Evaluation of Daphnid Grazing on Microscopic Zoosporic Fungi by Using Comparative Threshold Cycle Quantitative PCR

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ABSTRACT
Lethal parasitism of large phytoplankton by chytrids (microscopic zoosporic fungi) may play an important role in organic matter and nutrient cycling in aquatic environments by shunting carbon away from hosts and into much smaller zoospores, which are more readily consumed by zooplankton. This pathway provides a mechanism to more efficiently retain carbon within food webs and reduce export losses. However, challenges in accurate identification and quantification of chytrids have prevented a robust assessment of the relative importance of parasitism for carbon and energy flows within aquatic systems. The use of molecular techniques has greatly advanced our ability to detect small, nondescript microorganisms in aquatic environments in recent years, including chytrids. We used quantitative PCR (qPCR) to quantify the consumption of zoospores by Daphnia in laboratory experiments using a culture-based comparative threshold cycle (Ct) method. We successfully quantified the reduction of zoospores in water samples during Daphnia grazing and confirmed the presence of chytrid DNA inside the daphnid gut. We demonstrate that comparative Ct, qPCR is a robust and effective method to quantify zoospores and evaluate zoospore grazing by zooplankton and will aid in better understanding how chytrids contribute to organic matter cycling and trophic energy transfer within food webs.

IMPORTANCE
The study of aquatic fungi is often complicated by the fact that they possess complex life cycles that include a variety of morphological forms. Studies that rely on morphological characteristics to quantify the abundances of all stages of the fungal life cycle face the challenge of correctly identifying and enumerating the nondescript zoospores. These zoospores, however, provide an important trophic link between large colonial phytoplankton and zooplankton: that is, once the carbon is liberated from phytoplankton and organic matter in aquatic food webs through the liberation of organic matter from large hosts, which are generally inaccessible to small zooplankton. This pathway from large inedible algae to zooplankton via zoospores is called the “mycoloop” [see references 7 and 8]. Chytrid zoospores are rich in polyunsaturated fatty acids, making them nutritious food items for filter-feeding zooplankton. For example, in laboratory experiments, a diet of zoospores has been shown to improve the fitness and survival of the zooplankter Daphnia, compared to a diet of large colonial host diatoms (9). The potential importance of chytrids in aquatic food webs has been reviewed at length (3, 10–15); however, understanding of the role that chytrid zoospores play in food web dynamics is limited by the lack of robust methods to quantify parasitic zoosporic fungi in water samples and in the guts of consumers.

The difficulty in determining ecological roles of chytrid fungi arises in large part from the fact that their infective life stage is a small, morphologically nondescript, motile zoospore that resembles many other small microeukaryote taxa. The infective zoosporic life stage commences once zoospores have differentiated within the host-attached sporangium and are subsequently released into the environment (4). As opposed to the host-attached sporangium, which is easily recognizable using fluorescence microscopy (3, 16), the motile zoospore phase is more difficult to detect due to its small size (2 to 5 μm) and nondescript morphology (17). Success has been achieved through the combined application of molecular and microscopic approaches using fluorescence in situ hybridization (FISH), which has been employed to quantify zoospores in the field (18). In addition, quantitative PCR (qPCR) has been used to enumerate uncultured Rhizophyidae.
chrytrids semiquantitatively within mixed fungal assemblages based on 18S rRNA gene copy numbers (19), to quantify gene copies associated with the amphibian chrytrid parasite *Batrachocephrium dendrobatidis* (20–23), and to assess the grazing of *B. dendrobatidis* zoosporae by *Daphnia* (24–26). Although previous studies have provided quantitative estimates of chrytrid zoosporae, these methods are either time-consuming with high detection limits (FISH) or provide semiquantitative information (qPCR).

