Grazing by microzooplankton on *Pfiesteria piscicida* cultures with different histories of toxicity

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ABSTRACT: Susceptibility of actively toxic (TOX-A) zoospores, temporarily non-toxic (TOX-B) zoospores, and zoospores non-inducible to toxicity (NON-IND) of *Pfiesteria piscicida* to microzooplankton grazing was compared in a laboratory experiment. Zoospores from all cultures were ingested by microzooplankton, but community grazing coefficients for TOX-A were <20% of those for TOX-B or NON-IND zoospores in 6 h incubations. Tintinnids and strobilidiid ciliates that fed on *P. piscicida* declined in incubations containing TOX-A zoospores. There was no decline in a strombidid ciliate or heterotrophic dinoflagellate populations that fed on TOX-A zoospores. These data suggest that, although microzooplankton grazing on non-toxic zoospores can be a significant source of mortality to planktonic populations of *P. piscicida*, grazing on toxic or very recently toxic zoospores is relatively low.

KEY WORDS: *Pfiesteria piscicida* · Zoospores · Toxicity · Microzooplankton · Grazing

INTRODUCTION

*Pfiesteria piscicida* is a toxic dinoflagellate that can cause fish diseases and death in estuarine waters (Steidinger et al. 1996, Burkholder et al. 2001a). During toxic events, exposure to aerosols can affect human health (Burkholder et al. 2001a). Zoospores of *P. piscicida* vary in their toxicity to fish and potentially in their toxicity and palatability to microzooplankton grazers (Burkholder & Glasgow 1995, 1997a,b, Stoecker et al. 2000, Burkholder et al. 2001a). TOX-A zoospores are actively toxic, TOX-B zoospores are temporarily non-toxic in the absence of live fish, and non-inducible (NON-IND) zoospores have undetectable toxicity in the presence or absence of fish (Burkholder et al. 2001a,b). (See Glossary of *Pfiesteria*-Related Terms, *Pfiesteria* Interagency Coordination Workgroup, www.redtide.whoi.edu/pfiesteria/documents/glossary.html). In the presence of live fish, TOX-B can convert to TOX-A zoospores. In the absence of fish, some TOX-A zoospores (formerly called toxic zoospores [TZs] or toxic flagellated vegetative cells [TFVCs]) can transform to TOX-B zoospores or to amoebae or cysts (Burkholder & Glasgow 1997a,b, Marshall et al. 2000, Burkholder et al. 2001a). When cultured on algal prey and not exposed to fish, TOX-B zoospores can gradually lose their capability to become toxic and become non-inducible. In nature, toxic outbreaks are thought to be due to induction of toxicity in planktonic TOX-B populations of zoospores or to recruitment of toxic forms from the benthos (Burkholder et al. 1997a,b).

Microzooplankton grazers are important consumers of nanoplanckton, including small dinoflagellates such as *Pfiesteria piscicida*, and may be important in controlling populations of zoospores. Experiments with a NON-IND strain of *P. piscicida* indicate that potential...
grazing coefficients of natural microzooplankton assemblages on zoospores are often higher than the maximum growth coefficient of zoospores, 1 to 2 d⁻¹ (Stoecker et al. 2000, Stoecker & Gustafson 2002). Thus, it is possible that microzooplankton grazing is important in regulating the net growth of *P. piscicida* zoospores *in situ*. However, it was not known whether microzooplankton are capable of grazing, and potentially regulating, populations of TOX-A and TOX-B zoospores. Burkholder & Glasgow (1995) observed ciliates (including *Saprophiilus* and *Microthorax* spp., species that do not ingest zoospores) coexisting with TOX-A zoospores. The ciliate *Stylonchia putrina* and the rotifers *Brachionus* spp. have been observed to feed on toxic zoospores during and immediately after fish kills (Burkholder & Glasgow 1995). Mallin et al. (1995) found that toxic zoospores could be used as food by *Brachionus plicatilis* and by the copepod *Acartia tonsa*. However, exposure to toxic zoospores caused erratic swimming behavior in the copepod. Previous experiments comparing grazing by natural assemblages on TOX-B (Strain 271A-1) and NON-IND zoospores had contradictory results; in the first experiment TOX-B inhibited grazing, in the second it did not (Stoecker et al. 2000). Loss of toxicity between experiments may have accounted for this discrepancy. Toxicity declines rapidly in TOX-B zoospores cultures grown on algal food (Burkholder et al. 2001a).

