unique to eDNA filter samples. Replicate swab samples can vary a great in the amount of *Bsal* or *Bd* they recover from an animal. Moreover, there were several occasions where swabs did not detect any *Bsal* DNA but the eDNA filters did (Figure 6). Overall, our results suggest that at the level of individual animals, eDNA filtered from water housing the animal are similarly likely to detect *Bsal* DNA as swab samples (Figure 7).

Our goal, however, is not to identify another sample type with which to detect *Bsal* in individual animals, but rather to detect *Bsal*, or at least *Bsal* DNA, if it is present in a population. At this scale eDNA filters tend to perform much better than swabs. With a given number of samples eDNA filters had an equal or greater chance of detecting *Bsal* than swabs, across all time points in our study (Figure 8). As the size of the population being screened increases,

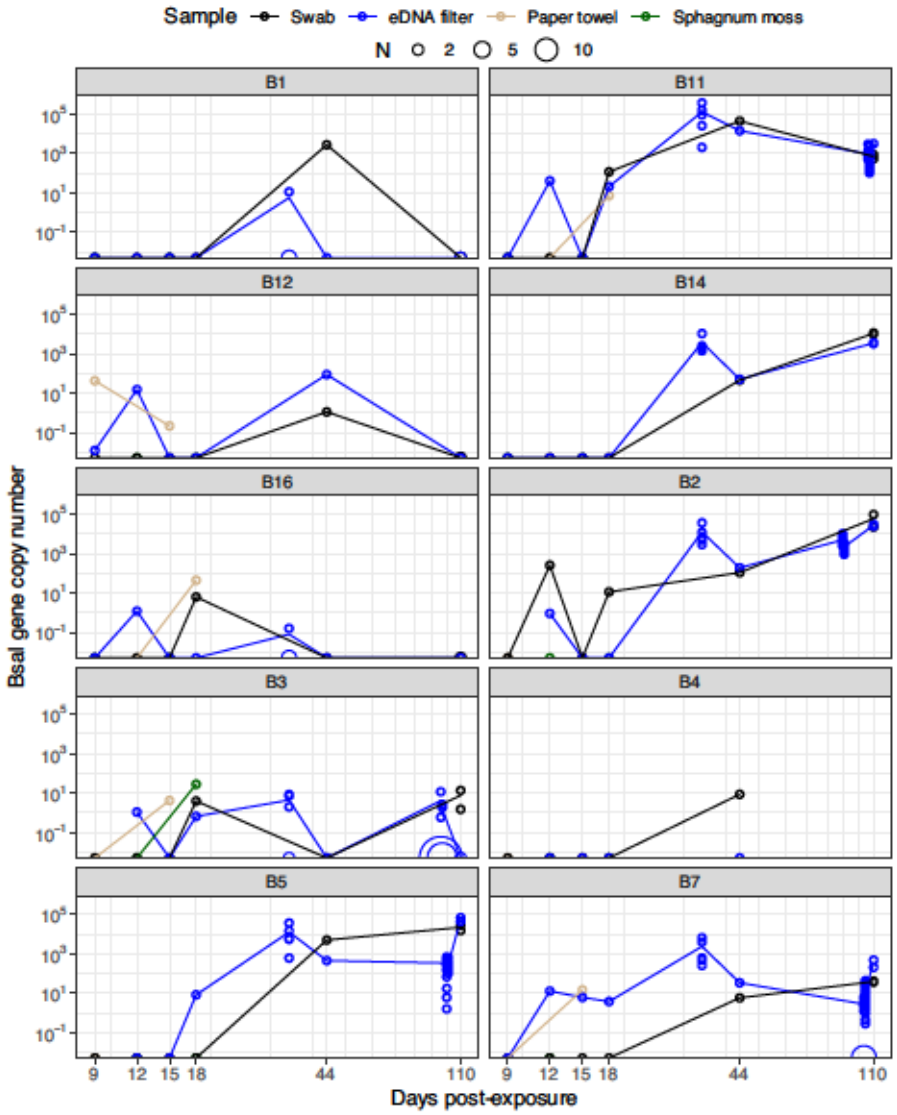

FIGURE 6 Estimated *Bsal* gene copy number from each sample type (colours) over time for each of the 10 *Bsal*-exposed animals with ≥1 positive sample (panels). Non-detections were aggregated in cases where multiple samples were collected (five eDNA filter samples in experiment 4 and 20 in experiment 5, two swabs at the end of the study) so that they are more visible along the abscissa.

