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Experimental Evidence of Rapidly Decaying Environmental DNA Highlights Infection Risk from Two Major Amphibian Pathogens

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ABSTRACT

Infectious diseases spread through international wildlife trade networks, presenting major conservation and welfare challenges. The diseases amphibian chytridiomycosis (caused predominantly by chytrid fungus *Batrachochytrium dendrobatidis, Bd*) and ranavirosis (caused by iridoviruses in the genus *Ranavirus, Rv*) are the result of infection by globally distributed pathogens. These pathogens spread internationally through live-animal trade networks and have driven population declines, mass mortalities, and community collapse for a broad range of amphibian species. Environmental (e)DNA methods may provide highly sensitive and non-invasive pathogen surveillance for traded or wild amphibians. To investigate the relationship between eDNA detection and environmental pathogen persistence, eDNA degradation rates were quantified across a range of temperatures (15°C–25°C) for both *Bd* and *Ranavirus*. Estimated decay rates suggest that overall pathogen eDNA concentration degrades by 99% between 18.9–52.4 h. Low levels of pathogen eDNA remained detectable for the duration of the experiment (> 28 days). Time was found to have a significant negative effect on eDNA concentration for both pathogens ($p < 0.001$). The negative effect of temperature on eDNA concentration was significant for both pathogens (20°C for *Rv*, $p < 0.05$; 25°C for *Bd/Rv* $p < 0.001$). We argue that high concentrations of eDNA represent viable pathogen in the environment, demonstrating the usefulness of eDNA for the monitoring of disease status of consignments of traded amphibians.

1 | Introduction

International wildlife trade facilitates the transport and transmission of infectious diseases that cause devastating impacts on human health and livelihoods (Morens, Folkers, and Fauci 2004; World Health Organization 2024), native wildlife, and broader ecosystem health (Karesh et al. 2005; Smith, Sax, and Lafferty 2006; Smith, Acevedo-Whitehouse, and Pedersen 2009), while amassing billions of dollars in damage to local economies around the world (Cunningham, Daszak, and Wood 2017; Narrod, Zinsstag, and Tiongco 2012). In many cases, trade constitutes the final opportunity to monitor and mitigate pathogen spread before potential spillover into natural systems, after which the costs imposed on society and the environment are multiplied greatly. Monitoring efforts are easily overwhelmed by the sheer number of animals and plants in legal and unregulated networks, plausibly in the hundreds of millions each year, although probably higher, as reporting is notoriously unreliable (Blundell and Mascia 2005; Harfoot et al. 2018; Scheffers et al. 2019). Currently, international exotic wildlife trade is regulated solely through the Convention on International Trade

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in Endangered Species of Wild Fauna and Flora (CITES), in which 75% of amphibian and 80% of reptile species are not listed by appendices despite being particularly vulnerable to transmitting infectious diseases through trade (although individual countries or unions may impose additional domestic regulations such as the USA or Australia; Green et al. 2020). Largely unquantified and unknown, infectious diseases in trade are serious conservation challenges that urgently need addressing.

Evidence points to trade in facilitating the intercontinental transmission and expansion of World Organization for Animal Health notifiable infectious diseases (WOAH 2024), such as *Ranavirus* (Brunner et al. 2015; Stöhr et al. 2015), amphibian chytridiomycosis (Fisher and Garner 2020), koi herpesvirus disease (Bergmann et al. 2020; Haenen et al. 2004) or crayfish plague (Mrugała et al. 2015). In addition to unknowns around the numbers of traded animals, data is also lacking on the number of infected shipments, despite evidence suggesting the major role of trade in the emergence of infectious diseases (Martel et al. 2014; Nguyen et al. 2017; O'Hanlon et al. 2018), their presence in aquaculture (Peters et al. 2018), and their impact on livestock and as zoonoses (Kestel et al. 2022).

For aquatic diseases, shipment water can facilitate environmental transmission between co-housed individuals, lead to cross-contamination between shipments, or, if improperly disposed, advance the spread into naïve wild populations (Kolby et al. 2014; Picco and Collins 2008; Schloegel et al. 2009). Rather than identifying high-risk species or individuals, monitoring infection using rapid methods of detection for whole consignments may prove more useful. Traded species fluctuate in demand and availability, so monitoring disease on a per-species basis is unlikely to be effective in controlling disease in trade. The sampling of shipment water from aquatic species may facilitate infection monitoring at the level of consignment rather than individual, and could save traders time, resources, and money, particularly for species regularly traded in large quantities (in aquaculture or for live-food markets) when compared to smaller shipments of a few animals (in the pet trade). For example, Schloegel et al. (2009) estimated that more than 56 million live amphibians are imported into the USA annually, including millions of American bullfrogs (*L. catesbeianus*) for human consumption from South America and Southeast Asia; infection prevalence was found to be high in market frogs, at 62% for *Bd* (306/493) and 8.5% for *Ranavirus* (50/588). Despite the large volume of trade, there is little documentation on exactly how different species are traded and how these practices differ between countries or domestically within localities. In the absence of documented substrates available to sample, it will be particularly challenging to develop methods for consignment-level sampling of terrestrial species transported without shipment water. Without scalable methods of surveillance, large-scale monitoring efforts can rapidly become economically unfeasible given the cost of processing samples from a sufficient proportion of individuals to detect rare infections within a consignment (Brunner 2020).

