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Needle in a Haystack: A Droplet Digital Polymerase Chain Reaction Assay to Detect Rare Helminth Parasites Infecting Natural Host Populations

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ABSTRACT

Helminths infect humans, livestock, and wildlife, yet remain understudied despite their significant impact on public health and agriculture. Because many of the most prevalent helminth-borne diseases are zoonotic, understanding helminth transmission among wildlife could improve predictions and management of infection risks across species. A key challenge to understanding helminth transmission dynamics in wildlife is accurately and quantitatively tracking parasite load across hosts and environments. Traditional methods, such as visual parasite identification from environmental samples or infected hosts, are time-consuming, while standard molecular techniques (e.g., PCR and qPCR) often lack the sensitivity to reliably detect lower parasite burdens. These limitations can underestimate the prevalence and severity of infection, hindering efforts to manage infectious diseases. Here, we developed a multiplexed droplet digital PCR (ddPCR) assay to quantify helminth loads in aquatic habitats using 18S rRNA target genes. Using *Schistocephalus solidus* and their copepod hosts as a case study, we demonstrate ddPCR's sensitivity and precision. The assay is highly reproducible, reliably detecting target genes at concentrations as low as 1 pg of DNA in lab standards and field samples (multi-species and eDNA). Thus, we provide a toolkit for quantifying parasite load in intermediate hosts and monitoring infection dynamics across spatio-temporal scales in multiple helminth systems of concern for public health, agriculture, and conservation biology.

1 | Introduction

From populations to landscapes, parasites can drastically alter the ecology of an ecosystem. Parasites with complex life cycles can have particularly drastic consequences for global health, as they infect hosts that span multiple trophic levels (Labaude et al. 2015; Barber et al. 2016). Utilising diverse hosts across their life cycle makes these parasites difficult to track, and considerable work has been invested to disentangle the transmission dynamics of parasites in multi-host systems (Fenton et al. 2015; Webster et al. 2017). Yet, a black box often exists around infection in first intermediate hosts in many host–parasite systems, due in

large part to the difficulty of detection and identification of microscopic larval parasites (Kurtz et al. 2002; Fenton et al. 2015; Bass et al. 2021; Klawonn et al. 2023). While a parasite's downstream effects can be quantified in second intermediate or definitive hosts (when parasites are mature and macroscopic), the evolutionary forces shaping initial infection and the ecology maintaining endemic infection levels (Benesh 2016) remain difficult to assess on a population level.

Here, we focus on helminth parasites (roundworms, tapeworms, and flukes), which remain understudied despite the enormous challenges they pose to global health and livestock sectors (Lustigman et al. 2012; Charlier et al. 2014). As a consequence of economic losses and compromised human and animal welfare, there have been repeated calls to improve detection of early infection and to quantify helminths in dynamic, ecologically realistic environments (Lustigman et al. 2012; Ngwese Mbong et al. 2020). However, helminths remain difficult to study and manage, in part because many begin their life cycles in invertebrate hosts, which are especially challenging to observe and control (Scholz et al. 2009).

For example, *Diphyllobothrium* has infected humans for millennia (its oldest identified human host was a mummified corpse from ancient Chile; Reinhard and Urban 2003). In spite of this long history of human infection, the multi-host life cycle of the parasite was only described in the 20th century, when copepods (an aquatic crustacean) were shown to be its first intermediate host (Scholz et al. 2009). An especially debilitating helminth parasite, *Dracunculus medinensis*, infected over 3.5 million humans in the 1980s (Cairncross et al. 2002). Extensive control efforts have failed to eliminate this tapeworm from animal reservoirs, in part because the initial zooplankton host is difficult to monitor (Goodwin et al. 2022). In these and other parasites with complex, multi-host lifecycles, monitoring infection levels and predicting epidemiological patterns continue to perplex theoreticians and epidemiologists alike (Fenton et al. 2015; Walker et al. 2017). A lack of resolved data across sequential hosts hinders model verification and refinement, and thus, effective parasite management.

Disentangling infection dynamics hinges on the ability to track host and parasite dynamics. Assessing these relationships in helminthiases remains challenging because, unlike many other parasites, helminths are difficult to culture and study under standard laboratory conditions (Brindley et al. 2009). Consequently, most of our knowledge surrounding helminth infections is based on patterns derived from the analysis of field samples. Conventional diagnostic methods primarily depend on microscopy for parasite identification. While arguably more affordable, microscopy is both labour-intensive and ineffective in reliably detecting early stages of infection within hosts and early life history stages of parasites (Boonham et al. 2020; Ngwese Mbong et al. 2020).

Molecular approaches such as qPCR have improved the specificity and sensitivity of diagnostic assays; however, they do not provide absolute quantification of targets, are prone to inhibition by environmental contamination (Shannon et al. 2007), and are unreliable when detecting minute amounts of DNA (Amoah et al. 2017). Additionally, critics of qPCR highlight that problems with data reproducibility underscore the need for new and improved quantitative methods (Hindson et al. 2011; Dijkstra et al. 2014; Bustin et al. 2013). A lack of quantification hinders disease containment efforts and restricts the empirical data required to predict environmental drivers associated with increased infections in nonhuman hosts (Boonham et al. 2020; Goodwin et al. 2022).

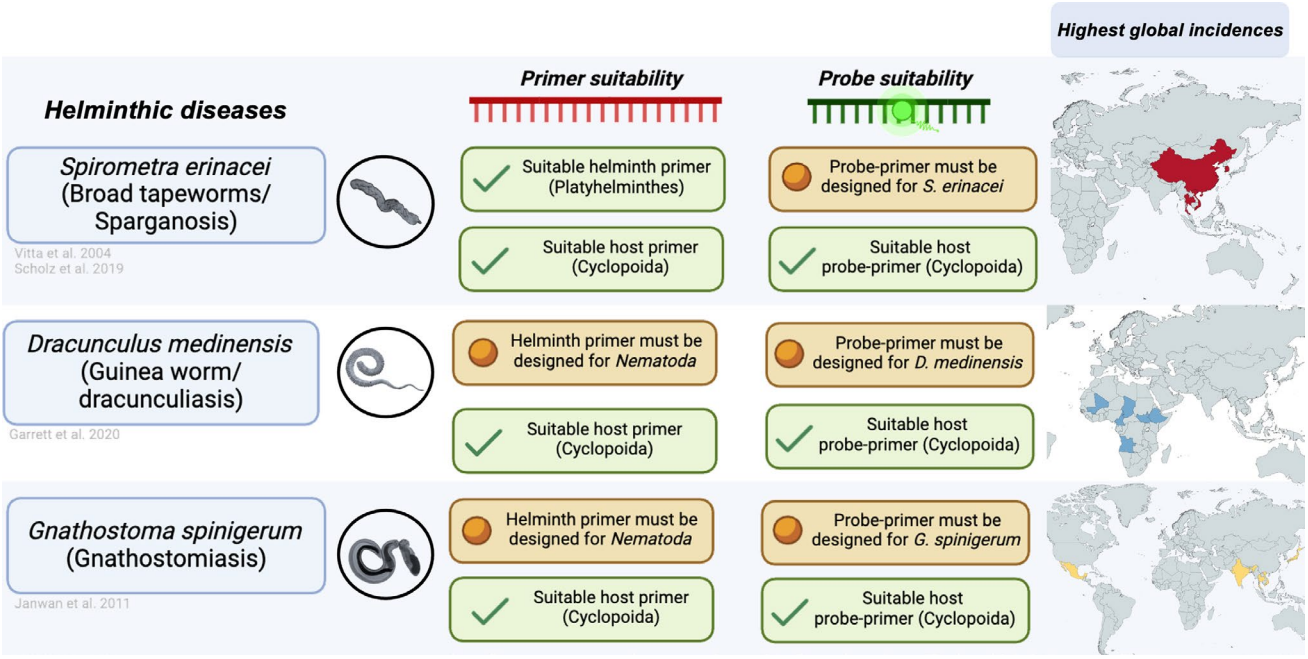
We use a model host–parasite system as a case study to develop and refine a sensitive molecular assay to quantify infection dynamics in wildlife hosts and across natural environments. *Schistocephalus solidus*, the first parasite described with a complex life cycle (Abildgaard 1790), uses the cyclopoid copepod *Acanthocyclops robustus* as one of its first intermediate hosts. With abundant genetic and ecological resources, *S. solidus* has become a powerful model for studying host–parasite coevolution (Barber and Scharsack 2009), and conserved immune responses such as fibrosis (Hund et al. 2022). Detecting early infections in copepods is critical for understanding natural transmission dynamics and parasite loads, and provides a tractable framework for addressing broader questions in disease ecology and public health. Digital PCR approaches (dPCR) enable absolute quantification of nucleic acid targets by partitioning the sample into discrete reaction compartments, allowing for high sensitivity and precision (Hou et al. 2023). Various dPCR platforms, including droplet-based and chip-based systems, support multiplexing and high-throughput analysis. In this study, we used droplet digital PCR (ddPCR) technology to design a multiplexed assay based on universal 18s rRNA primers.

