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Grazing on *Pfiesteria piscicida* by microzooplankton

Diane K. Stoecker*, Kristi Stevens, Daniel E. Gustafson Jr

University of Maryland Center for Environmental Science (UMCES), Horn Point Laboratory, PO Box 775, Cambridge, Maryland 21613, USA

ABSTRACT: The potential grazing pressure of natural assemblages of microzooplankton (<200 µm size fraction) on cultured non-toxic zoospores (NTZ) of *Pfiesteria piscicida* (Dinamoebiales, Dinophyceae, Pyrrhophyta) was measured approximately weekly during the summer of 1999 using surface water samples from a tidal tributary of the Chesapeake Bay (Chicamacomico River, MD, USA) in which fish kills associated with *Pfiesteria*-like dinoflagellates have occurred. NTZ of *P. piscicida* (strain FDEPMDR23, an apparently non-toxic strain) were stained with a vital green fluorescent dye, 5-chloromethylfluorescein diacetate, and added to treatments with (<200 µm) and without (<1.2 µm) the natural microzooplankton assemblage. The dominant micrograzers on *P. piscicida* NTZ were large tintinnids and oligotrichous ciliates. Grazing mortality depended on species composition as well as abundance of microzooplankton. The instantaneous rate of grazing mortality varied from 0 to 10.2 d^{-1} and was >2 d^{-1} in 6 out of 10 experiments. In previous studies, the maximum instantaneous rate of growth of NTZ was <2 d^{-1}; thus, microzooplankton grazing has the potential to prevent net growth of NTZ. However, in 1 out of 2 experiments in which grazing on different strains of *P. piscicida* were compared, grazing was inhibited with a recently toxic strain (271A-1) of *P. piscicida*. Intervals of low grazing pressure may present windows of opportunity for growth of *P. piscicida* NTZ, which, in some cases, can become toxic in the presence of fish.

KEY WORDS: Harmful algal blooms · Biological control · Dinoflagellates · Microzooplankton · Microzooplankton grazing · *Pfiesteria piscicida* · Tintinnids · Oligotrichs · Ciliates · CMFDA

INTRODUCTION

Pfiesteria piscicida (Dinamoebiales, Pyrrhophyta) is a mixotrophic dinoflagellate linked to toxic events and fish kills along the Atlantic coast of the USA (Burkholder et al. 1995, Burkholder & Glasgow 1997, Steidinger et al. 1996). The non-toxic zoospores (NTZ) prey primarily on small phytoplankters, but can also be photosynthetic due to kleptoplastidy (Lewitus et al. 1999). *P. piscicida* has a maximum specific growth rate of 1.1 d^{-1} when growing on *Cryptomonas* sp. (Cryptophyceae) as prey at 23°C and 15 psu under a 12:12 h light:dark cycle at 80 µE m^{-2} s^{-1} (Glasgow et al. 1998). In some cases, NTZ can become toxic in the presence of fish (Burkholder et al. 1995, Burkholder & Glasgow 1997).

In the Chesapeake Bay area, *Pfiesteria* was first identified in 1993 (Lewitus et al. 1995). In 1997 fish kills and lesions associated with *Pfiesteria*-like dinoflagellates were reported from the lower Pocomoke River, from Kings Creek, a tributary of the Manokin River, and from the Chicamacomico River, all on the eastern shore of the Chesapeake Bay in Maryland, USA (Maryland Dept. of Natural Resources 1998).

In most cases, planktonic algal blooms are linked to a combination of factors that enhance growth rate and/or suppress mortality (Smayda 1997). The factors responsible for enhanced growth have received the most attention. However, in most cases low mortality is also necessary for the net growth and maintenance of high populations. In the plankton, predation (grazing) is usually the major mortality factor (Kivi et al. 1996) although parasitism and algicidal bacteria and viruses may also be important when population density is high

*E-mail: stoecker@hpl.umces.edu

(Coats et al. 1996, Kim et al. 1998, Lovejoy et al. 1998). The factors that determine grazing impact, or lack thereof, on particular algal blooms are poorly understood (Buskey et al. 1997, Turner & Tester 1997).

Pfiesteria piscicida NTZ can be both prey and predators of microzooplankton (Burkholder & Glasgow 1995). In samples from estuaries during and after fish kills, Burkholder & Glasgow (1995) observed feeding by the benthic ciliate *Stylonichia putrina* and planktonic rotifers *Brachionus* spp. on various *P. piscicida* life stages. In laboratory experiments, both an estuarine rotifer and adult copepods were shown to ingest and grow when provided with *P. piscicida* as food, although the copepods exhibited erratic behavior when exposed to toxic zoospores (Mallin et al. 1995).

However, no quantitative information was available on predation on *Pfiesteria piscicida* NTZ by natural planktonic assemblages. This information is needed in order to understand natural controls on the proliferation of *P. piscicida* NTZ. It is possible that natural grazing pressure can suppress or limit growth of planktonic populations of *P. piscicida* under some conditions and not others. Changes in trophic structure could be important in allowing planktonic populations of *P. piscicida* to build to levels that could cause fish kills.

Existing information suggests that microzooplankton could be particularly important as predators on flagellated stages of *Pfiesteria piscicida.* NTZ are approximately 5 to 8 µm in size (Steidinger et al. 1996). This is within the prey size range of many microzooplankters (20 to 200 µm size fraction) but too large for many heterotrophic nanoflagellates (prey size ranges reviewed in Hansen et al. 1994). Most planktonic ciliates have optimum prey sizes in the nanoplankton range (2 to 20 µm), with most of the larger tintinnids and nonloricate choreotrichs (herein referred to loosely as 'oligotrichous' ciliates) capable of ingesting 5 to 8 µm prey (Heinbokel 1978, Jonsson 1986, Rassoulzadegan et al. 1988, Kivi & Setälä 1995). Heterotrophic and mixotrophic dinoflagellates have optimum prey sizes that are close to their own size (Hansen et al. 1994), and thus could be capable of ingesting *P. piscicida* NTZ. Protistan microzooplankton often exhibit high ingestion and growth rates when provided with dinoflagellates as prey (Stoecker et al. 1983, 1986, Hansen 1992). Evidence from field and laboratory studies suggest that protistan grazing can sometimes suppress or control dinoflagellate blooms, especially in their early stages (Watras et al. 1985, Stoecker et al. 1986, Hansen 1992, Jeong & Latz 1994, Kamiyama 1997, Matsuyama et al. 1999).

