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INTENSE GRAZING AND PREY-DEPENDENT GROWTH OF *PFIESTERIA PISCICIDA* (DINOPHYCEAE)[1]

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Grazing and growth of *Pfiesteria piscicida* (Pfiest) were investigated using batch and cyclostat cultures with *Rhodomonas* sp. (Rhod) as prey. Observed maximum growth rates (1.4 d^{-1}) and population densities (2×10^5 cells $\cdot$ mL^{-1}) corresponded to values predicted by Monod functions (1.76 d^{-1}; 1.4×10^5 cells $\cdot$ mL^{-1}). In batch cultures under a range of prey-to-predator ratios (0.1:1 to 180:1) and prey concentrations (1000–71,000 cells $\cdot$ mL^{-1}), *Rhodomonas* sp. was always depleted rapidly and *P. piscicida* concentrations increased briefly. The rate of *Rhodomonas* sp. depletion and the magnitude of *P. piscicida* population maxima depended on the prey-to-predator ratio and prey concentration. Starvation resulted in cell cycle arrest at G1 and G2 + M and ultimately the demise of both *P. piscicida* and *Rhodomonas* sp. populations, demonstrating the dependence of *P. piscicida* on the supply of appropriate prey. The depletion of *Rhodomonas* sp. populations could be attributed directly to grazing, because *P. piscicida* did not exert detectable inhibitory effects on the growth of *Rhodomonas* sp. but grazed intensely, with maximum grazing rates > 10 Rhod $\cdot$ Pfiest^{-1} $\cdot$ d^{-1} and with no apparent threshold prey abundance for grazing. The results suggest that 1) the abundance of appropriate prey may be an important factor regulating *P. piscicida* abundance in nature, 2) *P. piscicida* may control prey population, and 3) high growth and grazing potentials of *P. piscicida* along with cell cycle arrest may confer survival advantages.

Key index words: **cell cycle; flow cytometry; grazing; growth; heterotrophic dinoflagellate; *Pfiesteria piscicida*; prey dependence; *Rhodomonas* sp.**

Abbreviations: **LT Batch, long-term batch cultures; Pfiest, *Pfiesteria piscicida*; PPF, *P. piscicida* filtrate; Rhod, *Rhodomonas* sp.; SC Batch, semicontinuous batch cultures**

Pfiesteria piscicida Steidinger et Burkholder is an ambush predator dinoflagellate implicated in major fish kills in North Carolina and Maryland estuaries. The suspected ichthyotoxicity and potential consequences of this organism for public health have motivated intensive research to understand factors promoting its blooms (Burkholder et al. 2001). Research has focused on understanding the nutritional requirements and trophic interactions (Burkholder and Glasgow 1997, Lewitus et al. 1999, Seaborn et al. 1999, Feinstein et al. 2002). Although reported to feed by myzocytosis on moribund or caged fish (Burkholder et al. 1992, Glasgow et al. 2001, Berry et al. 2002), zoospore populations of *P. piscicida* are able to meet their nutritional requirements in diverse ways. They are capable of using dissolved organic and inorganic nutrients and can prey on organisms ranging from bacteria to mammalian tissues (Burkholder et al. 2001). It has been shown that *P. piscicida* feeds and grows efficiently on phytoplankton prey (Glasgow et al. 1998, Seaborn et al. 1999, Burkholder et al. 2001, Parrow et al. 2001,

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Feinstein et al. 2002). However, the quantitative relationship between *P. piscicida* growth and algal prey concentration has not yet been adequately assessed.

The capacity of *P. piscicida* grazing and its effect on prey populations also remain to be assessed. Grazing by heterotrophic and mixotrophic dinoflagellates has received increasing attention in recent years, but most studies focused on short-term grazing rates and growth efficiencies of the grazers (Strom 1991, 2002, Jeong and Latz 1994, Skovgaard 2000). Long-term observations and quantitative analyses of grazing and its effect on prey populations are largely unexplored for dinoflagellates, including *P. piscicida*. The scarcity of information is likely due to culturing difficulties (Skovgaard 2000). *Pfiesteria piscicida* provides an excellent model species for studying the effects of grazing on prey populations because laboratory cultures are robust. In this study, we designed a range of feeding experiments (both short and long term) to investigate 1) *P. piscicida* growth and grazing rates with respect to prey availability, 2) cell cycle responses of *P. piscicida* to prey deficiency, and 3) the effect of *P. piscicida* grazing on prey populations. Results will contribute to our general understanding of how *P. piscicida* natural populations may be regulated by prey availability, what strategies *P. piscicida* may have for surviving prey deficiency, and how dinoflagellate predators like *P. piscicida* can regulate the dynamics of algal prey.

MATERIALS AND METHODS

Cultures. *Pfiesteria piscicida* strains NCSU113–3 (Dr. J. M. Burkholder, North Carolina State University) and CCMP 1831 (Provasoli-Guillard National Center for Culture of Marine Phytoplankton at the Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA) were grown in 15 psu seawater at 20° C. *Rhodomonas* sp. (strain CCMP768), grown at the same salinity and temperature in seawater supplemented with f/2 nutrients, was supplied to *P. piscicida* as food. Light was supplied at 75 μmol photons $\cdot m^{-2} \cdot s^{-1}$ with cool white fluorescence lamps on a 12:12-h light:dark cycle. Both NCSU113-3 and CCMP1831 were verified to be *P. piscicida* by 18S rRNA sequencing (Oldach et al. 2000), and the identity of the cultures used in this study was further confirmed by *P. piscicida*-specific dual-gene PCR (Zhang and Lin 2002). Most results shown originate from strain CCMP1831; results from the other strain were comparable.

Experiments with long-term batch (LT Batch) cultures. Experiments were designed to determine how a single addition of prey affects the growth of *P. piscicida* and how the predator affects prey population growth. A range of initial prey concentrations and prey-to-predator ratios in the experimental cultures (Table 1) was achieved by adding different amounts of *Rhodomonas* sp. to *P. piscicida* cultures (schemes I and II) or by introducing different amounts of *P. piscicida* to *Rhodomonas* sp. cultures (schemes III and IV). In each experiment, predator and prey abundances were quantified microscopically using a counting chamber (Sedgwick-Rafter, Phyco Tech, Inc., St. Joseph, MI, USA) after preservation with Lugol's iodine solution. Over 200 cells were counted for each sample, except when low cell concentrations made it impractical, in which case 5–10 random swipes across the whole counting chamber were counted. Two replicate sets of counts made on each sample were averaged. If the two counts disagreed, additional counts were taken until the variation was within 20%.

Feeding experiments were set up with stock cultures 1–50×10^4 cells $\cdot mL^{-1}$ for *Rhodomonas* sp. and 5–10×10^3 cells $\cdot mL^{-1}$ for *P. piscicida*. In feeding scheme I, variations in prey abundance were achieved by adding 1, 3, 10, or 20 mL of *Rhodomonas* sp. stock culture to 30 mL of *P. piscicida* stock culture and adjusting the final volume to 50 mL. In scheme II, 5

TABLE 1. Parameters obtained from feeding schemes I through IV during the 45-day experimental period.