A multiplexed ddPCR enables rapid, high-throughput quantification of two distinct targets, allowing precise measurement of gene concentrations in both hosts and parasites. To refine a ddPCR method and demonstrate its utility, we use a model host–parasite system with abundant genetic and ecological resources. ddPCR has multiple advantages over other quantification methods. First, it provides a direct and independent quantification of DNA without standard curves (Hou et al. 2023). This approach is especially powerful for investigating newly emerging and relatively understudied parasites, when culturing and creating a known concentration of target DNA may be logistically impossible. Second, by sample partitioning and endpoint detection, ddPCR analytics quantify nucleic acids independent of reaction efficiency (Taylor et al. 2017). Free of this limitation, accurate detection can occur at much lower DNA concentrations in samples that do not require dilution to exclude possible contaminants. Third, the high sensitivity of ddPCR can capture target DNA spanning a wide gradient, especially at lower concentrations (Hiillos et al. 2021).

Despite being commercially available for over a decade (Hindson et al. 2011), the application of ddPCR remains primarily utilised in medical research. Meanwhile, the fields of disease ecology and epidemiology have called for a revamping of quantitative methods (Momčilović et al. 2019; Boonham et al. 2020), where fundamental disease questions, such as "when" and "where" disease outbreaks occur, demand sensitive and precise detection methods. Indeed, providing a solid quantitative toolkit for tracking infection levels across diverse hosts and heterogeneous environments could greatly bolster management efforts of Neglected Tropical Diseases (NTDs) globally (Brindley et al. 2009). As tracking infection dynamics across complex field samples is akin to finding "a needle in a haystack," ddPCR could provide a powerful solution to detecting rare DNA sequences in multispecies and environmental samples.

BOX 1 | Applications of ddPCR probe-primer design to parallel systems.

Cyclopoid copepods serve as initial hosts for diverse helminthic diseases distributed globally. The primers designed in this assay are suitable for other systems, with minimal work required for probe design specific to each helminth species.



More broadly, the *S. solidus*-copepod system shares similarities with other NTDs. Like other helminths (e.g., Guinea worms, Box 1), *S. solidus'* first intermediate host is also a cyclopoid copepod. As both an (ecto-)parasite and host to hundreds of parasite species, copepods profoundly affect wildlife, aquaculture, and humans (Bass et al. 2021). As in other systems, epidemiological studies in our focal system have focused primarily on vertebrate hosts, leaving critical gaps in understanding over the entire life cycle of the parasite. To address these gaps, we focus here on quantifying parasite loads in the first intermediate hosts (Anderson and May 1978). Finally, we outline applications of this ddPCR toolkit for other multi-host helminth parasites of global health concern, including relatives of *S. solidus* (family Diphyllobothriidae) that infect humans via farmed fish (Scholz et al. 2019). Together, our study highlights ddPCR as a uniquely reliable quantitative tool, offering new insights across the field of disease ecology.

2 | Materials and Methods

2.1 | Natural History of the Focal Host–Parasite System

Like many other helminths, *S. solidus* is characterised by a complex life cycle requiring transmission between more than one host species (Wedekind 1997). Tapeworm eggs hatch from freshwater substrate, yielding free-swimming larvae (coracidia) that are consumed by and infect multiple species of cyclopoid copepods (Wedekind 1997). This parasite develops into procercoids within copepods, becoming capable of infecting its secondary obligate host, the threespine stickleback (*Gasterosteus aculeatus*) (Nishimura et al. 2011). The final stage of the parasite's life cycle is reached when infected sticklebacks are consumed by piscivorous birds (Wedekind 1997).

2.2 | Field Sites and Field Sample Collections

We sampled three lakes from June 2023–June 2024 across Vancouver Island, B.C., Canada: Pachena Lake (GPS = 48.834893, −125.03362; 54.9 ha), Black Lake (GPS = 48.761545, −125.101215; 69.5 ha), and Blackwater Lake (GPS = 50.1684, −125.5916; 37.5 ha). While *S. solidus* naturally occurs in all three lakes, parasite loads in copepods have not been previously documented. Historical data on mean parasite load in the helminth's second intermediate host, the three-spined stickleback, span a gradient across lakes (File S1).

We visited lakes monthly, and at each visit, we collected zooplankton samples and eDNA. Zooplankton samples were obtained with vertical tows from the epilimnion (i.e., the upper, warmer layer of stratified lakes; Wetzel 2001). We used an EXO2 multiprobe sonde (YSI Incorporated, Ohio, USA) to identify the epilimnion. We pooled three vertical tows of a Wisconsin net (13 cm diameter, 80-μm mesh). Samples were immediately stored in 95% ethanol and transported to the University of Wisconsin, Madison, where they were stored at room temperature for 2 months and then moved to 4°C for long-term storage. We also collected eDNA samples during each sampling visit

TABLE 1 | Primers and probe-primers optimised for ddPCR assay.

Target	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon (bp)
18S rRNA primer Platyhelminthes	F517* (universal primer)-GCCAGCMGCCGCGGTAA	S708R-TGGCTGAAAGGTGACACCACC	208
18S rRNA primer cyclopoid copepods		C138R-TGGCTGAAAGGTGACACCACC	156
Probe-primer for *S. solidus*	**FAM**-CCCACCTACACCGACACAATCAGCC-**IBFQ**		
Probe-primer for cyclopoid copepods	**HEX**-CAGTCGCGTCAAATAACGGACCGCCC-**IBFQ**		

Note: Bolded values denote the relative positions of the fluorophore and quencher used in ddPCR probe-primers.

at each location used for the zooplankton tows. We vacuum filtered 4 L of lake water through a self-preserving 5-μM filter (filter: Smith-Root 11580-25, Pump: Smith-Root 12099; Thomas et al. 2018). Filters were stored at room temperature and transported to the University of Connecticut.

2.3 | DNA Extraction

DNA from zooplankton was extracted using PowerFecal Pro Kits (Qiagen 51804) following the manufacturer's protocol, with an additional 10-min 65°C heating step to help lyse zooplankton. DNA quantity and quality were measured using a NanoDrop (ND-1000) Spectrophotometer, and extracts were stored at −20°C. DNA used for this experiment came from 10 ng μL^{-1} aliquots. eDNA from water filters was extracted using the DNAeasy PowerWater kit (QIAGEN, United States; see Kraemer et al. 2020 for details). DNA extracted from water filters was visually inspected for intactness using agarose gels and quantified using a Qubit assay (Lumiprobe, United States).

2.4 | Design of 18S rRNA Primers for Both *Schistocephalus solidus* and Copepods

While *S. solidus* obligately infects threespine stickleback fish (Bråten 1966), there are a range of cyclopoid copepods that may eat larval stages of the parasite (Barber and Scharsack 2009). We designed primers for copepods that were broad enough to amplify diverse cyclopoids but excluded other zooplankton (Table 1). We tested primers that have been previously reported to amplify copepod sequences (Hubbard et al. 2016; Teterina et al. 2016; Mercado-Salas et al. 2021) but none either (a) consistently amplified copepod-rich samples from Vancouver Island lakes or (b) were the correct size and melting temperature required for ddPCR.

18S rRNA genes were chosen because they contain conserved sequences surrounding variable regions that are species-specific; for both *S. solidus* and copepods, we use the universal 18S rRNA primer, F517, as the forward sequence (Bates et al. 2012, Table 1). We designed primers for *S. solidus* using a complete 18S rRNA sequence available on NCBI (*GenBank: AF124460.1*). Having accessed this sequence, we aligned universal 18S rRNA primers to *S. solidus* (F517, R1119, Bates et al. 2012). We performed a local BLAST on the resulting amplicon against two assemblies of *S. solidus* available in GenBank (GCA_900618435.1, GCA_017591395.1) to identify 18S rRNA present in the assemblies. The resulting hits were aligned using AliView (v. 1.28, Larsson 2014). From these aligned assemblies, we visually identified a 21 bp conserved region common among platyhelminthes that serves as a reverse primer (S708R; 5′-TGGCTGAAAGGTGACACCACC-3′). This primer was designed to be broad, as the probe-primer of the ddPCR provides an additional level of specificity to bind only closely related *Schistocephalus* species (File S2).

Copepod primers were similarly designed using the same 18S rRNA universal forward primers used for *S. solidus* (Table 1). Using the 18S rRNA sequence available for the cyclopoid copepods, *Acanthocyclops viridis* (GenBank: AY626999.1), we identified the amplified region and BLASTed the amplicon against the nucleotide collection (nr/nt database) in NCBI. From selected alignments, we found a region where universal primers would bind to all aligned copepod sequences. We aligned the first 101 hits from this search and identified a conserved region to serve as the reverse primer (C138R, 5′-TGGCTGAAAGGTGACACCACC-3′). The validity of primers was assessed using primer BLAST on NCBI. PCR copepod primers will amplify diverse genera of copepods, though more strongly amplify cyclopoids (File S3).

