



# Isolation and Characterization of Rhizophydiales (Chytridiomycota), Obligate Parasites of *Planktothrix agardhii* in a Laurentian Great Lakes Embayment

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**ABSTRACT** *Planktothrix agardhii* dominates the cyanobacterial harmful algal bloom community in Sandusky Bay, Lake Erie (USA), from May through September. This filamentous cyanobacterium is host to a known obligate parasite, the chytrid *Rhizophyidium* sp. During the 2018 bloom season, by utilizing dilution and single-filament isolation techniques, 7 chytrid and 12 *P. agardhii* strains were isolated from Sandusky Bay. These 7 chytrids and a selection of *P. agardhii* hosts were then characterized with respect to infection rates. Infections by the isolated chytrids were specific to *Planktothrix* planktonic species and were not found on other filamentous cyanobacterial taxa present in the bay (*Aphanizomenon* sp. and *Cuspidothrix* sp.). Even among the potential *P. agardhii* host strains, individual chytrid isolates had different degrees of infectivity and showed preferences for different host isolates, suggesting possible ecological partitioning even within the same sample population. When mechanisms of chytrid pathogenesis were examined, the zoospores displayed a swarming pattern to attack and fracture the host filament and create new infection sites at the trichome termini. Infections by these parasitic chytrids also led to a release of intracellular microcystin toxins from the hosts. Additionally, infections were dependent on medium type, highlighting the importance of medium choice for experimental outcomes. Media in which chytrid swarming was observed closely matched the ionic strength of the natural environment. Understanding pathogenesis by fungal parasites will assist future efforts to determine environmental factors favoring loss mechanisms for *Planktothrix agardhii*-dominated blooms.

**IMPORTANCE** Whereas many studies have focused on the factors contributing to the establishment and persistence of cyanobacterial harmful algal blooms (cHABs), few studies have examined bloom pathogenesis. Chytrid fungi infect cyanobacteria and stimulate food web interactions through manipulation of previously hard-to-digest filaments and the release of nutrients to support heterotrophic microbes. Specifically, chytrids infective for filamentous *Planktothrix agardhii* exhibit a species-specific infection that fragments trichomes into shorter units that can be consumed more easily by grazers. Chytrid zoospores also serve as a high-quality food source for the lower food web. Understanding host-pathogen relationships and mechanisms of pathogenesis on cyanobacteria will be necessary to effectively model the ecology of cHABs.

**KEYWORDS** chytrid, cyanobacteria, harmful algal blooms

Sandusky Bay, Lake Erie (Laurentian Great Lakes, USA), is a 165-km<sup>2</sup> hypereutrophic embayment (1) that is subject to annual toxic cyanobacterial harmful algal blooms (cHABs). Unlike the annual *Microcystis*-dominated cHABs found in the open waters of western Lake Erie, Sandusky Bay is dominated by *Planktothrix agardhii* (2–4). While both species are common in this region and have the capability to produce microcystins,

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*P. agardhii* can initiate bloom formation earlier due to its tolerance to a wider temperature range than *Microcystis* (5–7). Due to this trait, and its enhanced capacity to tolerate nitrogen limitation arising from seasonal denitrification, blooms dominated by *P. agardhii* in this region start earlier and persist longer than the open-water *Microcystis*-dominated blooms, which make them an ongoing concern to lake management (3, 4, 8).

*Planktothrix* species are known to be host to a variety of pathogens, including the fungal parasites known as chytrids (Chytridiomycota) (9–12). Thus far, only strictly parasitic chytrids have been isolated from *Planktothrix rubescens* (10–12), and most of the current research has focused on understanding the role of chytrid parasites on the ecological food web. Parasitic chytrid infection in filamentous cyanobacteria such as *Planktothrix* can increase the edibility of low-quality food sources (like cyanobacteria). Chytrid infections can fragment the host, making them smaller and easier to consume and digest (13, 14). Infections can also promote the release of cellular material (organic carbon and nitrogen), which can act as alternative food sources for heterotrophic bacteria (13, 15). Additionally, the zoospores themselves can act as a food source for grazers (13, 14, 16, 17). Since there is evidence that suggests that chytrids are a significant link in the food web, we aimed to isolate chytrids from *P. agardhii*-dominated blooms in Sandusky Bay.

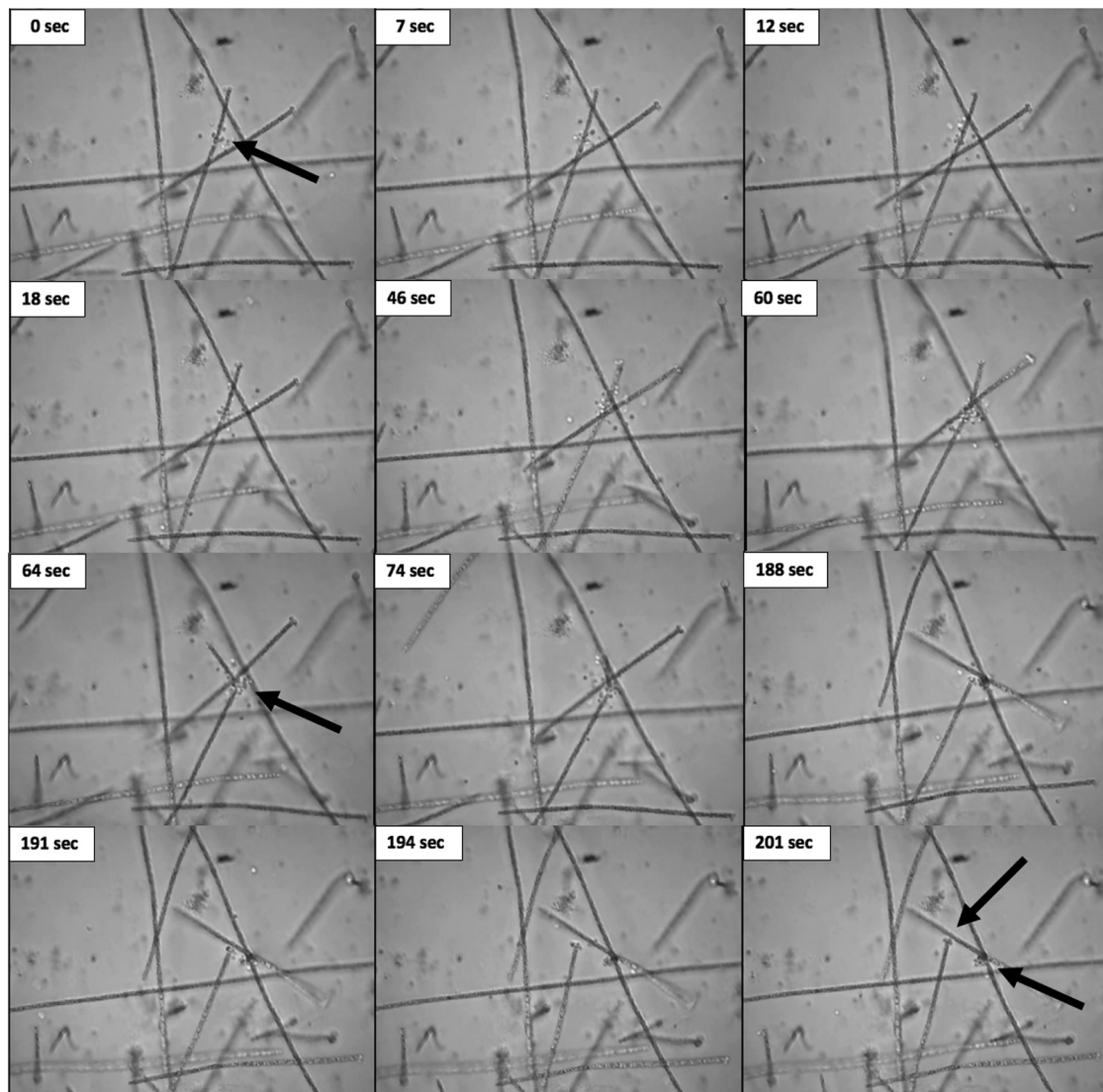
This work outlines our success in isolating multiple potential host strains and chytrid strains from *Planktothrix*-dominated blooms in Sandusky Bay, which is, to our knowledge, the first instance of multiple hosts and multiple chytrid pathogens isolated from the same source at the same time. We then characterized infection mechanisms and rates across multiple host strains to determine various fitness levels among the chytrid isolates. Finally, we tested the effects of infection on the release of microcystin toxins to determine if chytrid infections could be implicated in spikes of dissolved toxins released during the bloom season.