	Initial prey concentration (cells $\cdot mL^{-1}$)	Initial prey-to-predator ratio	Time for prey to decrease by 90% (τ)	2-Day average growth rate (d^{-1})[a]	Maximum *Pfiesteria piscicida* cell concentration (cells $\cdot mL^{-1}$)
Scheme I	1505	0.2	1	0.04	1025[b]
	4335	0.5	1	–0.24	2947[b]
	5225	1.2	1	0.13	6854
	15,326	3.0	1	0.27	9165
Scheme II	5969	1.0	1	0	4030
	3724	1.4	1	0.11	3724
	4183	7.5	1	0.43	3367
	4795	10.5	4	0.50	3520
Scheme III	38,979	22	4	0.21	19,642
	45,918	96	4	0.41	30,152
	71,156	116	4	0.82	25,765
	51,904	152	4	0.54	32,397
Scheme IV	9642	6.3	2	0.42	3673
	3418	6.7	2	0.31	1836
	4286	14	2	0.18	2499
	20,869	31.5	4	0.65	8928
	49,438	60.6	4	0.78	20,918
	12,859	63	5	0.27	9132
	21,938	107.5	5	0.74	11,071
	47,091	184.6	4	0.90	15,969

[a]Estimated as the slope of an ln(cell count) versus time (days) regression based on data from the second and third day since feeding.

[b]Highest cell concentrations other than the initial concentrations that were higher in these cases.

mL of a *Rhodomonas* sp. stock culture was added to a dilution series of *P. piscicida* cultures representing 10%, 25%, 50%, or 100% of the parent culture density to yield varying predator concentrations. In scheme III, uniform prey density was attempted by adding 1, 3, 5, or 15 mL of *P. piscicida* to 35 mL of *Rhodomonas* sp. derived from a single parent culture and adjusting the final volume to 50 mL. To achieve a uniform predator condition in the two experiments in scheme IV, 1 or 5 mL of *P. piscicida* was added to *Rhodomonas* sp. cultures representing a dilution series of 10%, 25%, 50%, or 100% of initial stock culture concentrations. The total volume for each culture in all feeding schemes was adjusted to 50 mL with autoclaved filtered seawater. Initial prey concentrations and prey-to-predator ratios in each experimental culture were measured from initial cell counts from each culture (Table 1). Because similar trends of predator and prey abundances were observed in all 20 treatments, their results were analyzed as replicates.

Effects of starvation on cell cycle and cell size. To determine how *P. piscicida* responds to prey depletion, cell cycle progression and cell size were examined in cultures that had been starved and then resupplied with prey. Triplicate batch cultures were grown to exponential phase by supplying excess *Rhodomonas* sp. Feeding was then discontinued for up to 9 days, during which time cell cycle and size were examined. In one experiment, samples were collected every 4 h for the first 2 days and less frequently for another 2 days thereafter. In another experiment, samples were collected on days 1, 2, 5, and 9, after which *Rhodomonas* sp. was resupplied, and samples were collected for an additional 2 days for cell cycle and size analyses. At each sampling point, one sample set was preserved with Lugol's iodine solution for cell counts of both predator and prey and cell size measurement of *P. piscicida*. A second set of samples was preserved for flow cytometric cell cycle analysis. Cells from 15-mL samples were harvested by centrifugation (4000*g* for 20 min at 18° C) and fixed in 4% PBS-buffered paraformaldehyde for 6 h at 4° C and stored in 0.5% PBS-buffered paraformaldehyde at 4° C until analysis.

Triplicate samples were combined before analysis of cell size and cell cycle. Cell size was measured, for > 200 cells in each sample, from apex to antapex of the cell using a microscope (model BX51, Olympus, Melville, NY, USA) equipped with Northern Eclipse imaging software (Empix, Ontario, Canada). *Pfiesteria piscicida* cell cycle was analyzed on a FACSort flow cytometer (Becton-Dickinson, San Jose, CA, USA) after samples were washed with PBS and incubated for 30 min at 37° C with 0.1 $g \cdot L^{-1}$ RNase (1:1 mix of RNase A and B), and cellular DNA was stained with SYBR Green I (10^5-fold dilution of commercial stock; Molecular Probes, Eugene, OR, USA) for 20 min (Jochem 2001). Samples were measured at a flow rate of 0.2 $\mu L \cdot s^{-1}$, and *P. piscicida* populations were gated on side-angle light scatter versus DNA (FL1; 535 ± 15 nm) fluorescence. Cell cycle phases (G1, S, G2 + M) were resolved from one-parameter histograms of DNA fluorescence of gated populations by ModFit LT (Verity Software, Topsham, ME, USA). *Pfiesteria piscicida* cells that contained 1N DNA were defined as G1, whereas those with 2N DNA were, as usual, referred to as G2 + M, because flow cytometric cell cycle assessment based on cellular DNA content cannot differentiate between cells in G2 or M phases. Starved *P. piscicida* cultures were essentially depleted of prey cells, and *P. piscicida* cells did not contain visible DNA from ingested *Rhodomonas*. In fed cultures, prey cells were present, but their DNA did not interfere with *P. piscicida* cell cycle analysis because DNA fluorescence from these *Rhodomonas* cells was generally dim and fell below the dynamic range set for DNA analysis in *P. piscicida*.

Effects of Pfiesteria piscicida *filtrate on* Rhodomonas *sp. growth.* To determine whether prey growth was inhibited by *P. piscicida* toxins or exudates, *Rhodomonas* sp. was grown with f/2 medium amended with filtrate from *P. piscicida* cultures. Triplicate 10-mL cultures of *Rhodomonas* sp. were grown in 15-mL centrifuge tubes under temperature and light conditions described above. Filtrates of a dense *P. piscicida* culture (1.2×10^5 cells $\cdot mL^{-1}$; PPF) were obtained using 0.45 μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA, USA). *Rhodomonas* sp. medium was amended either with 0% (control), 5%, or 50% PPF and final nutrient concentrations adjusted to f/2. Over a 10-day period, 1-mL samples were withdrawn periodically for cell counts, and after each sampling, 1 mL of the respective f/2 media (with 0, 5%, and 50% of PPF) was replenished.

Experiments with semicontinuous batch (SC Batch) cultures. To determine quantitatively the effect of prey density on *P. piscicida* grazing and growth, triplicate semicontinuous cultures were established with initial *P. piscicida* concentration of 1000 cells $\cdot mL^{-1}$ and initial prey concentrations of 100, 1000, 10,000, or 100,000 cells $\cdot mL^{-1}$. Prey concentrations were adjusted to those levels each day of examination by adding more *Rhodomonas* sp. (except for a few occasions in which prey concentration increased due to growth and no *Rhodomonas* sp. was added). Control *Rhodomonas* sp. cultures were grown at identical cell densities and nutrient concentrations as those in the *P. piscicida* cultures (natural seawater without addition of nutrients) to estimate growth in the absence of grazing. Grazing rates were calculated as in Frost (1972), based on comparison between Rhod (*Rhodomonas* sp.) concentrations in the Pfiest (*P. piscicida*) culture and in the control (Rhod without Pfiest).

Experiments with a two-stage cyclostat culture. To measure *P. piscicida* grazing and growth under equilibrium conditions, a two-stage cyclostat culture system was constructed (Fig. 1), essentially following Rhee et al. (1981) and Selph (1999). Culture vessels consisted of modified 2-L polycarbonate "roller bottles" (Fisher Scientific, Hampton, NH, USA) with overflow and sampling nipples affixed to their sides. Vessels were connected with sterile silicone tubing (Cole-Parmer Instrument Company, Vernon Hills, IL, USA), and the system was run in an environmental chamber (Hotpack, Philadelphia, PA, USA) at 20° C and 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$. A unialgal culture of *Rhodomonas* sp. (stage 1) was grown in f/2 medium at a dilution (= growth) rate of 1 d^{-1}. Media was supplied at a constant rate using a peristaltic pump (Cole-Parmer Instrument Company), and cultures were mixed by constant bubbling with sterile air. The prey culture (stage 1) was pumped into a bialgal culture of *Rhodomonas* sp. and *P. piscicida* (stage 2) or to the waste. Stage 2 was not stirred continuously because constant turbulence or shear inhibits the growth of dinoflagellates (Berdalet 1992), a result confirmed in preliminary experiments with *P. piscicida*. Instead, 10-min mixing was applied hourly when prey was pumped to stage 2. With the pumping rate kept constant, dilution rate (or food supply rate and hence grazer growth rate) for stage 2 was changed by varying the size of the culture vessel.

