Fungal parasites of a toxic inedible cyanobacterium provide food to zooplankton

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Abstract

During the end of spring and throughout summer, large-sized phytoplankton taxa often proliferate and form dense blooms in freshwater ecosystems. In many cases, they are inedible to zooplankton and prevent efficient transfer of energy and elements to higher trophic levels. Such a constraint may be alleviated by fungal parasite infections on large-sized phytoplankton taxa like diatoms and filamentous cyanobacteria, as infections may provide zooplankton with a complementary food source in the form of fungal zoospores. Zoospores have been shown to support somatic growth of large filter feeding zooplankton species. Here, we tested if selectively feeding zooplankton, more specifically rotifers, also can use fungal zoospores as a food source. Our results show that chytrid fungal parasites can indeed support population growth of rotifers (Keratella sp.). Specifically, in cultures of an inedible filamentous cyanobacterium (Planktothrix rubescens), Keratella populations rapidly declined, while in Planktothrix cultures infected with chytrids, Keratella population growth rate equaled the growth observed for populations fed with a more suitable green algal diet (Chlorella sorokiniana). Feeding of Keratella on zoospores was furthermore indicated by a reduced number of zoospores during the last sampling day. These findings not only imply that rotifers may survive on zoospores, but also that the zoospores can support high rotifer population growth rates. We thus show that fungal parasites of inedible cyanobacteria can facilitate grazers by providing them alternative food sources. Together, these results highlight the important role that parasites may play in the aquatic plankton food web.

During the onset of spring, phytoplankton communities in freshwater ecosystems usually contain a relatively large amount of small-sized edible algae, which are available as food to herbivorous zooplankton (Lampert et al. 1986; Sommer et al. 1986). Later in the season increased herbivory or competition for nutrients result in the replacement of the phytoplankton community by larger-sized groups, e.g., dinoflagellates and/or filamentous cyanobacteria (Sommer et al. 1986; De Senerpont Domis et al. 2013). Since these taxa may be toxic and are generally less easily ingested by zooplankton, they may represent trophic bottlenecks (Yasumoto and Murata 1993; Havens and East 1997; Sivonen and Jones 1999; Havens 2008). As a result, zooplankton may be forced to graze on other food sources such as bacteria (Christoffersen et al. 1990). Bacteria, however, generally represent low quality food (Demott and Müller-Navarra 1997; Schmidt and Jonasdottir 1997) because they lack essential sterols to sustain zooplankton growth (Martin-Creuzburg et al. 2011).

Besides grazing, other top-down factors can regulate phytoplankton growth and population densities. Viruses and fungal parasites are among the most common pathogenic agents involved in the regulation of phytoplankton blooms (Canter and Lund 1951; Brussaard et al. 1995; Brussaard 2004b; Ibelings et al. 2004; Tijdens et al. 2008; Sime-Ngando 2012; Gerphagnon et al. 2015; Frenken et al. 2016, 2017a). Through lysis of inedible phytoplankton cells, parasites may unlock nutrients that otherwise would remain unavailable to higher trophic levels. Fungal parasites belonging to the phylum Chytridiomycota, usually referred to as chytrids, are often involved in the decline of blooms of diatoms (Van Donk and Ringelberg 1983; Van Donk...
Fed with a suitable food source consisting of the green alga *Planktothrix* and with noninfected *Planktothrix*. This experiment was performed in six series of five 12-well plates (VWR, Amsterdam, The Netherlands). The second and third column of each well plate were left empty. The
remaining wells were randomly assigned to one of the six treatments using a random number generator (https://www.random.org/). Over the course of the experiment, five series of five replicates were used to sample Keratella, phytoplankton and bacteria every second day (further referred to as “destructive samples”). The sixth series of five replicates was maintained over the entire course of the experiment in which Keratella numbers were counted every day (referred to as “continuous samples”). See Supporting Information for a schematic overview of the experimental setup.

One day before the start of the experiment, Keratella individuals were picked from the stock cultures and washed three times in sterile WC medium to remove Chlorella. The wells that received grazing treatments were filled with 1 mL of WC-medium and 10 randomly picked individuals of Keratella. These individuals were left to starve overnight, after which the different food treatments were added reaching a total culture volume of 4 mL per well. All plates were incubated on a randomly assigned location within a temperature and light controlled incubator (Snijders Labs, Tilburg, The Netherlands) at 16°C and 5 μmol photons m⁻² s⁻¹ in a 14 : 10 light : dark cycle.