In recent years, the surveillance of wildlife diseases such as the causative agent of chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*), and *Ranavirus* using environmental (e)DNA has been documented in traded amphibian populations (Kolby et al. 2014, 2015) as well as in natural systems (Hyman and Collins 2012; Hall et al. 2020; Kaganer et al. 2021; Lastra González et al. 2021). Recent efforts to develop protocols for sampling trade-specific sources of *Batrachochytrium salamandrivorans* (*Bsal*) eDNA have included analysis of materials on which amphibians are traded, such as paper towels or sphagnum moss (Brunner et al. 2023). Broadly, however, we still lack a clear understanding of pathogen eDNA decay i.e., the rate at which pathogen eDNA signals decline in the environment, although some studies have aimed to determine persistence or the upper temporal limits of detection of the pathogen itself (Johnson and Speare 2003; Johnson and Brunner 2014; Munro et al. 2016). A poor understanding of the temporal dynamics of eDNA signals measured with typical molecular diagnostic techniques such as quantitative PCR (qPCR) limits the informative potential of eDNA methodologies for pathogen surveillance. For example, if DNA decay is very slow, eDNA signals may only reveal historical presence of infection rather than active shedding from hosts (Ruppert, Kline, and Rahman 2019). A positive detection of target DNA does not necessarily imply that the target is spatially (in the case of contaminated consignment water) or temporally (persisting but pathogenically inviable) present at the point of sampling, which may result in false positive data (Troth et al. 2021). Rapid degradation may facilitate better estimations of distribution and biomass in vertebrate populations (Jo et al. 2017), as eDNA signals are unlikely to accumulate asynchronously with target presence, although if pathogen eDNA signals decay too rapidly, it may lead to high-risk pathogens going undetected with devastating impacts on management (Trujillo-González et al. 2019), especially if sampling is performed outside an optimal timeframe.

Degradation rates are mechanistically accelerated and decelerated by environmental gradients. Two ubiquitous abiotic factors influencing eDNA are temperature (Harrison, Sunday, and Rogers 2019) and substrate (Buxton, Groombridge, and Griffiths 2017; Koziol et al. 2019). Their effect on eDNA availability can be usefully understood within the framework of eDNA ecology (Barnes and Turner 2016). These factors also influence the growth, reproduction, persistence, and pathogenicity of *Bd* (Johnson and Speare 2003; Turner et al. 2021) and *Ranavirus* (Brunner and Yarber 2018; Nazir, Spengler, and Marschang 2012). Some environmental effectors may be more relevant to eDNA research than directly to trade, but they are critical to understand as they determine eDNA detection, quantification, and analysis (Stewart 2019).

Here, we experimentally measured eDNA decay rates of the two most common pathogens in amphibian trade, *Bd* and *Ranavirus* (the latter also affects consignments of reptiles and fish). In this experiment we addressed (1) What are the degradation rates of Bd/Rv pathogen eDNA and (2) What is the effect of temperature on pathogen eDNA decay?

2 | Materials and Methods

2.1 | Pathogen Culturing

Bd-GPL (isolate 343 *'Artlet.K'*; from *Alytes obstetricans* in Lac d'Artlet, Pyrenees National Park, France, 2015) was cultured and maintained (Longcore 2000). Briefly, cultures were grown

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and maintained in liquid TGhL media at 20°C. To harvest zoospores, cultures were transferred to 1% TGhL agar plates, and at least 5 plates flooded with liquid media after 5 days (see Longo et al. 2013). Approximately 10 mL of distilled water was added to each plate, and following 10 min incubation at room temperature, the resulting solution was pooled and counted three times with a hemocytometer. Counts were averaged to determine zoospore concentration.

Ranavirus FV-3 type (isolate RUK-13, see Cunningham 2001; Hyatt et al. 2000) was cultured following (Price et al. 2018). Briefly, *Epithelioma papulosum cyprini* (EPC, derived from fathead minnow fish, *Pimpephales promelas*; ECACC 93120820) cells were grown at 28°C with 5% CO_2 and maintained in EMEM (w/ 10% FBS, 1% L-Glutamine) before inoculation with *Ranavirus* viral genome copies. *Ranavirus* was then grown at 24°C in a non-CO_2 incubator. Upon harvest, media and cells were separated by centrifugation at 1000 g for five minutes and harvested *Ranavirus* stored in 50 mL Falcon tubes at −20°C. *Ranavirus* yield was quantified using the tissue culture 50% infective dose (TCID50) assay, in which a serial dilution of viral stocks (10^{-1} to 10^{-17}) was inoculated into culture media, and after five to six days, cytopathic effect was recorded (Reed and Muench 1938).

2.2 | Effects of Temperature on Pathogen eDNA Degradation

To investigate the temporal dynamics of pathogen eDNA detection, degradation rates of pathogen eDNA were estimated across a range of environmentally representative temperatures (15°C, 20°C, and 25°C, ±1°C) where *Bd*/*Rv* coinfection would be expected to occur (Brunner et al. 2015; Gajewski et al. 2021). Cultured pathogen mixtures were spiked into polypropylene containers of sterile water and maintained for four weeks, as animals are highly unlikely to remain in transport between locations beyond this timeframe. We constructed low-cost climate-controlled units based on Price et al. (2019). Each unit (hereafter referred to as 'polybox') comprised a 54 L polystyrene box (Custompac) encasing the following: a 6 L stacking container filled with 4 L of reverse osmosis (RO) water (Rako Euro GUM3211, drop-on lids GUM3214P0), a temperature-controlled heat mat (EuroRep, 20 W, 43 × 28 cm), a thermostat (Swell Reptiles SWLR0268), a temperature logger (Signatrol SL51T) and a computer fan for airflow (Arctic F8). Experimental design is shown in Figure 1. The experimental room was first cleaned with 50% bleach (v/v, W1 Thin Bleach, 4.53% Sodium hypochlorite) to denature any residual DNA, before wiping with RO water to remove any residue. When dry, surfaces were wiped with 70% ethanol prior to commencing polybox construction. Polybox position and sampling order was initially randomized with the resulting order maintained throughout the experiment. Shoe coverings, labcoats, and nitrile gloves were worn throughout, and replaced between sampling events. The experiment ran from 18:00 on 16/02/2022 to 12:00 on 17/03/2022 inclusive and all internal polybox temperatures were logged at 20 min intervals for the duration of the experiment. The experimental room was maintained at 15°C and 50% humidity, with a 12:12 light–dark cycle. Polyboxes maintained at 15°C still contained heatmats although they were deactivated.