The microzooplankton also includes rotifers, copepod nauplii and benthic invertebrate larvae in estuarine waters. The size range of NTZ, 5 to 8 µm (Steidinger et al. 1996), is within the prey size range for many estuarine rotifers and meroplankton (reviewed in Hansen 1994). Nauplii of the 2 most common copepod genera in estuaries, *Acartia* and *Eurytemora*, ingest particles in the size range of NTZ and are known to feed on some dinoflagellates (Berggreen et al. 1988, Merrell & Stoecker 1998). When metazoan zooplankton are abundant, they can have an important grazing impact on nanoplankton such as NTZ (Merrell & Stoecker 1998, Suzuki et al. 1999).

We investigated the potential grazing impact of microzooplankton (<200 µm fraction) on cultured *Pfiesteria piscicida* NTZ using surface water samples from the Chicamacomico River. We also conducted experiments to compare predation by natural assemblages of microzooplankton on cultured strains of *P. piscicida* with different histories of toxicity. The objectives were (1) to quantify temporal changes in potential grazing during the summer, (2) determine if grazing mortality had the potential to balance growth and thus prevent or suppress blooms of *P. piscicida*, and (3) identify natural predators on this dinoflagellate.

MATERIALS AND METHODS

Culture and staining of *Pfiesteria piscicida*. Cultures of *Pfiesteria piscicida* were obtained from Dr K. A. Steidinger on 10 December 1998 (strain FDEPMDR23) and from Dr J. M. Burkholder and Dr H. B. Glasgow Jr. on 4 August 1999 (strain 271A-1). Strain FDEPMDR23 had been isolated from a stored sample from the Chicamacomico River, Maryland, USA, grown on an algal enrichment. Its identification as *P. piscicida* had been confirmed using SEM (scanning electron microscopy) (Steidinger pers. comm). Strain FDEPMDR23 has not been shown to be toxic in bioassays with fish and is thus considered 'non-inducible' with regard to toxicity (Burkholder pers. comm.).

Strain 271A-1 was derived from samples collected during a fish kill on the Neuse River, North Carolina, USA. It was established as a clonal culture from toxic fish bioassay no. 263 on 18 June 1999 and verified as *Pfiesteria piscicida* by PCR (polymerase chain reaction) and SEM (Glasgow & Burkholder pers. comm.). This culture was considered 'inducible' with regard to toxicity when we received it.

Both cultures were maintained at Horn Point Laboratory in media prepared using GF/F filtered, diluted seawater (15 psu) at 20°C on a 12:12 h light:dark cycle at an irradiance of 50 mmol photons m^{-2} s^{-1}. Algal prey *Storeatula major* (Cryptomonadales, Chromophyta) were added to *Pfiesteria piscicida* cultures every 3 to 4 d at concentrations of ~10^4 cells ml^{-1}.

Approximately 4 d after prey were last added, dense cultures (10^3 to 10^4 cells ml^{-1}) of *Pfiesteria piscicida* with low concentrations of algal prey (<50 *Storeatula major* cells ml^{-1}) were stained with the vital green fluorescent stain CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes Inc.) (Haugland 1994) using the protocol described by Li et al. (1996). Stained cells of *P. piscicida* appear to swim normally and are capable of division (Stoecker pers. obs.); thus, our staining protocols do not appear to have an adverse effect on *P. piscicida* cultures. Although we have not compared grazing on stained and unstained *P. piscicida*, Kamiyama (2000) has compared grazing by a tintinnid ciliate on unstained and CMFDA-stained cells of the dinoflagellate *Heterocapsa circularisquama* and found no difference.

Pfiesteria piscicida were incubated with 1 µM CMFDA at room temperature in the dark for 1 h. Then, a small subsample of the stained culture was fixed with cold glutaraldehyde (final concentration, 1%) and examined with epifluorescent microscopy as described below. Under the microscope, brightness of staining was checked and cell concentrations were determined. Aliquots of the stained cultures were used within 15 min to start the grazing experiments.

Enumeration of *Pfiesteria piscicida* stained with CMFDA. To enumerate cells in stained cultures, 3 to 5 ml fixed samples were gently filtered (<15 mm Hg pressure) onto 2 µm pore size black membrane filters and then mounted on glass slides with immersion oil (Resolve) under a cover slip. Slides were enumerated with epifluorescence microscopy at 200× with a Nikon Eclipse standard microscope (Nikon filter set EF-4 B-2A; exciter filter 450 to 490 nm, dichromatic beam splitter 500 nm, barrier filter 515 nm). With this filter set, CMFDA-stained cells have a bright green fluorescence. The procedure for enumerating stained *P. piscicida* in samples from the grazing experiments was similar, except that 3 ml samples were filtered and slides were stored frozen at –20°C until counted.

Observation of microzooplankton and enumeration of ingested CMFDA-stained *Pfiesteria piscicida*. Samples from the experimental incubations were fixed with cold glutaraldehyde (final concentration 1%) and stored at 4°C in the dark until they were concentrated by sedimentation and observed. Depending on the abundance of microzooplankton and detritus, 10 to 50 ml aliquots were settled in Utermöhl chambers in the dark and then examined using epifluorescence microscopy on a Nikon Eclipse inverted microscope (Nikon filter set B-2E/C; exciter filter 465 to 495 nm, dichromatic beam splitter 505 nm, barrier filter 515 to 555 nm). With this filter set, the green fluorescence of CMFDA-stained cells was less than with the filter set used on the standard microscope, but the narrower range barrier filter reduced the natural red and yellow fluorescence of phytoplankton and some microzooplankon, and therefore made it easier to observe ingested *Pfiesteria piscicida* cells. The dominant tintinnids were identified based on lorica morphology using Gold & Morales (1975) and Snyder & Brownlee (1991).