Microzooplankton populations might be incapable of regulating toxic (TOX-A) or potentially toxic (TOX-B) zoospores for several reasons: they do not ingest them, and they ingest toxic cells and then stop feeding, stop growing or die. To explore these possibilities, we did experiments with 3 assemblages of microzooplankton to compare grazing on TOX-A, TOX-B, and NON-IND cultured zoospores and to determine whether microzooplankton populations were reduced after exposure to TOX-A, TOX-B, and NON-IND zoospores. We tested the following hypotheses: (1) toxicity reduces microzooplankton community grazing coefficients, and grazing on TOX-A < TOX-B < NON-IND; (2) microzooplankton populations exposed to toxic zoospores decrease in abundance; and (3) species that ingest zoospores are more affected by toxicity than species of microzooplankters that do not ingest zoospores.

**MATERIALS AND METHODS**

**Cultures.** The *Pfiesteria piscicida* used in this experiment was from a clonal culture that had been obtained using flow cytometric procedures (Glasgow et al. 2001b) from a water sample from the Middle River, Maryland, USA, collected 27 August 1999. The organism was identified from scanning electron microscopy on suture-swollen cells (Steidinger et al. 1996, Glasgow et al. 2001b) and polymerase chain reaction (PCR) molecular probes (Rublee et al. 1999, Glasgow et al. 2001b). Clonal status was cross-confirmed by D. Oldach using the heteroduplex mobility assay (Oldach et al. 2000).

The actively toxic isolate (CAAE #416T) of *Pfiesteria piscicida* used in this experiment (TOX-A) was maintained in a specialized biohazard III facility and verified as ichthyotoxic using standardized fish bioassays (Burkholder & Glasgow 1997a, Marshall et al. 2000, Burkholder et al. 2001a,c). Briefly, the culture was maintained at 23°C, in a 12:12 h light-dark cycle, and 80 µmol photons m⁻² s⁻¹, in a 9 l culture vessel filled with sterile-filtered synthetic seawater (0.2 µm porosity; deionized water + CORALIFE® Scientific Grade Marine Salts) at 15 PSU, and fed 2 to 3 juvenile tilapia (*Oreochromis mossambicus*, total length 5 to 7 cm) daily. Ammonia concentrations in the vessel with fish were monitored and maintained at ca. 130 µg NH₄⁺ l⁻¹. CAAE culture #416T was a highly toxic isolate, capable of causing fish death in standardized fish bioassays (Burkholder et al. 2001c) within <1 to 2 h at densities as low as 800 TOX-A zoospores ml⁻¹. For further details on the standardized fish bioassay procedure and the environmental conditions that were maintained, see Burkholder et al. (2001c). Prior to use in the experiment, the culture was gently cleaned using the flow cytometric procedures of Glasgow et al. (2001b). Of the cleaned TOX-A culture, 7.5 ml was added to 150 ml of microzooplankton assemblage, so carry-over of material, other than zoospores, from the culture with fish was minimal.

The TOX-B *Pfiesteria piscicida* culture was removed from the TOX-A culture, *P. piscicida* #416T, 2 wk before the experiments and was maintained during that period on cryptomonad prey (*Rhodomonas* sp., cloned from commercial source CCMP757, Bigelow Laboratory) in 15 psu f/2-Si media (Guillard 1975) in darkness. Lack of active production by the TOX-B sub-culture was verified using the standardized fish bioassay procedure (Burkholder et al. 2001c, Cancelieri et al. 2001). The TOX-B culture grew well with fish, but 28 d were required for that sub-culture to regain fish-killing activity (TOX-A mode). Thus, this culture was temporarily not producing toxin but eventually regained ichthyotoxic activity, demonstrating that it was not NON-IND.

NON-IND *Pfiesteria piscicida* culture CAAE #416A was from the same field isolate but had been maintained in batch-mode culture with *Rhodomonas* sp. similarly to the TOX-B *P. piscicida*. This sub-culture, *P. piscicida* #416A, had lost its ability to cause fish disease and death over time in culture with algal prey,
as verified by repeated testing in standardized fish bioassays over the previous 3 mo. It was grown for 10 mo on cryptomonad prey prior to these experiments.