The cultures were grown with both stages under a 12:12-h light:dark regime. Cell concentrations in both chambers were measured daily, and experiments were performed once populations in both chambers were stable for at least 5 days (at least three generations). *Pfiesteria piscicida* abundance in stage 2 fluctuated only slightly once steady state was achieved. Therefore, *P. piscicida* growth rates could be approximated by the dilution rate. Daily average grazing (I) was estimated as

$$I = \frac{S1 - S2}{XT} = \frac{(S1 - S2)D}{X} \quad (1)$$

where $S1$ and $S2$ are steady-state *Rhodomonas* sp. concentrations in stage 1 and stage 2, respectively; T is residence time; D is the dilution rate or inverse of T; and X is the *P. piscicida* concentration in stage 2.

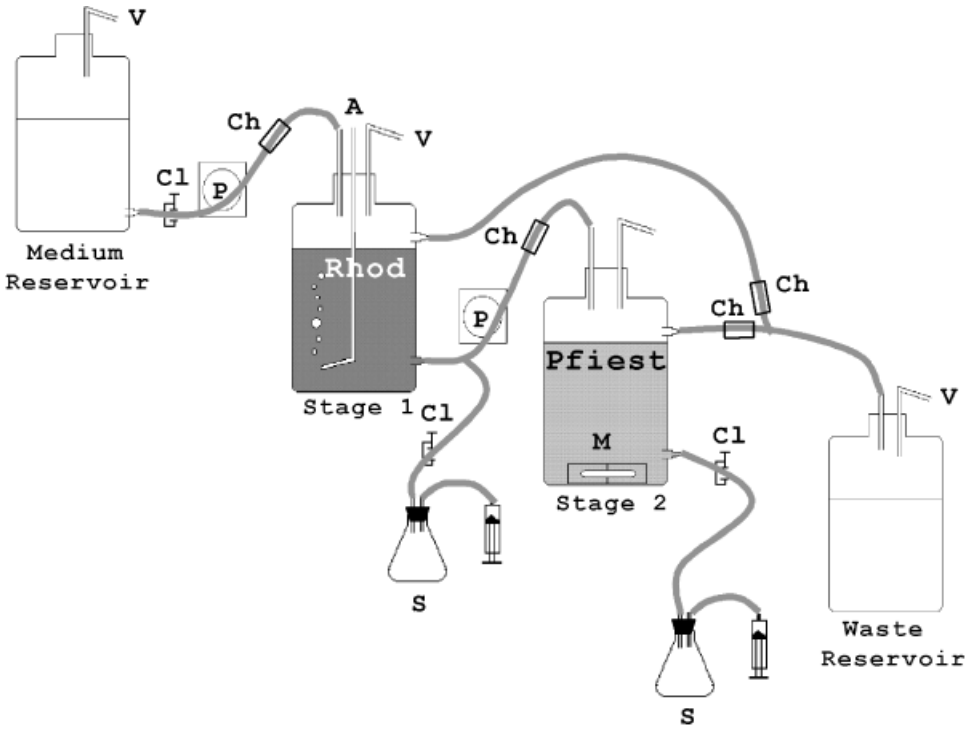

FIG. 1. Two-stage cyclostat culture system. A, filtered air; Ch, check valve; Cl, tubing clamp; M, low-speed magnetic stirring bar for tissue culture applications; P, peristaltic pump; S, sterile sampling port; stage 1, *Rhodomonas* sp. (Rhod); stage 2, *P. piscicida* fed with *Rhodomonas* sp. (Pfiest); V, filtered air valve.

Grazing by *P. piscicida* was further examined at shorter time intervals. After prey addition, cell concentrations of the prey (*Rhodomonas* sp.) were determined every 10 min for nearly 1 h. Sampling was repeated for three 50-min periods, and cell concentrations for each time point (minutes since feeding) were averaged.

Data analysis. Dynamics of *P. piscicida* in the LT Batch cultures were fitted to a "peak" function when a peak occurred or to a polynomial function (power of 3) when a peak was lacking. Prey (*Rhodomonas* sp.) dynamics in these cultures was fitted to an exponential decay function. Growth rates of *P. piscicida* were calculated assuming exponential growth, that is, $\mu = (\ln N_t - \ln N_0)/t$, where μ is the specific growth rate (d^{-1}), and N_t and N_0 are the cell concentrations at times t and 0, respectively. Average growth rates over 2 or more days were derived from the slope of the linear regression of ln(cell concentration) with time. "Carrying capacity" was defined as the maximum *P. piscicida* concentration sustained by each prey supply level and calculated as the average cell concentration over the period in which *P. piscicida* populations reached steady levels (days 8–10 in the SC Batch cultures and days 3–5 in the cyclostat system). Functional and numerical responses were analyzed by correlating grazing and growth rates to the prey-to-predator ratio (prey concentration). Daily prey supply rather than the amount remaining in the culture bottle was used in the analysis because most of the prey was removed rapidly in most treatments. Calculations thus did not account for prey growth in the bottles containing prey. The amount of prey added to batch cultures was measured directly by cell counts and that added to the cyclostat cultures was calculated as the product of prey concentration in stage 1 (*Rhodomonas* culture) and dilution rate. Therefore, the prey-to-predator ratio equaled the prey density at the time of supply divided by predator density for the batch cultures and (Rhod in stage 1 × dilution rate)/(Pfiest in stage 2) for the cyclostat. Linear, exponential, and Michaelis-Menten (M-M) functions were first tested in the regression analyses, and the function that best fit the data was chosen. Regression analyses were performed using SigmaPlot 8.0.2 (SPSS Science, Chicago, IL, USA), and statistical tests for difference followed Sokal and Rohlf (1995).

RESULTS

Pfiesteria piscicida growth and Rhodomonas *sp. in batch cultures.* In the LT Batch experiments, a brief population increase of *P. piscicida* was observed in all feeding schemes typically 2 or 3 days after addition of *Rhodomonas* sp. prey, both for individual treatments (Fig. 2 as a typical example) and pooled data (Fig. 3). As shown in Table 1, the growth rate, averaged for 2 days (second and third days since feeding), was negligible at low prey concentrations (<5000 Rhod cells $\cdot$ mL^{-1}) and prey-to-predator ratios (<5) (schemes I and II) but increased with food abundance (schemes III and IV). Accordingly, *P. piscicida* maximum cell concentrations increased with prey concentration and prey-to-predator ratio.