Determination of potential grazing on *Pfiesteria piscicida*. Grazing experiments were conducted with 10 surface water samples collected from the Chicamacomico River, a humic-rich tidal tributary of the Chesapeake Bay, USA (Table 1). Surface water samples were collected between 07:30 and 11:00 h at approximately weekly intervals during the summer of 1999 from the base of the bridge at Bestpitch, Maryland, USA (38.4172° N, 75.9930° W). On one morning, 13 August, a water sample was also collected at Decoursey Bridge (38.4653° N, 76.0001° W), which is upstream from Bestpitch. Samples were collected with a clean plastic bucket and passed through a 200 µm mesh to remove larger zooplankton as the water was gently poured into a 1 l capacity wide-mouth polycarbonate bottle. At the time of sample collection, water temperature and salinity were measured using a YSI salinometer.

The water samples were transported in a cooler to Horn Point Laboratory and the grazing experiments initiated within 2 h of sample collection. All incubation bottles were made of polycarbonate and had been acid

Table 1. Experiments to determine potential grazing on *Pfiesteria piscicida* nontoxic zoospores (NTZ) (strain FDEPMDR23) using surface water from the Chicamacomico River, 1999. B = Bestpitch Bridge, D = Decoursey Bridge, n = number of sampling times

Date	Sampling location	Salinity (psu)	Water temp. (°C)	NTZ[a] (cells ml^{-1})	Duration of incubation (h)	n
16 Jun	B	14.3	21.4	75	3.0	7
23 Jun	B	12.5	21.2	19	5.0	9
02 Jul	B	14.3	29.7	308	5.0	9
09 Jul	B	14.4	29.8	760	5.0	9
17 Jul	B	14.7	26.7	353	5.0	8
26 Jul	B	12.7	29.3	477	5.0	8
13 Aug	B	16.3	27.9	784	3.0	6
	D	15.1	28.6	552	3.0	6
20 Aug	B	14.7	28.2	339	3.0	6
27 Aug	B	15.4	25.5	408	3.0	6

[a]At $t = 0$ h

washed, rinsed 4× with distilled water, and sterilized before use. For each experiment there were 2 treatments, with duplicate incubation bottles for each treatment. The <200 µm treatment contained the natural assemblage of bacteria, algae, protozoa and other microzooplankton. The <1.2 µm treatment was the same, except that the water was gently filtered through a GF/C glass fiber filter to remove algae, protozoa and other microzooplankton.

Two hundred ml of the <200 or <1.2 µm filtered water was added to each 250 ml polycarbonate bottle. At $t = 0$, aliquots of the stained *Pfiesteria piscicida* culture (strain FDEPMDR23; the 'non-inducible' strain) were added to each replicate. The incubation bottles were gently mixed by inverting them several times and then subsamples were immediately withdrawn and fixed for determination of initial concentrations of stained *P. piscicida* NTZ (Table 1). The bottles were then incubated in the dark in a cooler at 22 to 24°C.

In the first 5 experiments, samples were withdrawn at 15 and 30 min, and in the remaining 5 experiments at 10 and 20 min, for enumeration of microzooplankton grazers of *Pfiesteria piscicida* and for counting stained NTZ both free in the water and within grazers. An additional 3 to 6 samples were withdrawn over the 3 to 5 h incubation for enumeration of stained NTZ free in the water (Table 1).

For determination of taxon-specific grazing on *Pfiesteria piscicida* NTZ, data from the initial and first time points (10 or 15 min) from the <200 µm treatment were used. For each taxon and replicate, the average number of ingested NTZ individual^{-1} was calculated and then converted to an hourly ingestion rate. Average clearance per individual was calculated as the ingestion rate (NTZ individual^{-1} h^{-1}) divided by the average concentration of free NTZ at $t = 0$ (there was no significant differences in concentrations of NTZ between the $t = 0$ and first time point in the incubations). Mean abundance of NTZ and mean clearance for each experiment were calculated using data derived from the duplicate <200 µm treatment bottles. Estimated grazing impact (EGI) of a taxon was calculated as the product of the mean number of individuals of the taxon and the mean individual clearance (Sherr & Sherr 1993).

Community growth or grazing coefficient for each replicate was calculated using data on free NTZ concentrations from all time points using the approach developed by Frost (1972) for measuring zooplankton grazing. For each replicate, the slope of a regression of the natural log (ln) of NTZ ml^{-1} versus time was calculated. The slope of the <200 µm treatment is an estimate of net growth rate, K, of NTZ in the presence of the natural microzooplankton assemblage. The slope for the <1.2 µm treatment is an estimate of the rate of increase or decrease, μ, of NTZ in the absence of grazing. The estimate of μ is a minimum estimate since the 1.2 µm filtration removed potential prey for *Pfiesteria piscicida* as well as its potential predators. To determine if grazing significantly changed the abundance of *P. piscicida* in an experiment, the slopes of the <200 µm (microzooplankton assemblage present) and the <1.2 µm treatment (microzooplankton absent) were compared using single classification analysis of variance (ANOVA). A minimum estimate of the grazing coefficient, g, for each replicate of the <200 µm treatment was calculated as $\mu - K$; the average μ for the <1.2 µm treatment was used in the calculation.