**Sample analysis**

In the continuous samples, Keratella abundance was determined daily using a dissecting microscope at a magnification of 15X (Leica WILD MZB, Leica Microsystems B.V., Son, The Netherlands). In the destructive samples, Keratella abundances were determined every second day after which the entire sample was preserved immediately using 25% glutaraldehyde (Merck, Darmstadt, Germany) to a final concentration of 0.5% (v/v). We used these samples to assess biovolume and population densities of the phytoplankton, zoospore density, prevalence of chytrid infection, and bacteria density. Planktothrix biovolume and Chlorella population density were determined in triplicate on a CASY Cell Counter (Schärfe System GmbH, Reutlingen, Germany).

Density of zoospores was determined using a protocol modified from Gsell et al. (2013), where every sample was counted in duplicate until at least 250 counts or 20 fields of view were reached, using an inverted microscope (DMI 4000B, Leica Microsystems CMS GmbH, Mannheim, Germany). Infections were counted as a categorical variable: at least 100 infected (i) or uninfected (ui). The prevalence of infection was subsequently calculated as \( P = i/(i + u) \).

In short, samples were diluted at least 10 times in a Tris-EDTA buffer (pH 8.2) and stained with the DNA stain SYBR® Green I nucleic acid gel stain (Molecular Probes, Invitrogen, Paisley, UK) for 10 min in the dark at 80°C (final concentration of 5 × 10⁻⁵ of commercial stock). Just before analyses, the sample was left to cool down at room temperature for 5 min after which 5 μL of a red fluorescent bead solution was added as a control (Fluoresbrite® Polychromatic Red Microspheres 2.0 μm, Polysciences, Hirschberg an der Bergstraße, Germany). This suspension was analyzed on a MoFlo XDP cell sorter (Beckman Coulter Nederland BV, Woerden, The Netherlands). On the last day of the experiment, all Keratella individuals were photographed on the inverted microscope, after which body length and width were measured using the program ImageJ 1.48v (ImageJ, National Institutes of Health). Rotifer body volume was calculated assuming a cylindrical shape.

**Data analysis**

To test if the time course of the different measured variables showed differences between treatments, a repeated measurements ANOVA (RM ANOVA) was performed in the statistical package Statistica 12.5 (Statsoft Europe, Hamburg, Germany). Variables were tested for normality and equal variance using the Shapiro-Wilk and Levene’s test, respectively. Bacteria density was square root transformed before analyses to improve normality. Keratella net population growth rates (\( \mu \)) were calculated as the change in the population count between the start and end of the experiment according to \( \mu = \ln(N_{\text{end}}/N_{\text{start}})/(t_{\text{end}} - t_{\text{start}}) \), where \( N_{\text{end}} \) and \( N_{\text{start}} \) refer to the population counts at time points \( t_{\text{end}} \) and \( t_{\text{start}} \), respectively (Weisse and Frahm 2002). Growth rates, body length, body width, and body volume were compared between treatments using one-way ANOVA in the statistical program SigmaPlot for Windows version 13 (Systat Software, London, UK). Pairwise comparisons were conducted using the Tukey post hoc test.

**Results**

Keratella population development differed significantly between the different food treatments (Fig. 1). Keratella density in the uninfected *Planktothrix* treatment showed a strong
decrease, especially after 6 d and reached very low levels by the end of the experiment (< 0.3 individuals mL$^{-1}$). In contrast, population densities of *Keratella* increased similarly in the treatments with *Chlorella* and infected *Planktothrix* (Tables 1, 2). Body length of *Keratella*, body width, and volume showed no differences between treatments (Table 1).

When *Keratella* was absent, *Chlorella* density increased strongly during the course of the experiment (Fig. 2a; Table 2). In presence of *Keratella*, *Chlorella* growth was lower ($F_{1,8} = 643.3$, $p < 0.001$). Population growth of infected *Planktothrix* was lower than that of uninfected *Planktothrix* ($F_{1,16} = 146.8$, $p < 0.001$). *Keratella* had no influence on *Planktothrix* biovolume (Fig. 2a,b; Table 2) in either of these treatments ($F_{1,16} = 0.2$, $p = 0.702$). Chytrid zoospore density increased strongly 5 d after the start of the experiment (Fig. 3a). As a result, infection prevalence of *Planktothrix* increased rapidly from about 20% to almost 70%. Overall, *Keratella* presence had no significant effect on prevalence of infection ($F_{1,8} = 1.8$, $p = 0.217$). Zoospore density remained unaffected by *Keratella* ($F_{1,8} = 5.2$, $p = 0.052$) except during the last time interval when densities were lower in the presence than in the absence of *Keratella* (Fig. 3a; Table 2).