Rhodomonas sp. populations declined immediately after they were supplied to *P. piscicida* cultures in all feeding schemes, exhibiting a trend of exponential decay. This was evident in both individual treatments (Fig. 2 as a typical example) and in the pooled data (Fig. 3). The rate of prey depletion was assessed as the time period (τ) in which the prey population declined by 90% (Table 1). In feeding scheme I with low initial

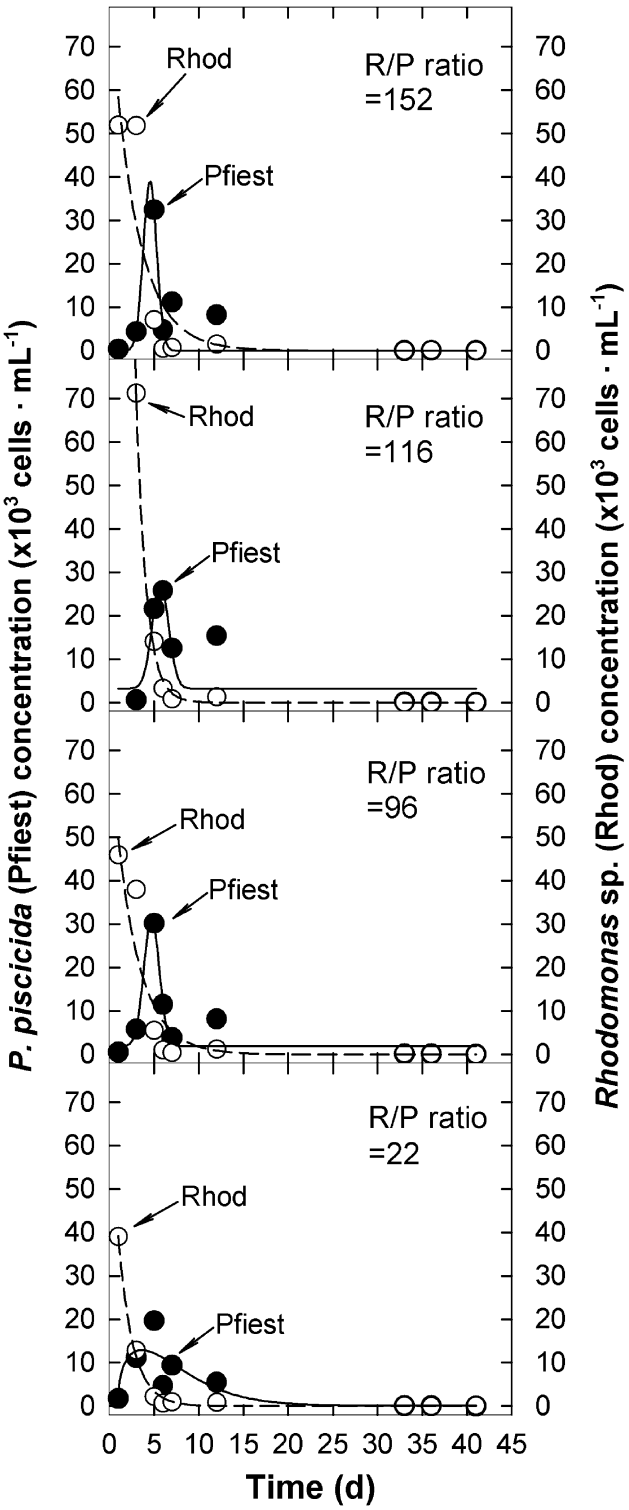

FIG. 2. Temporal variation in cell concentrations of *Pfiesteria piscicida* (Pfiest) and *Rhodomonas* sp. (Rhod) in LT Batch cultures. Shown as an example are results from feeding scheme III. Results from schemes I, II, and IV showed similar trends, although prey depletion time and *P. piscicida* population maxima varied with initial prey-to-predator (R/P for Rhod/Pfiest) ratio and prey concentration (see Table 1).

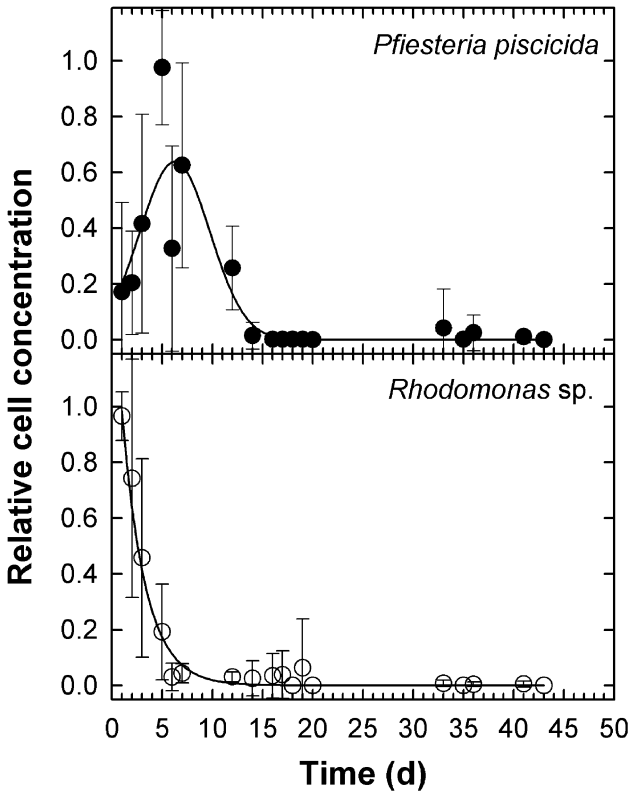

FIG. 3. Temporal trend in averaged cell concentrations of *Pfiesteria piscicida* (Pfiest) and *Rhodomonas* sp. (Rhod) from 22 LT Batch cultures; mean ± SD (error bar).

prey concentration (mostly <5000 cells $\cdot$ mL^{-1}) and prey-to-predator ratio (<10), τ equaled 1 day. In feeding scheme II, *Rhodomonas* sp. was supplied at low concentrations, and τ equaled 1 day, except in the treatment of initial prey-to-predator ratio >10, in which τ prolonged to 4 days (Table 1). In contrast, when initial prey concentration and prey-to-predator ratio were high ($>20{,}000$ cells $\cdot$ mL^{-1} and >20, respectively), as in feeding scheme III and parts of IV, the prey was depleted more slowly ($\tau = 4$–5 days). In scheme IV, low or moderate prey concentrations were accompanied by moderate or low prey-to-predator ratios, and τ amounted to 2 days. In general, τ increased with prey concentration and prey-to-predator ratio (Table 1).

In all four feeding schemes, both *P. piscicida* and *Rhodomonas* sp. declined to low or undetectable levels, and neither population recovered during the 45-day experimental period (Figs. 2 and 3).

Cell cycle arrest by starvation. In exponentially growing batch cultures fed at a prey-to-predator ratio of about 3, the prey declined exponentially, as observed in the other experiments, and was depleted within 12 h (Fig. 4A). During this period, the percentage of *P. piscicida* S- and G2 + M-phase cells (S and G + M fractions) increased (from 21.5% to 28.1% and 31.2% to 35.8%, respectively), whereas the G1 fraction