Comparison of grazing on cultures with different histories of toxicity. On 20 October and 18 November 1999, grazing experiments were performend to determine if community g were similar for NTZ of strain 271A-1 (which was last exposed to fish and demonstrated to be toxic in June 1999) and the non-inducible (non-toxic) strain FDEPMDR23. The salinity of the Chicamacomico River at Bestpitch was ≤10 psu in October and November 1999, and our cultures of *Pfiesteria piscicidia* were growing in 15 psu media. Rapid decreases in salinity can cause considerable mortality to cultured *P. piscicida* (Gustafson pers. obs.). Therefore we decided to use natural assemblages from the Choptank River since it had surface salinity closer to 15. Water was collected from the Choptank River at the Horn Point dock for the first comparative experiment in October, when the surface salinity was ~12 and the water temperature was 16°C. Water was collected for the second experiment in November, when the salinity was ~12 and the water temperature was 9°C. On each date, separate incubations were set up with addition of strain FDEPMDR23 and strain 271A-1 to the same assemblage. Experimental procedures were similar to those described for the Chicamacomico experiments. For each date, g with the 2 *P. piscicida* strains was compared by single classification ANOVA.

RESULTS

Environmental and experimental conditions

Nine grazing experiments were performed with water from the Bestpitch site and 1 with water from the Decoursey bridge site (Table 1). Water temperatures at time of collection varied from 21 to 30°C and salinity varied from 12.5 to 16.3. Initial concentrations of CMFDA-stained *Pfiesteria piscicida* NTZ were between 308 and 784 cells ml^{-1}, except in the experiments in June, when they were <100 ml^{-1}. Unstained NTZ in the natural samples were at concentrations of <5 ml^{-1} (Stoecker unpubl. data).

Grazers on *Pfiesteria piscicida*, individual clearing rates and grazing impacts

Examination of samples from the first 2 time points revealed that a variety of protistan grazers consumed the added *Pfiesteria piscicida* (Fig. 1). The most common grazers were certain tintinnids and oligotrichous ciliates. Herein we use the category 'oligotrichous ciliates' for all non-loricate members of the subclass Choreotrichida (Small & Lynn 1985) because with epifluorescence microscopy it is not always possible to distinguish members of the orders Choreotrichida and Oligotrichida. Among the tintinnids, stained NTZ were not ingested by species with oral diameters (o.d.) <21 µm; therefore data are only presented for species with o.d. ≥21 µm. Genera that consumed NTZ included *Tintinnopsis*, *Tintinnidium*, *Favella* and *Nolaclusilis* (Ciliophora, Choreotrichida Small & Lynn 1985). *Tintinnopsis* spp. dominated the tinntinnid assemblage on all dates and often several different species co-dominated. Mean tintinnid abundance and species composition varied greatly among dates, with the lowest abundance from mid-July to mid-August (Fig. 2). Mean specific clearance of tintinnids also varied from experiment to experiment, but the species with wider o.d. tended to have higher individual clearance than species with narrower o.d. (Table 2). Average clearance individual^{-1} for tintinnids with o.d. >21 µm varied from <0.001 to > 0.200 ml individual^{-1} h^{-1} (Fig. 3). The EGI of tintinnids, based on 10 to 15 min incubations, ranged from 4 d^{-1} on 16 June to <0.5 d^{-1} from mid-July through late August 1999 (Fig. 4).