The objectives of this study were (i) to develop a robust method to quickly and accurately quantify chrytrid zoosporae using a calibrated qPCR assay in laboratory experiments and (ii) to confirm the presence of chrytrids in zooplankton guts by molecular identification. We first evaluated a new method to quantify zoosporae from a chrytrid parasitic on the diatom *Asterionella formosa*, using a culture-based comparative threshold cycle (C\text{t}) qPCR (27) approach. We used standards of known concentrations of chrytrid zoosporae from an established cultured isolate to quantify multiple zoosporae suspensions in the laboratory, a method previously successful at quantifying harmful algal bloom species in the field (28). We then evaluated the application of the calibrated method to assess consumption of chrytrid zoosporae by *Daphnia* in grazing experiments. Due to the importance of direct comparison between treatments in laboratory experiments, we added an external DNA control (lambda phage) to correct for variation in extraction efficiency of samples in order to accurately compare experimental treatments. By targeting a chrytrid parasitic to phytoplankton, this method supplies a new approach to quantify zoosporae and will aid in studies that seek to determine the transfer of organic material from primary producers to consumers through parasitic pathways.

**MATERIALS AND METHODS**

**Preparation of zoosporae for qPCR.** Isolation of the diatom host and chrytrid parasitic was described previously (29). In brief, a single chrytrid sporangium attached to the host diatom, *A. formosa*, was isolated by micropipette from a whole water sample collected in the lower Columbia River in April 2011 and maintained by subsequent weekly transfers to uninfected host colonies. The culture was nonaxenic; however, the culture was free of other flagellates, and care was taken to avoid bacterial contamination. Based on sequencing of three regions of the 18S ribosomal DNA (rDNA) (accession no. KJ480959 and KJ480958), the chrytrid isolate was 99\% similar to *Zygorhizidium planktonicum* in the NCBI GenBank database (29), although this submission was later determined to be *Rhizophydiunm planktonicum* (unpublished observation). Further microscopic analysis of the culture used in this study also determined the chrytrid to be *R. planktonicum*. To obtain zoosporae suspensions, highly infected diatom stellate host colonies (average abundance of 22,033 zoospores determined using microscopy, i.e., 1:5 dilution of sample 7/20/2012) of known zoospore abundance were placed in the dark overnight until a dense concentration of zoospores from an established cultured isolate to quantify multiple zoosporae suspensions in the laboratory, a method previously successful at quantifying harmful algal bloom species in the field (28). We then evaluated the application of the calibrated method to assess consumption of chrytrid zoosporae by *Daphnia* in grazing experiments. Due to the importance of direct comparison between treatments in laboratory experiments, we added an external DNA control (lambda phage) to correct for variation in extraction efficiency of samples in order to accurately compare experimental treatments. By targeting a chrytrid parasitic to phytoplankton, this method supplies a new approach to quantify zoosporae and will aid in studies that seek to determine the transfer of organic material from primary producers to consumers through parasitic pathways.

**Evaluation of PCR amplification efficiency.** An assumption of the comparative C\text{t} method is that the PCR amplification efficiencies of the chrytrid DNA (the target) and the lambda phage external DNA (the control) are similar in that they will amplify at the same rate in each PCR cycle (27, 33). To evaluate the similarity between the amplification efficiencies of the target DNA and control DNA, one extracted DNA sample containing chrytrid zoosporae (target) plus lambda phage (control) was diluted with nuclease-free water (included in each 48-well plate) to either 10\% or 1:50, followed by 45 to 400 cycles (ranging from 10 to 50 cycles) of PCR amplification. The PCR efficiencies (E) were calculated from the slopes (10\(^{-1/slope}\)) of each of the standard curves.