## RESULTS

**Chytrid infection characteristics.** All chytrid isolates obtained from 2018 Sandusky Bay (Ohio, USA) sampling surveys (see Materials and Methods for details) had morphologies, infection patterns, and internal transcribed spacer (ITS) sequence identical to those of chytrid isolate Chy-Kol2008 (10), which makes it likely that these Sandusky Bay isolates are *Rhizophydium megarrhizum*, as described by Canter and Lund (9). Zoospores encyst only on the termini of *Planktothrix* sp. trichomes and mature into sporangia at the same locations. Frequently, no more than two mature sporangia were located on the same apex, and trichomes could have active infections at either apex (see Fig. S1 in the supplemental material). Infected filaments sank in stationary-phase cultures and could be seen to decay over time until only the cell walls remained visible by microscopy. Free-swimming zoospores were spherical with a single posterior flagellum. Infections did not occur in all of the filaments in each culture (infection rates depended on the susceptibility of the host and culture conditions, as described below), but infections were lethal for those that were infected. No resting states for the chytrids were observed in culture.

During infection, zoospores were prevalent enough that attachment events could be captured; these involved zoospores swarming around trichome termini until one or more attached while the rest dispersed. This swarming event also occurred at specific sites along the *Planktothrix* trichome (Fig. 1; Movie S1). These regions were attacked by a zoospore swarm until the filament was cleaved, creating two new apex locations for attachment and maturation of the chytrid zoospores. The attack and cleavage of the filament routinely occurred in less than 10 min (Movie S1). Due to the fragmentation of filaments during infection, resistant host filaments were longer, and some strains (e.g., 1026) formed aggregates where elongated filaments wrapped around each other (Fig. S2; Movie S2).

**Host and medium specificity.** The Sandusky Bay chytrid isolates are species specific, infecting only *Planktothrix agardhii* and *Planktothrix rubescens* cultures (Table 1). The chytrids were not infective on the other two *Planktothrix* cultures tested:



**FIG 1** Time series of a chytrid swarm on a *Planktothrix agardhii* filament (screen captures from Movie S1). The swarm homes in on a specific point along the filament; after ca. 10 min, the filament breaks at that site, and both new free ends of the filament serve as an attachment point for some of the swarm. The swarm disperses to find another host.

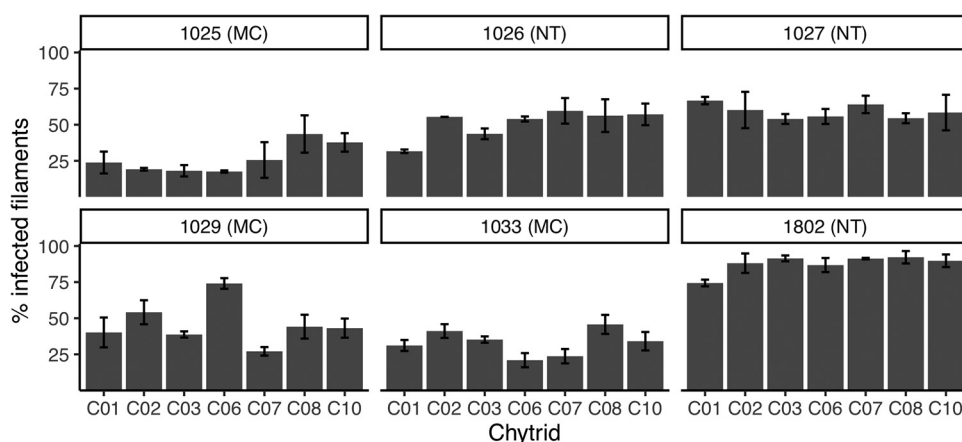
*Planktothrix sarta* PCC 8927 and *Planktothrix paucivesiculata* PCC 8926, which are benthic *Planktothrix* isolates from Berre Pond, France, and Marne River, France, respectively (18). Additionally, the chytrids did not attach to a series of *Aphanizomenon* isolates from Sandusky Bay, a *Cuspidothrix* isolate from Sandusky Bay, or *Microcystis aeruginosa* LE3, a Lake Erie isolate (19). The chytrid isolates have not yet been tested on *P. agardhii* isolates from locations other than Sandusky Bay, but the *P. rubescens* isolates were isolated from lakes in Europe (*P. rubescens* 72 from Zürichsee, Switzerland, *P. rubescens* 34 from Ammersee, Germany, and *P. rubescens* 21/1 from Figur, a pond near Vienna, Austria). Due to the lack of polymorphisms between *P. agardhii* and *P. rubescens* 16S rRNA genes and other sites and to the ability for recombination between the species, it is believed that *P. agardhii* and *P. rubescens* are conspecific (20).

Even though the chytrid isolates are all likely the same species based on PCR targeting of the ITS sequence, they infected the different hosts at different rates and prevalences (Fig. 2). Infections were terminated after 7 days, which seemed to correlate to peak infectivity under the chytrid propagation conditions, and while the chosen

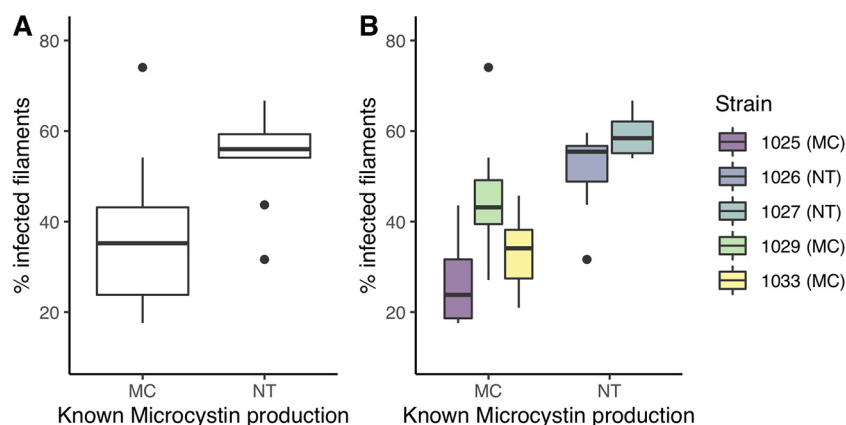
**TABLE 1** Tested cyanobacteria cultures and their susceptibility to chytrid infections