PCR assays using 18S rRNA primers were conducted on genomic DNA samples to ensure primer specificity. PCR reactions were performed in a total volume of 25 μL [12.5 μL Master Mix, 8 μL PCR water, 1.25 μL forward and reverse primer (10 μmol L^{-1} conc.), 2 μL DNA (10 ng μL^{-1} conc.) (or 2 μL of PCR water for NTC)] in a BioRadT100 thermal cycler. Taq Master Mix was supplied by PR1MA (Standard Taq, PR1MA PR1001-R, MidSci, Catalogue No PR1001-R-1000). PCR conditions included a denaturation step of 5 min at 95°C, followed by an annealing and elongation step of 40 cycles at 95°C for 45 s, 54°C for 45 s, 72°C for 60s, and a final step of 72°C for 7 min with a 1°C s^{-1} ramp speed. PCR products were run on a 1.5% agarose gel using SYBR safe staining (Invitrogen). After gel visualisation, the appropriately sized bands were excised and purified from the gel using the Zymoclean Gel DNA Recovery Kit (Cat No. D4001S, LOT No. 228633) following the manufacturer's protocol. Purified PCR products were then sequenced by Functional BioSciences (Madison, WI) to ensure primers were amplifying the desired targets; sequencing data showed that these PCR products contained target segments. Primer specificity was also internally

validated by using DNA extracts from lab-reared *A. robustus* that were experimentally exposed to larval *S. solidus*.

Sequenced *S. solidus* amplicons were identical to the genome assembly available on NCBI Gene Bank (assembly number: GCA_017591395.1). Cyclopoid primers successfully amplified both lab-reared copepods and wild copepods from mixed-species zooplankton tows (where zooplankton identification in paired samples all contain cyclopoid copepods, Srinivas et al. unpublished data).

2.5 | ddPCR Assays

Our goal was to produce an assay that could be used to detect and estimate parasite loads (Anderson and May 1978) of a helminth parasite from total DNA extracts that contained both diverse zooplankton and target parasite DNA. Following Anderson and May (1978), we define mean parasite load as the total parasite population divided by the total host population at a given time: *P(t)/H(t)* where *P(t)* is the total parasite population and *H(t)* is the total host population at time *t*. Importantly, *H(t)* refers to the entire host population, not only the infected individuals. It is important to note that this metric reflects the average parasite burden across the total host population, without distinguishing how parasites are distributed among individual hosts.

ddPCR requires probe-primers to bind within the amplicon and cannot overlap either of the amplification primers. This provides an additional degree of specificity to the reaction (Hou et al. 2023). Primer-probe suitability (e.g., melting point, GC-content, secondary structure) was checked using the OligoCalc Tool (biotools.nubic.northwestern.edu; starting settings recommended by BioRad: 300 nM primer, 50 mM salt, using nearest neighbour melting temperature). Here, the F517 universal primer (Bates et al. 2012) was shortened by 2nts at the 5′ end to lower its melting temperature (renamed F517*). Based on the broad primers for platyhelminthes, we designed probe-primers to be specific to *S. solidus*. Experimental testing of the primers demonstrates distinct amplification of *S. solidus* from the locally co-occurring helminth, *S. cotti* (File S2). These primers do amplify the sister species *S. pungitii*, which differs by a two-nucleotide gap from *S. solidus*; however, these helminths have yet to be observed in the Vancouver Island area (Daniel I. Bolnick, personal observations). For copepods, we selected a 26 nt probe-primer within the amplified region based on manual inspection of the alignment. Copepod probe-primers are specific to cyclopoids and will not amplify other genera, such as calanoids (see File S3 for empirical support). *S. solidus* probe-primers were labelled with a 5′ FAM fluorophore (5′-CCCACC TACACCGACACAATCAGCC-3′) and copepod primers were labelled 5′ HEX fluorophore (5′-CAGTCGCGTCAAATAACG GACCGCCC-3′) to allow multiplexed reactions. Probe-primers were designed and ordered from Integrated DNA Technologies (IDT), these included the 5′ fluorophores specified above and ZEN Double-Quenched Probes, which contain a 3′ Iowa Black FQ (IBFQ) quencher and a proprietary internal ZEN quencher.

ddPCR was performed using Bio-Rad's QX-200 Droplet Digital PCR System. Across the ddPCR run, we conducted both multiplex and singleplex reactions to address detection and inhibition questions. The multiplexed assay was used to detect both copepods and *S. solidus* within samples to better identify true positives (e.g., a positive signal for *S. solidus* without a signal for copepods is a false positive). To ensure that multiplexing reactions did not bias 18S rRNA quantification, we included singleplex reactions for both host (HEX) and parasite (FAM) targets. For parasites, this is considered the quantification of parasite 18S rRNA genes from singleplex (FAM) and multiplex (FAM + HEX) reactions from infected copepod standards. For hosts, we compared 18S rRNA gene quantification from the same infected copepod standards (FAM + HEX) and unexposed copepod adult standards ($n = 100$ copepods) in a singleplex reaction (HEX). The reaction mix was prepared to a volume of 22 μL per sample. This was composed of 11 μL of 2X ddPCR supermix for probes (BioRad), 2.2 μL of FAM- and/or HEX-labelled primer/probe mixes (900 nM primers/250 nM probes, depending on single- or multiplex reaction), and 8.8 μL of DNA template (serially diluted from original concentration of 10 ng μL^{-1} or RNase/DNase-free water for NTC). Serial dilutions and zooplankton tows were run in triplicate for method validation. Samples were mixed within wells by pipetting.

Once combined, 20 μL of the reaction mix and 70 μL of Droplet Generation Oil (Bio-Rad) were loaded into their appropriate wells in a single-use DG8 cartridge. Cartridges were loaded into a QX200 Droplet Generator (Bio-Rad), where samples are partitioned into nanoliter-sized droplets. 40 μL of the resulting emulsion was manually transferred to a ddPCR 96-well PCR plate (Bio-Rad), which was heat-sealed with a foil cover. The droplets were then subject to thermocycling using a Bio-Rad C1000 thermocycler with a ramp rate of 1°C s^{-1} using the following specifications: a 10 min enzyme activation step at 95°C, followed by 40 cycles of 30 s at 94°C (denaturation) and 1 min at 62.5°C (annealing/extension), followed by a 10 min hold at 98°C. Amplification efficiency was optimised over a temperature gradient (54.6°C–65°C), where we found the ideal optimal temperature for both primer probes at 62.5°C. All experiments included both a negative control containing nucleotide-free water and a double-positive control containing *S. solidus*-infected copepods. Following thermocycling, the droplets were immediately read with Bio-Rad's Droplet Reader.

One of the advantages of ddPCR is that technical replicates are not needed, as there can be more than 15,000 PCR reactions in a single well (Bio-Rad Laboratories 2017). Within a single oil droplet the presence of target DNA is assessed based on fluorescence which is 'binned' as either positive or negative. From this, Poisson statistics are used to estimate the absolute copy number of target DNA based on the proportion of positive droplets in the entire reaction (Jones et al. 2014). As concentration estimates hinge on droplet counts, the precision of calculations is more accurate in wells with more successfully generated/processed droplets; as such, we excluded wells with low droplet counts (< 10,000 droplets) from further analysis. In the context of this study, we repeated three true technical replicates in order to quantify the repeatability of detection in samples with extremely rare (< 1 picogram of total DNA) events. A threshold to separate the target positive and negative droplets is initially suggested by the ddPCR QuantaSoftware. We manually adjusted this threshold to be above the negative amplitude of both dyes (excluding more of the "rain" from Poisson calculations) for a more conservative estimate of target concentration (Figure 1), although