At time point 0 h, 4 L of RO water housed in each polybox ($n = 4$ at each temperature, $n = 12$ in total) was spiked with 1.27×10^7 zoospores of *Bd* and 8×10^6 TCID50 of *Rv*. These concentrations were the maximum amount of cultured

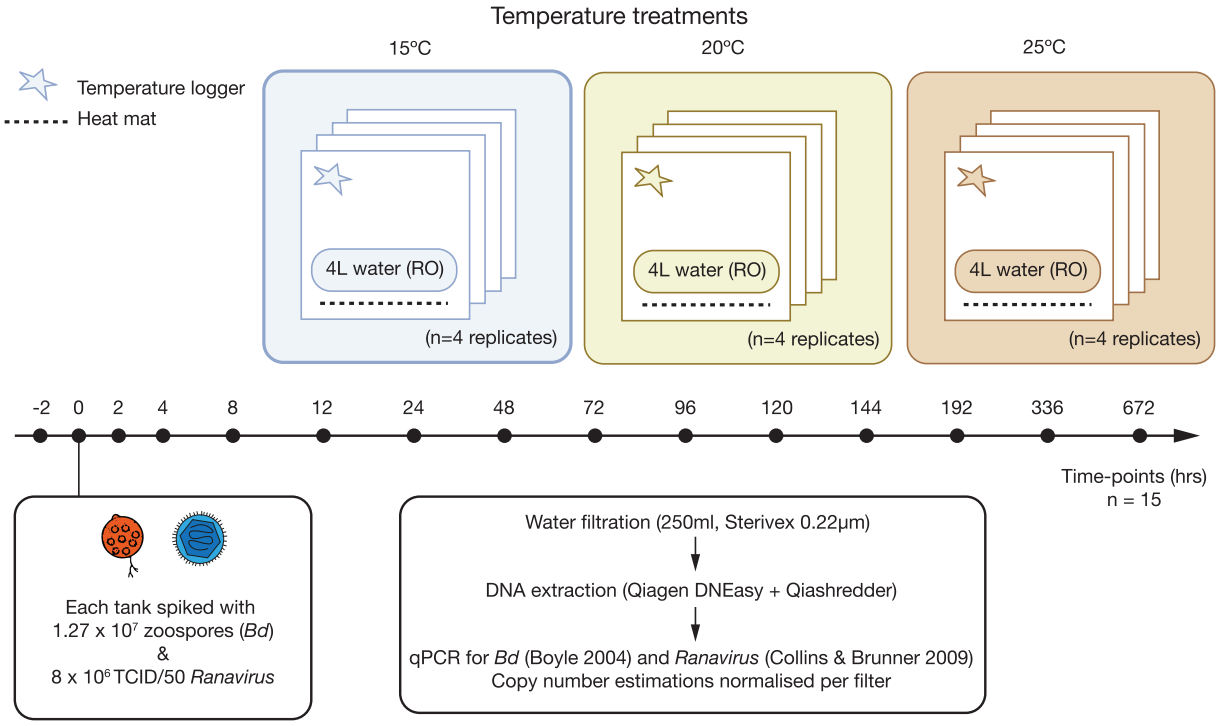

FIGURE 1 | Experimental design for eDNA degradation experiment. Simplified schematic of replicate polyboxes showing inclusion of temperature logger, heatmat, polypropylene container and 4 L RO water. Color warmth represents treatment temperature, where warmer (more orange) colors represent higher temperatures. Timeline is non-linear and shows time points where eDNA samples were collected ($n = 15$).

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pathogen available for use at the time of the experiment and may be much higher concentrations than those seen in trade. For example, between 5.7–16,887 zoospore equivalents were detected in ~300–500 mL of water from shipments of amphibians from Hong Kong. Initial spiking with low concentrations were avoided to minimize the risk of non-detections too early into the experiment to sufficiently estimate decay rates. Immediately following spiking, the water was homogenized for 30 s using a stick blender (sterilized with 50% bleach and rinsed with RO water to remove bleach residue in-between usage) and a sample was drawn with sterile serological pipette tips (Starlab) and a AppJet power pipette (Appleton) until a total of 250 mL was collected into a sterile beaker. Homogenization was undertaken prior to the collection of all subsequent samples, which were taken at 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 360, and 672 h after spiking (resulting in a total number of 13 events, 12 treatments, and 156 samples). Additionally, control samples were taken from each polybox 2 h prior to spiking to ensure absence of pathogen DNA from all units at the beginning of the experiment.

In conclusion, experiment shutdown measures prioritized biosecurity when dismantling tanks and disposing of pathogen mixtures to ensure that any remaining pathogen was inactivated. Firstly, all water was treated overnight with Virkon Disinfectant tablets (Rely+On) to inactivate both *Bd* and *Ranavirus* (Bryan et al. 2009; Gold et al. 2013). Tanks were then soaked in 50% bleach, rinsed with RO water, and dried before removal from the experimental room. All surfaces and equipment used in sample collection were wiped with 50% bleach, wiped down with RO water, and subsequently wiped with 70% ethanol as described before, and equipment was irradiated with UV for at least 30 min following removal.