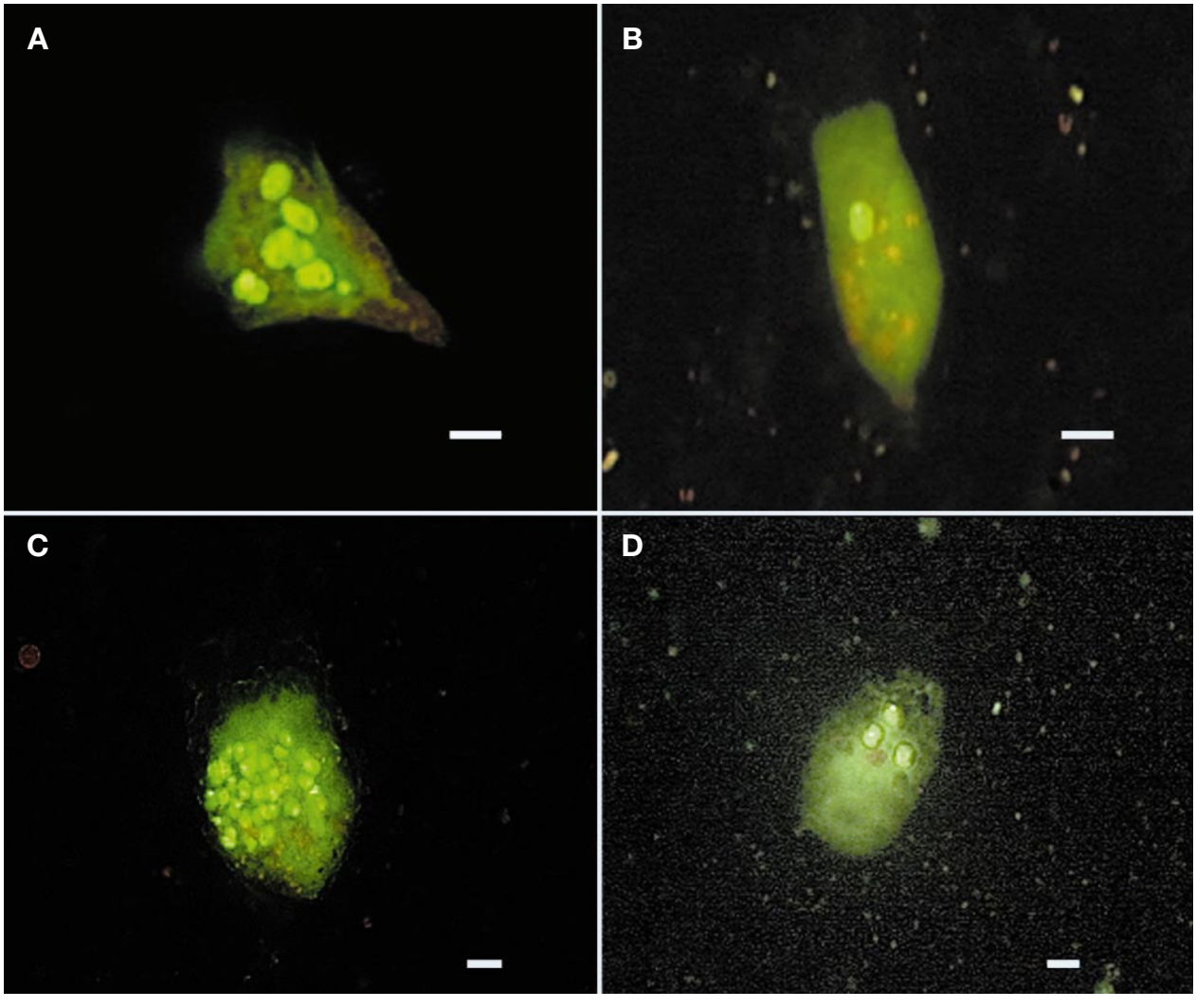

Fig. 1. Epifluorescent micrographs of microzooplankton with ingested 5-chloromethylfluorescein diacetate(CMFDA)-stained non-toxic zoospores (NTZ) of *Pfiesteria piscicida*. (A) Plastidic *Strombidium* sp. with 6 ingested NTZ; (B) *Tintinnopsis* sp. with 1 ingested NTZ; (C) an unidentified tintinnid with many ingested NTZ; (D) *Polykrikos* sp. with 3 ingested NTZ. Scale bars = 10 µm

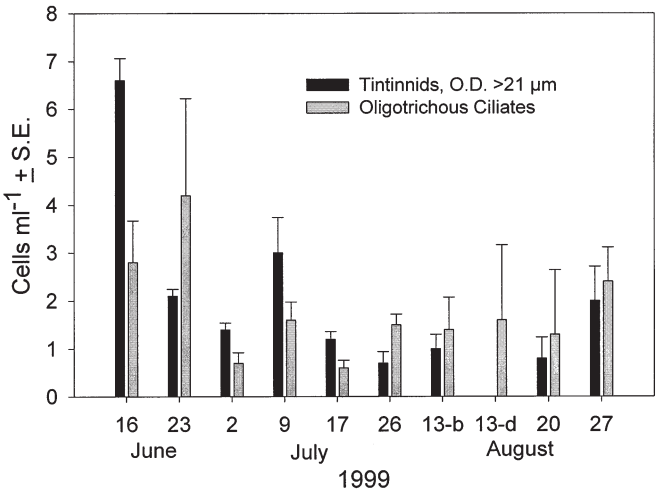

Fig. 2. Mean (±SE) abundance of tinntinnids with an oral diameter (o.d.) ≥21 µm and oligotrichous ciliates from surface waters of the Chicamacomico River, Maryland, USA, at the beginning of the experimental incubations. Incubations used surface water collected at the Bestpitch site, except on 13 August, when water was collected at Bestpitch (b) and Decoursey (d) sites

Some oligotrichous ciliates also ingested *Pfiesteria piscicida* NTZ (Fig. 1). For oligotrichous species, ability to feed on *P. piscicida* did not seem to be determined by ciliate cell size. For example, although *Strombidium* spp. A, B and C (Ciliophora, Oligotrichida) ingested NTZ readily (Table 3), other similar-sized *Strombidium* spp. did not ingest them at all. *Strobilidium* sp. (Ciliophora, Choreotrichida), *Strombidinopsis* spp. (Ciliophora, Choreotrichida) and *Laboea strobila* (Ciliophora, Oligotrichida) ingested NTZ, but ingestion appeared to be low except for a large *Strobilidium* sp. (data not shown). *Tontonia* spp. (Ciliophora, Oligotrichida) were not observed to ingest the stained NTZ. Capacity to ingest NTZ did not appear to be related to trophic type. Some plastidic (mixotrophic) and some non-plastidic oligotrichous species ate NTZ whereas others did not (Table 3). Among the common oligotrichous species that consumed NTZ, measured clearance varied from 0.004 to 0.063 ml ind.$^{-1}$ h^{-1} (Table 3).

Average abundance of oligotrichous ciliates and their mean individual clearance rates for NTZ were in the same range as the >21 µm o.d. tintinnids (Figs. 2 & 3). In most experiments, except the first, the EGI of the oligotrichous ciliates was in the same range as the tintinnids (Fig. 4). Similarly to the tintinnids, the EGI of oligotrichous ciliates was low from mid-July until the end of August 1999.

Another common planktonic ciliate that was observed to ingest *Pfiesteria piscicida* was *Mesodinium pulex* (Ciliophora, Haptorida). *M. pulex* appeared to have low clearance rates for *P. piscicida* compared to tintinnids and oligotrichs (data not shown). Scuticociliates were abundant in some incubations, but ingested, stained NTZ were not observed.

The heliozoan *Actinophyrs sol* (Sarcodina) was common in some samples and appeared to be able to prey on the NTZ. NTZ were sometimes observed attached to the outside of the heliozoan and a green fluorescent inclusion about 5 to 10 µm in size was occasionally observed within a heliozoan cell.