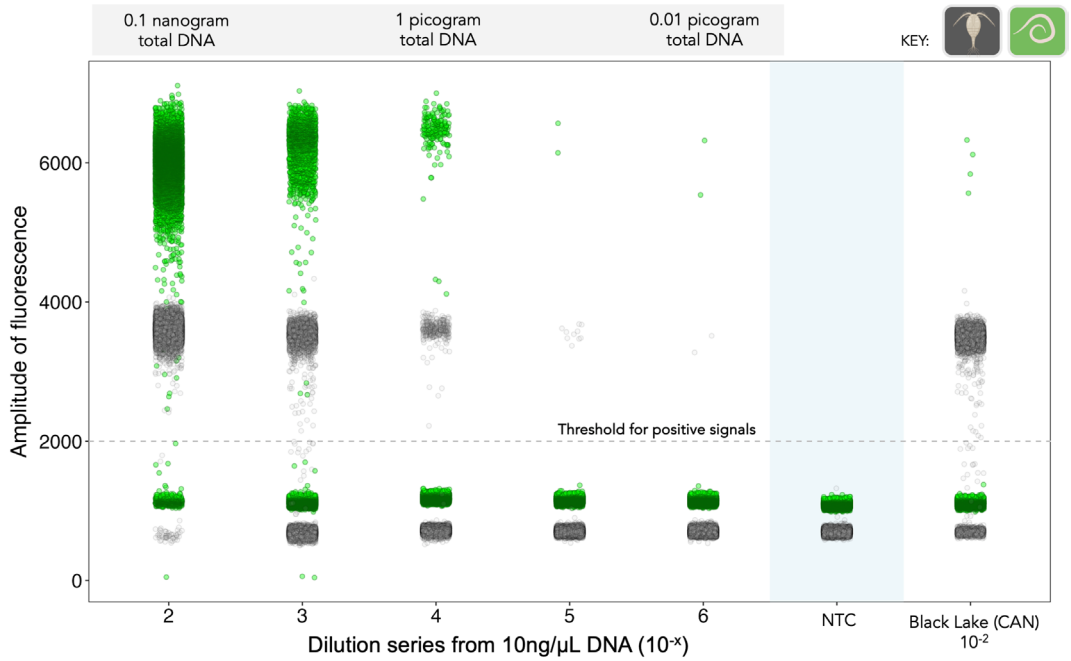


FIGURE 1 | 1-D plot of the limit of detection in a multiplexed ddPCR assay. Infection standard consisted of 100 singly infected copepods. Last column is a field sample included as a comparison to the lab standard. The panel highlighted in blue is the negative template control. Positive reactions and negative reactions (light green and black, respectively) are separated by a manually set threshold (dashed line, at amplitude of 2000). Green points are FAM-fluorescently labelled *S. solidus* droplets and grey points are HEX-fluorescently labelled copepod droplets. Differences in fluorescence amplitude signal of each target dye (here FAM (green) shines brighter than HEX (black)) allow spatial differentiation of the droplet clusters. Each point is an individual reaction.

these isolated droplets have little effect on an estimate of more than 10,000 points.

2.6 | Quantification of Target Gene Measures

Absolute quantification of target gene copies was done with default ABS settings in QuantaSoft Analysis Pro 2.0 software (Bio-Rad). ddPCR reactions occur in droplets, which, after being amplified to endpoint, are assigned as positive or negative for target genes, based on their fluorescence. The fraction of positive partitions within a well is used to estimate target gene concentration by modelling as a Poisson distribution, which is reported in target gene copies per µL. In laboratory standards and zooplankton tows from the field, undiluted DNA resulted in zero negative droplets. Without separation, it is impossible to estimate the target copy number. We found samples diluted to 10^{-2} ($0.1\,ng\,\mu L^{-1}$) exhibited enough separation required for ddPCR to estimate gene concentrations. In eDNA samples, where both host and parasite targets were presumed to be rare and total sample DNA was under $0.01\,ng\,\mu L^{-1}$, undiluted samples were suitable for ddPCR reactions.

2.7 | Limit of Detection (LOD) Experiment

In order to inspect the LOD of this ddPCR assay, we did a 10-fold dilution series (10^{-2} to 10^{-6}) from $10\,ng\,\mu L^{-1}$ DNA stocks. Assay repeatability was determined by both (1) the % coefficient of variation (%CV = concentration standard deviation/concentration mean × 100) between the replicates and (2) the linearity of the dilution assay.

2.8 | Experimental Infection Assays

Positive controls for ddPCR were generated using a 1:1 infection standard derived from experimental infections. Standards were composed of 100 individual adult *Acanthocyclops robustus* (Cyclopidae) copepods confirmed to be infected with a single *S. solidus* coracidium ($n = 100$) from exposure 7 days prior. In parallel, we also ran a "control" standard of 100 unexposed adult copepods to ensure that multiplexing ddPCR reactions did not impact the quantification of copepod genes.

All hosts used for the infection assay were selected from existing cultures isolated from Echo Lake (Vancouver Island, B.C., Canada) in 2015/2016. Prior to beginning the infection assays, laboratory cultures of copepod hosts were maintained in 1 L flasks at 19°C with a 16:8 L:D cycle with 900 mL of standard (low-hardness) COMBO water for animals (artificial lake water media, Kilham et al. 1998). Animal stocks were fed freeze-dried crushed *Artemia* (AMZEY Natural Artemia; $0.022\,mg\,L^{-1}$) every other day.

Parasite eggs were originally collected from Kjerag Fjord, Norway (8 September 2022, GPS: 67.501487, 14.742647). To minimise fungal growth, eggs were washed several times with sterile water (Weber et al. 2017) and maintained in long-term storage in the dark at 4°C in 15 mL Falcon tubes at the University of Wisconsin, Madison. To stimulate egg hatching, 200 µL of the egg suspension was aliquoted into a single well of a foil-covered 24-well microtiter plate with 2 mL of COMBO media and incubated in the dark at 18°C for 7 days. Following this, egg plates were moved to room temperature (25°C–26°C) and placed under full-spectrum grow lights (GT-Lite LED Grow Bulb; 13.09 PPF,

8.5 W; SKU:GR-A19) with a 16:8 L:D cycle (Jakobsen et al. 2012; Weber et al. 2017).

On the day prior to parasite exposure, single adult copepod hosts were isolated individually and maintained in the 24-well plates with 1.5 mL COMBO at 19°C under a 16:8 L:D cycle (Jakobsen et al. 2012). To improve infection success rates, which rely on hosts ingesting parasite eggs, hosts were maintained without food for 24 h before exposure. Following starvation, one coracidium was placed in each well for a 24-h exposure period. Immediately following the 24-h exposure, isolated individuals were fed aliquots of 1 mL of *Artemia* suspension from a 0.022 mg L^{-1} stock solution every other day. Single infections were confirmed 7 days post-exposure using a compound microscope (Leica M80 at 60× magnification using a Leica KL 300 LED). Infected hosts were placed individually into a 1.5 mL Eppendorf tube and stored at −20°C until DNA extraction.

3 | Results

3.1 | Experiment 1: What Is the Limit of Detection of Rare DNA From a Known Infection Dose in a Helminth–Copepod System?

In our LOD experiment, we reliably (e.g., positive *S. solidus* in every replicate) detected *S. solidus* DNA in total concentrations of less than one picogram of total DNA (a 10^{-5} dilution, see Table 1 and Figure 1), otherwise expressed as a mean of 0.187 18S rRNA copies per µL. When run in triplicate, the probability of detection increases, allowing detection of true positives of *S. solidus* in as little as 0.01 picograms of DNA. The assay was reliable, demonstrating linearity across the dilution series ($r^2 = -0.98$, $p < 0.001$, for both targets; see Figure 2). The %CV was below 0.01 for both *S. solidus* and *A. robustus* targets. Droplet counts for the multiplexed standard experiment ranged from 12,423 to 15,834.

We used three independent standards to quantify zooplankton gene concentration: an infection standard that contained 100 adult *A. robustus* copepods individually infected with a single *S. solidus* and two independent standards (created from different lab lines) of 100 adult unexposed *A. robustus* copepods. We did not detect a significant difference in estimates of copepod numbers across standards (ANOVA, F-value = 1.88, $p = 0.18$), indicating no effect of multiplexing or infection on copepod 18s rRNA gene quantification. When averaging copepod gene concentration of each standard at each dilution step, all estimates were on the same order of magnitude after correction (10^{-4} = 474,333 copies µL^{-1}, 10^{-5} = 330,625 copies µL^{-1}, 10^{-6} = 251,250 copies µL^{-1}). Taking the average across all three standards after correcting for dilution factor, we estimate 100 adult copepods to have 340,955 (SE = 64,421) 18S rRNA gene copies µL^{-1}.

The estimate of *S. solidus* DNA was based solely on the 1:1 infection standard. Despite the %CV within each dilution step being very low, when correcting for the dilution factor, we find that *S. solidus* gene estimates are an order of magnitude smaller in more dilute samples (i.e., 10^{-5} and 10^{-6}, Table 1). Based on the corrected average from the less diluted replicates (10^{-2}–10^{-4}) from both singleplexed and multiplexed reactions, we estimate 100 encysted *S. solidus* to have an average of 159,857 (SE = 22,887) 18S rRNA gene copies µL^{-1}.