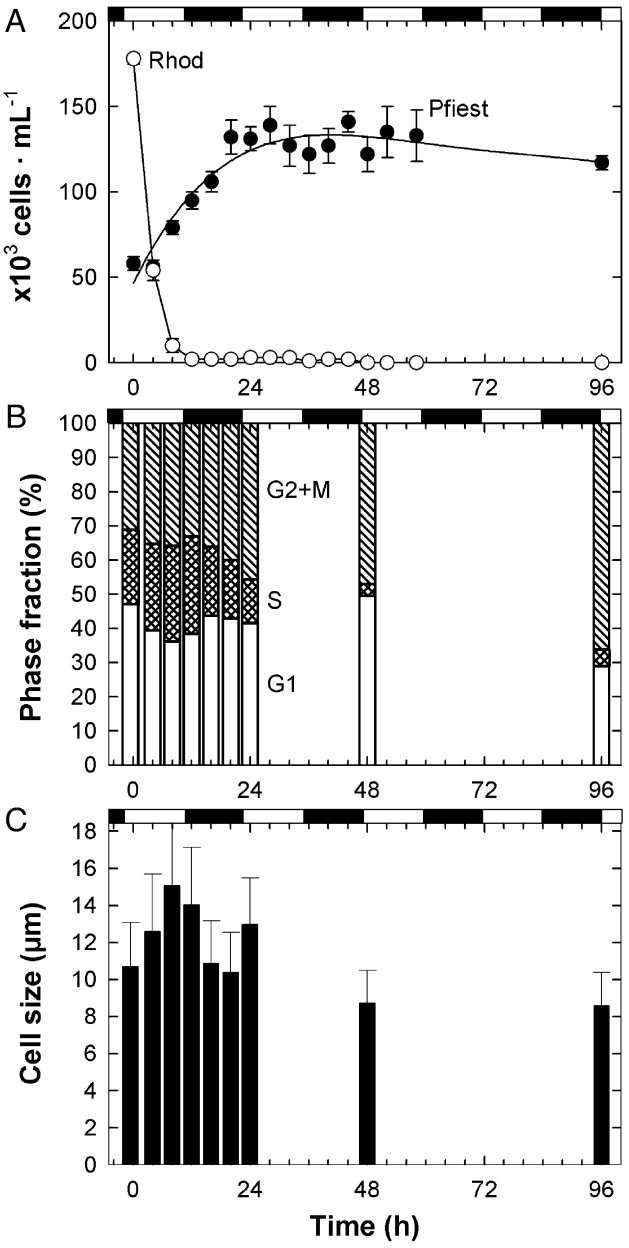

FIG. 4. Effects of short-term starvation on cell concentration (A), cell cycle (B), and cell size (C) of *Pfiesteria piscicida*. Bars on top indicate light (open) and dark (closed) periods. Data points in A are means ± SD (error bar) derived from triplicates (in some cases SD and the error bars are smaller than symbol size) and those in B and C are from analysis of combined triplicate samples with cell size measured from >200 cells (C, mean ± SD).

decreased (from 47.0% to 36.1%) (Fig. 4B), an indication of active cell growth in preparation for cell division. Active cell growth was also evident by the increase in cell size, from 10.7 ± 2.4 (n = 211) at the beginning of the experiment to 15.1 ± 3.4 (n = 204) at hour 8 (Fig. 4C). After 12 h, the S fraction decreased (Fig. 4B), coinciding with rapid cell division as mirrored in the growth curve (Fig. 4A) and cell size reduction (from 14.0 ± 3.1 [n = 201] to 10.4 ± 2.2 µm [n = 220]) (Fig. 4C). After 24 h of starvation, *P. piscicida* population growth ceased (Fig. 4A), and the S fraction continued to decline and reached a minimum after 48 h (3.0%) (Fig. 4B), a sign that the cell cycle was stalled. Meanwhile, a large fraction of the *P. piscicida* population remained in G1 or G2 + M (50% and 47%, respectively) (Fig. 4B), suggesting a cell cycle arrest in these two phases. At hour 96, the G2 + M fraction had increased further, and cell size was reduced to 57% of the largest measured cell size (8.6 ± 1.8 µm; Fig. 4C).

The effects of prey availability on the cell cycle and cell size were further examined in a longer term batch culture experiment (Fig. 5). Two to 5 days after feeding, the fraction of S-phase cells and cell size decreased, whereas the fraction of G1 cells increased (Fig. 5). When prey deficiency was prolonged (9 days), cell size and cell concentrations decreased further, the fraction of S-phase cells remained low, and the G2 + M-phase cell fraction increased (Fig. 5A). Upon resupply of prey (day 9), a rapid recovery was observed. The fraction of S-phase cells and cell size increased markedly, with the latter nearly doubling in just 1 day. Concomitantly, the fraction of G1-phase cells decreased dramatically and the G2 + M fraction increased, indicating progression of cells from the G1 to the S and G2 + M phases. Because of resumed cell division, cell abundance increased (Fig. 5C). Two days later, without further prey supply, the S fraction and cell size declined again with a substantial increase in the G1 fraction.

Effects of Pfiesteria piscicida *on* Rhodomonas *sp. growth.* Throughout the 10-day experimental period, *Rhodomonas* sp. cultures amended with different amounts of *P. piscicida* filtrate all grew well until the last day, when cell abundance started to decrease in all cases. Average specific growth rates during the exponential growth phase (days 1 through 7) were 0.90 ± 0.01, 0.90 ± 0.00, and 0.90 ± 0.01 d^{-1}, respectively, for the control, 5%, and 50% PPF treatments. Growth rates did not differ among treatments ($P > 0.05$; t-test). Growth rates then slowed, and cell concentrations for the three treatments increased from similar levels on day 7 to their maximum on day 9 of $1.3 \times 10^6 \pm 7.8 \times 10^4$, $1.2 \times 10^6 \pm 1.5 \times 10^5$, and $8.6 \times 10^5 \pm 1.5 \times 10^4$ cells · mL^{-1}, respectively. It is not clear why cell concentrations were lower on day 9 in the cultures with 50% filtrate, but it might be related to reduced nutrient availability and/or increased microbial activity. Nevertheless, final cell concentration in the 50% PPF treatment ($8.3 \times 10^5 \pm 8.6 \times 10^4$ cells · mL^{-1}) was not lower than in the control ($7.0 \times 10^5 \pm 5.8 \times 10^4$ cells · mL^{-1}) or the 5% PPF treatment ($8.4 \times 10^5 \pm 2.8 \times 10^5$ cells · mL^{-1}). Overall, no direct impact of *P. piscicida* filtrate on *Rhodomonas* sp. growth was evident.

Grazing and growth in the cyclostat and the SC Batch cultures. Two experiments were designed to quantify grazing and growth of *P. piscicida*. In the cyclostat culture system, the outflow of *Rhodomonas* sp. from

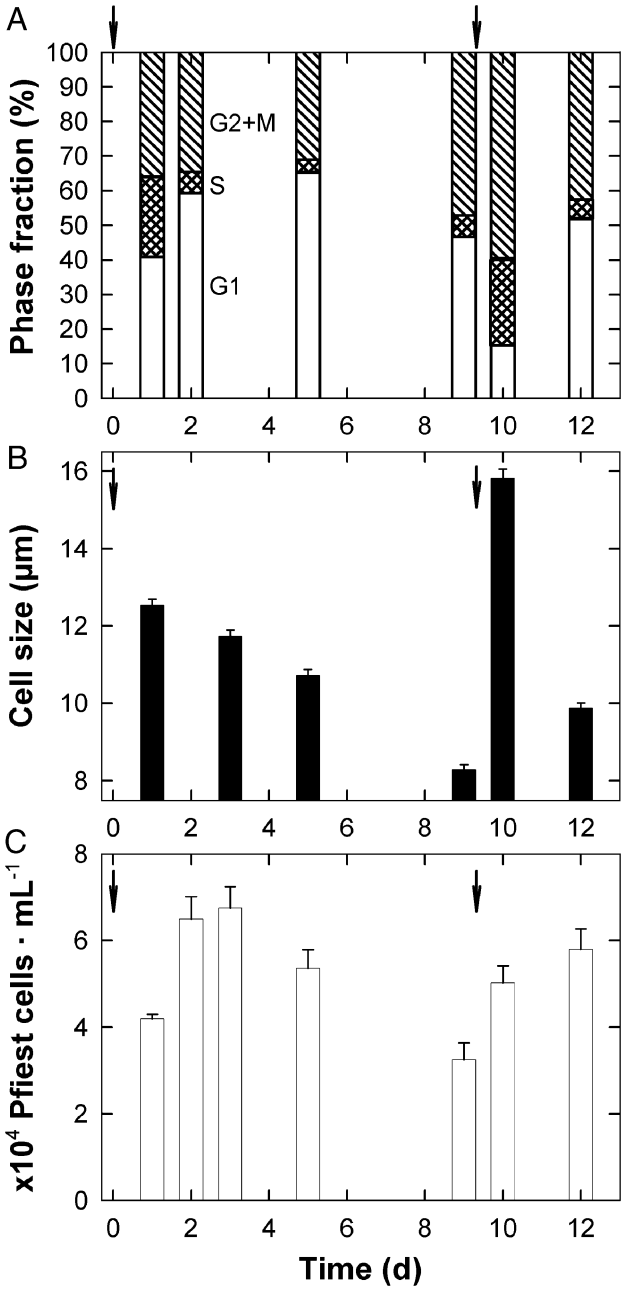

FIG. 5. Effects of prolonged starvation and refeeding on the cell cycle (A), cell size (B), and cell concentration (C) of *Pfiesteria piscicida*. Each data point in A originates from the analysis of combined triplicates, and B and C present the mean of the triplicates ± SD. Arrows indicate addition of the prey alga *Rhodomonas* sp.