Phylum	Order	Genus	Species	Strain designation	Host to all 7 chytrids	Origin region			
Cyanobacteria	Oscillatoriales	Planktothrix	P. agardhii	1025	+	Sandusky Bay, Lake Erie			
				1026	+	Sandusky Bay			
				1027	+	Sandusky Bay			
				1029	+	Sandusky Bay			
				1030	+	Sandusky Bay			
				1031	+	Sandusky Bay			
				1032	+	Sandusky Bay			
				1033	+	Sandusky Bay			
				1801	+	Sandusky Bay			
				1802	+	Sandusky Bay			
				1803	+	Sandusky Bay			
				1804	+	Sandusky Bay			
				1805	+	Sandusky Bay			
				1806	+	Sandusky Bay			
				1807	+	Sandusky Bay			
				1808	+	Sandusky Bay			
				1809	+	Sandusky Bay			
				1810	+	Sandusky Bay			
				1811	+	Sandusky Bay			
				1812	+	Sandusky Bay			
				Nostocales	Aphanizomenon	P. rubescens	72	+	Zürichsee, Switzerland
							34	+	Ammersee, Germany
							21/1	+	Figur, Austria
	P. sarta	PCC8927	—				Berre Pond, France		
	P. paucivesiculata	PCC8926	—				Marne River, France		
	Aphanizomenon sp.	SB02	—				Sandusky Bay		
		SB04	—				Sandusky Bay		
		SB06	—	Sandusky Bay					
		SB07	—	Sandusky Bay					
		SB10	—	Sandusky Bay					
	Chroococcales	Cuspidothrix	Cuspidothrix sp.	SB09	—	Sandusky Bay			
		Microcystis	M. aeruginosa	LE3	—	Lake Erie			

isolates had minor differences in growth rates (Fig. S3), the total number of filaments in each treatment was not significantly different ( $P > 0.05$ ) (Fig. S4). *Planktothrix agardhii* 1025 was the least susceptible strain tested (Fig. 2), with most of the chytrid isolates averaging less than 25% infected filaments (chytrid isolates C01, C02, C03, C06, and C07) and reaching maximum infectivity with C08 at  $43.5 \pm 12.9\%$  infected filaments. *P. agardhii* 1802 was the most susceptible host strain, with an average filament infection rate of  $87.7 \pm 6.2\%$  (Fig. 2). There was also variability in chytrid host preference; C06



**FIG 2** Percentage of infected *P. agardhii* filaments in culture after 7 days of infection with each chytrid isolate. MC, cultures known to produce microcystins; NT, nontoxic cultures (those that do not produce microcystins).



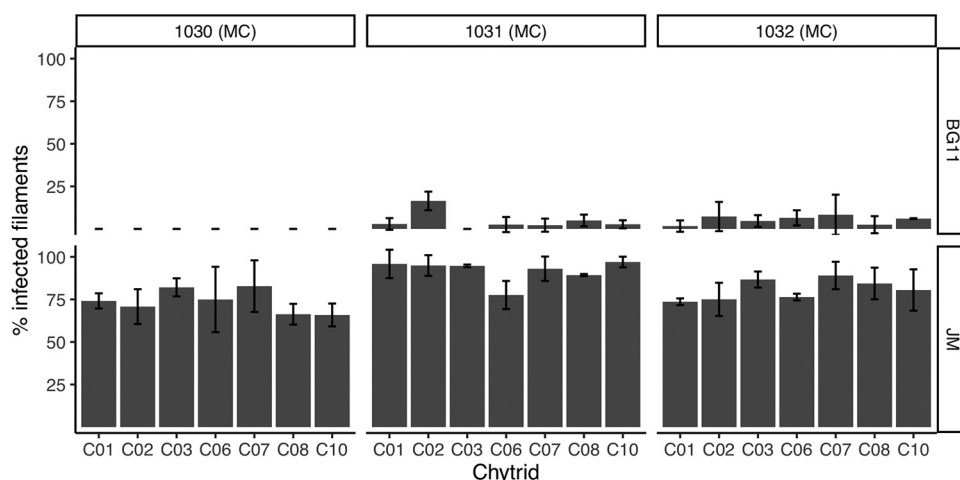
**FIG 3** Box plot analysis of the number of infected *P. agardhii* filaments after 7 days of infection separated based on the cultures' ability to produce microcystins (MC) or not (NT [nontoxic]). (A) Toxin producers and non-toxin producers combined for an average effect. (B) Cultures separated to show outlier cultures and individual effects.

was the most infective chytrid isolate on *P. agardhii* 1029 ( $74.0 \pm 3.7\%$ ) and the least infective isolate on both *P. agardhii* 1025 ( $17.6 \pm 0.8\%$ ) and 1033 ( $21.0 \pm 4.9\%$ ).

There was a difference in infection rates between *Planktothrix* hosts that can produce microcystins and those that cannot (Fig. 3), although currently there is no evidence to suggest that microcystins are antifungal compounds. The three cultures known to produce some microcystin congeners all had a lower average percent infection after 7 days than those with no detectable microcystin production (Fig. 3) ( $P < 0.001$ ). The combined percent infection by microcystin producers averaged around 35%, whereas infection by nonproducers averaged around 65% (Fig. 3A). When the strains are analyzed individually (Fig. 3B), the infection percentage average among the microcystin producers is increased by an outlier strain, 1029 (due to infection by C06 [Fig. 2]), and the nonproducers' average is lowered by outlier strain 1026 (due to infection by C01 [Fig. 2]). These outliers highlight the variability in host susceptibility to each specific chytrid isolate, while the overall trend tends toward separation in infection rates between microcystin producers and nonproducers. Finally, there is a large difference between the infection rate of SB1802 and those of all the other tested strains, which may reflect a specificity for host isolates from the same year as the chytrid isolates.

Infection rates depend on the type of medium in which the host is growing (Fig. 4). Infections in BG-11 medium were significantly lower ( $P < 0.001$ ) than those using the same host/chytrid combination in Jaworski's medium (JM); in BG-11, the average infection rate was 3.2%, barely above the inoculation concentration, and in JM, the average infection rate was 82.1%, which shows that medium composition is an important consideration when analyzing host pathogen responses in culture. BG-11 contains more nitrogen and more sodium (which accompanies the extra nitrogen) than JM, as well as a few trace metals (Table S1).