Bacteria density showed significant differences between treatments (Fig. 4; Table 2). Generally, bacteria densities were lowest in the *Chlorella* treatment, higher in the *Planktothrix* treatment and highest in the infected *Planktothrix* treatment. Bacteria density increased in time in both the *Planktothrix* treatments. In the *Planktothrix* cultures, bacteria density was higher during infections ($F_{1,15} = 109.6$, $p < 0.001$). In the presence of *Keratella*, bacteria density was lowered in the *Chlorella* treatment ($F_{1,21} = 7$, $p = 0.015$) but not in the *Planktothrix* treatments.

### Discussion

Our results clearly demonstrate that chytrid infections supported *Keratella* population growth when the food source is

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**Table 1.** *Keratella* population growth rate ($\mu$), body length ($L$), body width ($W$), and body volume ($V$). Values denote average with standard deviation between brackets. Superscript letters represent summary output of the pairwise comparison between treatments (Tukey test, $\mu$: $F_{2,14} = 106.6$, $p < 0.001$; $L$: $F_{2,14} = 0.259$, $p = 0.776$; $W$: $F_{2,14} = 2.281$, $p = 0.145$; and $V$: $F_{2,14} = 1.636$, $p = 0.235$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\mu$ (d$^{-1}$)</th>
<th>$L$ ((\mu)m)</th>
<th>$W$ ((\mu)m)</th>
<th>$V$ (10$^3$ (\mu)m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella</em></td>
<td>0.07 (0.02)$^a$</td>
<td>107.9 (2.8)$^a$</td>
<td>61.8 (3.3)$^a$</td>
<td>325 (44)$^a$</td>
</tr>
<tr>
<td><em>Planktothrix</em></td>
<td>−0.03 (0.08)$^b$</td>
<td>109.4 (3.7)$^a$</td>
<td>65.3 (4.1)$^a$</td>
<td>369 (56)$^a$</td>
</tr>
<tr>
<td>Infected <em>Planktothrix</em></td>
<td>0.06 (0.01)$^a$</td>
<td>108.6 (3.1)$^a$</td>
<td>65.6 (1.6)$^a$</td>
<td>368 (24)$^a$</td>
</tr>
</tbody>
</table>

**Table 2.** Output of the RM ANOVA reporting the significance, degrees of freedom, and the $F$ value of treatment effect on different variables. Values in bold denote a significant effect ($\alpha < 0.05$).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Variable</th>
<th>Effect</th>
<th>$df$</th>
<th>$F$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Keratella</em></td>
<td><em>Chlorella</em> density</td>
<td>Treatment</td>
<td>1</td>
<td>643.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td><strong>Keratella</strong> density</td>
<td>Time × treatment</td>
<td>3</td>
<td>96.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td><strong>Planktothrix</strong> biovolume</td>
<td>Treatment</td>
<td>1</td>
<td>0.2</td>
<td>0.702</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>4</td>
<td>0.3</td>
<td>0.886</td>
</tr>
<tr>
<td></td>
<td><em>Bacteria</em> density</td>
<td>Treatment</td>
<td>1</td>
<td>7.0</td>
<td><strong>0.015</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>3</td>
<td>1.4</td>
<td>0.266</td>
</tr>
<tr>
<td></td>
<td><em>Zoospore</em> density</td>
<td>Treatment</td>
<td>1</td>
<td>5.2</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>3</td>
<td>7.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td><em>Prevalence of infection</em></td>
<td>Treatment</td>
<td>1</td>
<td>1.8</td>
<td>0.217</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>3</td>
<td>0.6</td>
<td>0.594</td>
</tr>
<tr>
<td><em>Food source</em></td>
<td><strong>Keratella</strong> density</td>
<td>Treatment</td>
<td>2</td>
<td>59.2</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>18</td>
<td>54.3</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td><em>Bacteria</em> density</td>
<td>Treatment</td>
<td>2</td>
<td>1305.3</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>6</td>
<td>2.8</td>
<td>0.176</td>
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<tr>
<td><em>Chytrid</em></td>
<td><strong>Planktothrix</strong> biovolume</td>
<td>Treatment</td>
<td>1</td>
<td>146.8</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>4</td>
<td>90.2</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Keratella</strong> density</td>
<td>Treatment</td>
<td>1</td>
<td>156.4</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>9</td>
<td>130.6</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Bacteria</strong> density</td>
<td>Treatment</td>
<td>1</td>
<td>109.6</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time × treatment</td>
<td>3</td>
<td>2.6</td>
<td>0.068</td>
</tr>
</tbody>
</table>
dominated by inedible filamentous cyanobacteria. In the absence of chytrid infections, *Keratella* populations that were exclusively fed with *Planktothrix* declined rapidly to very low levels (Fig. 1). In contrast, when exposed to *Planktothrix* that were infected with chytrids, *Keratella* abundances increased and net population growth was comparable to that observed for populations grown on the green alga *Chlorella*. Specifically, population growth rates of *Keratella* ranged between 0.05 d\(^{-1}\) and 0.09 d\(^{-1}\) when fed on