the number of swab samples has to increase dramatically to achieve the same probability of detecting a rare infection, simply because the chance an infected animal is among those swabbed declines as the population size increases, while the performance of eDNA filters becomes a question of dilution (Brunner, 2020) and so depends on the conditions in which those larger populations are housed. Indeed, there are elements of control when trying to detect *Bsal* using eDNA filters that are unavailable in a swab-based approach. Larger volumes of water can be filtered, up to the point at which filters clog, and animals can be held in water for longer durations before sampling, both of which tend to increase the amount of *Bsal* eDNA collected (Figures 3 and 4). The faeces, microbes, and other host materials shed into the water do not appear to cause issues with *Bsal* eDNA recovery (Figure 5). We also expect that under realistic settings where eDNA is allowed to accumulate (e.g. between water changes or during a shipment) the eDNA filter method would have even greater power to detect *Bsal*. Thus eDNA-based sampling appears to be a more efficient method of screening captive populations and shipments for *Bsal* infection.

However, there are important caveats to these generally positive conclusions. First, it is simpler and, apparently, more effective to collect eDNA from water, and so eDNA may be most useful for screening aquatic or semi-aquatic amphibians as opposed to strictly terrestrial stages and species. While we were able to collect eDNA and recover *Bsal* DNA from paper towels, especially by soaking the paper towels in water and then filtering the water, we had considerable issues with PCR inhibition and non-detections with eDNA collected from sphagnum moss (Table 1, Figure 6). There are likely other substrates that are similarly problematic. Further research aimed at establishing the substrate types, conditions and protocols that are or are not effective would be helpful.

Second, and more importantly, our results, like those of prior studies (e.g. Blooi et al., 2015; Gray et al., 2023), illustrate how much the intensity of *Bsal* infections can change over time. In the weeks after exposure animals tended to have very low level, often undetectable infections and some animals reduced if not cleared their infections by the end of the study (Figure 6). Collectively this suggests that *when* captive populations or shipments are screened for