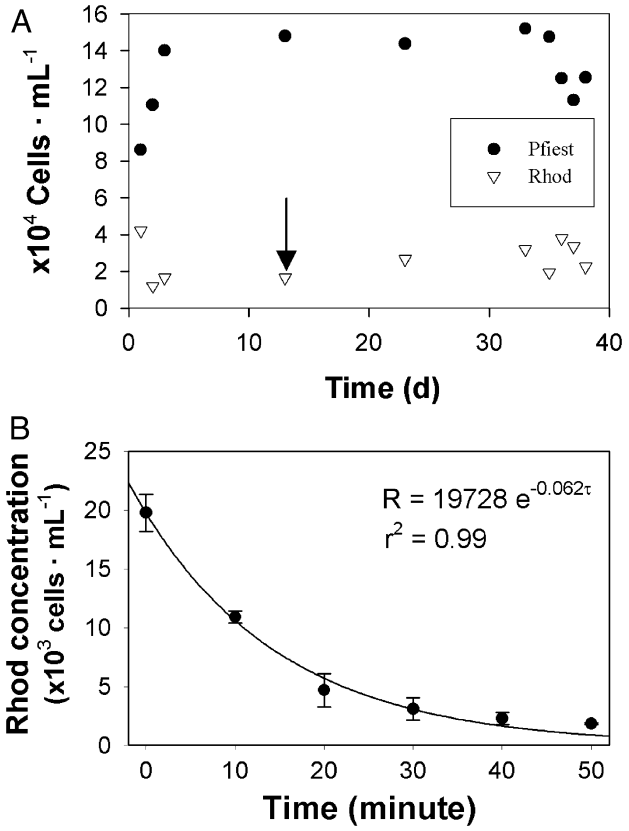

FIG. 6. Dynamics of *Pfiesteria piscicida* (Pfiest) and *Rhodomonas* sp. (Rhod) in the cyclostat culture system. (A) Periodic cell counts of prey and the predator verifying the steady-state condition of the culture system (days 5 through 30). Arrow indicates the date of short-term grazing (shown in B) measurement. (B) Exponential decline of Rhod during the 1-h feeding interval; each data point represents the mean of triplicates ± SD.

stage 1 was fairly constant throughout each experiment. Once reaching steady state, *Rhodomonas* sp. and *P. piscicida* concentrations in stage 2 were stable, with *Rhodomonas* sp. present at low levels (Fig. 6A). *Pfiesteria piscicida* growth rate was assumed to equal the dilution rate of stage 2. Hourly supplied *Rhodomonas* sp. rations were removed rapidly, resulting in an exponential decline of the prey population in stage 2 (Fig. 6B). In one experiment, at a *P. piscicida* growth rate of one doubling per day, *Rhodomonas* sp. concentrations declined from 20,000 to 10,000 cells $\cdot$ mL^{-1} within 10 min and down to 5000 cells $\cdot$ mL^{-1} in 20 min (Fig. 6B). After 50 min, >90% of *Rhodomonas* were removed. Integration of the exponential decay function fitted to the dynamics of *Rhodomonas* sp. gave an average concentration of about 5000 cells $\cdot$ mL^{-1}. For the experiments conducted at three *P. piscicida* growth rates (0.5, 0.75, and 1 doubling $\cdot$ d^{-1}), grazing rates ranged from 1 to 8 Rhod $\cdot$ Pfiest^{-1} $\cdot$ d^{-1} and averaged 1.94 ± 0.42, 2.44 ± 0.46, and 4.61 ± 1.44 Rhod $\cdot$ Pfiest^{-1} $\cdot$ d^{-1}, respectively.

In the SC Batch culture system, *P. piscicida* growth and final population density depended directly on the amount of prey provided daily (Fig. 7A). In treatment R1 (*Rhodomonas* sp. concentration adjusted to 100 cells $\cdot$ mL^{-1} daily), *P. piscicida* did not grow. A small increase in the *P. piscicida* population was attained by the daily prey supply of 1000 cells $\cdot$ mL^{-1} (R2). The population increase was more remarkable when prey supply was 10^4 and 10^5 cells $\cdot$ mL^{-1} (R3 and R4, respectively) (Fig. 7A). *Pfiesteria piscicida* concentrations reached steady state after 8 days, except for R4, which

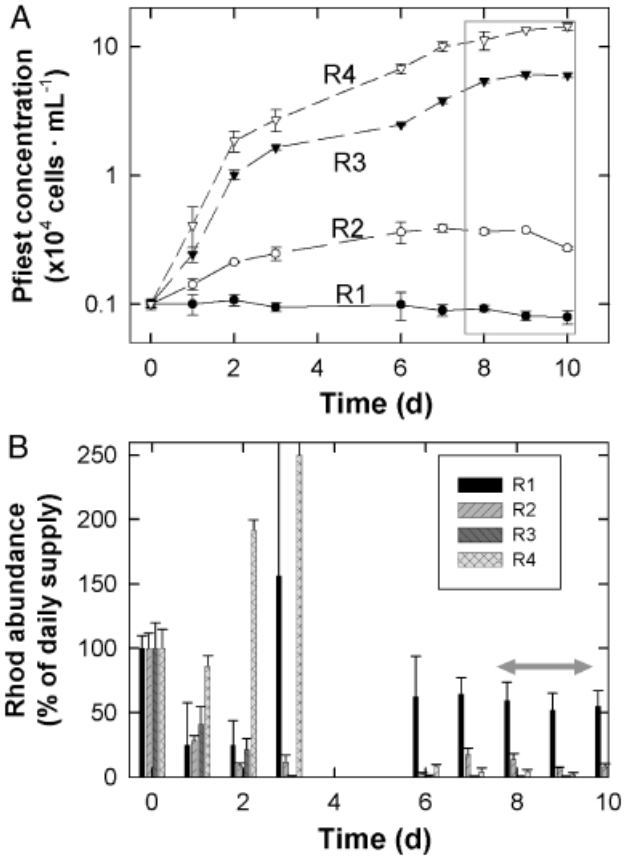

FIG. 7. Dynamics of *Pfiesteria piscicida* (Pfiest) and *Rhodomonas* sp. (Rhod) in the SC Batch culture system. Prey was replenished daily to maintain constant levels in each treatment: R1, 100 cells · mL^{-1}; R2, 1000 cells · mL^{-1}; R3, 10,000 cells · mL^{-1}; R4, 100,000 cells · mL^{-1}. Error bar is SD from the triplicates. (A) Pfiest growth curves under the four feeding conditions; rectangle box indicates the period during which steady state was assumed and the time for which average maximum Pfiest concentrations (carrying capacity) were calculated for each prey supply level (shown in Fig. 10). (B) Rhod concentrations as percent of the daily ration. The apparently high percentage in R1 on days 6–10 represents a low prey concentration ($\sim$ 50 cells · mL^{-1}). Arrowed bar indicates the steady-state period shown in A.