\[\text{Chlorella} \ (\text{a}) \ \
\text{Uninfected Planktothrix} \ (\text{b}) \ \
\text{Infected Planktothrix} \ (\text{c}) \]

**Fig. 2.** Population densities of *Chlorella* (a) and biovolume of uninfected (b) and infected *Planktothrix* (c), in treatments with and without *Keratella*. Values denote mean ± standard error (n = 5).

\[\text{Without Keratella} \ \
\text{With Keratella} \]

**Fig. 3.** Zoospore densities (a) and prevalence of infection (b) in the treatments with and without *Keratella*. Values denote mean ± standard error (n = 5).

\[\text{Chlorella} \ (\text{a}) \ \
\text{Uninfected Planktothrix} \ (\text{b}) \ \
\text{Infected Planktothrix} \ (\text{c}) \]

**Fig. 4.** Bacteria densities in treatments with *Chlorella* (a), uninfected *Planktothrix* (b) and infected *Planktothrix* (c), with and without *Keratella*. Values denote mean ± standard error (n = 5).
Chlorella, and between 0.04 d−1 and 0.07 d−1 when fed with infected Planktothrix cultures. These growth rates are largely in line with earlier reported values of 0.08–0.10 d−1 for Keratella feeding on a good quality food source (Walz 1983, 1987). Zoospore density at the end of the experiment was lower in the treatments where Keratella was present, as compared to the treatments where Keratella was absent. These results thus support our hypothesis that Keratella could directly feed on chytrid zoospores (Frenken et al. 2016), and confirm that the mycooel may transfer nutrients from large inedible algae to zooplankton (Kagami et al. 2007a). As a result, cyanobacterial blooms may not represent trophic bottlenecks, since chytrid parasites recoup primary producers to secondary consumers.

In the Planktothrix treatment, rotifer populations rapidly declined over the course of the experiment, with over 50% of the population dying within 2–5 d of exposure. This is comparable to earlier observations, where Keratella were fed with the filamentous cyanobacterium Dolichospermum flos-aquae (Gilbert and Durand 1990). Filamentous cyanobacteria generally cannot serve as food for rotifers, or at least poorly support rotifer population growth (Gilbert and Durand 1990; Rothhaupt 1991; Weithoff and Walz 1995; Fey et al. 2010). Keratella preferentially consume living algae in the size range < 10 μm, but their diets can also include detritus, bacteria, and yeast (Pourriot 1965, 1977; Lindström and Peijer 1975; Peijer 1977; Gilbert and Bogdan 1981; Stemberger 1981; Bogdan and Gilbert 1982; Walz 1983; Ronneberger 1998).

Cyanobacteria may affect higher trophic levels generally in two ways. First, they can directly affect zooplankton grazing by interfering with the feeding process or filtering apparatus (Soares et al. 2009; Panosso and Lürling 2010). Rotifers, however, are considered to be more selective in choosing the particles they ingest, e.g., the rotifer Brachionus calyciflorus can actively reject food particles at multiple stages during the feeding process (Gilbert and Starkweather 1977). Consequently, Keratella may not have fed on Planktothrix and thus suffered from starvation in the uninfected Planktothrix treatment.

Second, cyanobacteria have biochemical properties that either lack essential elements needed for zooplankton growth (Elert et al. 2003; Martin-Creuzburg et al. 2005; Wacker and Martin-Creuzburg 2012) or might result in toxic effects on the grazer (Haney 1987; Lampert 1987; De Bernardi and Giussani 1990). When Keratella was fed with a small unicellular cyanobacterium (Microcystis), survival was lower as compared to an unfed control, which could indicate possible toxic effects of cyanobacteria on Keratella (Fulton and Paerl 1987). A major difference with our study, however, is that Planktothrix is a large and filamentous cyanobacterium which is likely unsuitable for ingestion by rotifers, since Keratella seems to prefer food particles with a diameter < 10 μm (Ronneberger 1998). Furthermore, in our cultures, no negative effect of Keratella grazing on Planktothrix biomass build-up is apparent.