All cultures were sub-sampled daily for 6 d prior to the experiments. The sub-samples were preserved with acidic Lugol’s solution (Vollenweider et al. 1974) and were analyzed for cell abundance in Palmer chambers (Wetzel & Likens 1991) using light microscopy at 400× magnification with phase contrast (Olympus BH2 microscope).

Microzooplankton assemblages. In order to obtain robust results, 3 different estuarine microzooplankton assemblages were used in the experimental incubation. To obtain the assemblages, surface water samples were gently screened through a 200 µm mesh. Assemblage CC was derived from a 10 psu surface water sample collected from the Horn Point Laboratory (HPL) dock on the Choptank River in Cambridge, Maryland, USA, on 13 June 2000, 14 d before the experiment. The <200 µm fraction was enriched with a mixture of Isochrysis galbana, Thalassiosira pseudonana, and Heterocapsa pygmaea and maintained at 20°C on a 14:10 h light:dark cycle. Assemblage BR was collected on 26 June, the day before the experiment, from 12 psu surface waters along the shore of Brannock’s Bay on Chesapeake Bay in Dorchester County, Maryland, USA. Assemblage NE was collected on 24 June, 3 d before the experiment, at the Carolina Pines site (12 psu) on the Neuse River in North Carolina, USA. The assemblages were transported in coolers to the Center for Applied Aquatic Ecology, North Carolina State University.

Microzooplankton grazing and toxicity experiment. The experiment was conducted on 27 June 2000. Aliquots of 50 to 80 ml from cultures of TOX-A (2.0 × 10^4 cells ml^-1), TOX-B (3.5 × 10^4 cells ml^-1), and NON-IND (3.2 × 10^4 cells ml^-1) were incubated with 1 µM 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes), a vital green fluorescent stain, for 1 h in the dark at room temperature using the protocols described in Stoecker et al. (2000). Individual grazers were fixed with cold glutaraldehyde (final concentration) and stored in the dark at 4°C. Labeled zoospores free in the water were enumerated in the preserved samples from each time point. Five milliliter fixed samples were gently filtered (<15 mm Hg pressure) onto 2 µm pore size black membrane filters and then enumerated using epifluorescent microscopy as described in Stoecker et al. (2000).

Experimental or control water (150 ml) was added to polycarbonate incubation bottles. At t = 0, aliquots of the appropriate stained Pfiesteria piscicida cultures were added to each replicate to achieve a calculated zoospore density of 500 zoospores ml^-1. The incubation bottles were inverted several times in order to gently mix their contents. The bottles were incubated in the dark at a cooler at 22 to 24°C for 6 h. At the beginning of the incubation and after 1 and 3 h, 20 ml samples were taken from each bottle. At the end of the incubation, 60 ml samples were taken. Samples were fixed with 1% glutaraldehyde (final concentration) and stored in the dark at 4°C.

Labeled zoospores in the water were enumerated at the controls bottles from each time point. Five milliliter fixed samples were gently filtered (<15 mm Hg pressure) onto 2 µm pore size black membrane filters and then enumerated using epifluorescent microscopy as described in Stoecker et al. (2000). Because prey size can influence grazing rates (Hansen 1992, Kivi & Setälä 1995), the length of 50 zoospores of each type was measured using a calibrated ocular micrometer. Community grazing coefficients (g) were calculated from changes in prey concentrations in the experimental and control bottles for each combination of microplankton assemblage and Pfiesteria piscicida culture using the approach developed by Frost (1972) for measuring zooplankton grazing (see Stoecker et al. 2000). g was calculated as follows:

\[ g = K - \mu \]

where K is the instantaneous rate of change of prey concentrations in the experimental bottles and µ is the instantaneous rate of change of prey concentrations in the controls bottles without grazers.

To determine whether toxicity of the different cultures had an effect on microzooplankton populations, the dominant heterotrophic protists ≥20 µm in size in each assemblage were enumerated in the 0 and 6 h samples. Fixed samples were concentrated by sedimentation in Utermöhl chambers in the dark and then examined using transmitted light microscopy (200 or 400× magnification). To estimate concentrations at the beginning of the incubation, three 10 ml volumes, one from each culture treatment, were enumerated for each assemblage. To estimate concentrations of microzooplankton after exposure to zoospores, 25 ml from the 6 h samples of each experimental bottle was settled and enumerated.