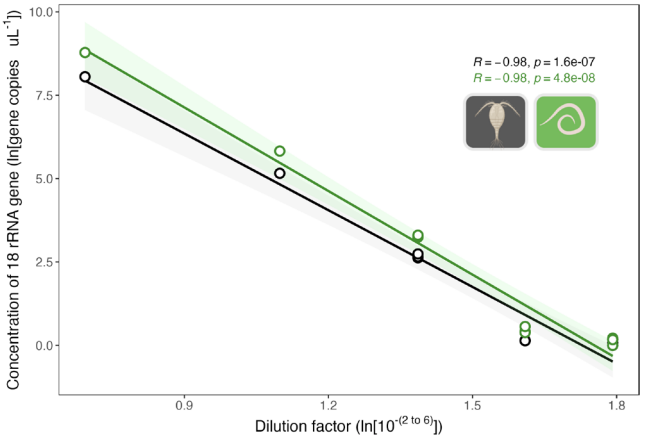


FIGURE 2 | Linear relationship between dilution factor and gene concentration. There exists a significant correlation ($r^2 = -0.98$, $p < 0.001$ for both targets) between dilution and absolute concentration, demonstrating a reliable linearity of the dilution assay.

3.2 | Experiment 2: Does Multiplexing ddPCR Reactions Inhibit the Detection of Rare DNA?

Using a t-test, we found that there is no statistical difference between the amount of target *S. solidus* 18S rRNA detected across a dilution series (0.001–0.00001 ng µL^{-1} DNA) between ddPCR assays with single versus multiplexed reactions ($t = 0.0759$, df = 15.955, p-value = 0.940; Table 2).

3.3 | Experiment 3: How Can We Use Infection Standards to Track Infection Dynamics in Natural Systems?

In natural systems where the mean infection load is unknown, running both 10^{-1} and 10^{-2} dilutions was ideal for both helminth detection and host quantification. We found that a 10^{-1} dilution removes potential inhibitors from a sample with minimal compromise to parasite detection. A 10^{-2} dilution generated enough separation between positive and negative droplets to quantify host density.

Using both water filters (eDNA) and zooplankton tows to groundtruth this methodology, this ddPCR assay has the sensitivity required to detect *S. solidus* within their cyclopoid copepod host from a large mixed species population (Figure 3). In a zooplankton tow from June 2023, Pachena Lake (Vancouver Island, B.C.), we found an average of 13 copies of *S. solidus* 18S rRNA per µL (SE = 0.58, corrected for dilution factor) within 108,700 copies of cyclopoid host 18S rRNA per µL (SE = 961, corrected for dilution factor). The following spring, we found zooplankton tows from Black Lake (Vancouver Island, B.C.; sampled March 2024) contain a mean of 67.7 copies of *S. solidus* 18S rRNA per µL (SE = 2.6, corrected for dilution factor) within 205,100 copies of cyclopoid 18S rRNA per µL (SE = 7970, corrected for dilution factor).

Using this detection system as a proof-of-concept, we leveraged lake-level estimates of both parasites (Figure 4A) and hosts (Figure 4B) to calculate mean parasite load across sampling sites (Figure 4C). Following the Anderson and May (1978) definition, we can estimate the mean parasite load from zooplankton tows. Pachena Lake had a mean parasite load of 1.20×10^{-4} copies μL^{-1} and Black Lake had a mean parasite load of 3.30×10^{-4} copies μL^{-1}. Multiplexed ddPCR analysis of eDNA from filtered lake water (Blackwater Lake, Vancouver Island B.C.) also showed positive signals for both host and parasite 18S rRNA. The eDNA sample was run only once and undiluted. ddPCR detected 0.42 copies of *S. solidus* 18S rRNA μL^{-1} and 1767 copies μL^{-1} of cyclopoid host 18S rRNA in the environment.

4 | Discussion

Despite enormous control efforts, helminth parasites continue to infect billions of humans and animals annually (Lustigman et al. 2012; WHO 2017). One of the leading factors responsible for ineffective management is the failure to monitor parasites in their initial hosts (Webster et al. 2015). This gap in knowledge is central

TABLE 2 | Detection of *S. solidus* DNA in single versus multiplexed reactions using an infection standard (100 copepods exposed and infected with 1 *S. solidus* parasite).

Treatment	Dilution (10^{-x})	Total DNA ($ng\,\mu L^{-1}$)	Mean *18S rRNA* concentration ($copies\,\mu L^{-1}$)	Standard error	Mean accepted droplets
Multiplex *S. solidus*	2	0.1	3150[a]	—	13,680[a]
	3	0.01	173[a]	—	14,469[a]
	4	0.001	13.5	0.504	14,640
	5	0.0001	0.153	0.003	15,462
	6	0.00001	0.09	0.055	13,867
Singleplex *S. solidus*	4	0.001	12.8	0.504	14,829
	5	0.0001	0.187	0.074	15,004
	6	0.00001	0.053	0.027	14,811

Note: Samples were diluted from $10\,ng\,\mu L^{-1}$ stock.
[a]Samples without true technical replicates.

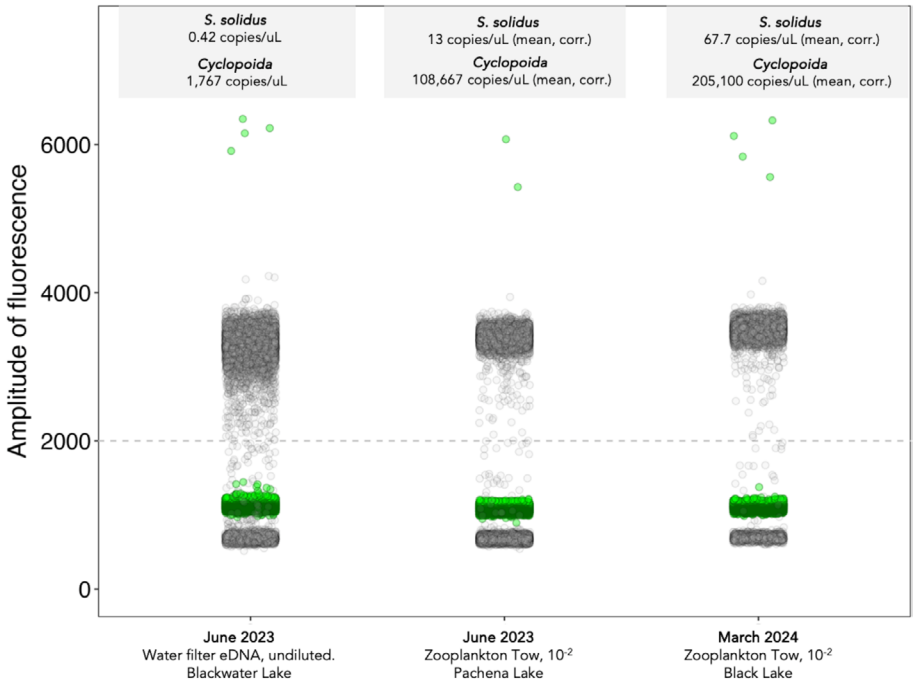


FIGURE 3 | Host–parasite detection in environmental samples. In multiplexed ddPCR reactions, we find positive results in (1) diverse lakes in different months and (2) in both water samples and zooplankton tows. Single representative amplification wells are visualised here, but samples were run in triplicate across the dilution series (except for the eDNA sample, which was run only once). Averages in grey boxes are corrected to full strength.