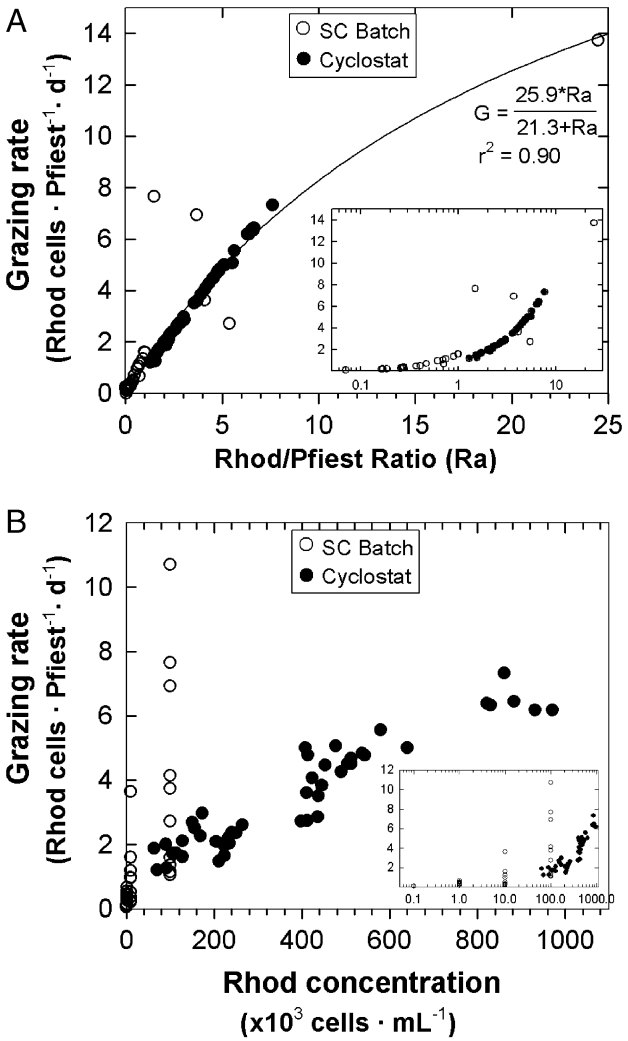

FIG. 8. *Pfiesteria piscicida* grazing rate under different prey-to-predator ratios (A) and daily prey supplies (B). (A) The correlation between grazing rate (G) and prey-to-predator ratio (Ra) best fit the Michaelis-Menten function as depicted by the regression line and equation. Shown are original data pooled from SC Batch and cyclostat cultures. (B) The correlation between grazing rate and Rhod concentration exhibits some difference between the two culture systems (see text for more details). Insets, redrawn plots using log-scale for the abscissas to show the wide range of prey conditions.

continued to increase slowly. *Pfiesteria piscicida* cell concentrations from day 8 through day 10 averaged 844 ± 69, 3377 ± 71, $58{,}267 \pm 2167$, and $130{,}104 \pm 9615$ cells · mL^{-1}, respectively, for R1 through R4.

Population dynamics of *Rhodomonas* sp. within *P. piscicida* cultures are shown in Figure 7B. During the first 4 days, most of the prey was grazed in R1 through R3, whereas R4 experienced net growth of *Rhodomonas* sp. Subsequently, as *P. piscicida* cell concentrations increased, *Rhodomonas* sp. became essentially depleted within a day of its resupply in all treatments. For the lowest prey concentration, although about 50% of the prey supply remained, prey concentrations were very low, <50 cells · mL^{-1}. Grazing rates in these cultures ranged from 0.1 to 10.7 Rhod · $Pfiest^{-1} \cdot d^{-1}$ and averaged 0.07 ± 0.04, 0.41 ± 0.14, 0.96 ± 1.18, and 4.14 ± 3.74 Rhod · $Pfiest^{-1} \cdot d^{-1}$, respectively, for R1, R2, R3, and R4.

Functional and numerical responses in the cyclostat and the SC Batch cultures. *Pfiesteria piscicida* grazing rates increased with prey concentration and prey-to-predator ratio (Fig. 8). Under the prey conditions used, maximum grazing rate was 10.7 Rhod · $Pfiest^{-1} \cdot d^{-1}$. Among the different models tested, the commonly used M-M model seemed to best fit the prey-to-predator ratio data pooled from the SC Batch and the cyclostat experiments, although the resulting asymptote lacked sufficient data support (Fig. 8A). A maximum grazing rate of 25.9 Rhod · $Pfiest^{-1} \cdot d^{-1}$ resulted from regression analysis ($r^2 = 0.90$), which might be a conservative estimate, considering the weak data support for the asymptote. The apparent

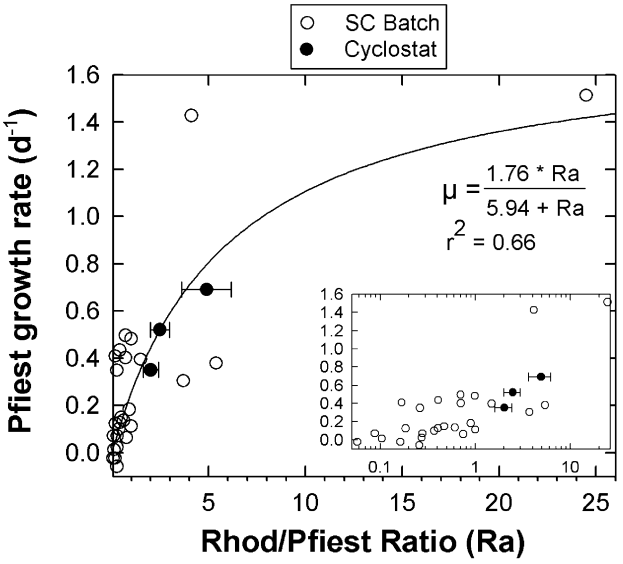

FIG. 9. *Pfiesteria piscicida* growth rate under different prey-to-predator ratios. Inset: Redrawn plot using log-scale for the abscissa to show the wide range of prey-to-predator ratios. Data from the SC Batch culture are original, whereas those from the cyclostat are the means and SDs (error bars) from multiple (5–10) data points obtained for each fixed growth rate.

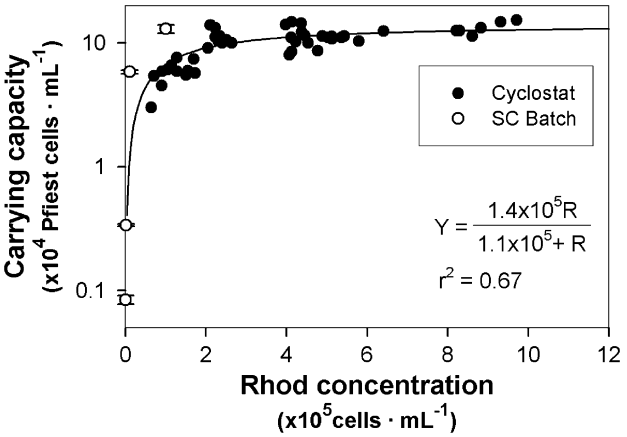

FIG. 10. Maximum *Pfiesteria piscicida* concentration (carrying capacity) for each level of daily prey supply. Data originate from both the steady state cyclostat system and SC Batch cultures; data points for SC Batch cultures are mean ± SD (error bar) derived from triplicates and multiple observations made during the steady-state period shown in Figure 7. The equation (Monod model) indicates the regression line.

half-saturation coefficient from the same regression was 21.3. Imposing a threshold prey-to-predator ratio on the regression analyses, that is, replacing R_a in the correlation equation in Figure 8A with $(R_a - b)$, decreased the correlation coefficient remarkably (r^2 from 0.9 to 0.7), and the resulting b value was negative. Forcing a linear or an exponential regression also yielded no positive intercept. Therefore, no significant feeding threshold was apparent. Correlation between grazing rate and prey concentration was less consistent between the SC Batch and the cyclostat experiments (Fig. 8B). Grazing rates increased faster at lower prey concentrations in the SC Batch cultures than in the cyclostat.