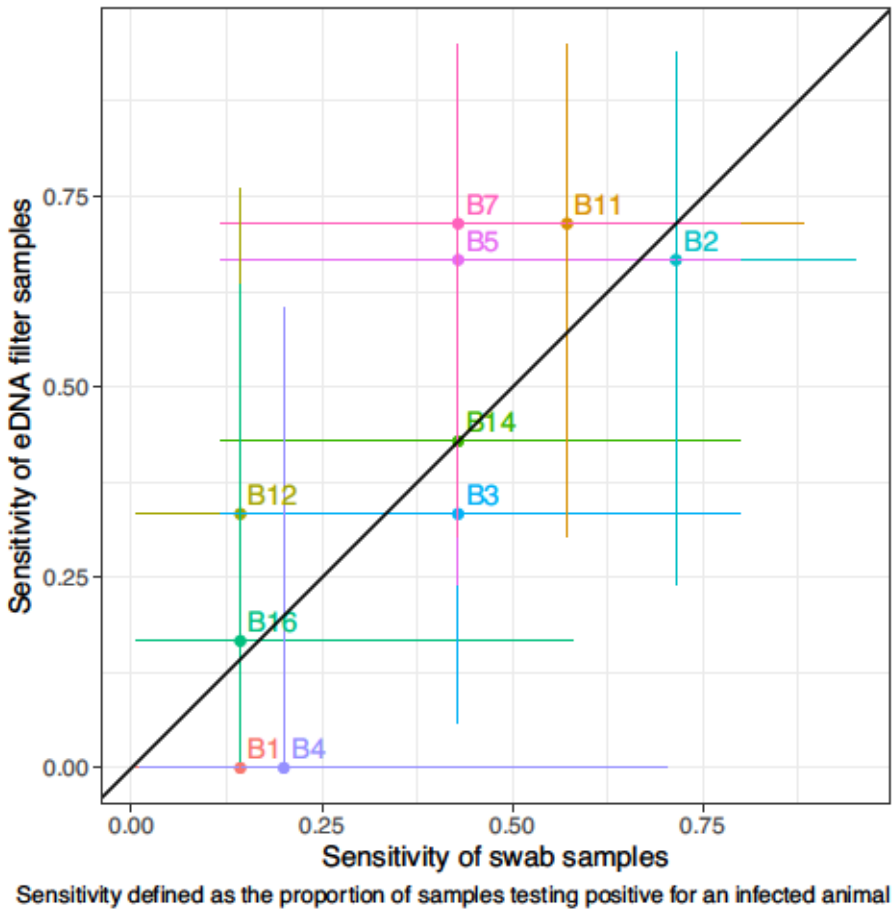

FIGURE 7 Estimated diagnostic sensitivity and 95% CI for eDNA filter samples relative to swab samples for each individual animal. Sensitivity was defined as the proportion of samples collected from an infected animal that tested positive. We restricted these the eDNA filter samples to those with matching swab sample time points.

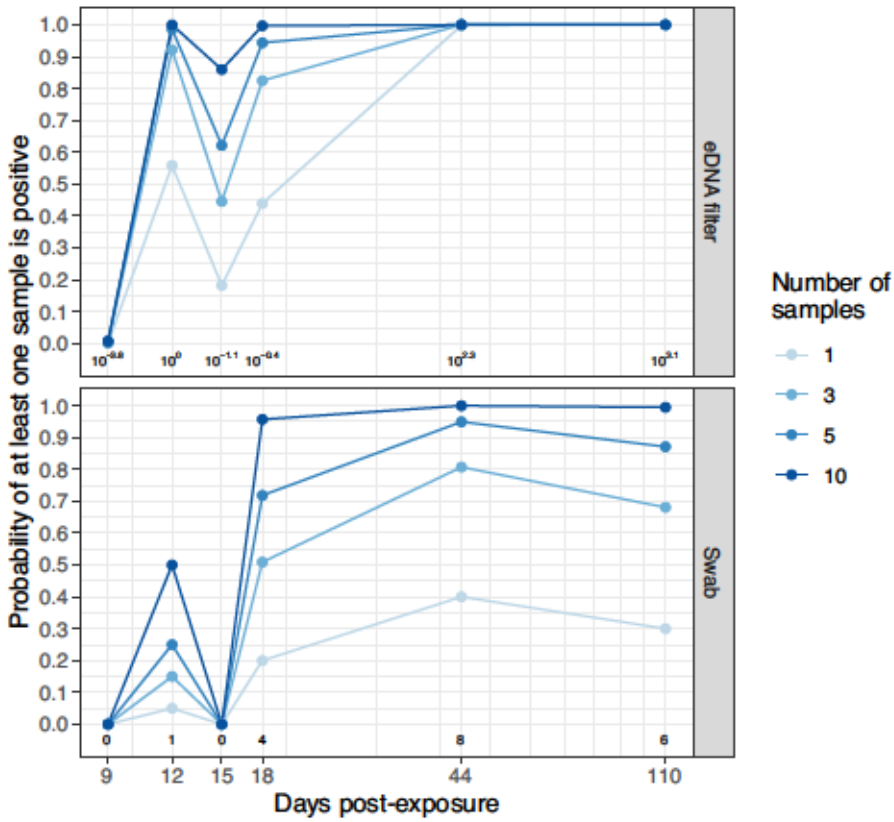

FIGURE 8 The probability of detecting *Bsal* in populations of 20 newts in which 10 are infected in the manner observed in our study. The numbers along the abscissa are the relevant average concentrations of *Bsal* DNA in eDNA filter samples (top panel) or numbers of positive-testing animals using swabs (bottom panel).

infections may be as or more important than the particular type of sample used (Figure 8). Moreover, the myriad of factors that make infections more or less intense also make them more or less detectable (Brunner, 2020). We thus caution against using single values of diagnostic sensitivity to establish sampling protocols or freedom from infection, at least without careful consideration.