2.3 | Environmental DNA Capture and Extraction

Environmental DNA was captured by filtration of water through Sterivex analytical filters (0.22 μm pore-size, Millipore, SVGP01050) with a Geopump II peristaltic pump (Geotech). Sterivex filters were chosen due to their enclosed filter design, to reduce contamination risk from handling of filter units during sample collection (Spens et al. 2017; Wong, Nakao, and Hyodo 2020). The 0.22 μm pore-size was chosen to improve the capture of smaller size fractions of DNA-bearing particles, which may have otherwise been lost in larger pore-sizes (for testing of mock eDNA samples see Supporting Information Methods S2). Briefly, a sterile Sterivex unit was attached to a length of MasterFlex pump tubing (MasterFlex 96410-15), and 250 mL of water filtered at a low flow rate into a waste-collection flask. This relatively small volume was used to simulate the low volumes of water available for sampling in traded amphibian shipments (though exact volumes are poorly documented, and likely to vary between species and shipment). Sterivex filters were then stored individually in zip-lock bags to reduce cross-contamination and kept at −20°C until extraction. Tubing was wiped with 50% bleach and rinsed with RO water between uses to denature potentially contaminating DNA to undetectable levels. Environmental DNA was extracted from filters using the Qiagen DNeasy Blood & Tissue kit + Qiashredder (Qiagen), with protocol modifications by Doble et al. (2019), and with additional steps for extraction from Sterivex filters by Hallam et al. (2021). All eluted DNA from samples obtained at time point 0 h were separately treated with Zymo OneStep PCR Inhibitor Removal (Zymo Research, Invine, CA, USA) following the manufacturers protocol to determine whether PCR inhibitors were present in the pathogen mixture upon spiking and whether all subsequent samples would require inhibitor-removal treatment.

2.4 | Real-Time qPCR Reaction Setup and Thermal Cycling Conditions

Pathogen eDNA concentration was determined using an Applied Biosystems StepOne-Plus (Life Biosystems, Carlsbad, CA) real-time qPCR system. Plate setup and sample addition were performed in separate laboratory spaces in order of increasing DNA exposure to minimize contamination risk. Plate setup was performed in a DNA-free lab, and then eDNA samples and positive controls were added in a pre-PCR space where the handling of low-copy DNA samples is permitted. In both laboratory spaces, surfaces were routinely cleaned with 50% bleach, rinsed with distilled water, wiped with ethanol, and, where possible, UV irradiation.

Quantification of *B. dendrobatidis* DNA was achieved using the probe-based TaqMan assay developed by (Boyle et al. 2004) targeting the internal transcribed spacer (ITS) region: forward primer ITS1-3 Chytr (5'-CCTTGATATAA TACAGTGTGCCATATGTC-3′), reverse primer 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA-3′) and 6FAM-labeled minor groove binding probe (5'-6FAM-CGAGTCGAACAAA AT-MGBNFQ-3′). Reactions contained 7.5 μL of 2× TaqMan Universal PCR Master Mix (ThermoFisher Scientific), forward and reverse primers at 900 nM, probe at 250 nM and 2 μL of template DNA resulting in a total reaction volume of 15 μL. Reaction conditions began with an initial two-minute hold at 50°C, a subsequent hold for ten minutes at 95°C for polymerase activation followed by 50 cycles of 15 s at 95°C and one minute at 60°C. eDNA concentrations are reported in copy-number and not zoospore equivalents, due to the highly variable number of ITS copies between *Bd* isolates (Longo et al. 2013).

Quantification of *Ranavirus* DNA was achieved using the probe-based TaqMan assay developed by (Brunner and Collins 2009), targeting the major capsid protein (MCP) gene: forward primer rtMCP-for (5'-ACACCACCGCCCAAAAGTAC-3′), reverse primer rtMCP-rev (5'-CCGTTCATGATGCGGATAATG-3′) and 6FAM labeled minor groove binding probe (5'-6FAM-CC TCATCGTTCTGGCCATCAACCAC-MGBNFQ-3′). Reactions contained 7.5 μL of 2x TaqMan Universal PCR Master Mix (ThermoFisher Scientific), forward and reverse primers at 900 nM, probe at 250 nM and 2 μL of template DNA resulting in a total reaction volume of 15 μL. Reaction conditions began with a 2-min hold at 50°C and subsequent hold for 10 min at 95°C for polymerase activation, followed by 50 cycles of 15 s at 95°C, 20 s at 54°C and 30 s at 72°C.

Samples were analyzed with eight qPCR replicate reactions, given the preferred approach of high-replicate analysis to

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increase detection power (Lesperance et al. 2021). eDNA copies were quantified using a relative standard-curve ranging from 2×10^8 copies to 0.032 copies per reaction, using the 'eLowQuant' method (Matthias et al. 2021; Robinson et al. 2022; see Supporting Information Methods S1). Each plate included duplicate positive controls containing 20 copies of gBlock synthetic ds-DNA fragments (IDT Technologies, Coralville, Iowa, USA) comprising the amplicon of each qPCR assay. Duplicate negative controls comprising Invitrogen UltraPure water (Fisher Scientific) were included on each plate to account for possible contamination during setup. Positive controls were typically within Ct ± 1 between plates; if significantly greater variation was seen, a new aliquot of gBlocks was used and samples reanalyzed. Prior to statistical analysis, copy-number values for replicate reactions were averaged per sample. After non-detection of pathogen eDNA was observed in a replicate treatment, subsequent readings from that treatment were discounted from analysis to account for zero-inflation (on a per-pathogen basis). Samples from time 0 h were also tested after treatment with the Zymo OneStep PCR Inhibitor Removal kit; samples would be considered inhibited if a shift in Ct values was observed between reactions pre- and post-treatment.