Dinoflagellates occasionally ingested the CMFDA-stained *Pfiesteria piscicida* (Fig. 1). However, estimation of taxon-specific grazing rates was not usually possible because of the low ingestion rates and low densities. A small (20 to 30 µm) heterotrophic nonthecate dinoflagellate was abundant (mean 25, SE 6.0 cells ml^{-1}) in the August incubation from the Decoursey site. In this experiment, it had an estimated clearance of 0.0009 ml ind.$^{-1}$ h^{-1}, resulting in an EGI of 0.56 d^{-1}, whereas in all the other incubations it

Table 2. Abundances and grazing rates on *Pfiesteria piscicida* NTZ by the dominant tintinnids. Bestpitch site, Chicamacomico River, 1999. Mean ± SE. n = number of ciliates examined at t = 10 or 15 min for ingested NTZ, EGI = estimated grazing impact, o.d. = oral diameter, L = length of lorica

Species	o.d. (µm)	L (µm)	Date	No. ml^{-1}	n	Clearance (ml ind.$^{-1}$ h^{-1})	EGI (g d^{-1})
Tintinnopsis cf. *baltica*	35–40	55–66	16 Jun	3.5 ± 0.17	42	0.019 ± 0.0010	1.59
			02 Jul	0.2 ± 0.03	38	0.026 ± 0.0010	0.12
			17 Jul	0.5 ± 0.00	26	0.014 ± 0.0025	0.17
Tintinnopsis cf. *dadayi*	59–60	66–74	23 Jun	0.8 ± 0.16	33	0.148 ± 0.0345	2.84
Tintinnopsis sp. A	29–30	70–80	02 Jul	0.6 ± 0.13	55	0.024 ± 0.0060	0.34
			09 Jul	1.3 ± 0.34	70	0.012 ± 0.0005	0.37
Tintinnopsis cf. *tubulosides*	37	110–120	09 Jul	1.0 ± 0.13	60	0.026 ± 0.0040	0.62
Tintinnopsis sp. B	21	59–70	27 Aug	0.5 ± 0.03	22	0.005 ± 0.0010	0.06
Favella sp.	60	100–150	13 Aug	0.04 ± 0.039	2	0.065	0.06

occurred at densities <3 cells ml^{-1}, and had a minor potential impact.

The heterotrophic dinoflagellate *Polykrikos* sp. (Gymnodiniales, Pyrrhophyta) was also observed with ingested CMFDA-stained *Pfiesteria piscicida* (Fig. 1). The heterotrophic dinoflagellate *Oblea* sp. (Peridiniales, Pyrrhophyta) and the mixotrophic dinoflagellate *Ceratium* sp. (Gonyaulacales, Pyrrhophyta) were observed with round green fluorescent inclusions, rather than with stained *Pfiesteria*-shaped cells inside, after exposure to the CMFDA-stained NTZ. It was not feasible to determine grazing rates of these species on *P. piscicida* because these grazers occurred at low densities (<0.1 ml^{-1}).

The <200 µm treatment contained small Metazoa including rotifers, copepod nauplii and larvae of benthic invertebrates. In the first 5 experiments, rotifers occurred at densities of <0.1 ml^{-1}, and at densities of 0.4 to 1.5 ml^{-1} from late July through the end of August. Copepod nauplii occurred at densities of ≤0.1 ml^{-1} during June and July, and at densities of 0.1 to 0.5 ml^{-1} during August 1999. Invertebrate larvae occurred at densities ≥1 ml^{-1} on 13, 20 and 27 August in water from the Bestpitch site and were at densities of <0.1 ml^{-1} in the other incubations. Glutaraldehyde-induced background green fluorescence made enumeration of CMFDA-stained prey difficult or impossible in the Metazoa, therefore it was not possible to determine their individual clearance rates or grazing impact.

Table 3. Abundances and grazing rate on *Pfiesteria piscicida* NTZ by the dominant oligotrichous ciliates that consume NTZ. Bestpitch site, Chicamacomico River, 1999. Mean ± SE. n = number of ciliates examined at t = 10 or 15 min for ingested NTZ, EGI = estimated grazing impact, p = plastidic species; np = non-plastidic species

Species	Date	No. ml^{-1}	n	Clearance (ml $ind.^{-1}$ h^{-1})	EGI (g d^{-1})
Strombidium sp. A	23 Jun	1.9 ± 0.31	59	0.063 ± 0.0020	2.87
(37–40 µm, conical, p)	02 Jul	0.6 ± 0.13	59	0.034 ± 0.0015	0.49
Strombidium sp. B (44–52 µm, ovoid, np)	09 Jul	1.3 ± 0.13	59	0.021 ± 0.0020	0.66
Strombidium sp. C (30–40 µm, ovoid, p)	26 Jul	1.0 ± 0.36	53	0.004 ± 0.0015	0.10

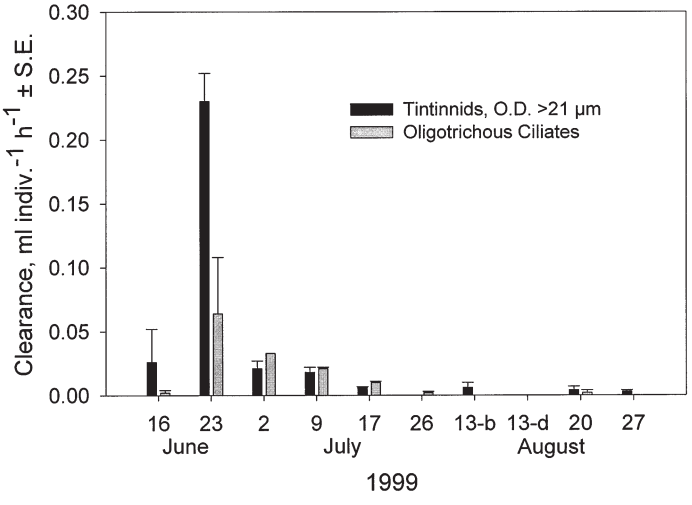

Fig. 3. Mean (±SE) clearance of tinntinnids with an oral diameter ≥21 µm and oligotrichous ciliates from surface waters of the Chicamacomico River for NTZ of *Pfiesteria piscicida*. Incubations used surface water collected at the Bestpitch site, except on 13 August, when water was collected at Bestpitch (b) and Decoursey (d) sites. Rates were calculated from ingestion of CMFDA-stained prey during the first 10 or 15 min of the incubations