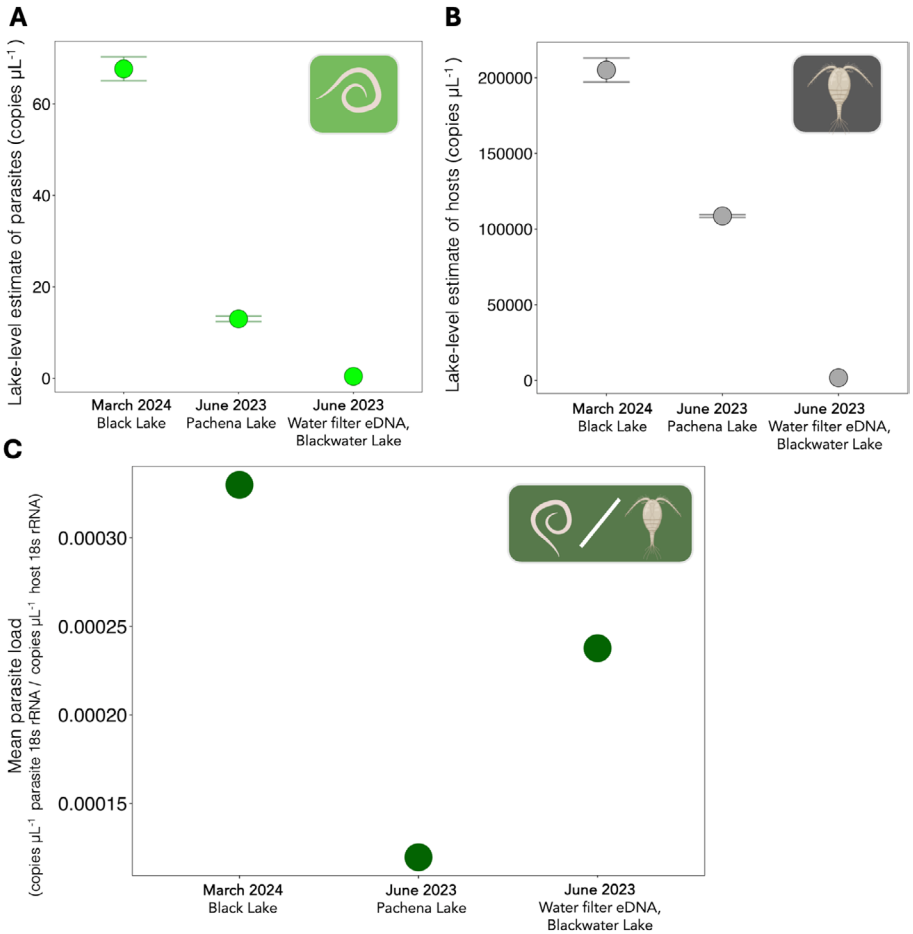


FIGURE 4 | Lake-level estimates of parasites (A), hosts (B), and mean parasite load (C) varies substantially across sites. Parasite load is highest when both parasite and host DNA copy numbers are high, as observed in Black Lake. In contrast, when parasites are less abundant relative to hosts, as in Pachena Lake, parasite load is lower. Blackwater Lake, where both parasite and host levels are relatively low, exhibits intermediate parasite loads. Error bars represent SEs between replicates ($n = 3$).

to understanding the factors that modulate the establishment and transmission of helminths. In this work, we designed and applied a ddPCR approach to detect and quantify host and parasite loads from environmental samples. We used a zooplankton-tapeworm model system as a case study to demonstrate the application of ddPCR as a valuable epidemiological tool. Small zooplankton (copepods, *A. robustus*, ~1.3 mm) are the initial hosts to the larval stages of the helminth tapeworm *S. solidus*. Our assay can reliably detect infection in initial hosts starting from 0.1 picograms of total DNA. The assay is robust, demonstrating successful detection of both host and parasite 18S rRNA in both aquatic field samples (multi-species zooplankton tows) and eDNA samples (water filters). Our multiplexed ddPCR approach unites a cutting-edge method that has yet to be widely applied in disease ecology or epidemiological contexts. Together, we provide a solid framework for environmental detection and tracking that could greatly bolster management efforts of NTDs globally (Brindley et al. 2009).

4.1 | A Tool for Population-Wide Assessment (of Both Hosts and Parasites)

ddPCR enables the quantification of mean parasite loads in initial hosts at both the individual and population levels. Such data are crucial for understanding, predicting, and managing disease outbreaks. However, previous tools have failed to provide the level of quantitative detail required for such analyses. In helminths, for example, previous epidemiological efforts estimated infection dynamics in natural populations by quantifying zooplankton abundance (e.g., zooplankton density, Stutz et al. 2014) or using visual screening of infected zooplankton (e.g., Rusinek et al. 1996; Dörücü 1999; Hanzelová and Gerdeaux 2003) to estimate infection prevalence and transmission rates in primary hosts. While these data are fundamental for parameterising transmission models, estimating infection using these methods is labour-intensive and time-consuming. Our method helps address these logistical challenges by providing a high-throughput and cost-effective toolkit that can not only rapidly assess parasite loads across multiple scales of biological organisation but also detect low levels of infection that are often missed by canonical methods.

Previous studies have established data for *S. solidus* infection in secondary hosts (stickleback fish: Marcogliese 1995; Fuess et al. 2021). Our study contributes a fundamental piece of the puzzle to fill key gaps relating to initial infection and transmission dynamics of *S. solidus* and has the capacity to do the same for related host–parasite systems. This work will be applicable to estimating copepod contributions to R_0, as we can leverage ddPCR outputs to estimate parasite loads in the initial host

(Fenton et al. 2015). For example, we observed notable variation in mean parasite loads across different months and sampling sites (Pachena Lake mean June = 1.20×10^{-4} copies μL^{-1} vs. Black Lake mean March = 3.30×10^{-4} copies μL^{-1}). Ultimately, evolutionary processes occur at the population level; without understanding infection dynamics in initial host populations, we miss critical eco-evolutionary processes that drive the entire infection sequence (e.g., contact probability, dilution effect). This principle is relevant for all helminthiases and is a key barrier to the successful management of many NTDs.

4.2 | Applications to Other Helminth Systems

Our goal was to establish a generalisable toolkit that can serve as a foundation for future research on helminths dependent on copepods as initial hosts. The primers and probe-primers we have designed here lay the groundwork for broader research in a diverse array of other helminth systems (Box 1). Our assay targets the 18S rRNA gene of both *S. solidus* and cyclopoid copepods, leveraging conserved sequences found across the animal kingdom. Since *S. solidus* is known to infect various cyclopoid species (Wedekind 1997), we designed the cyclopoid primer to encompass ecologically relevant hosts, such as *Acanthocyclops* sp. and *Macrocyclops* sp. These primers and probe-primers can be immediately applied to other helminthic diseases involving cyclopoids, including guinea worms and gnathostomiasis (Box 1; Nithiuthai et al. 2004). Additionally, our parasite primers broadly amplify the phylum *Platyhelminthes*, making them suitable for detecting other cestode helminths, such as broad tapeworms. Given the projected increase in helminth infections due to climate change and land-use disturbance, it is crucial to quantify animal reservoirs of these parasites (Blum and Hotez 2018).

4.3 | Limitations

Previous work optimising ddPCR similarly found assays to be reproducible and sensitive in detecting rare or cryptic symbionts (Yang et al. 2014; Hiillos et al. 2021). While our assay demonstrated a strong linearity across the dilution gradient for both targets, the reliability of target quantification strongly decreased in lower concentrations of total DNA (i.e., <1 pg. of total DNA, helminth gene estimates differed by an order of magnitude when corrected compared to less diluted samples). For this reason, when handling field samples with unknown host/parasite concentrations, it is important to establish adequate dilution to ensure proper droplet separation while maximising target detection. In a limnological context, we found that field samples diluted to 1 and 0.1 ng μL^{-1} of total DNA resulted in the best detection and most reliable gene quantification within the ddPCR framework.

It is worth mentioning that one trade-off of this assay compared to manual inspection of copepods is that we do not know the stage-structure or the sex of detected cyclopoids. If a genetic marker between males and females were known, then it could potentially be included as another element of ddPCR reaction. This could be achieved by using different probe concentrations in order to generate distinct amplitude peaks without having to add an additional fluorescent dye. As in other systems, infection prevalence in copepods varies significantly across males and females (females: Hanzelová and Gerdeaux 2003, males: Rusinek et al. 1996; Wedekind and Jakobsen 1998). Based on laboratory studies, male *A. robustus* appears more susceptible to infection than females (Ipsita Srinivas, Chloe A. Fouilloux, unpublished data) but the role of infection on downstream effects, such as reproduction and sex determination remains unknown.

4.4 | Future Applications of ddPCR

Helminthiases are notoriously difficult to track in the environment. Due to the relative rarity of helminth infections in the wild (Marcogliese 1995), a multiplexed ddPCR design quantifying both host and parasite prevalence allows for the reduction of false positives (e.g., non-ingested helminths) and the quantification of mean parasite load in a sample. We found that multiplexing samples does not impede rare target detection. We use the multiplexed approach by using two dyes (HEX and FAM) to detect two targets; while most ddPCR instruments typically have only two fluorescence detection channels, it is possible to detect more targets by discriminating between the amplitude threshold of different target sequences. For example, we can discriminate between different, closely related helminth species (*S. solidus* and *S. cotti*) based on variations in fluorescence amplitude, even though both species are labelled with FAM dye (File S2, Figure 2). An additional advantage of targeting the 18S rRNA gene is that it is present in multiple copies within an organism, which enhances detection sensitivity, particularly when the source is present in low abundance. This sensitivity is especially valuable for detecting infection in natural populations, as published infection prevalences in copepods by other orders of cestodes are low (0.13%–0.21%; Rusinek et al. 1996; Hanzelová and Gerdeaux 2003). While we do not report infection prevalence directly, the mean parasite load estimated from our field samples is similarly low (0.011%–0.033%, Figure 4C). However, more extensive sampling will be necessary to capture infection dynamics across both spatial and temporal scales.

We show here that ddPCR is a powerful detection tool across diverse environmental samples. ddPCR offers applications of broad interest to ecologists beyond this scope, such as quantifying gene expression. This is especially applicable in helminth systems, as there has been a significant uptick in the analysis of functional genomics in these worms that infect over 20% of the world's population (Jolly et al. 2007). For instance, measuring growth-related gene expression in helminths (such as TRIP12 in *Schistosoma*, Gobert et al. 2006) could indicate their readiness for transmission to the next host, providing in situ insights on parasite life history and transmission dynamics. Additional genome sequencing and gene expression studies of initial hosts will also set the foundation for many studies relevant to population ecologists. For example, identifying growth markers in copepods (e.g., pre vs. post metamorphosis) would also open up avenues to adding stage-structured analysis to field samples, allowing researchers to consider population demographics of diverse hosts in natural populations.