**Fate of microcystins during infection.** Because infections are lethal for the host tri-chome, we followed the release of toxins during chytrid infections on microcystin-producing strains of *P. agardhii*. *Planktothrix agardhii* 1031 produces two demethylated congeners of microcystin and was susceptible to the most robust chytrid isolate, C02. On day 0, dissolved (extracellular) toxin concentrations measured  $0.85 \mu\text{g liter}^{-1}$  in the uninfected culture and  $3.4 \mu\text{g liter}^{-1}$  in the infected culture, a difference likely attributable to inoculation with chytrid-infected culture (day 7 culture at a 1:10 [vol/vol] dilution). Within 4 days after inoculation with the chytrid, dissolved toxin concentrations reached  $20 \mu\text{g liter}^{-1}$ , and within 9 days, they reached  $30 \mu\text{g liter}^{-1}$  (Fig. 5A). This release in toxin levels corresponds to a high prevalence of chytrid infection (Fig. 5B). Indeed, infections of *Planktothrix agardhii* 1031 started to reach late log phase on days 9 and 11, which corresponds to a similar trend in dissolved microcystin concentrations



**FIG 4** Chytrid infections in two freshwater media: BG-11 and Jaworski's medium (JM). Medium composition for each can be found in Table S1.

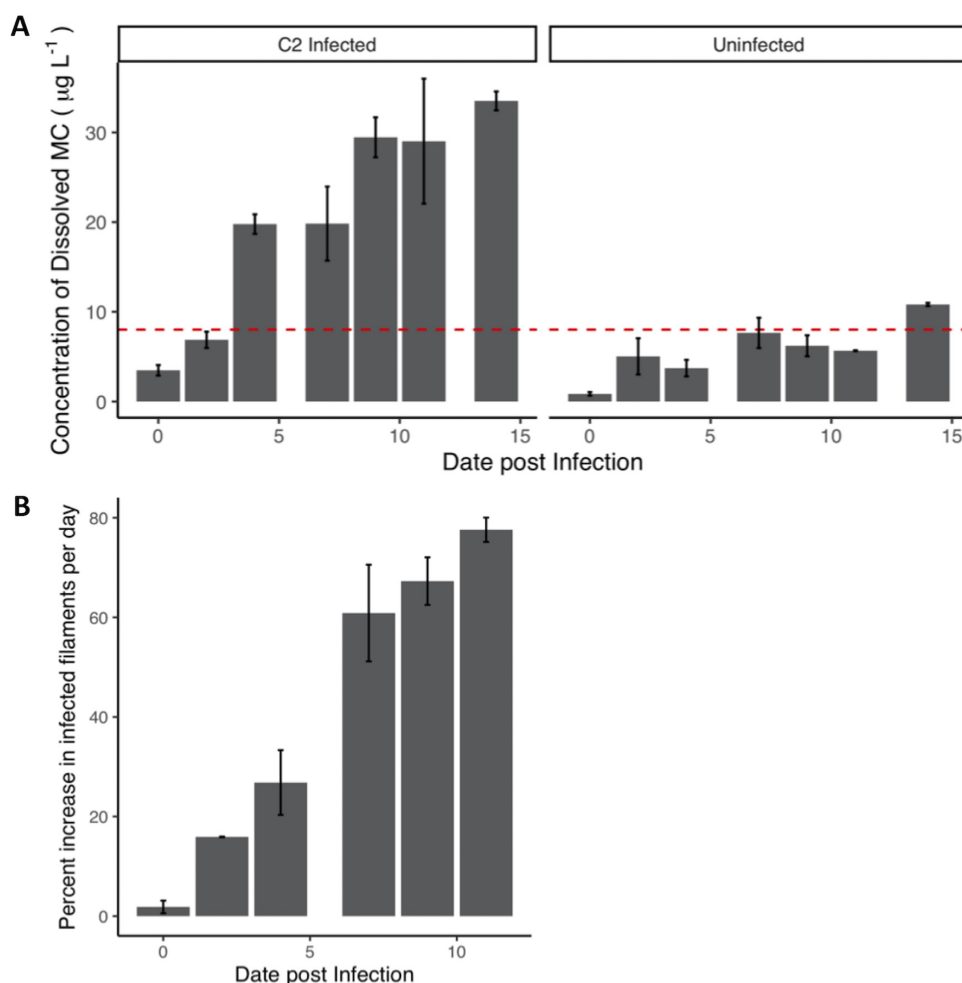
(at approximately  $30 \mu\text{g liter}^{-1}$ ). In comparison, an uninfected culture yielded a maximum dissolved microcystin concentration of  $10 \mu\text{g liter}^{-1}$  after 2 weeks. Under lab conditions, infected cultures can release up to 4 times as much microcystins as uninfected cultures.

**Zoospore persistence.** To determine how long zoospores can survive without a host, we separated the zoospores from an infected culture and inoculated them at 24-h intervals over 2 days (Fig. 6). Zoospores by themselves lost viability with a half-life of 16.6 h. Two different chytrid isolates (C2 and C10) with different starting densities decayed at about the same rate, which was a loss of  $18.4$  infective zoospores  $\text{h}^{-1}$  ( $r^2 = 0.8988$ ;  $P < 0.005$ ).

**Host extract effects on infectivity of chytrids.** Since the types of compounds that promote or inhibit chytrid infections are unknown, strains were tested to identify isolates that may inhibit infections as a potential source of antifungal compounds (Fig. 7). Without the addition of any crude lysate, or with the addition of the crude lysate of the same host culture (*P. agardhii* SB1802, a non-microcystin producer), the percentage of filaments infected was 87.5%. Strain extracts that yielded a 25 to 50% reduction in infected SB1802 filaments (between 65.6 and 43.8% total infected filaments) were those from *P. agardhii* 1027 and *P. agardhii* 1033, and strain extracts that yielded a  $>50\%$  reduction in infected SB1802 filaments were those from *P. agardhii* SB1811 and *P. sarta* 8927.

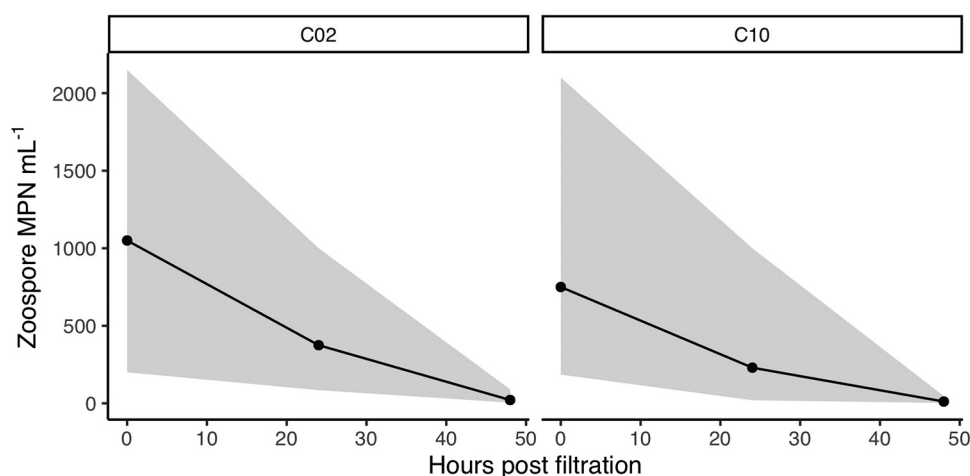
## DISCUSSION

This work outlines the isolation and characterization of seven new Chytridiomycota isolates of the genus *Rhizophydium* from Sandusky Bay, Lake Erie, with the ability to infect multiple host isolates of both *Planktothrix agardhii* and *Planktothrix rubescens*. *P. rubescens* and *P. agardhii* are similar enough to both be hosts (even though *P. rubescens* is not found in Sandusky Bay), but other *Planktothrix* species are not (Table 1). Recent work by Pancrace et al. (18) on the genomics of the genus *Planktothrix* found that planktonic strains belong to a single species, whereas benthic and biphasic strains are split into four different species. Indeed, they consider that red and green *Planktothrix* strains should be considered different ecotypes, occupying different niches, which is supported by other work (20–22). Since early studies on chytrid-cyanobacterium interactions suggested that chytrid fungi have narrow host ranges (23), infection of both *P. agardhii* and the *P. rubescens* strains provides further evidence of their close phylogenetic affinity (as shown by Sønstebo and Rohrlack [10] and Kurmayer et al. [24]). Further experimentation on both *P. agardhii* and *P. rubescens* could lead to better understanding on local adaptation (or lack thereof) to these chytrids depending on the geographical origins of the host.