**Comparative C\text{t} analysis.** For each qPCR plate, a calibrator sample (i.e., 1:5 dilution of sample 7/20/2012) of known zoospore abundance (average abundance of 22,033 zoospores determined using microscopy, with a lower range of 13,235 and an upper range of 30,832 zoospores) was used as a standard to calculate the abundance of zoospores in all unknown samples (27), using the following sequential equations:

\[
\Delta C_t^{\text{sample}} = C_t^{\text{sample, chrytrid}} - C_t^{\text{sample, lambda}}
\]

\[
\Delta\Delta C_t = \Delta C_t^{\text{sample}} - (C_t^{\text{standard, chrytrid}} - C_t^{\text{standard, lambda}})
\]

\[
\text{No. of zoospores (sample)} = 2^{-\Delta\Delta C_t} \times \text{no. of zoospores (standard)}
\]
ences in the efficiency of DNA extraction. The calculation of \( \Delta \Delta C_T \) standardizes all chytrid samples relative to the known zoospore standard (from which lambda control DNA has also been subtracted: i.e., \( C_T \) standard_chytrid - \( C_T \) standard_lambda). Since the abundance of zoospores in the standard is a known value, the number of zoospores in unknown samples can be determined by multiplying the relative amount of chytrid DNA present in the sample (2^{\Delta\Delta C_T}) by the number of zoospores present in the known standard. Zoospore abundances in the unknown samples were calculated from each of the qPCR triplicate reactions and then averaged to yield a single value with standard deviations reported.

**Evaluation of comparative \( C_T \) method.** Two 10-fold zoospore concentration series (\( 10^2, 10^3, 10^4, \) and \( 10^5 \)) were analyzed by qPCR, and zoospore concentrations were calculated according to equations 1 to 3 using the average, lower, and upper ranges for microscopic counts of the zoospore standard (i.e., 1:5 dilution of sample 7/20/12). These values were compared to microscopic counts in order to validate the robustness and repeatability of the method.

**Grazing experiments.** For preparation of zoospores in the grazing experiments, infected diatoms were grown on Chu-10 medium (34) in the laboratory at 18°C at 40 \( \mu \)mol quanta m\(^{-2}\) s\(^{-1}\) on a 14-h/10-h light/dark cycle. Fresh live zoospore suspensions were prepared as described in the previous section (30, 31). *Daphnia pulex* and *Daphnia similis* were isolated from Lake Inba, Japan, in 2012 and propagated in the laboratory in dechlorinated tap water with the green alga *Scenedesmus* sp. as a food source. Prior to the experimental setup, 48 *D. pulex* (5 to 8 days old, female) and 24 *D. similis* (6 days old, female) daphnids were transferred once every hour for 3 h to fresh dechlorinated tap water at 18°C to rinse out the remaining algae from the medium. This was done in order to ensure that zoospores were the only food source available to daphnids during the experiment. The dry weight (micrograms) of each daphnid was calculated from body length measurements (millimeters) and daphnid-specific coefficients determined by Bottrell et al. (35). *D. similis* (2.71 ± 0.83 mm) was significantly larger than *D. pulex* (1.38 ± 0.15 mm), and therefore, fewer animals were used in the *D. similis* bottles. Data for *Daphnia* dry weights between all treatments passed a normality test (Shapiro-Wilk) and an equal variance test, and different treatments were compared using a one-way analysis of variance (ANOVA) with Holm-Sidak all pairwise multiple-comparison test. Dry weights for each species were compared with type 2 two-tailed \( t \) tests. Within-species differences in dry weight among individuals across treatments were not significant (Table 1).

For each *Daphnia* species, triplicate controls (Chu-10 medium only) and triplicate zoospore-fed treatments (zoospore suspensions) were prepared in glass vials with a 50-ml total volume (filled and capped with minimal air space). To control for biomass, either eight *D. pulex* or four *D. similis* daphnids were added to each of the control and zoospore-fed treatment bottles. Two bottles containing zoospores only (i.e., without daphnids) were prepared to assess zoospore decline in the absence of daphnid grazing. Vials were wrapped in aluminum foil and placed on their sides at 18°C for 4.5 h. After the 4.5 h of incubation, the vials were thoroughly mixed, and daphnids were individually removed in a minimal volume of medium (<1 ml) with a dropper pipette and rinsed with fresh Chu-10 medium. For each bottle, half the total number of daphnids (4 *D. pulex* or 2 *D. similis* daphnids) were immediately placed in a 1.5-ml Eppendorf tube (whole-body treatment) and frozen at −80°C. The carapace was carefully removed from the second half of the daphnids with insect pins, and the guts were placed in a 1.5-ml Eppendorf tube (carapace-removed treatment) and frozen at −80°C.