2.5 | Statistical Analysis, Calculations of Estimated Decay Rate, and T90/99

Statistical analysis was performed in R Studio 2022.07.2 (Build 576) using R version 4.2.0. Decay rate constants were estimated for each pathogen at each temperature treatment by fitting the data to a first-order log-linear decay (i.e., decay rate estimates were calculated for 15°C, 20°C, and 25°C data, separately). eDNA has been often reported as having first-order exponential decay (characterized by a constant decay rate; Andruszkiewicz et al. 2021; Jo et al. 2019; Kasai et al. 2020; Tsuji et al. 2017), following the equation $N = N0 * exp(-k*t)$, where N is eDNA copies, $N0$ is starting eDNA copies, $-k$ is decay constant, and t is time-since spiking. Following Jo et al. (2019), the data was modeled using the *nls* function in R *stats* (version 4.2.0). The following formula was used: $propMax \sim exp(-k * timeSinceMax)$, where *propMax* is the DNA concentration at a time-point normalized as a proportion of maximum DNA concentration for that treatment replicate, and *timeSinceMax* is the time in hours since this maximum DNA concentration was detected. Due to an initial delay of approximately 4–12 h from pathogen spiking to maximum DNA concentration, the model was fitted using the time from maximum DNA concentration (i.e., maximum DNA concentration becomes time 0 h, per replicate). Time until reduction in 90% and 99% of original eDNA concentrations (T90/T99, respectively) for each temperature treatment were then calculated using the following formulae: $(-log(1-(90/100)))/k)$ or $(-log(1-(99/100)))/k)$, where k is the estimated decay rate.

A linear model was used to test the effects of time and temperature on eDNA copy number for both pathogens, using the *lm* function in R *stats* (version 4.2.0). Here the following formula was used: $log10(copies) \sim time + temp$, where *copies* are eDNA copies, *time* is time-since spiking, and *temp* is the temperature treatment of each group. To calculate decay rates, some early time points were removed out of necessity, but here all time points were included, and absolute copy number was used. To test for serial correlation, Dubin-Watson statistics were calculated using the *orcutt* package.

3 | Results

3.1 | Pathogen eDNA Degradation

Due to evaporation of water, a final sample could not be obtained for one polybox (25°C replicate 'd' at 672 h). All control samples tested negative for both pathogens, confirming the absence of eDNA prior to spiking. In total, 123/156 samples tested positive for Bd and 143/156 for Rv over the course of the experiment. Total eDNA ranged from 0 to 1.03×10^9 copies of *Bd* ITS region and 0–1.38×10^{10} for *Rv* MCP region normalized per 250 mL sample. Non-detections were only observed from 360 h after spiking *Rv*, whereas non-detection was observed as early as 72 h after spiking *Bd* (although samples from these tanks subsequently tested positive). Additionally, all qPCR negative controls failed to amplify for *Bd* and *Rv* DNA suggesting that there was no evidence of sample contamination during this experiment or during analysis.

Observed *Bd* and *Rv* eDNA concentrations reduced rapidly, with estimated T99 values within 18.9–52.4 h (from point of maximum DNA concentration). Decay rate constant (k) estimations were generally more rapid than previously reported (see Table 1). Linear models showed evidence of serial correlation with significant Durbin-Watson statistics for both *Bd* (1.08, $p < 0.001$) and *Rv* (0.622, $p < 0.001$), and errors exhibited an AR(1) pattern and were therefore transformed to remove serial correlation using the Cochrane-Orcutt procedure (*Bd*, 2.48, $p = 0.997$; *Rv* 2.80, $p = 1$). Linear models showed that time had a significant negative effect on the degradation of both *Bd* and *Rv* eDNA ($p < 0.001$). At time 0 h, immediately after spiking with pathogen cultures, estimated eDNA copies per 250 mL sample ranged from 7.79×10^5–1.21×10^8 for *Bd* and 7.05×10^7–5.42×10^9 for *Rv*. Here, contrary to expectation, maximum eDNA concentrations were not observed. Instead estimated eDNA copies were observed to increase until reaching a maximum value between 4 and 12 h, depending on replicate treatment. As a result, decay rate estimates were calculated instead using time-since-maximum eDNA concentration on a per-replicate basis, and concentrations were normalized as a proportion relative to maximum concentration (i.e., between 1 and 0, Figure 2). Treatment of 0 h samples with inhibitor removal did not result in a shift in Ct values, suggesting that no PCR inhibitors were present in initial samples; since no additional substances were added to treatment tanks following spiking, subsequent samples were also considered free from PCR inhibitors. After reaching maximum eDNA concentration, eDNA estimates began to decline rapidly. Small concentrations of *Bd* eDNA remained detectable up to 15 days (4/4 15°C, 1/4 20°C treatments, 0/4 25°C) whilst *Rv* eDNA remained detectable up to 28 days (end of experiment, 2/4 20°C, 1/3 25°C treatments; Figures S4 and S6).

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TABLE 1 | Summary of pathogen persistence estimations from this study and previous investigations by other authors.