**FIG 5** Effect of chytrid infection on toxin-producing strains of *Planktothrix agardhii*. (A) Release of toxins due to chytrid infection in a susceptible microcystin-producing host strain. The red dashed line represents the U.S. EPA maximum concentration for recreational waters ( $8 \mu\text{g liter}^{-1}$ ). (B) Chytrid infection prevalence as a function of percent infected filaments to relate to toxin release.

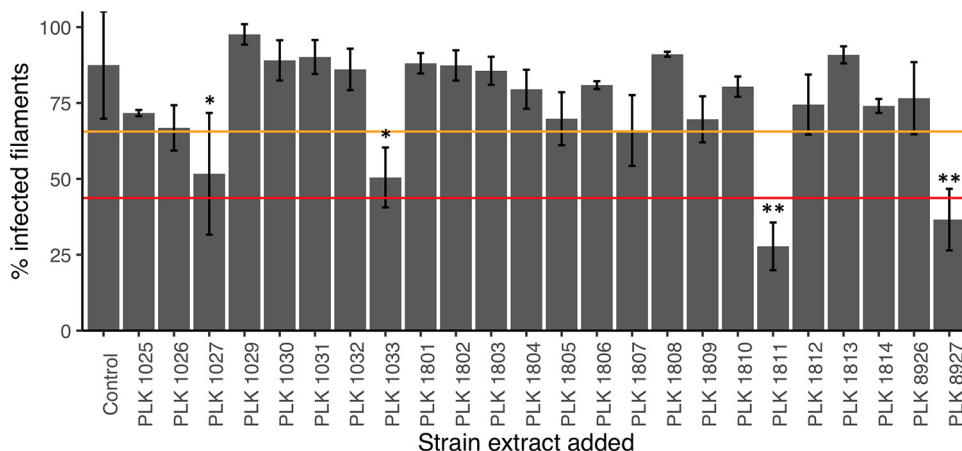
The chytrid isolates described here display different infection rates and show a preference for different host isolates, suggesting possible ecological partitioning even within the same sample population (Fig. 2). The differences in rates of infection likely reflect differences in physiological traits, such as zoospore production and release, or host specificity. The coexistence of distinct chemotypes of *Planktothrix* sp. in lakes in Scandinavia suggests that the chytrids isolated from these regions are one potential driving force behind the division of *Planktothrix* populations (10). Selective parasitism on one strain or species will favor the development of other strains and species (25). Indeed, sediment cores showed that there was a correlation between changes in host chemotype and presence of chytrid parasites but that the chytrid-driven diversity was not maintained over time due to other environmental factors (12). Temperature variation is one such environmental factor, as the temperature range of infection can be narrower than the temperature at which the host can survive, creating ecological safe zones (26). Studies using chytrid species closely related to the ones identified here show that some lakes may allow the presence of a cold thermal refuge, where the host is able to survive but chytrid infections are difficult to maintain (11). Further, chytrids have the ability to adapt to novel hosts rapidly but can be thwarted by cyanobacterial diversity, suggesting that some resistance to chytrid infection on the host is possible in diverse populations (27). Here, we describe a diverse population of hosts and a diverse population of chytrid parasites, suggesting a constant pressure on both organisms to



**FIG 6** Zoospore persistence without an available host under laboratory conditions, calculated as most probable number (MPN) per milliliter. The shaded regions represent 95% confidence intervals for each point.

adapt and diversify. Additional studies could also explore mechanisms of resistance that may arise due to host filament length, zoospore exclusion by host filament aggregation (Fig. S2; Movie S2), and other host phenotypic traits.

Among the limited number of *Planktothrix agardhii* isolates that were tested, there was a difference in rates of infection between isolates known to produce at least one congener of microcystin and isolates known to produce no congeners (Fig. 2 and 3). While our data suggest that strains that can produce microcystins are less susceptible to infection, Sønstebo and Rohrlack (10) found that there was a positive correlation between the severity of infections and the production of certain oligopeptides, including microcystins, but they studied only one strain that did not produce microcystins (Cht7e). Indeed, a later study using one of the same chytrids from the 2011 study showed that the chytrid was most virulent on the wild-type host compared to knock-out mutants incapable of producing desmethyl microcystin LR and desmethyl microcystin RR (28). Even if there is a correlation between the production of microcystins and lower rates of chytrid infections, there is still the possibility that the lower rates of infection are due to the production of other oligopeptides or potential antifungal compounds. When the crude lysate of host isolates was tested for antifungal compounds,



**FIG 7** Effect of crude host extract on the prevalence of chytrid infections on a highly susceptible host strain, *Planktothrix agardhii* SB1802. Strain designations show the hosts from which the lysate was taken; the control had no lysate added. The lysates from *P. agardhii* 1027 and *P. agardhii* 1033 showed a 25 to 50% reduction in infected filaments (orange line), and strains that exhibited a >50% reduction in infected filaments (red line) were *P. agardhii* SB1811 and *P. sarta* 8927. \*\*,  $P < 0.001$ ; \*,  $P < 0.05$ .

the strains that exhibited a >50% reduction in infected filaments were *P. agardhii* SB1811 and *P. sarta* 8927 (Fig. 7). *P. agardhii* SB1811 does not produce microcystins (Table S2), whereas *P. sarta* 8927 was previously shown to possess a novel antifungal biosynthetic gene cluster for hassallidin E (18). Whole-genome sequencing of our *P. agardhii* isolates should aid in the discovery of other novel antifungal compounds or allow a better understanding of some oligopeptide functions in *P. agardhii*.

A novel finding in this study is the characterization of the infection mechanism, in which zoospores swarm to fragment the long filaments to yield new foci for pathogenesis at the filament termini. Considering the swarming behavior exhibited by zoospores, it is likely that they find their host through chemotaxis (Fig. 1; Movie S1). It has been hypothesized that zoospores respond to both chemo- and phototactic cues (29, 30), where phototaxis directs zoospores to the photic zone occupied by their hosts and chemotaxis directs them to the infection site. Recently, whole-cell extracts and mixtures of carbohydrates (polyunsaturated fatty acids, phenols, and aldehydes) were shown to attract chytrid zoospores infective for marine diatoms (31). It is unclear what the cue is for the chytrid zoospores in this study, as we are not certain if the swarm attacks host cells that are already damaged (and releasing intracellular compounds) or if they are attracted to compounds excreted by the chytrids themselves to signal to the others to join the swarm. Additionally, we should consider the possibility that the zoospores are attracted to regions along the filament that are undergoing active division. The swarm may respond to the leaking of cellular contents at a point of filament division, thus triggering their chemotactic response. Further experiments are needed to determine the chemical cue behind the swarm behavior and the response by zoospores responsible for fragmenting trichomes.