Zoospore suspensions (9 to 10 ml) were filtered onto 25-mm 0.6-μm-porosity polycarbonate filters at time zero (\( T_0 \)) and after 4.5 h (\( T_{4.5} \)) from the duplicate zoospore-only bottles and at \( T_{4.5} \) from the triplicate zoospore-fed *Daphnia* treatments. DNA from the filters was extracted with the ISOIL for Bead Beating kit with lambda phage DNA providing an external control, as described in the previous section. DNA from the whole body and guts of *D. pulex* and *D. similis* were extracted with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Briefly, frozen suspended daphnids were heated at 60°C in 1.5-ml Eppendorf tubes until the total volume was evaporated to 20 to 30 μl, and then lambda phage (10^6 particles) was added. Tubes were then incubated at 56°C for 3 to 4 h in 180 μl Qiagen ATL buffer and 20 μl proteinase K (provided in the DNeasy blood and tissue kit) until the daphnids were visually lysed. The extraction procedure followed the manufacturer’s instructions, and all DNA samples were eluted in a final 50 μl of Qiagen AE buffer and stored at −20°C.

**Comparative \( C_T \) analysis of grazed zoospores.** Zoospore abundances in the grazing experiments were calculated from \( C_T \) values generated from qPCR triplicate reactions using equations 1 to 3 and the average microcopy count of the zoospore standard (i.e., a 1:5 dilution of sample 7/20/12 average count is equal to 22,033 zoospores). The zoospore abundances in bottles incubated with daphnids at \( T_{4.5} \) (after 4.5 h of incubation) were compared against the \( T_0 \) control (bottle at the beginning of the experiment with no daphnids) using type 2 two-tailed \( t \) tests (since data passed Shapiro-Wilk normality and equal variance tests). The \( T_{4.5} \) zoospore control (bottle with no daphnids after 4.5 h of incubation) was compared to the \( T_0 \) control with a Mann-Whitney rank sum test rather than a parametric procedure, since although the data passed a Shapiro-Wilk normality test, the data failed an equal variance test.

Since the qPCR standards were based on DNA extracted from whole zoospore particles and how digestion affects this value is unknown, the zoospores associated with daphnids are reported as \( \Delta C_T \) (\( C_T \) sample - chytrid - \( C_T \) sample - lambda), a value that incorporates DNA extraction efficiency and is inversely related to the concentration of chytrid DNA detected by qPCR. If chytrid DNA was undetected in grazing experiments, then the \( C_T \) for the chytrid sample was set to the maximum cycle number (i.e., 40) in order to calculate the \( \Delta C_T \) of the sample. The \( \Delta C_T \) data passed a Shapiro-Wilk normality and an equal variance test, and a one-way ANOVA with post hoc testing (Holm-Sidak all pairwise multiple-comparison test) was performed. All statistical analyses were performed using SigmaPlot v12.5, and significance levels were set at \( \alpha = 0.05 \).

**RESULTS**

Zoospore quantification using comparative \( C_T \). We evaluated the use of a comparative \( C_T \) qPCR method to quantify chytrid

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**TABLE 1 Average dry weight of *Daphnia pulex* and *Daphnia similis* daphnids from triplicate experimental bottles and treatments**