Study	Pathogen	Temperature	Environment	Decay constant (k ± SE)	T90	T99.9	Survival	Method used
This study	Rv	15°C	RO water	0.08791 (± 0.034)	26.192 h	52.383 h		qPCR
	Rv	20°C	RO water	0.19873 (± 0.018)	11.586 h	23.173 h		qPCR
	Rv	25°C	RO water	0.21669 (± 0.028)	10.626 h	21.251 h		qPCR
Johnson and Brunner 2014	Rv	22°C–24°C	Unmanipulated pondwater		20.1 h			qPCR
	Rv	22°C–24°C	UV-treated pondwater		1.582 days			qPCR
	Rv	22°C–24°C	Filtered pondwater		8.018 days			qPCR
Nazir, Spengler, and Marschang 2012	Rv	20°C	Sterilized pondwater		22–31 days			TCID/50
Reinauer, Böhm, and Marschang 2005	Rv	23°C	Unmanipulated lakewater			56 days		TCID/50
	Rv	23°C	Drinking water			28 days		TCID/50
Munro et al. 2016	Rv	20°C	Filtered lakewater		28 days			TCID/50
	Rv	15°C	Filtered lakewater		66 days			TCID/50
This study	Bd	15°C	RO water	0.2435 (± 0.0063)	9.456 h	18.913 h		qPCR
	Bd	20°C	RO water	0.12375 (± 0.022)	18.607 h	37.214 h		qPCR
	Bd	25°C	RO water	0.23439 (± 0.021)	9.824 h	19.648 h		qPCR
Walker et al. (2007)	Bd	18°C	Autoclaved sediment				12 weeks	qPCR
Johnson and Speare 2003	Bd	23°C	Tap water				3 weeks	microscopic zoospore activity
	Bd	23°C	Deionized water				4 weeks	microscopic zoospore activity
	Bd	23°C	Lake water				7 weeks	microscopic zoospore activity
Johnson and Speare, 2005	Bd	23°C	Sterile moist sand				12 weeks	microscopic zoospore activity

Note: Methods used and environmental conditions are also reported. Estimates of pathogen eDNA decay rate constants (k) from this study are presented for each temperature treatment, including confidence intervals, and time taken for 90% and 99% reduction in concentrations in hours (T90/99).

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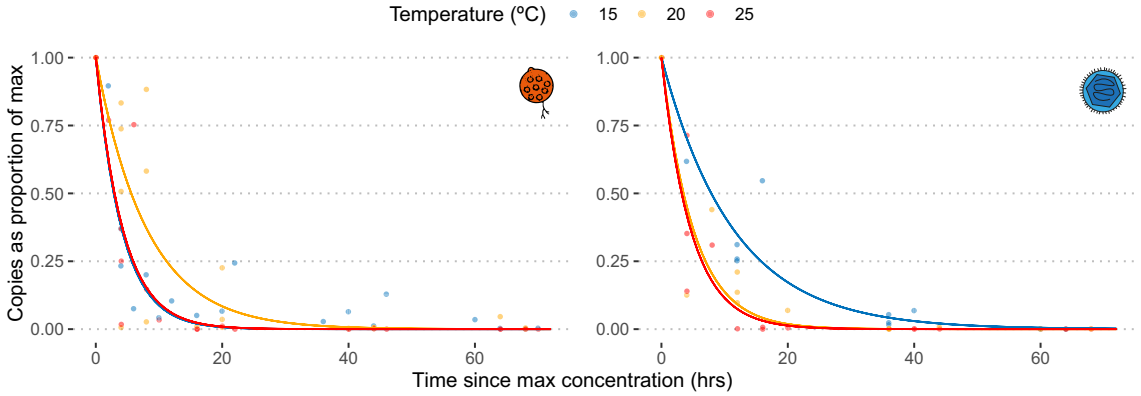

FIGURE 2 | Results from the first 72 h eDNA degradation data for *Bd* (left) and *Ranavirus* (right). Copy number was adjusted as a proportion of the maximum eDNA concentration recorded in each replicate experimental treatment. Color represents temperature treatment, with increasingly warm colors corresponding to warmer temperature treatments. Curves follow first-order decay, using exponential decay constants (k) shown in Table 1; Table S2.

TABLE 2 | Showing Cochrane-Orcutt transformed linear model for *Bd* data, showing the effects of time and temperature on log10 eDNA copies.

Bd	Estimate	Std. Error	CI		*t* value	*p* value
Intercept	7.604	0.361	6.890	8.319	21.069	< 0.001
Time	−0.016	0.003	−0.022	−0.010	−5.483	**< 0.001**
Temperature						
20°C	−0.404	0.270	−0.939	−0.131	−1.496	0.137
25°C	−1.420	0.264	−1.946	−0.900	−5.388	**< 0.001**

Note: 95% confidence intervals for estimates are shown.

Temperature averages across the experiment, as monitored by data loggers at each treatment level, were 15.424 (se ± 0.04), 19.494 (se ± 0.07) and 24.891°C (se ± 0.08), respectively, keeping within ~1°C of target temperature. Linear regression models revealed negative significant effects of temperature at 25°C treatments on eDNA concentrations for both pathogens ($p < 0.001$) and at 20°C for *Rv* ($p < 0.05$) when compared to 15°C (Tables 2 and 3). We found significant fits ($p < 0.001$) to first-order decay models for both pathogens at all three temperatures (Table S2). Decay rates (k) for *Rv* eDNA were estimated to be more rapid at warmer temperatures. The same trend was not observed for *Bd* eDNA, where decay rates are similar for 15/25°C treatments ($k = 0.243$ vs. 0.234) compared to a much lower estimate for 20°C treatments ($k = 0.124$), although this trend may be an artifact due to the removal of early time points where eDNA concentrations were below the detected maximum. In 15°C Bd treatments, decay rate was calculated for some treatments with 0, 2, 4, and 8 h data points removed. If our estimated decay rate constants for each temperature are applied to the maximum detected concentrations, the time to read LOQ for each assay (2.5 ITS copies, 1 MCP copy; see Supporting Information Methods S1) would range between 54.5–110,8 h for *Bd* and 75.7–204.7 h for *Rv*; though low levels were detectable for longer (Table S2), further suggesting that the removal of early time points may have led to more rapid decay rate estimations.