While there are some questions regarding the swarming cues, fragmentation of host filaments (Fig. 1; Movie S1) play an essential role in aquatic ecosystem function, likely providing a more edible food source for zooplankton. The zoospores themselves are edible, possibly serving as an important food source and providing essential lipids when CHABs are dominant and zooplankton grazing rates are reduced (32). Indeed, zoospores have been shown to be capable of sustaining populations of grazers such as *Daphnia* (16) and *Keratella* (33), even to the point where grazing was not sufficient to limit chytrid infections in the host cyanobacteria (34). Lysis by chytrids may also introduce previously inaccessible nutrients from cyanobacterial hosts and, through the swarming mechanism reported here, may fragment filaments into smaller and more readily consumable pieces, which can be utilized by grazers (14). Together, these processes form the mycoloop, a food web pathway in which fungal infections facilitate a transfer of nutrients between trophic levels ("trophic transfer") utilizing large and/or otherwise inedible phytoplankton (35).

The results observed here were obtained by monitoring infections of the same chytrid-host pair in two different growth media (Fig. 3). Previous studies discussing the isolation of and experimentation with *Planktothrix*-specific chytrids utilized two freshwater media: Z8 (10) and BG-11 (11, 28). Since the conductivity of Jaworski's medium (JM) more closely matches the ionic strength of Sandusky Bay, it was tested against BG-11 (Table 2; Table S1). The greatest difference between the two media is the amount of NaNO<sub>3</sub> added, which is nearly 19 times greater in BG-11 than in JM. While altering the nitrogen-to-phosphorus ratio between the two media, it also greatly increases the ionic strength in BG-11 compared to JM. Since both nitrogen and phosphorus are replete nutrients in both media, the stoichiometric ratio of nitrogen to phosphorus can alter cellular processes, such as phosphorus uptake (36), and production of certain secondary metabolites, like microcystin toxins (37, 38). A greater concern is the higher ionic strength in BG-11, as deviations from the salinity of the chytrids local environment is known to inhibit growth and infectivity of chytrids (31, 39, 40). It is possible that the differences in osmotic gradient from the original culture to the inoculation in BG-11 initially caused osmotic shock to the zoospores and disrupted the chemical signals used by zoospores released later from the already-attached sporangia.

**TABLE 2** Sampling information in Sandusky Bay, Lake Erie<sup>a</sup>

Site	Location (latitude, longitude [°])	Water temp (°C)	Conductivity (μS/cm)	pH
ODNR4	41.453333, -82.960767	21.9	448	9.31
ODNR6	41.457300, -82.898655	21.5	431	9.32
Edison Bridge	41.480156, -82.834328	21.7	414	9.37
ODNR2	41.479817, -82.782867	21.5	413	9.4
ODNR1	41.477367, -82.739783	20.5	342	9.24
EC1163	41.469000, -82.715000	19.2	274	8.65

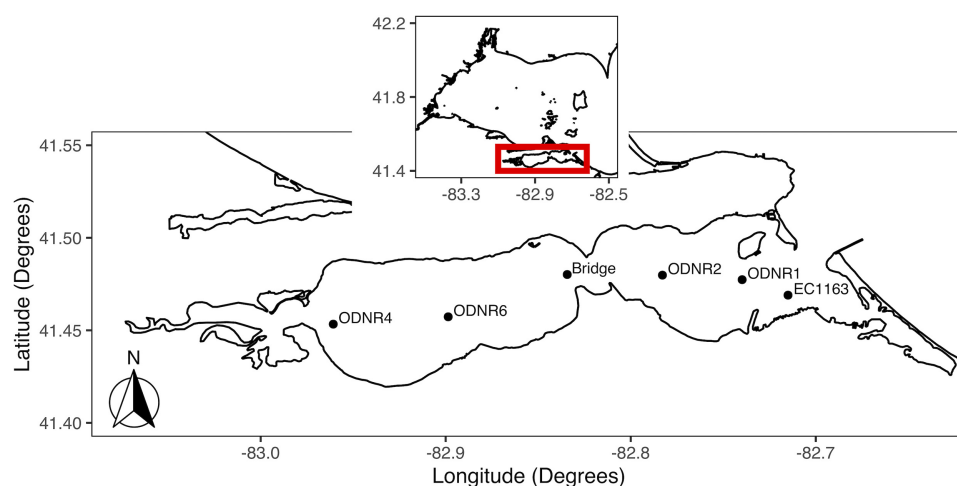
<sup>a</sup>All samples were taken at a depth of 1 m on 5 June 2018.

Further work is needed to determine if the differences in infection rates between the two media are due to host uptake and production of different chemicals or due to disruption of zoospore signaling.

While it was already known that chytrids can infect toxin-producing hosts (10), this study quantifies toxin release due to infection in a lab setting. It is worth noting that chytrid infection rates are likely to be much lower in the wild than in the lab, and these experiments are meant to show the ability of chytrids to serve as a mediator for toxin release. Lysis events involving the release of intracellular toxins from blooms carry implications for water treatment utilities and the safety of potable water for livestock and pets, a phenomenon already documented to occur as a consequence of viral attack (41, 42). Chytrid-mediated toxin release may also play a role in *Dolichospermum* (*Anabaena*)-dominated blooms, as some of these species are known to be hosts to chytrid species (43–45). *Dolichospermum*-dominated blooms have been linked to poisoning events that included animal deaths after drinking from a reservoir during a bloom (46) and have been found in other lakes around water treatment plant intakes (47). The host of our specific chytrids, *Planktothrix agardhii*, is known to form perennial blooms in a number of lakes used for recreation in Europe (48–50) and the United States (3), where infections can lead to a release of dissolved toxins, which could challenge operations at water treatment facilities.

Under lab conditions, where infection rates are optimized and many inhibitory interactions, such as predation and nutrient limitation, are controlled, zoospores do not retain their infectivity for long (Fig. 5). Under such favorable conditions, the zoospore life span is very short, raising the question of their ability to survive in the wild. Perhaps what we observed are the conditions in which chytrids produce plentiful and small zoospores that exhaust their nutrient reserves quickly, given that hosts are frequent and abundant. The opposite of that would then be that under the stress of non-optimal conditions, chytrids form few and large zoospores that preserve their nutrient reserves. Under phosphorus-limited conditions, chytrids infective on *Asterionella* produce fewer zoospores that take longer to develop (51, 52), which may result in zoospores better adapted to persisting for longer before finding a viable host. Indeed, chytrids infecting *Planktothrix rubescens* were found to contain more carbon, nitrogen, and phosphorus under nutrient-limited conditions (53), allowing them to persist without a host longer. Further, zoospores were found to be smaller when grown at higher temperatures, indicating a relationship between reproductive output and the ability of the zoospores to survive that changes under various environmental conditions (54). In the wild, zoospores are likely not as plentiful and may survive longer than the isolates observed here under lab conditions. Unfortunately, at this time, no resting spores were formed in culture conditions, leaving how the chytrids survive overwintering in Sandusky Bay as another point for further research.