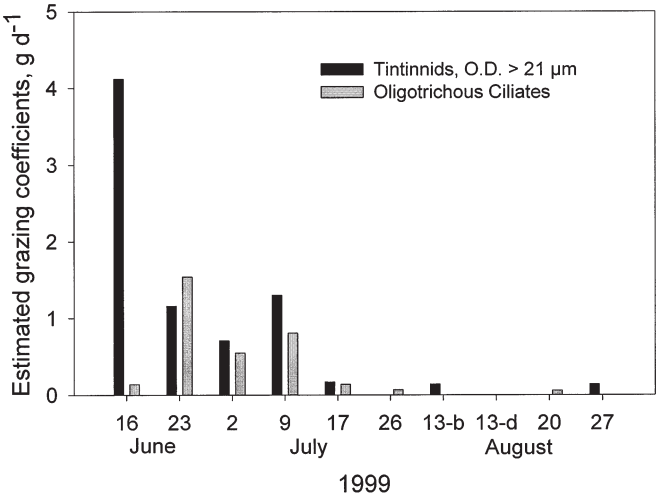

Fig. 4. Estimated potential grazing impact (EGI) of tintinnids and oligotrichous ciliates from surface waters of the Chicamacomico River for NTZ of *Pfiesteria piscicida*. Incubations used surface water collected at the Bestpitch site, except on 13 August, when water was collected at Bestpitch (b) and Decoursey (d) sites. Rates were calculated as the product of mean ciliate densities (Fig. 2) and mean specific clearance (Fig. 3). Data are from first 10 or 15 min of the incubations

Potential grazing by microzooplankton on *Pfiesteria piscicida*

Based upon changes in the concentrations of stained NTZ in the <200 and <1.2 µm treatments, potential community g for *Pfiesteria piscicida* were determined (Fig. 5). In the June experiments, potential grazing on

P. piscicida was high, with *g* ranging from 8.5 to 10.2 d^{-1}. In July, potential grazing was extremely variable, with estimated *g* of 0.4 d^{-1} on 2 and 17 July and the differences between the treatments with and without the microzooplankton fraction not statistically significant (Fig. 5). However, on 9 and 26 July, *g* was >6.7 d^{-1} (Fig. 5). On 13 August, *g* was 3.7 to 7.6 d^{-1}, but on 20 August, although *g* were relatively high, the effect on NTZ populations was not statistically significant, probably due to the high variability among replicates (Fig. 5). On 27 August, *g* was <2.0 d^{-1} (Fig. 5).

Comparison of grazing on strains with different histories of toxicity

The 2 experiments in which grazing on strain 271A-1 and strain FDEPMDR23 of *Pfiesteria piscicida* were compared are contradictory. In the 20 October experiment, both strains appeared to be initially grazed, but after 30 min, removal of strain 271A-1 declined, whereas the non-inducible strain FDEPMDR23 continued to be grazed at a high rate (Fig. 6). Over the 5 h incubation, the *g* with strain FDEPMDR23 was 7.4 d^{-1}, SE 0.48, and with strain 271A-1 was 3.7 d^{-1}, SE 0.10. The difference in *g* was significant ($p < 0.01$). Using only data from 30 min to 5 h, *g* with strain FDEPMDR23 was 5.7 d^{-1}, SE 0.64 and *g* with strain 271A-1 was 2.3 d^{-1}, SE 0.90. Again, the difference in mean *g* was significant ($p < 0.05$). In the 18 November experiment, both strains were grazed. The *g* were again significantly different but the *g* for strain FDEPMDR23 as prey (mean 2.2 d^{-1}, SE 0.63) was lower than for strain 271A-1 (mean 5.1 d^{-1}, SE 0.22) (Fig. 6).

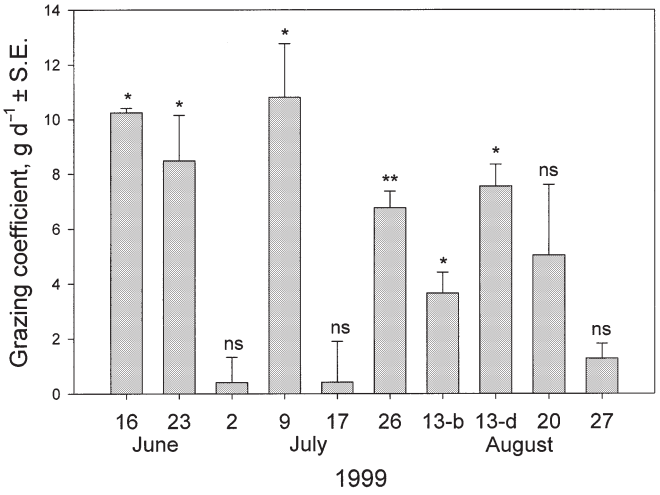

Fig. 5. Mean (±SE) potential grazing coefficient of microzooplankton assemblages (<200 µm fraction) from the Chicamacomico River on NTZ of *Pfiesteria piscicida*. Incubations used surface water collected at the Bestpitch site, except on 13 August, when water was collected at Bestpitch (b) and Decoursey (d) sites. Difference between rates in <200 and <1.2 µm fractions significant at $0.01 < p < 0.05$ (*) or $p < 0.01$ (**). ns = non-significant. Calculations based on changes in NTZ concentrations over the entire 3 to 6 h incubation

DISCUSSION

For net increase in a population, the growth rate has to be higher than the mortality rate, unless recruitment is supplying new individuals. In culture, the maximum reported growth rate for *Pfiesteria piscicida* NTZ was 1.1 d^{-1} (Glasgow et al. 1998). We have observed slightly higher growth, 2.0 d^{-1}, with strain FDEPMDR23 fed *Storeatula major* daily and maintained at 20°C (Stoecker & Gustafson unpubl. data). In the experimental incubations with Chicamacomico River surface waters, the potential predation by microzooplankton on NTZ was usually higher than 2 d^{-1} during the summer (Fig. 5). Net increases in NTZ populations would not have been possible. In a longitudinal study of samples collected from multiple stations on the Chicamacomico River from March to October 1999, *P. piscicida* was detected in surface waters from late July through October. *P. piscicida* DNA was detected in filtered water samples using a highly sensitive and specific 'real-time' PCR assay (Bowers & Oldach pers. comm.). However, densities of *Pfiesteria*-like dinoflagellates were low, with 0 to 58 cells ml^{-1} in our samples (Stoecker unpubl. data). The low populations of *P. piscicida* could reflect control by grazing as well as factors influencing growth rate. In coastal waters, the structure of nanoplankton (2 to 20 µm) assemblages depends on the composition of the grazer populations (Kivi et al. 1996).