Screening collections or consignments of amphibians for pathogens such as *Bsal* is not intended to guarantee every infected individual is identified, but rather to improve to some reasonable, if arbitrary level the chances of detecting the pathogen before it spreads or spills over to naïve populations and species. Even imperfect methods of screening can be helpful in minimizing this risk and preventing harm to captive populations and wildlife alike. Our results help establish that eDNA sampling can be a useful tool for screening captive populations, one that often increases the chances of detecting *Bsal* in a population relative to similar numbers of swab samples collected from individual animals. We hope that this work brings routine *Bsal* surveillance into reach in a greater number of settings and facilities, and spurs work on other pathogens moved in the live animal trade.

AUTHOR CONTRIBUTIONS

Jesse L. Brunner conceived the ideas; Jesse L. Brunner and Christian M. Yarber designed the experiments; Christian M. Yarber conducted the experiments; Christian M. Yarber and Robert A. I. Pearhill collected the data with the aid of Caren S. Goldberg; Jesse L. Brunner analysed the data and led the writing of the manuscript with important contributions from Caren S. Goldberg and Robert A. I. Pearhill.

ACKNOWLEDGEMENTS

We thank Toad Evergreen for help with animal care and Allan Pessier and members of the Office of the Campus Veterinarian at WSU for their help in treating chytridiomycosis. This work was supported by Morris Animal Foundation grant D19ZO-010 and National Science Foundation DEB grant 1754474.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to report.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/2041-210X.14217.

DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository: https://doi.org/10.5061/dryad.hdr7sqvp5 (Brunner & Yarber, 2023).

ORCID

Jesse L. Brunner https://orcid.org/0000-0003-4894-8274
Caren S. Goldberg https://orcid.org/0000-0002-0863-9939

REFERENCES

Altmann, M. C. G., & Kolby, J. E. (2017). Trends in US imports of amphibians in light of the potential spread of chytrid fungus, (Bd), and implications for conservation. *Journal of International Wildlife Law & Policy*, *20*, 226–252.

Basanta, M. D., Avila-Akerberg, V., Byrne, A. Q., Castellanos-Morales, G., González Martínez, T. M., Maldonado-López, Y., Rosenblum, E. B., Suazo-Ortuño, I., Parra Olea, G., & Rebollar, E. A. (2022). The fungal pathogen *Batrachochytrium salamandrivorans* is not detected in wild and captive amphibians from Mexico. *PeerJ*, *10*, e14117.

Blooi, M., Martel, A., Haesebrouck, F., Vercammen, F., Bonte, D., & Pasmans, F. (2015). Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. *Scientific Reports*, *5*, 8037.

Blooi, M., Pasmans, F., Longcore, J. E., Spitzen-van der Sluijs, A., Vercammen, F., & Martel, A. (2013). Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. *Journal of Clinical Microbiology*, *51*, 4173–4177.

Brunner, J., & Yarber, C. (2023). Data from: Environmental DNA-based detection of *Batrachochytrium salamandrivorans* in captive settings. *Dryad Digital Repository*. https://doi.org/10.5061/dryad.hdr7sqvp5

Brunner, J. L. (2020). Pooled samples and eDNA-based detection can facilitate the "clean trade" of aquatic animals. *Scientific Reports*, *10*, 10280.

Canada Border Services Agency (CBSA). (2018). Environment and Climate Change Canada (ECCC)'s import restrictions on salamanders. Customs Notice 17-17. https://www.cbsa-asfc.gc.ca/publications/cn-ad/cn17-17-eng.html

Connelly, P. J., Ross, N., Stringham, O. C., & Eskew, E. A. (2023). Ongoing amphibian trade into the United States threatens salamander biodiversity. *bioRxiv* https://doi.org/10.1101/2023.01.05.522946

Cunningham, A. A., Daszak, P., & Rodriguez, J. R. (2003). Pathogen pollution: Defining a parasitological threat to biodiversity conservation. *Journal of Parasitology*, *89*, S78–S83.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife—Threats to biodiversity and human health. *Science*, *287*, 443–449.

Eskew, E., & Carlson, C. J. (2020). Overselling wildlife trade bans will not bolster conservation or pandemic preparedness. *Lancet Planetary Health*, *4*, e215–e216.

Fèvre, E. M., Bronsvoort, B. M., Hamilton, K. A., & Cleaveland, S. (2006). Animal movements and the spread of infectious diseases. *Trends in Microbiology*, *14*, 125–131.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*, 423–425.