5 | Conclusions

This study demonstrates multiplexed ddPCR as a highly sensitive and repeatable method to simultaneously quantify parasite and

host genes from multi-species, environmental samples. We present a toolkit of primers and probes that are applicable to a range of helminth species, offering a flexible toolkit for studying NTDs and host–parasite interactions in natural systems. Future work may build upon this methodology by considering additional target species (i.e., co-infection) by varying probe concentrations in addition to multiplexing assays. By bridging molecular precision with ecological (and societal) relevance, this study contributes to promoting the early detection and quantification of helminthiases globally.

Author Contributions

C.A.F. wrote the initial manuscript, all authors contributed to manuscript revisions and approved the final version of the submission. C.A.F. and E.N.E. designed molecular components of the ddPCR assay and performed molecular analysis. C.A.F. designed the figures. I.S. performed infection assays and led the generation of infection standards, supported by J.S. and J.S.C., C.W. and J.W. contributed helminth tissues and live cyclopoid copepods and cestodes. A.H., J.B.III, H.A., E.C., D.I.B., and J.L.H. conducted field studies and sample collection (eDNA and zooplankton tows).

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Disclosure

Benefits generated: The research addresses a priority concern of identifying tapeworm helminths of global health concern in natural habitats. All data will be shared with the broader public via appropriate biological databases. All undergraduate collaborators of this manuscript are included as co-authors, supporting the early career of our next generation of scientists.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw ddPCR data and R code are available in the Environmental Data Initiative (EDI) repository (Fouilloux et al. 2025, DOI: 10.6073/pasta/029b089ae5fbad09515c19e2e8bc3331) and CAF's GitHub (https://github.com/chloefouilloux/18s_ddPCR) for ease of access. All data is publicly available, ensuring that researchers can access, reuse, and reproduce the analyses.

References

Abildgaard, P. C. 1790. "Almindelige Betragtninger over Indvolde-Orme, Bemaekningerved Hundstellens Baendelorm, og Beskrivelse med Figurer af nogel nye Baendelorme." *Skrivter Af Naturhistorie-Selskabet* 1: 26–64.

Amoah, I. D., G. Singh, T. A. Stenström, and P. Reddy. 2017. "Detection and Quantification of Soil-Transmitted Helminths in Environmental Samples: A Review of Current State-of-the-Art and Future Perspectives." *Acta Tropica* 169: 187–201.

Anderson, R. M., and R. M. May. 1978. "Regulation and Stability of Host-Parasite Population Interactions: I. Regulatory Processes." *Journal of Animal Ecology* 47: 219–247.

Barber, I., B. W. Berkhout, and Z. Ismail. 2016. "Thermal Change and the Dynamics of Multi-Host Parasite Life Cycles in Aquatic Ecosystems." *Integrative and Comparative Biology* 56: 561–572.

Barber, I., and J. P. Scharsack. 2009. "The Three-Spined Stickleback-*Schistocephalus solidus* System: An Experimental Model for Investigating Host-Parasite Interactions in Fish." *Parasitology* 137: 411–424.

Bass, D., S. Rueckert, R. Stern, et al. 2021. "Parasites, Pathogens, and Other Symbionts of Copepods." *Trends in Parasitology* 37: 875–889.

Bates, S. T., D. Berg-Lyons, C. L. Lauber, W. A. Walters, R. Knight, and N. Fierer. 2012. "A Preliminary Survey of Lichen Associated Eukaryotes Using Pyrosequencing." *Lichenologist* 44: 137–146.

Benesh, D. P. 2016. "Autonomy and Integration in Complex Parasite Life Cycles." *Parasitology* 143: 1824–1846.

Blum, A. J., and P. J. Hotez. 2018. "Global "Worming": Climate Change and Its Projected General Impact on Human Helminth Infections." *PLoS Neglected Tropical Diseases* 12: e0006370.

Boonham, N., J. Tomlinson, S. Ostoja-Starzewska, and R. A. McDonald. 2020. "A Pond-Side Test for Guinea Worm: Development of a Loop-Mediated Isothermal Amplification (LAMP) Assay for Detection of *Dracunculus medinensis*." *Experimental Parasitology* 217: 107960.

Bråten, T. 1966. "Host Specificity in *Schistocephalus solidus*." *Parasitology* 56: 657–664.

Brindley, P. J., M. Mitreva, E. Ghedin, and S. Lustigman. 2009. "Helminth Genomics: The Implications for Human Health." *PLoS Neglected Tropical Diseases* 3: e538.

Bustin, S. A., V. Benes, J. Garson, et al. 2013. "The Need for Transparency and Good Practices in the qPCR Literature." *Nature Methods* 10: 1063–1067.

Cairncross, S., R. Muller, and N. Zagaria. 2002. "Dracunculiasis (Guinea Worm Disease) and the Eradication Initiative." *Clinical Microbiology Reviews* 15: 223–246.

Charlier, J., M. van der Voort, F. Kenyon, P. Skuce, and J. Vercruysse. 2014. "Chasing Helminths and Their Economic Impact on Farmed Ruminants." *Trends in Parasitology* 30: 361–367.

Dijkstra, J. R., L. C. van Kempen, I. D. Nagtegaal, and S. A. Bustin. 2014. "Critical Appraisal of Quantitative PCR Results in Colorectal Cancer Research: Can We Rely on Published qPCR Results?" *Molecular Oncology* 8: 813–818.

Dörücü, M. 1999. "Seasonal Variation of Pseudophyllidean Cestode, *Diphyllobothrium* spp. Infection in *Cyclops strenuus abyssorum* (Copepoda) in Loch Lomond." *Turkish Journal of Zoology* 23: 85–91.

Fenton, A., D. G. Streicker, O. L. Petchey, and A. B. Pedersen. 2015. "Are All Hosts Created Equal? Partitioning Host Species Contributions to Parasite Persistence in Multihost Communities." *American Naturalist* 186: 610–622.

Fouilloux, C. A., E. Neeno-Eckwall, I. Srinivas, et al. 2025. "A Droplet Digital Polymerase Chain Reaction Assay to Detect Rare Helminth Parasites Infecting Natural Host Populations (Vancouver Island 2023, University of Wisconsin Madison Laboratory Colony 2024)." Environmental Data Initiative.

Fuess, L. E., J. N. Weber, S. den Haan, N. C. Steinel, K. C. Shim, and D. I. Bolnick. 2021. "Between-Population Differences in Constitutive

and Infection-Induced Gene Expression in Threespine Stickleback." *Molecular Ecology* 30: 6791–6805.

Gobert, G. N., R. McInnes, L. Moertel, et al. 2006. "Transcriptomics Tool for the Human Schistosoma Blood Flukes Using Microarray Gene Expression Profiling." *Experimental Parasitology* 114: 160–172.

Goodwin, C. E. D., M. Léchenne, J. K. Wilson-Aggarwal, et al. 2022. "Seasonal Fishery Facilitates a Novel Transmission Pathway in an Emerging Animal Reservoir of Guinea Worm." *Current Biology* 32: 775–782.e4.

Hanzelová, V., and D. Gerdeaux. 2003. "Seasonal Occurrence of the Tapeworm *Proteocephalus longicollis* and Its Transmission From Copepod Intermediate Host to Fish." *Parasitology Research* 91: 130–136.

Hiillos, A. L., A. Thonig, and K. E. Knott. 2021. "Droplet Digital PCR as a Tool for Investigating Dynamics of Cryptic Symbionts." *Ecology and Evolution* 11: 17381–17396.

Hindson, B. J., K. D. Ness, D. A. Masquelier, et al. 2011. "High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number." *Analytical Chemistry* 83: 8604–8610.

Hou, Y., S. Chen, Y. Zheng, X. Zheng, and J. M. Lin. 2023. "Droplet-Based Digital PCR (ddPCR) and Its Applications." *TrAC – Trends in Analytical Chemistry* 158: 1–21.

Hubbard, I. M., K. M. Hill-Spanik, D. Knott, and I. De Buron. 2016. "Development of *Anguillicoloides crassus* in a Cyclopoid Copepod From the *Acanthocyclops robustus-Americanus-vernalis* Complex in South Carolina, U.S.A." *Comparative Parasitology* 83: 192–196.

Hund, A. K., L. E. Fuess, M. L. Kenney, et al. 2022. "Population-Level Variation in Parasite Resistance due to Differences in Immune Initiation and Rate of Response." *Evolution Letters* 6: 162–177.