However, on 3 or 4 out of 10 experimental dates, potential microzooplankton grazing pressure was <2 d^{-1} (Fig. 5). Temporal windows of opportunity for proliferation of NTZ may occur; however, this cannot be evaluated without coincident data on potential macrozooplankton grazing on NTZ. Previous laboratory studies have indicated that estuarine copepods ingest *Pfiesteria piscicida* zoospores (Mallin et al. 1995).

In our investigation, ciliates were important grazers of *Pfiesteria piscicida* NTZ, but the community *g* was not significantly correlated with total ciliate density ($p > 0.05$). This was probably because not all ciliates consume *Pfiesteria*; the ingestion of NTZ was species specific. In addition, non-ciliate taxa were also consumers of the NTZ. It may be difficult to predict *g* of microzooplankton for *P. piscicida* because of differences in feeding habits and clearance among species and because of the ephemeral nature of ciliate and heterotrophic dinoflagellate blooms.

It is interesting that, although the optimum salinity for growth and toxicity of *Pfiesteria piscicida* zoospores

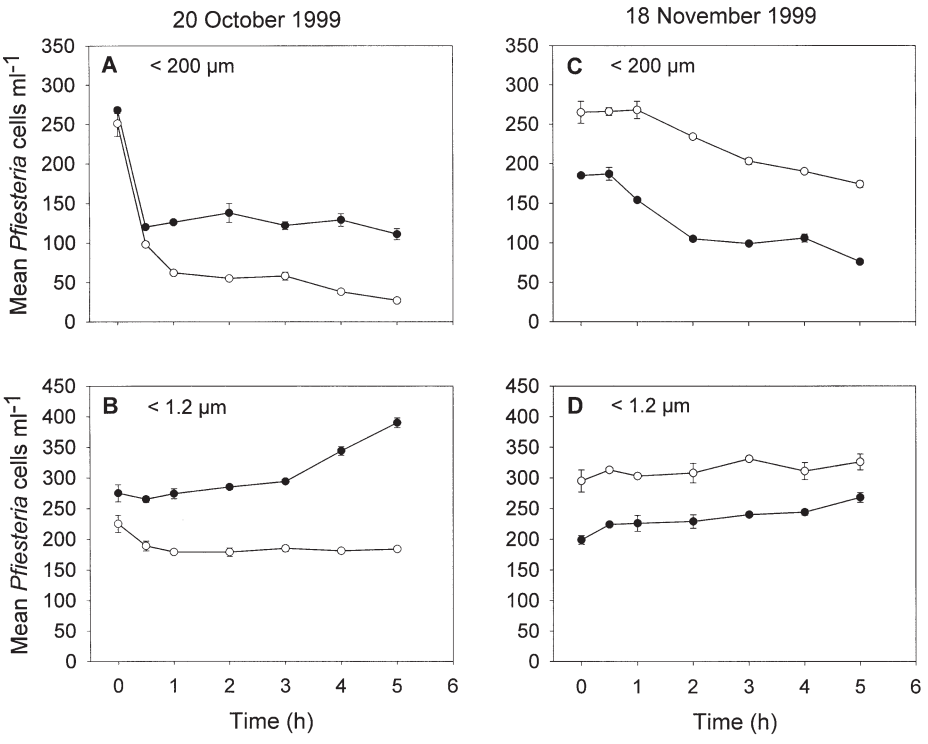

Fig. 6. Comparison of grazing on NTZ of strain FDEPMDR23 (○) and strain 271A-1 (●) of *Pfiesteria piscicida*. Changes in *P. piscicida* populations during incubation in the presence of microzooplankton (<200 µm treatment) (A,C) and in the absence of microzooplankton (<1.2 µm treatment) (B,D)

is ~15 psu (Burkholder et al. 1995), most *Pfiesteria*-related fish kills have occurred at much lower salinity (Burkholder et al. 1995). For example, the fish kill area on the Chicamacomico River during 1997 was mostly 0.5 to 5 psu (Maryland Dept. of Natural Resources 1998). This could be due to the presence of large congregations of susceptible fish, higher prey or nutrient concentrations or reduced grazing in the upper, lower salinity parts of the river. No information is currently available on the distribution of grazers or potential grazing pressure in the Chicamacomico River.

An important question that our investigation did not answer is whether some NTZ from strains that can be induced to produce toxin in the presence of fish are actually toxic or inhibitory to potential grazers. The results of the comparative experiments were contradictory (Fig. 6). The inducible strain (271A-1) was obtained on 4 August 1999 from Drs Burkholder and Glasgow at the North Carolina State University Center for Applied Aquatic Ecology after it had been tested for toxicity to fish on 3 June 1999. In the 20 October experiment, it appeared to inhibit sustained microzooplankton grazing but in the experiment 1 mo later it did not inhibit grazing (Fig. 6). It is possible that this strain lost toxicity in our laboratory between the first and second experiments. If more 'toxic' strains of NTZ inhibit grazing, and occur in nature after fish kills, then they may be able to proliferate at times when growth of less-toxic strains would be regulated by grazing. Our experiments with Chicamacomico River water were carried out with a strain of *Pfiesteria piscicida* which in laboratory trials was not toxic to fish, and thus our results may reflect the 'best case' scenario for control of NTZ by microzooplankton grazing.

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