In summary, we isolated several related chytrids from the same location, displaying a diversity in host preference and infectivity which promotes a better understanding of how fungal pathogens can assert selective pressure on the *Planktothrix agardhii* community found in Sandusky Bay. Due to their mechanism of infection, involving breakage of large filaments into smaller, more easily consumable fragments, these fungal pathogens likely play an important role in the aquatic food web of this region, as



**FIG 8** Map of sampling locations in Sandusky Bay. The inset shows the location of Sandusky Bay in relation to the western basin of Lake Erie.

severe *Planktothrix*-dominated cyanobacterial blooms recur annually. More work is needed in establishing the genetic profiles of both host and pathogen, which will help fill some gaps in our understanding of how these relationships coevolve in the environment.

## MATERIALS AND METHODS

**Sampling sites and water processing.** At the southeast region of the western Lake Erie is the shallow (mean depth, 2.6 m), hourglass-shaped Sandusky Bay (Fig. 8), which is divided into an outer bay (eastern half) and an inner bay (western half) (Fig. 8). In conjunction with the Ohio Department of Natural Resources biweekly water quality surveys of Sandusky Bay, six sampling sites were chosen as part of this work; ODNR4, ODNR6, Edison Bridge, ODNR2, ODNR1, and EC1163 (Table 2) (55). At each site, in addition to other parameters collected, 1 liter of water from a 1-m depth was collected using a Van Dorn sampler and stored in a cooler with ice for transport to the laboratory. Once in the laboratory, samples were stored for up to 24 h at 4°C. Samples were processed by concentrating 250 ml of raw water to 1.5 ml using a Whatman 4 filter (GE Healthcare Bio-Sciences, Pittsburgh, PA). A portion of the concentrate was added to Jaworski's medium (JM) (Table S1) and monitored for visible chytrid infections or serially diluted to obtain single-filament *Planktothrix* isolates for micropipette isolation (56).

**Cyanobacterial cultures and Sandusky Bay isolates.** Sandusky Bay *Planktothrix agardhii* strains (strain numbering 18XX or SBXX) (Table 1) were isolated during the 2018 sampling season. To accomplish this, samples from each site were serially diluted until fewer than 10 filaments remained in a well. Single filaments were pulled from the lowest dilution using a capillary tube and placed in a clean well containing JM. Plates with single filaments were incubated for several weeks and were monitored by microscopy for growth and contamination from other phytoplankton. Successful isolates were scaled up and maintained in batch cultures. Isolates were confirmed to be *Planktothrix* sp. through morphological observation (no heterocytes or akinetes; blue-green filaments without sheaths; long with no constrictions at cross-cell walls [57]) and PCR with the *Planktothrix agardhii*-specific PCR primers rpoC1\_Plank\_F271 (5'-TGTTAAATCCAGGTAAGTATGACGGCCTA-3') and rpoC1\_P\_agardhii\_R472 (5'-GCGTTTTGTCCCTTAGCAACGG-3') (58).

*Planktothrix agardhii* 10XX-series isolates and *Planktothrix rubescens* strains were obtained from Rainer Kurmayer (Universität Innsbruck) (Table 1; Table S2). The *P. agardhii* 10XX strains were isolated from Sandusky Bay mixed cultures by Kurmayer during summer 2016. *Microcystis aeruginosa* LE3 [19] was obtained from NOAA-GLERL (Ann Arbor, MI, USA). *Planktothrix sarta* PCC 8972 and *Planktothrix paucivesiculata* PCC 8926 were obtained from the Pasteur Culture Collection (Paris, France).

Cyanobacterial strains were grown as unialgal, nonaxenic batch cultures in JM. The cultures were maintained in 125-ml glass flasks at 22°C. Light was supplied by warm-white fluorescent tubes at a light-dark cycle of 12 h–12 h at a photosynthetic photon flux density (PPFD) of 10  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Cultures of *Planktothrix rubescens* were covered with a black mesh to further reduce PPFD to about 6  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

**Chytrid infections.** (i) **Isolation and genetic characterization.** Concentrated field samples (as described above) from several sites were inoculated in JM in 24-well plates in replicate from the 26 June 2018 sampling date (Table 2). Samples were monitored daily by microscopy for several days for *Planktothrix* filaments showing signs of chytrid infection (Fig. S1). Only samples from site ODNR2 on this date contained enough infected filaments for isolation, which consisted of serially diluting the sample until fewer than five filaments were visible in a field of view. Single infected filaments were removed

from the diluted sample with a capillary tube and placed into separate dense cultures of *Planktothrix agardhii* 1029 or 1031, following the methods of Sønstebo and Rohrlack (10). Upon establishment of the newly infected chytrid culture, the chytrid was again transferred on the host strain (1029 or 1031) using the single-infected-filament method described above to yield a pure culture of host and chytrid. The identity of the chytrid was determined by PCR using Rhizophydiales strain Chy-Kol2008-specific ITS primers as described by Sønstebo and Rohrlack (10). In brief, DNA was extracted from infected host biomass using the DNeasy PowerWater Sterivex DNA isolation kit (Qiagen, Germantown, MD) following the manufacturer's instructions. PCR was performed using the primers ITS5 (5'-GGAAGTAAAGTCGTAACAAGG-3') (59) and ITS4Chytrid (5'-TTTCCCGTTTCATTCGCCA-3') (60) for the ITS sequence and 5.8S-R (5'-TCGATGAAGAACGCAGC-3') and LR7 (5'-TACTACCACCAAGATCT-3') (61) for the 5' end of the 28S gene. PCR was performed in a 25- $\mu$ l reaction volume using the DreamTaq Hot Start Green PCR master mix (Thermo Fisher Scientific, Waltham, MA) and a 400 nM concentration of each primer. The thermocycling conditions were 95°C for 1 min, followed by 35 cycles of denaturing (95°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 2 min) and a final extension step (72°C for 2 min). The PCR products were sequenced on a NovaSeq6000 (Discovery Life Sciences, Huntsville, AL). The chytrid sequences were aligned with the sequences of Chy-Kol2008 and Chy-Lys2009 and other closely related sequences as determined by a BLAST search (Table S3).

**(ii) Chytrid culture maintenance.** Infections on the host strains were conducted in 24-well plates, under the same incubation conditions as the cyanobacterial cultures described above. Chytrid isolates were maintained using cultures of *Planktothrix agardhii* 1029, which exhibits a low infection rate, where chytrid sporangia were viable for passage for up to 3 weeks, and *Planktothrix agardhii* SB1802, which yields faster infections, during which chytrid sporangia were viable for passage for only up to 2 weeks, at which point the infectivity of the cultures was significantly reduced. Infections were monitored every other day until day 7, at which point the host culture was severely infected (i.e., the percent infected filaments with visible sporangia was above 70% in SB1802), and infections were then diluted 1:100 in fresh dense host cultures.