Jakobsen, P. J., J. P. Scharsack, K. Hammerschmidt, P. Deines, M. Kalbe, and M. Milinski. 2012. "In Vitro Transition of *Schistocephalus solidus* (Cestoda) From Coracidium to Procercoid and From Procercoid to Plerocercoid." *Experimental Parasitology* 130: 267–273.

Jolly, E. R., C. S. Chin, S. Miller, et al. 2007. "Gene Expression Patterns During Adaptation of a Helminth Parasite to Different Environmental Niches." *Genome Biology* 8: R65.

Jones, M., J. Williams, K. Gärtner, R. Phillips, J. Hurst, and J. Frater. 2014. "Low Copy Target Detection by Droplet Digital PCR Through Application of a Novel Open Access Bioinformatic Pipeline, 'defineth-erain'." *Journal of Virological Methods* 202: 46–53.

Kilham, S. S., D. A. Kreeger, S. G. Lynn, C. E. Goulden, and L. Herrera. 1998. "COMBO: A Defined Freshwater Culture Medium for Algae and Zooplankton." *Hydrobiologia* 377: 147–159.

Klawonn, I., S. Dunker, M. Kagami, H. P. Grossart, and S. Van den Wyngaert. 2023. "Intercomparison of Two Fluorescent Dyes to Visualize Parasitic Fungi (Chytridiomycota) on Phytoplankton." *Microbial Ecology* 85: 9–23.

Kraemer, S. A., N. Barbosa da Costa, B. J. Shapiro, M. Fradette, Y. Huot, and D. A. Walsh. 2020. "A Large-Scale Assessment of Lakes Reveals a Pervasive Signal of Land Use on Bacterial Communities." *ISME Journal* 14: 3011–3023.

Kurtz, J., I. T. Van Der Veen, and M. Christen. 2002. "Fluorescent Vital Labeling to Track Cestodes in a Copepod Intermediate Host." *Experimental Parasitology* 100: 36–43.

Labaude, S., T. Rigaud, and F. Cézilly. 2015. "Host Manipulation in the Face of Environmental Changes: Ecological Consequences." *International Journal for Parasitology: Parasites and Wildlife* 4: 442–451.

Laboratories, B.-R. 2017. "Droplet Digital™ PCR Applications Guide." https://bif.wisc.edu/wp-content/uploads/sites/389/2017/11/ddPCR_Applications_Guide.pdf.

Larsson, A. 2014. "AliView: A Fast and Lightweight Alignment Viewer and Editor for Large Data Sets." *Bioinformatics* 30, no. 19: 3276–3278.

Lustigman, S., R. K. Prichard, A. Gazzinelli, et al. 2012. "A Research Agenda for Helminth Diseases of Humans: The Problem of Helminthiases." *PLoS Neglected Tropical Diseases* 6: 1–13.

Marcogliese, D. J. 1995. "Comparison of Parasites of Mummichogs and Sticklebacks From Brackish and Freshwater Ponds on Sable Island, Nova Scotia." *American Midland Naturalist* 133: 333–343.

Mercado-Salas, N. F., S. Khodami, and P. M. Arbizu. 2021. "Copepods and Ostracods Associated With Bromeliads in the Yucatán Peninsula, Mexico." *PLoS One* 16: e0248863.

Momčilović, S., C. Cantacessi, V. Arsić-Arsenijević, D. Otranto, and S. Tasić-Otašević. 2019. "Rapid Diagnosis of Parasitic Diseases: Current Scenario and Future Needs." *Clinical Microbiology and Infection* 25: 290–309.

Ngwese Mbong, M., G. P. Manouana, P. A. N. Moure, M. Ramharter, M. Esen, and A. A. Adégnika. 2020. "Diagnostic Techniques of Soil-Transmitted Helminths: Impact on Control Measures." *Tropical Medicine and Infectious Disease* 5: 93.

Nishimura, N., D. C. Heins, R. O. Andersen, I. Barber, and W. A. Cresko. 2011. "Distinct Lineages of Schistocephalus Parasites in Threespine and Ninespine Stickleback Hosts Revealed by DNA Sequence Analysis." *PLoS One* 6: e22505.

Nithiuthai, S., M. T. Anantaphruti, J. Waikagul, and A. Gajadhar. 2004. "Waterborne Zoonotic Helminthiases." *Veterinary Parasitology* 126: 167–193.

Reinhard, K., and O. Urban. 2003. "Diagnosing Ancient Diphyllobothriasis From Chinchorro Mummies." *Memórias do Instituto Oswaldo Cruz* 98: 191–193.

Rusinek, O. T., M. P. Bakina, and A. V. Nikolskii. 1996. "Natural Infection of the Calanoid Crustacean *Epischura baicalensis* by Procercoids of Proteocephalus sp. in Listvenichnyi Bay, Lake Baikal." *Journal of Helminthology* 70: 237–247.

Scholz, T., H. H. Garcia, R. Kuchta, and B. Wicht. 2009. "Update on the Human Broad Tapeworm (Genus *Diphyllobothrium*), Including Clinical Relevance." *Clinical Microbiology Reviews* 22: 146–160.

Scholz, T., R. Kuchta, and J. Brabec. 2019. "Broad Tapeworms (Diphyllobothriidae), Parasites of Wildlife and Humans: Recent Progress and Future Challenges." *International Journal for Parasitology: Parasites and Wildlife* 9: 359–369.

Shannon, K. E., D. Y. Lee, J. T. Trevors, and L. A. Beaudette. 2007. "Application of Real-Time Quantitative PCR for the Detection of Selected Bacterial Pathogens During Municipal Wastewater Treatment." *Science of the Total Environment* 382: 121–129.

Stutz, W. E., O. L. Lau, and D. I. Bolnick. 2014. "Contrasting Patterns of Phenotype-Dependent Parasitism Within and Among Populations of Threespine Stickleback." *American Naturalist* 183: 810–825.

Taylor, S. C., G. Laperriere, and H. Germain. 2017. "Droplet Digital PCR Versus qPCR for Gene Expression Analysis With Low Abundant Targets: From Variable Nonsense to Publication Quality Data." *Scientific Reports* 7: 2409.

Teterina, V. I., Y. A. Galimova, N. G. Sheveleva, et al. 2016. "A Simple and Effective Method for Assessing Chromatin Diminution Values in Copepods Using qPCR." *Russian Journal of Genetics* 52: 1200–1203.

Thomas, A. C., J. Howard, P. L. Nguyen, T. A. Seimon, and C. S. Goldberg. 2018. "eDNA Sampler: A Fully Integrated Environmental DNA Sampling System." *Methods in Ecology and Evolution* 9: 1379–1385.

Walker, J. G., M. Plein, E. R. Morgan, and P. A. Vesk. 2017. "Uncertain Links in Host-Parasite Networks: Lessons for Parasite Transmission in

a Multi-Host System." *Philosophical Transactions of the Royal Society B: Biological Sciences* 372: 20160095.

Weber, J. N., M. Kalbe, K. C. Shim, et al. 2017. "Resist Globally, Infect Locally: A Transcontinental Test of Adaptation by Stickleback and Their Tapeworm Parasite." *American Naturalist* 189: 43–57.

Webster, J. P., A. Borlase, and J. W. Rudge. 2017. "Who Acquires Infection From Whom and How? Disentangling Multi-Host and Multimode Transmission Dynamics in the 'Elimination' Era." *Philosophical Transactions of the Royal Society, B: Biological Sciences* 372, no. 1719: 20160091.

Webster, J. P., C. M. Gower, S. C. L. Knowles, D. H. Molyneux, and A. Fenton. 2015. "One Health – An Ecological and Evolutionary Framework for Tackling Neglected Zoonotic Diseases." *Evolutionary Applications* 9: 313–333.

Wedekind, C. 1997. "The Infectivity, Growth, and Virulence of the Cestode *Schistocephalus solidus* in Its First Intermediate Host, the Copepod *Macrocyclops albidus*." *Parasitology* 115: 317–324.

Wedekind, C., and P. J. Jakobsen. 1998. "Male-Biased Susceptibility to Helminth Infection: An Experimental Test With a Copepod." *Oikos* 81: 458–462.

Wetzel, R. G. 2001. *Limnology: Lake and River Ecosystems*. Elsevier.

World Health Organization (WHO). 2017. *Guideline: Preventive Chemotherapy to Control Soil-Transmitted Helminth Infections in At-Risk Population Groups*. WHO. Licence: CC BY-NC-SA 3.0 IGO.

Yang, R., A. Paparini, P. Monis, and U. Ryan. 2014. "Comparison of Next-Generation Droplet Digital PCR (ddPCR) With Quantitative PCR (qPCR) for Enumeration of Cryptosporidium Oocysts in Faecal Samples." *International Journal for Parasitology* 44: 1105–1113.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.