4 | Discussion

4.1 | Pathogen eDNA Degradation Rates and Effect of Temperature

We experimentally measured the decay rate of eDNA for two significant amphibian pathogens, the chytrid fungus *Bd* and the DNA virus *Ranavirus*, which have spread through international trade routes. Whilst the detection of pathogen eDNA occurred over extended periods (4 weeks), we found that high concentrations do not remain detectable for long as they rapidly decay and transition to lower concentrations through the breakdown of DNA molecules in sterile water. This was observed across the range of temperatures at which infection with either pathogen has been reported (Brunner et al. 2015; Piotrowski, Annis, and Longcore 2004). Even at the slowest observed rate, pathogen eDNA was found to decay by 99% in a little over two days. This would suggest that in environments without sustained input of pathogen eDNA (i.e., contaminated shipment water, or shipments retaining eDNA signals after hosts have cleared infection), pathogen eDNA signals would be expected to rapidly decay. It is also possible that pathogen eDNA may not strictly follow a first-order decay pattern, as we continued to detect eDNA after we would expect levels to fall below detectable levels (although this may also be due to the removal of early time points for some treatments).

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TABLE 3 | Showing Cochrane-Orcutt transformed linear model for *Ranavirus* data, showing the effects of time and temperature on log10 eDNA copies. 95% confidence intervals for estimates are shown.

Ranavirus	Estimate	Std. Error	CI		*t* value	*p* value
Intercept	8.315	0.478	7.369	9.261	17.358	<0.001
Time	−0.008	0.002	−0.013	−0.004	−3.760	**<0.001**
Temperature						
20°C	−0.464	0.193	−0.846	−0.082	−2.403	**<0.05**
25°C	−1.382	0.175	−1.728	−1.037	−7.920	**<0.001**

Temperature was found to have a significant negative effect on pathogen eDNA concentration. To better understand the availability of pathogen eDNA in the environment across the range of temperatures at which infection may occur, future work could characterize the effects of host abundance, infection load, and per-species shedding rates on eDNA accumulation. If strong signals of pathogen eDNA (i.e., >1000 copies) represent high concentrations of environmental pathogen load, they may only be sustained, with continual inputs derived from the signal of pathogenic agents (zoospores or viral particles) which have been shed from individuals with active infections. The sources of pathogen eDNA in natural systems are more diverse than in trade. For example, the abundance (and infection status) of contributing hosts is unknown, animals that died from disease may continue to release pathogen eDNA during decomposition, or eDNA may be transported across or between localities. Pathogen eDNA detection in natural settings is further complicated by variable climatic conditions, habitat types, and the presence of organic inhibitors and dynamic microbial communities. Although in trade settings, conditions are more controlled, and the number of contributing hosts, is limited to those within consignments (in theory).

Shedding is a mechanism by which both *Bd* and *Ranavirus* spread through the environment (Brunner and Yarber 2018; Fisher, Pasmans, and Martel 2021), and we expect this to be the main source of eDNA in a trade setting. In this sense, eDNA can usefully serve as a proxy measure of pathogen transmission between infected individuals. We argue that, especially over repeated samples, high concentrations of eDNA can serve as a proxy for 'risk' of infection in traded consignments as otherwise the strength of these signals soon diminishes without sustained shedding from hosts, or as infections clear and shedding ceases. Whilst low concentrations of persisting pathogen eDNA do not allow us to confidently interpret the stage in infection dynamics, or whether the signal is derived from viable infective pathogen, they can still be useful for ascertaining presence.

4.2 | Contextualizing Infection Risk from eDNA Surveillance

Importantly, however, risk must be appropriately contextualized. Risk of infection between individuals varies considerably between species (Woodhams et al. 2007), life-stages (Searle, Xie, and Blaustein 2013), pathogen (Van Rooij et al. 2015), pathogen lineage (Doddington et al. 2013), and temperature (Berger et al. 2004; Brand et al. 2016). Management of risk should consider these factors to minimize the impact of pathogen pollution through trade, possibly by considering a 'threshold' of risk i.e. a minimum detectable load at which a shipment is considered risky. For example, a threshold of risk for species with known low infection tolerance may be lower than for widely traded, infection-tolerant species, accounting for a greater vulnerability to disease (or the spread of infection). Similarly, shipments which are destined for localities where they may come into contact with other species of low tolerance or of high conservation concern should also be treated with greater caution (e.g., if destined for zoological collections with captive assurance colonies, or if consignments include CITES species). Infection-tolerant species traded from a southeast Asian or South African origin may be expected to harbor *Bd* infections due to their endemic exposure to the fungus (Farrer et al. 2011; O'Hanlon et al. 2018) and therefore may be treated with a higher threshold of risk. Though in practice, it may prove challenging to track the origin of individual shipments, as some countries act as indirect importers. Shipments destined for localities without documented infection outbreaks should be treated with greater caution to avoid the risk of accidental exposure of endemic populations to new pathogens (Friday et al. 2020), or novel isolates that pose a risk of recombination events resulting in novel hypervirulent lineages (Farrer et al. 2011).