<table>
<thead>
<tr>
<th>Bottle</th>
<th>No. of daphnids/bottle</th>
<th>Dry wt/bottle (μg)(^a)</th>
<th>Treatment</th>
<th>Dry wt/treatment (μg)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. pulex</em> control</td>
<td>8</td>
<td>82 ± 9</td>
<td>Whole body</td>
<td>45 ± 7</td>
</tr>
<tr>
<td><em>D. pulex</em> + zoospores</td>
<td>8</td>
<td>92 ± 12</td>
<td>Carapace removed</td>
<td>41 ± 11</td>
</tr>
<tr>
<td><em>D. similis</em> control</td>
<td>4</td>
<td>291 ± 12</td>
<td>Whole body</td>
<td>49 ± 7</td>
</tr>
<tr>
<td><em>D. similis</em> + zoospores</td>
<td>4</td>
<td>293 ± 7</td>
<td>Carapace removed</td>
<td>44 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Whole body</td>
<td>150 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carapace removed</td>
<td>145 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Whole body</td>
<td>147 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carapace removed</td>
<td>144 ± 7</td>
</tr>
</tbody>
</table>

\(^a\) Dry weight (micrograms) represents \( e^{1.468+2.829 \log \text{(body length)}}\) (35).
zoospores in laboratory grazing experiments using *Daphnia*. The difference in PCR amplification efficiencies between the target (chytrid) and the control (lambda phage) DNA sequences was small (7%) (Fig. 1), indicating that lambda phage DNA could be effectively used as a reference to quantify chytrid DNA using the comparative $C_T$ method.

To quantify zoospores in unknown samples, we then used a calibrator specific to our sample type (i.e., DNA from a known concentration of chytrid zoospores determined directly through microscopy) on each qPCR plate. There was a strong positive relationship between zoospores counted by microscopy and zoospores quantified by the comparative $C_T$ method ($r^2 = 0.92$), even when samples from two dilution series prepared on different dates were included (Fig. 2).

Application of $C_T$ method to grazing experiments with *Daphnia*. Zoospore abundance, as determined by comparative $C_T$ qPCR, decreased significantly in the presence of *D. pulex* and *D. similis* after the 4.5 h of incubation compared to the $T_0$ control ($P < 0.05$), while zoospore abundances in the control bottles at $T_0$ and $T_{4.5}$ were not statistically different from each other (Fig. 3A).

In order to determine the likelihood of zoospore ingestion versus nonspecific association or attachment to the carapace or appendages of *Daphnia*, we compared the amount of chytrid DNA in whole-body samples versus those in samples of daphnids with the carapace removed in treatments where the daphnids were exposed to zoospores (Fig. 3B). Chytrid DNA concentrations were greater in the carapace-removed zoospore-fed treatments than those in controls ($P < 0.05$) for *D. pulex*. For *D. similis*, both the whole-body and carapace-removed zoospore-fed specimens contained significantly more chytrid DNA than the control ($P < 0.05$).

**DISCUSSION**

Previous studies have shown that *Daphnia* is able to graze upon zoospores in the laboratory, resulting in greater fitness and survival than individuals fed a diet of host diatoms alone (9). However, although the disappearance of zoospores from the medium...
can be determined using microscopy, the consumption of zoospores by zooplankton is difficult to confirm using this method. In addition, the disappearance of zoospores is difficult to determine in mixed plankton assemblages due to their small size and nondescript morphologies, particularly when it is important to distinguish among particular taxa. In this study, we addressed these challenges by developing a quantitative assay targeting gene sequences from a parasitic chytrid (Rhizophydium planktonicum) of a widespread diatom (Asterionella formosa). Specifically, we (i) quantified abundances of chytrid zoospores in laboratory experiments where zoospores were introduced to containers with individual zooplankton and (ii) confirmed the presence of chytrids within zooplankton guts. The addition of an external DNA control (lambda phage) enabled direct comparison among sample treatments by accounting for potential differences in DNA extraction efficiencies (36). The comparative $C_T$ qPCR approach is useful for studying grazing relationships between chytrid zoospores and zooplankton in the laboratory to investigate potential effects such as size, food quality, and competition on rates of consumption. Comparative $C_T$ qPCR ($2^{-\Delta C_T}$) makes use of a calibrator to avoid biases associated with variations in gene copy number when calculating abundances of an organism of interest (28). This approach is advantageous over semiquantitative methods because (i) it avoids the need to generate a plasmid containing the target sequence in the standard curve method and (ii) the results produce ecologically relevant zoospore concentrations without requiring a conversion from a gene copy number. We used DNA extracted from known quantities of cultured chytrid zoospore isolates as a calibrator, which, once established, can be used to quantify zoospores of the same species from the environment. The isolation and cultivation of chytrid species has been reported in the Columbia River (29) and elsewhere (30, 31), providing guidelines for investigators interested in applying this method to other taxa. While we recognize that many chytrid taxa may not be readily cultivable (and thus not appropriate for comparative $C_T$ qPCR), the method presented here allows for the provision of quantitative data, which is an advantage over shotgun methods.