An earlier study showed that the intracellular microcystin content is lower in Planktothrix cultures exposed to chytrids as compared to unexposed treatments, which might indicate microcystin leakage into the water column (Frenken et al. 2017b). Negative effects of microcystin on zooplankton growth, however, are not unambiguous. Despite some reports on negative effects (Fulton and Paerl 1987; Rohrlack et al. 1999; Zurawell et al. 2005), other studies reported lack of adverse effects of microcystins on a range of zooplankton species, including rotifers (Porter and Orcutt 1980; Nandini and Rao 1997; Soares et al. 2010). Furthermore, Keratella were able to grow in the infected cultures where also uninfected Planktothrix filaments were present. Thus, toxic effects of Planktothrix on Keratella in our experiment are very unlikely.

Most probably, Keratella in our experiments fed on a diet of zoospores released from the infections, and bacteria growing on organic cell debris and lysates (Cole 1982; Middelboe et al. 1996; Gobler et al. 1998). Earlier work showed that bacteria can be of significant importance as a source of phosphorus to bacterivore zooplankton species (Hessen and Andersen 1990). The presence of Keratella in our experiment, however, had no observable effect on the number of bacteria in the Planktothrix treatment. Therefore, it is unclear to which extent Keratella grazed on bacteria. However, since the rotifers were unable to survive in the uninfected Planktothrix cultures, where the amount of bacteria was relatively high (5.6 × 106 cells mL−1), it seems unlikely that bacteria served as the sole food source for Keratella. This is in line to findings by Agha et al. (2016), which show that for cladocerans, survival, and reproduction are higher in cultures with both chytrids and bacteria present as compared to cultures with bacteria alone. This might be explained by the lack of essential sterols in bacteria, and thus their low nutritional quality to zooplankton (Martin-Creuzburg et al. 2011; Freese and Martin-Creuzburg 2013). In cultures where also chytrids were present, the zooplankton diet might have been complemented by chytrid zoospores, which are rich in polyunsaturated fatty acids and sterols, and thus provide a high quality food source supporting zooplankton growth and reproduction via the mycooel (Kagami et al. 2007a,b).

Chytrid zoospores are indeed presumed to be a high-quality food source for daphnids and copepods, in terms of size and shape, as well as their nutritional composition (Kagami et al. 2007b, 2011, 2017). Moreover, it has been demonstrated that zooplankton can lower the density of zoospores detected in the water (Searle et al. 2013; Schmeller et al. 2014). As hypothesized before (Frenken et al. 2016), chytrid zoospores may serve as an alternative food source to Keratella as well. With this experiment, we not only show that Keratella abundances increased in the infected treatments resulting in an increased grazing pressure, but also that zoospore concentrations were lower at the end of the experiment. Zooplankton grazing on zoospores may also feedback on the infections, and consequently on new zoospore production. We did not observe a

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decrease in prevalence of infection. Yet, this may have occurred if the experiment would have lasted longer. Moreover, we note that our experiments were performed in small chambers without mixing, which may have affected some aspects of the observed dynamics. Future studies under more natural conditions are required to corroborate the relevance of the mycello, and quantify trophic transfer of nutrients by for instance stable isotope probing (Frenken et al. 2017a).

Until now, it was shown that grazing on zoospores can enhance body length of cladocerans (Kagami et al. 2007b) and can sustain a higher percentage of surviving copepods (Kagami et al. 2011). Also, it has been shown that multiple *Daphnia* genotypes can survive and reproduce on a diet of chytrid zoospores and heterotrophic bacteria (Agha et al. 2016). With our study, we provide first evidence that chytrids can support population growth of rotifers, a more selectively feeding group of zooplankton. In other words, our results demonstrate that trophic links between primary producers and secondary consumers that are limited during presence of large inedible cyanobacteria can be re-established by fungal parasites. Our results thus highlight the important function of diseases and the role they can play in food webs. Not only can pathogens strongly regulate their host populations, they can also facilitate growth and reproduction of other trophic levels.

**References**


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Conflict of Interest

None declared.