Fitzpatrick, L. D., Pasmans, F., Martel, A., & Cunningham, A. A. (2018). Epidemiological tracing of *Batrachochytrium salamandrivorans* identifies widespread infection and associated mortalities in private amphibian collections. *Scientific Reports*, *8*, 443.

Forzán, M. J., Gunn, H., & Scott, P. (2008). Chytridiomycosis in an aquarium collection of frogs: Diagnosis, treatment, and control. *Journal of Zoo and Wildlife Medicine*, *39*, 406–411.

Garner, T. W., Stephen, I., Wombwell, E., & Fisher, M. C. (2009). The amphibian trade: Bans or best practice? *EcoHealth*, *6*, 148–151.

Goldberg, C. S., Pilliod, D. S., Arkle, R. S., & Waits, L. P. (2011). Molecular detection of cryptic vertebrates in stream water: A demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PLoS One*, *6*, e22746.

Gray, M. J., Carter, E. D., Piovia-Scott, J., Cusaac, J. P. W., Peterson, A. C., Whetstone, R. D., Hertz, A., Muniz-Torres, A. Y., Bletz, M. C., Woodhams, D. C., Romansic, J. M., Sutton, W. B., Sheley, W., Pessier, A., McCusker, C. D., Wilber, M. Q., & Miller, D. L. (2023).

Broad host susceptibility of north American amphibian species to *Batrachochytrium salamandrivorans* suggests high invasion potential and biodiversity risk. *Nature Communications*, *14*, 3270.

Hall, E. M., Crespi, E. J., Goldberg, C. S., & Brunner, J. L. (2016). Evaluating environmental DNA-based quantification of ranavirus infection in wood frog populations. *Molecular Ecology Resources*, *16*, 423–433.

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F., & Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, *73*, 175–192.

Hyman, O. J., & Collins, J. P. (2012). Evaluation of a filtration-based method for detecting *Batrachochytrium dendrobatidis* in natural bodies of water. *Diseases of Aquatic Organisms*, *97*, 185–195.

Kawato, Y., Mekata, T., Inada, M., & Ito, T. (2021). Application of environmental DNA for monitoring Red Sea bream Iridovirus at a fish farm. *Microbiology Spectrum*, *9*, e0079621.

Klocke, B., Becker, M., Lewis, J., Fleischer, R. C., Muletz-Wolz, C. R., Rockwood, L., Aguirre, A. A., & Gratwicke, B. (2017). *Batrachochytrium salamandrivorans* not detected in U.S. survey of pet salamanders. *Scientific Reports*, *7*, 13132.

Kuiken, T., Leighton, F. A., Fouchier, R. A., LeDuc, J. W., Peiris, J. S., Schudel, A., Stöhr, K., & Osterhaus, A. D. (2005). Public health. Pathogen surveillance in animals. *Science*, *309*, 1680–1681.

Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A., & Nguyen, T. T. (2017). *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports*, *7*, 44443.

Lastra González, D., Baláž, V., Solský, M., Thumsová, B., Kolenda, K., Najbar, A., Najbar, B., Kautman, M., Chajma, P., Balogová, M., & Vojar, J. (2019). Recent findings of potentially lethal salamander fungus *Batrachochytrium salamandrivorans*. *Emerging Infectious Diseases*, *25*, 1416–1418.

Lesperance, M. L., Allison, M. J., Bergman, L. C., Hocking, M. D., & Helbing, C. C. (2021). A statistical model for calibration and computation of detection and quantification limits for low copy number environmental DNA samples. *Environmental DNA*, *3*, 970–981.

Lötters, S., Wagner, N., Albaladejo, G., Böning, P., Dalbeck, L., Düssel, H., Feldmeier, S., Guschal, M., Kirst, K., Ohlhoff, D., Preissler, K., Reinhardt, T., Schlüpmann, M., Schultze, U., Schulz, V., Steinfartz, S., Twietmeyer, S., Veith, M., Vences, M., & Wegge, J. (2020). The amphibian pathogen *Batrachochytrium salamandrivorans* in the hotspot of its European invasive range: Past–present–future. *Salamandra*, *56*, 173–188.

Mahon, A. R., Horton, D. J., Learman, D. R., Nathan, L. R., & Jerde, C. L. (2018). Investigating diversity of pathogenic microbes in commercial bait trade water. *PeerJ*, *6*, e5468.