4.3 | Challenges of eDNA Detection in Wildlife Trade

Timing of sampling is important to improve chances of pathogen detection (Hyman and Collins 2012). eDNA detection is seasonally variable in natural environments, due to breeding and amphibian development, and timing of sampling should be adjusted to maximize the probability of detection (De Souza et al. 2016; Troth et al. 2021). Infection dynamics within trade may be less predictable though still seasonal (Schloegel et al. 2009). Due to the rapid decay observed in our experiments, eDNA signals informative of active shedding should be measured within the first 48 h. Because of this temporality, sampling strategies should consider this timeframe and avoid failing to detect shedding in shipments with high risk of transmission. For example, temporal sampling may be required to increase probability of detection, or to determine whether shedding dynamics are increasing or diminishing (Vilaça et al. 2020). This may be challenging in commercial trade, where maintaining the flow of shipments is important, and quarantining may be considered an obstacle to traders. However, consumers appear willing to pay more for animals that come certified pathogen-free (Cavasos, Adhikari,

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et al. 2023), offsetting the cost to traders. Although there is a risk that higher costs could entice some more cynical consumers to turn to cheaper black-market options; illegal wildlife trade is already highly valued (see Karesh, Smith, and Asmussen 2012). Indeed, *Bd* was documented in illegally traded *Telmatobius spp.* confiscated by the Administration of Forestry and Wildlife in Peru, emphasizing the threat of pathogen spread through unregulated trade networks (Zevallos et al. 2016). However, consumers appear to prioritize individual animal health, wanting to feel confident in the health of their animal (aside from acting in good faith; Cavasos, Poudyal, et al. 2023). Certified pathogen testing may facilitate longer quarantine periods and repeated sampling, but also reduce the volume of trade without impacting the economies of wholesalers or retailers, transitioning toward a 'degrowth' of live-animal trade and a more sustainable future in the industry (Garner et al. 2009). In practice, this could help meet WOAH international regulations and may function similarly to the Sanitary and Phytosanitary (SPS) agreement by World Trade Organization (WTO) members (Abdisa, Getu, and Etana 2023), which uses certification to assess health risks within the trade of plants, animals, and derivative products in the food and agriculture industries.

In our experiment, a delay of approximately 4–12 h after spiking was observed before maximum DNA concentration was recorded, further emphasizing the effect of timing on detection (Figures S3 & S5). This phenomenon has also been observed in previous eDNA degradation experiments (Eichmiller, Best, and Sorensen 2016; Lance et al. 2017). eDNA molecules may be initially unavailable for capture if they are absorbed onto surfaces of housing and after a short period of time desorb, resulting in a spike in captured eDNA (Eichmiller, Best, and Sorensen 2016). As RO water is hypotonic, the release of DNA copies from osmotically lysed zoospores or cultured cells in addition to the rapid absorption of eDNA to the polypropylene walls of the water housing could have resulted in a temporary unavailability of pathogen eDNA to be captured by filtration (Gaillard et al. 1999). The properties of housings or substrates available for sampling in trade are also highly varied, and the capacities of trade-specific samples to detect pathogen eDNA should be interrogated and validated to maximize detection probability (see Brunner et al. 2023). More broadly, shedding rates vary between host species, size, life-stages, temperatures (Andruszkiewicz et al. 2021) so it would be important to determine appropriate quarantine periods to allow for the sufficient build-up of aquatic eDNA before sampling.

In isolation, low-level detection of pathogen eDNA could be misleading and perform poorly as a proxy of environmental pathogen load. In practice, low concentrations may represent historical shedding events or more recent ones with diminished genetic signals due to influences not measured in this study such as transport/removal, microbial activity, UV-exposure etc. (Barnes and Turner 2016). Previous estimations of pathogen persistence are typically longer than those presented in this study and vary with substrate (Table 1). Estimated T90s for *Ranavirus* were found to support previous findings of rapid degradation in unmanipulated pondwater, however estimations for UV-treated and filtered water sources were much longer than those found in this study. Other investigations reported much longer T90s from sterilized pondwater, drinking water and lake water. Previous estimates of *Bd* persistence (measured as zoospore survival) have been reported for weeks in tap water, deionized water, lake water, and autoclaved sediment. Many of these investigations aim to determine the upper-limit of survival and infectivity, and typically use methods which measure the viability of each pathogen i.e. viral titration TCID/50 assays (Munro et al. 2016; Nazir, Spengler, and Marschang 2012; Reinauer, Böhm, and Marschang 2005) or microscopic observations of zoospore activity (Johnson and Speare 2003). Measurements from these methods and qPCR may not be comparable, as qPCR methods quantify genetic material from both viable and nonviable pathogen. Given that pathogen presence is typically ascertained using molecular diagnostics, it may be more useful to consider decay estimates based on qPCR methods when interpreting eDNA data.

5 | Conclusions

We find that eDNA from both pathogens degrades rapidly across the range of temperatures in which coinfection may occur. We believe that eDNA methods could be used routinely in import and quarantine environments to monitor and assess the risk of infection in traded amphibians and begin to collect data on high-risk shipments and their source suppliers. Future efforts should emphasize improved interpretations of low-copy eDNA detection, and a better understanding of the environmental factors affecting eDNA availability in non-natural environments such as housing material, substrate, and spatiality of detection. We consider eDNA as a highly relevant and useful tool for the management and mitigation of infectious diseases in live-animal trade, particularly for amphibians and aquatic organisms.

Author Contributions

J.D.T., T.W.J.G., and J.J.D. designed the study; J.D.T. conducted the labwork and experimental work; J.D.T. and D.J.M. conducted the statistical analyses; J.D.T. wrote the first manuscript draft, and all authors contributed edits.

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Ethics/Integrity Policies

This manuscript has not been submitted elsewhere, and all research meets the ethical guidelines of the study country. No animal or human subjects were involved in this study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available on the UCL Data Research Repository at http://doi.org/10.5522/04/26069098.v1.

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