Using comparative $C_T$ qPCR, we observed a significant reduction in chytrid zoospore abundances between 0 and 4.5 h in the presence of D. pulex or D. similis in the laboratory ($P < 0.05$), and the number of zoospores reduced in the medium was congruent with filtering rates of daphnids of similar body size at similar temperatures (37). Since zoospore abundances did not decrease significantly in the absence of daphnids, we infer that minimal natural zoospore mortality occurred during the experiment, which was within the time (2.8 days) that R. planktonicum zoospores are known to remain active at 18°C (38).

We quantified chytrid DNA in both whole-body daphnids and daphnids with the carapace removed to determine if zoospores had been actively consumed (i.e., chytrid DNA was present inside the gut) as opposed to nonspecifically associated with external appendages. There was a significant difference between the zoospore-fed treatments and controls in the carapace-removed specimens for both D. pulex and D. similis, indicating that consumption of chytrids had in fact occurred. However, in the whole-body specimens, a significant difference between the zoospore-fed treatments and controls was only detected in D. similis and not in D. pulex. Given that chytrid DNA was detected in the individuals without carapaces, this result is somewhat puzzling. However, it is likely that there is significant individual-to-individual variability in grazing behavior, which may explain the lack of grazing evidence in some D. pulex individuals. It could also be that the chytrid zoospores were consumed very quickly and were passed through the gut during the incubation period, since daphnids are able to pass the contents of their intestinal tract within 15 to 30 min (39, 40), which is much shorter than the 4.5 h of the incubation period. Thus, only a fraction of the chytrids grazed during the incubation period may have been detected within the animals by qPCR. Furthermore, the process of degradation during digestion likely decreases the amount of DNA that is detectable by qPCR (41). Since the main purpose of this work was to demonstrate that DNA from chytrid zoospores could be detected within daphnids using qPCR, delving into rigorous explanations for these findings is beyond the scope of this discussion.

Using comparative $C_T$ qPCR, we were able to quantify the abundance of free-swimming chytrid zoospores in different treatments and within the daphnids themselves. The consumption of zoospores by zooplankton is known to vary depending on the particular zooplankton species examined (26, 43), the density of zooplankton, and/or the presence of additional food sources (26). Thus, additional studies are necessary to investigate how the individual size and species of zooplankton influence the number of zoospores grazed and to determine variability in grazing rates among different individuals.

Comparative $C_T$ qPCR provides an additional tool to characterize the role that chytrids play in food webs and organic matter cycling in targeted laboratory experiments. For example, quantification of free-living and grazed zoospores by comparative $C_T$ qPCR permits the detection of concurrent grazing of zoospores by multiple zooplankton species and may allow an investigator to identify selective versus passive filter feeding of zoospores within mixed assemblages, which is not feasible using light microscopy. In order to adapt this method for use in natural systems, the following are required: (i) a counted calibrator sample derived from cultured isolates, (ii) addition of an external DNA control (lambda phage) prior to DNA extraction, and (iii) specific primer sets targeting organisms of interest. Although the establishment of cultured isolates required for standardization in the comparative $C_T$ analysis may often be nontrivial, culture-based molecular methods can be extremely valuable for carrying out hypothesis-driven studies on target model species in the laboratory and in the natural environment.

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