**(iii) Infection assay design.** Each cyanobacterial culture tested was infected while the organisms were in mid-exponential growth phase. Infections in each culture were initiated with a starting density of 8,000 filaments  $\text{ml}^{-1}$ . Infections occurred with peak chytrid-infected *Planktothrix agardhii* 1029 culture at a 1:10 (vol/vol) dilution (final concentration, approximately 2% infected filaments). Day 7 was chosen as the endpoint for the majority of the characterization experiments outlined below (10). Experimental samples were investigated for incidence of infection as a proportion of infected filaments in the population, and there were no noticeable differences in the intensity of infection (number of mature sporangia per filament) for each isolate. The abundance of infected and uninfected *Planktothrix agardhii* were counted under an inverted microscope at a magnification of  $\times 400$ . Replicate samples were fixed with glutaraldehyde (0.5% final concentration) and were counted in duplicate using a hemocytometer.

**Host and medium specificity.** Cyanobacterial cultures of *Planktothrix agardhii* (20 strains), *Planktothrix rubescens* (3 strains), *Aphanizomenon* sp. (5 strains), *Cuspidothrix* sp. (1 strain), *Microcystis aeruginosa* (1 strain), *Planktothrix sarta* (1 strain), and *Planktothrix paucivesiculata* (1 strain) (Table 1) were tested as possible hosts for each of the chytrid isolates. Cultures in triplicate were visually monitored every other day for 2 weeks for signs of infection and once a week after that for 4 weeks. Strains were considered possible hosts if there was evidence of zoospore attachment and maturing sporangia.

After hosts were identified, differences in the rate of infections was tested among the chytrid isolates by using the *Planktothrix agardhii* 10XX series (1025, 1026, 1027, 1029, and 1033) and SB1802 (Table 1), ensuring that equal numbers of the chosen cultures were known to either produce or not produce microcystins. The 10XX series was preferentially chosen for these experiments because of the characterization done by the Kurmayer lab in regard to the congener production of microcystin toxins (Table S2). Growth rates among these strains vary slightly (Fig. S3); therefore, triplicate mid-exponential-growth-phase cultures were infected as described above, and after 7 days, the samples were fixed with glutaraldehyde at a final concentration of 0.5% and stored at 4°C until processing.

Infections on three 10XX strains (1030, 1031, and 1032) were also tested in triplicate in two different freshwater cyanobacteria media to determine if there were distinct nutrient requirements for infections. These cultures were established on BG-11 (ATCC medium 616) and were allowed to acclimate to JM for 2 weeks prior to infection with the chytrid cultures, which were maintained on JM. BG-11 and JM were made according to the recipes listed in Table S1. Infection experiments were carried out as described above; infected cultures were fixed after 7 days with glutaraldehyde at a final concentration of 0.5% and stored at 4°C until processing.

**Fate of microcystins during infection.** *Planktothrix agardhii* 1031 is a strain that produces desmethyl microcystin RR and desmethyl microcystin LR (R. Kurmayer, unpublished data) (Table S2) and is highly susceptible to chytrid infections. Mid-exponential-growth-phase cultures of *Planktothrix agardhii* 1031 were infected with chytrid isolate C2 under standard conditions at a 1:10 (vol/vol) dilution. Samples for dissolved microcystin toxins were filtered into glass vials through 0.2- $\mu$ m-pore-size Nuclepore Track-Etch membrane filters (MilliporeSigma, Burlington, MA). All toxin samples were stored at  $-20^{\circ}\text{C}$  until analysis. The concentrations of dissolved microcystin toxins (reported as microcystin-LR equivalents) were measured using the Ohio Environmental Protection Agency (EPA)-approved Abraxis Microcystins-ADDA enzyme-linked immunosorbent assay (ELISA) (Abraxis LLC; Warminster, PA; EPA method 546) following the protocols of Fischer et al. (62). The Ohio EPA kit has the addition of a low-calibration-range check (LCRC) for further kit quality control. The LCRC is a provided control at the low end of the concentration curve ( $\geq 0.30 \mu\text{g liter}^{-1}$  and  $\leq 0.50 \mu\text{g liter}^{-1}$ ), used to ensure the acceptability of the curve at the low end. The assay is congener

independent, as it detects the invariant ADDA (3S-amino-9S-methoxy-2S,6,8S-trimethyl-10-phenyldeca-4E,6E-dienoic acid) moiety of microcystins. The assay detection limit was  $0.04 \mu\text{g liter}^{-1}$ .

**Zoospore persistence.** To determine how long the zoospores survive without a host, zoospore suspensions of chytrid isolates C2 and C10 were obtained by filtering from a peak-infected culture. Zoospores were prefiltered through 11- $\mu\text{m}$ -pore-size Nylon Net (MilliporeSigma), followed by triple filtration through 5- $\mu\text{m}$ -pore-size Nylon Net (MilliporeSigma). The resulting filtrate suspension was stored in the dark at room temperature (22.5°C) for the duration of the experiment.

Daily, the zoospore suspension was added to triplicate cultures of *Planktothrix agardhii* 1031 in a serial dilution series per the most-probable-number (MPN) method (63). Infected cultures were maintained in a lighted incubator under the light and temperature conditions described above for the maintenance of the cyanobacterial hosts. After a period of 7 days from inoculation, the samples were marked for visible infections, and the MPN and 95% confidence interval (CI) were calculated based on the MPN table.

**Host extract effects on infectivity of chytrids.** To test the effects of host intracellular metabolites on the chytrid infections, crude cell extracts were added in duplicate to a susceptible host strain, *Planktothrix agardhii* SB1802, that was newly infected with chytrid C2 at a 1:10 (vol/vol) dilution per previous experiments outlined above. The crude cell extracts were generated by adapting a protein extraction method from the work of Ivleva and Golden (64). In brief, dense cultures of *Planktothrix agardhii* (Table 1) were combined with 1 mM phenylmethylsulfonyl fluoride and 0.3 g of 0.1 mm glass beads (Research Products International Corp., Mount Prospect, IL). The samples were then frozen at  $-80^{\circ}\text{C}$  for 10 min, followed by a thaw at  $37^{\circ}\text{C}$  in a water bath to start the mechanical lysis process. Samples were then vortexed at maximum speed for 10 min in cycles of 30 s shaking and 30 s storage on ice. Immediately following, samples were centrifuged at  $10,000 \times g$  for 5 min, and the supernatant was saved. The crude lysate supernatant was added to the infected host mixture in duplicate at a ratio of 1:4 (vol/vol). After 7 days, samples were fixed with glutaraldehyde at a final concentration of 0.5% and stored at  $4^{\circ}\text{C}$  until counts for both total and infected filaments could occur.

**Statistics.** Statistical analyses for trends and regression analyses were performed using RStudio version 1.0.153 working on R version 3.6.1 (5 July 2019). Differences in prevalence of infection in microcystin producers compared to non-microcystin producers and differences in prevalence of infection depending on medium type were analyzed using the Welch two-sample *t* test in the Stats package of R.

**Data availability.** The ITS-rRNA sequences for the chytrid isolates were uploaded to GenBank under the accession numbers MW192423 to MW192429.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.8 MB.

**SUPPLEMENTAL FILE 2**, MP4 file, 8.4 MB.

**SUPPLEMENTAL FILE 3**, MP4 file, 6.9 MB.

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