To determine whether the dominant species of microzooplankton ingested TOX-A zoospores, the 1 h experimental samples from the treatments with TOX-A were examined using epifluorescence microscopy as described in Stoecker et al. (2000). Individual grazers were counted and the number of fluorescently labeled zoospores in each grazer was recorded.
RESULTS

Mean lengths (± standard deviation [SD]) of TOX-A, TOX-B, and NON-IND zoospores used in the experiment were 10.1 ± 1.45, 9.8 ± 1.63, and 9.8 ± 1.63 µm, respectively. Differences in zoospore length were not statistically significant (single classification ANOVA, \( n = 50, p = 0.5721 \)). Mean grazing coefficients (g), ± standard error (SE), for the 6 h incubation with Assemblages CC, BR, and NE were 4.6 ± 1.13, 4.2 ± 1.05, and 2.94 ± 1.15 d\(^{-1}\), respectively. There was no significant effect of assemblage on mean grazing coefficient (single classification ANOVA, \( p > 0.05 \)), so data from the 3 assemblages were combined in testing for differences between cultures in their susceptibility to grazing. Mean microzooplankton community grazing on TOX-A was significantly lower than on TOX-B or NON-IND zoospores (Fig. 1). Although grazing appeared to be slightly higher on NON-IND than on TOX-B, the difference was not statistically significant (Fig. 1).

Assemblage BR contained a large amount of detritus and was difficult to examine microscopically, so further analyses were restricted to the treatments with Assemblages CC and NE. For these assemblages, grazing coefficients for the first 3 h were compared to the grazing coefficients for the second 3 h of the incubation (Fig. 2). With all 3 types of *Pfiesteria piscicida* zoospores as prey, grazing coefficients were higher during the first half of the incubation than during the last half, but with TOX-A this trend was very pronounced (Fig. 2). After 3 h, grazing on TOX-A zoospores was almost undetectable (Fig. 2).

Abundant microzooplankters, defined as species that occurred at initial densities >1.5 ind. ml\(^{-1}\), were enumerated at the beginning and end of the incubations with Assemblages CC and NE. Tintinnid and choerotrich ciliate populations that consumed zoospores (Table 1) all declined in the treatments exposed to TOX-A zoospores and were significantly lower than in treatments with TOX-B or NON-IND zoospores (Table 2). The large strombidiid ciliate from the Choptank River consumed TOX-A zoospores but did not appear to be adversely affected (Tables 1 & 2). Surprisingly, cf. Mesodinium pulex, although only about 20 µm in size, ingested zoospores (Table 1). This species increased in abundance during incubation with all 3 zoospore types (Table 2). The <20 µm scuticociliates did not consume zoospores. Zoospore culture type did not affect scuticociliate populations (Table 2). The heterotrophic non-thecate dinoflagellates included Oxyrrhis sp. and Gyrodinium spp.; both taxa were observed with ingested TOX-A zoospores (Table 1).
Exposure to TOX-A zoospores did not have an adverse effect on dinoflagellate grazers during the 6 h incubation (Table 1).

**DISCUSSION**

In the experiment reported herein, the microzooplankton assemblages were ‘semi-natural’, having been held in the laboratory from 1 d to 2 wk prior to the grazing experiment. The community grazing coefficients with the NON-IND and TOX-B zoospores averaged ~5 to 6 d–1 (Table 1). In experiments with natural summer microzooplankton assemblages from the Chincamico River, Maryland, potential grazing mortality of NON-IND zoospores ranged from 0 to 10 d–1 (Stoecker et al. 2000). In similar experiments using assemblages from the Pocomoke River in Maryland, USA, potential community microzooplankton grazing ranged from 0 to 7.6 d–1 (Stoecker & Gustafson 2002). Thus, rates presented herein are within the range obtained using natural assemblages of micrograzers. The maximum growth rates of *Pfiesteria piscicida* cultures on algal prey are 1 to 2 d–1 (Stoecker et al. 2000, Burkholder et al. 2001a); thus, microzooplankton grazing should often be able to prevent the net growth of ‘non-toxic’ zoospore populations.