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., Farrer, R. A., Schmidt, B. R., Tobler, U., Goka, K., Lips, K. R., Muletz, C., Zamudio, K. R., Bosch, J., Lötters, S., Wombwell, E., Garner, T. W. J., Cunningham, A. A., Spitzen-van der Sluijs, A., … Pasmans, F. (2014). Recent introduction of a chytrid fungus endangers Western palearctic salamanders. *Science*, *346*, 630–631.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F., & Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 15325–15329.

Nguyen, T. T., Nguyen, T. V., Ziegler, T., Pasmans, F., & Martel, A. (2017). Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. *Amphibia-Reptilia*, *38*, 554–556.

Peeler, E. J., & Feist, S. W. (2011). Human intervention in freshwater ecosystems drives disease emergence. *Freshwater Biology*, *56*, 705–716.

Robinson, K. A., Pereira, K. E., Bletz, M. C., Carter, E. D., Gray, M. J., Piovia-Scott, J., Romansic, J. M., Woodhams, D. C., & Fritz-Laylin, L. (2020). Isolation and maintenance of *Batrachochytrium salamandrivorans* cultures. *Diseases of Aquatic Organisms*, *140*, 1–11.

Sabino-Pinto, J., Veith, M., Vences, M., & Steinfartz, S. (2018). Asymptomatic infection of the fungal pathogen *Batrachochytrium salamandrivorans* in captivity. *Scientific Reports*, *8*, 11767. https://doi.org/10.1038/s41598-018

Sieber, N., Hartikainen, H., & Vorburger, C. (2020). Validation of an eDNA-based method for the detection of wildlife pathogens in water. *Diseases of Aquatic Organisms*, *141*, 171–184.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., Hines, H. B., & Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, *4*, 125–134.

Smith, K. F., Behrens, M., Schloegel, L. M., Marano, N., Burgiel, S., & Daszak, P. (2009). Reducing the risks of the wildlife trade. *Science*, *324*, 594–595.

Stan Development Team. (2023a). RStan: The R interface to Stan. 2.26.13.

Thomas, V., Blooi, M., Van Rooij, P., Van Praet, S., Verbrugghe, E., Grasselli, E., Lukac, M., Smith, S., Pasmans, F., & Martel, A. (2018). Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*. *Transboundary and Emerging Diseases*, *65*, e478–e488.

Trujillo-González, A., Edmunds, R. C., Becker, J. A., & Hutson, K. S. (2019). Parasite detection in the ornamental fish trade using environmental DNA. *Scientific Reports*, *9*, 5173.

United States Fish and Wildlife Service (USFWS). (2016). Listing Salamanders as injurious due to risk of Salamander Chytrid Fungus (January 12, 2016).

Waddle, J. H., Grear, D. A., Mosher, B. A., Grant, E. H. C., Adams, M. J., Backlin, A. R., Barichivich, W. J., Brand, A. B., Bucciarelli, G. M., Calhoun, D. L., Chestnut, T., Davenport, J. M., Dietrich, A. E., Fisher, R. N., Glorioso, B. M., Halstead, B. J., Hayes, M. P., Honeycutt, R. K., Hossack, B. R., … Winzeler, M. E. (2020). *Batrachochytrium salamandrivorans* (Bsal) not detected in an intensive survey of wild north American amphibians. *Scientific Reports*, *10*, 13012.

Yap, T. A., Nguyen, N. T., Serr, M., Shepack, A., & Vredenburg, V. T. (2017). *Batrachochytrium salamandrivorans* and the risk of a second amphibian pandemic. *EcoHealth*, *14*, 851–864.

Yuan, Z., Martel, A., Wu, J., Van Praet, S., Canessa, S., & Pasmans, F. (2018). Widespread occurrence of an emerging fungal pathogen in heavily traded Chinese urodelan species. *Conservation Letters*, *11*, e12436.

How to cite this article: Brunner, J. L., Yarber, C. M., Pearhill, R. A. I., & Goldberg, C. S. (2023). Environmental DNA-based detection of pathogens in trade and captive settings: Best practices and validation for *Batrachochytrium salamandrivorans*. *Methods in Ecology and Evolution*, *14*, 2787–2799. https://doi.org/10.1111/2041-210X.14217