Community grazing (g) decreased in the second half of the 6 h incubation (Fig. 2), indicating that daily grazing was over-estimated. This was particularly true for the TOX-A treatment, in which community grazing decreased to close to zero after 3 h (Fig. 2). The decrease in grazing after 3 h in the TOX-B and NON-IND treatments may have been due to grazer saturation at zoospore concentrations (ca. 500 cells ml–1) used in the experiments. The digestion times of most microzooplankton grazers are less than 3 h at temperatures >20°C (Sherr et al. 1988, Capriulo 1990, Dolan & Coats 1991) so during the second half of the experiments, grazing should have been approaching steady state. Grazing on TOX-B and NON-IND zoospores was still greater than 2 d–1. These data, along with previous experiments (Stoecker et al. 2000, Stoecker & Gustafson 2002), suggest that potential microzooplankton grazing on NON-IND and TOX-B *Pfiesteria piscicida* zoospores is often >2 d–1 and thus should have an important impact on net growth of planktonic non-toxic zoospore populations.
However, TOX-B zoospores may differ in their residual toxicity. In the experiments reported herein, the TOX-B zoospores were last exposed to fish 2 wk prior to the incubation. Microzooplankton grazing on TOX-B zoospores appeared to be slightly lower than on NON-IND zoospores of the same isolate. The difference in susceptibility to microzooplankton grazing probably depends on how long the TOX-B zoospores have been separated from fish, the Pfiesteria piscicida isolate, and environmental variables. The experiments of Springer (2000) indicate that TOX-B zoospores can retain residual toxicity. Eastern oyster Crassostrea virginica larvae were killed in <1 h when placed in containers with dialysis bags containing TOX-A zoospores; there was ~50% mortality in a similar treatment with TOX-B zoospores (separated from live fish for 6 wk) and 100% survival in controls. In estuaries, exposure to live fish or their exudates may maintain some low level of toxicity in Pfiesteria sp. in the absence of toxic events.

Large choreotrich ciliates, including tintinnids, Strobilidium spp., and Strobilidium-like spp., are important grazers on Pfiesteria piscicida zoospores (Stoecker et al. 2000, Stoecker & Gustafson 2002), but populations of these ciliates declined within a few hours of ingesting TOX-A zoospores. Other types of planktonic ciliates and some dinoflagellates were able to ingest TOX-A zoospores and survive. In our incubation, in which we examined effects on only a few types of common ciliates and dinoflagellates, all the micrograzers that were negatively affected by exposure to TOX-A zoospores were species that ingested them. It is likely that TOX-A zoospores during a fish kill are more toxic than our TOX-A culture that had been separated from fish for a day before the experiment, so it is possible that during fish kills more severe effects on microzooplankton occur. The mixture of prey available to the micrograzers may also influence their susceptibility to P. piscicida toxins. In our experiments we used an initial zoospore density of ca. 500 cells ml−1, within the range of zoospore densities (generally 300 to 1000 cells ml−1) usually observed during toxic outbreaks (Burkholder et al. 2001a, Glasgow et al. 2001a), and thus our results are relevant to in situ conditions. However, the potential for a concentration-dependent effect of toxic P. piscicida on community clearance and microzooplankton species composition remains to be examined.

Although some microzooplankton species in these short-term experiments were able to grow when exposed to TOX-A Pfiesteria piscicida, the question remains as to whether these species would maintain growth if fed TOX-A zoospores for a longer period (e.g., days to weeks). Toxic Pfiesteria sp. outbreaks generally are ephemeral, lasting hours to days, but have persisted in some events for weeks (Burkholder et al. 2001a, Glasgow et al. 2001a). Thus, the response of microzooplankton species over longer intervals has ecological relevance. The presence of TOX-A zoospores in the plankton may select for growth of micrograzers, such as cf. Mesodinium pulex and some heterotrophic dinoflagellates, which can use them as food while having a negative impact on their competitors. Thus, microzooplankton grazing on TOX-A zoospores in a community adapted to the presence of TOX-A may be higher than we found in our experiment. No data are available on the composition of microzooplankton or on grazing by microzooplankton on zoospores during toxic P. piscicida outbreaks.

Microzooplankton community grazing can equal or exceed growth rates of dinoflagellates and may be a natural control that suppresses or controls blooms, especially in their early stages (Watras et al. 1985, Hansen 1992, Jeong & Latz 1994, Kamiyama 1997, Matsuyama et al. 1999). Data on the effects of variation in toxicity of dinoflagellates on grazing are scarce (Huntley et al. 1986, Hansen 1989, Kamiyama & Arima 1997, Turner & Tester 1997, Teegarden 1999). Information is needed on the complex interactions between toxic dinoflagellates and their grazers in order to understand natural controls on bloom formation and how these may be disrupted or enhanced.

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