Pfiesteria piscicida growth rate also increased consistently with prey-to-predator ratio and prey concentration. Similar to grazing rates, growth rates from both SC Batch and cyclostat experiments fit an M-M (commonly referred to as Monod in the case of growth rate) model in relationship with prey-to-predator ratio (Fig. 9), but the asymptote also lacked strong data support. Although the highest observed specific growth rate was 1.5 d^{-1}, the Monod model predicted a maximum rate of 1.76 d^{-1} (and a half-saturation prey-to-predator ratio of 5.94; $r^2 = 0.66$), which might again represent a conservative estimate, provided the data did not offer strong support for the asymptote. Regression of growth rate with prey concentration was less strong due to wider scatter of data (not shown).

Maximum Pfiesteria piscicida *population density.* The average steady-state *P. piscicida* concentration (after three generations in the cyclostat and during days 8–10 in the SC Batch culture) was considered the carrying capacity at the provided prey abundance. The carrying capacity increased with prey supply following a Monod model (Fig. 10), with a rapid increase at low prey concentration and an asymptote after prey concentration exceeding 2×10^5 cells $\cdot$ mL^{-1}, suggesting some intrinsic maximum growth capacity. The Monod function resulting from pooled data predicted a half-saturation prey concentration of 1.1×10^5 cells $\cdot$ mL^{-1} and a maximum carrying capacity (i.e. potential population maximum) of 1.4×10^5 cells $\cdot$ mL^{-1} ($r^2 = 0.67$).

DISCUSSION

Prey dependence of Pfiesteria piscicida *growth.* *Pfiesteria piscicida* blooms have been observed in areas adjacent to animal farms in Maryland and North Carolina, leading to the supposition that *P. piscicida* outbreaks are linked to nutrient inputs (Burkholder and Glasgow 1997, Burkholder et al. 2001). However, because this organism preys on other algae, it has been unclear whether nutrient enrichment stimulates *P. piscicida* growth directly or through enhanced growth of algal prey. Earlier reports of kleptoplastidic photosynthetic capability (Lewitus et al. 1999) suggested that *P. piscicida* could grow photoautotrophically and thereby possibly benefit directly from inorganic nutrient inputs. However, the photosynthetic capability was later found to be limited and to depend on the continuous presence of chloroplast donors, that is, the prey algae (Feinstein et al. 2002), suggesting that photoautotrophic growth by this species may be rare. Observations and numerical analyses of the relationship between *P. piscicida* growth and *Rhodomonas* sp. availability in this study support the hypothesis that nutrients stimulate *P. piscicida* growth indirectly by fostering the growth of algal prey species. These results also indicate that higher population maxima (carrying capac-

ity) of *P. piscicida* can be attained when appropriate prey is supplied at high rates.

Strong dependence of *P. piscicida* on the supply of algal prey is further supported by the modulation of cell cycle behavior by prey availability. For eukaryotes, cells undergo G1 (1N genome status), S (DNA replication or synthesis phase), G2 (2N genome status), and M (mitosis) phases before cell division, and entry into the S phase usually indicates commitment of the cells to division (Murray and Hunt 1993). The fraction of *P. piscicida* cells in the S phase was elevated whenever *Rhodomonas* sp. was provided as prey. After prey depletion, advancement of *P. piscicida* cells into the S phase decreased rapidly, in parallel with a reduction in cell size (an indicator of cellular growth) (after 48 h) and the arrest of a large fraction of cells in the G1 and G2 phases. Theoretically, zygotes formed during sexual reproduction also contain 2N or more DNA sets (Parrow and Burkholder et al. 2002) and could be compounded with G2 + M cells in our analysis. However, there is no evidence that starvation induces sexual reproduction in *P. piscicida* (Litaker et al. 2002). It is therefore most likely that the 2N cells observed under starvation in this study represented G2 + M cells. Although these G1- and G2 + M-arrested cells could theoretically be temporary cysts (Litaker et al. 2002, Parrow et al. 2002), no cysts were discovered during microscopic examination of the starved samples. Rapid recovery of cell cycle progression upon refeeding indicates that prey availability modulates the cell division cycle of *P. piscicida* closely.

Effects of Pfiesteria piscicida *grazing on prey populations.* Previous studies showed that *P. piscicida* is capable of grazing on phytoplankton (Burkholder et al. 2001). The array of feeding experiments in this study revealed that *P. piscicida* populations could not continue to grow without a resupply of prey and that both the algal prey and the grazer populations could not rebound without additional seed populations. This result contrasts classical prey–predator oscillations documented for micrograzers (Shertzer et al. 2002, Yoshida et al. 2003), including a *Pfiesteria*-like dinoflagellate (CCMP1828; unpublished data). The lack of effect of *P. piscicida* filtrate on the growth of *Rhodomonas* sp. noted in this study excluded the possibility of prey mortality or growth inhibition from toxic agents possibly produced and released by *P. piscicida*. Grazing by *P. piscicida* seemed to annihilate a prey population. This rapid and voracious feeding behavior is corroborated by video observations demonstrating swift clearance of prey by *P. piscicida* cultures. Our video recording (available at http://www.blackwellpublishing.com/products/journals/suppmat/jpy/jpy03217/jpy03-217 clip.avi) showed that a *P. piscicida* cell, using its peduncle to acquire the cellular contents of the prey, could consume one *Rhodomonas* sp. in less than a minute (not shown), a rate similar to a *Crypthecodinium*-like peduncle-dependent feeding dinoflagellate (Ucko et al. 1997) but two to three times faster than in the mixotrophic gymnodinioid, *Karlodinium micrum* (Li et al. 1999).

The exponential decline of prey was observed consistently in various *P. piscicida* cultures. In the LT Batch cultures, prey was generally depleted within 1 to 5 days, depending on the supply level. In some cases, prey depletion was observed within 8 to12 h (Feinstein et al. 2002). In addition, results from both the SC Batch and the cyclostat cultures revealed a maximum grazing rate of 10.7 Rhod cells $\cdot$ Pfiest^{-1} $\cdot$ d^{-1}, whereas the M-M function derived from the data predicted a maximum rate of 25.9 Rhod cells $\cdot$ Pfiest^{-1} $\cdot$ d^{-1}. Assuming *P. piscicida* and *Rhodomonas* sp. C contents of 206 and 48 pg $\cdot$ cell^{-1}, respectively (Feinstein et al. 2002), the maximum grazing rate amounted to 0.51–1.24 ng C $\cdot$ Pfiest^{-1} $\cdot$ d^{-1} or a specific grazing rate of 2.5–6.0 d^{-1}, which exceeds the *P. piscicida* specific growth rate markedly. This range of rates is higher than many reported maximum ingestion rates for other dinoflagellates. For instance, *Protoperidinium pellucidum* grazed at a maximum rate of 0.14 to 0.78 cells $\cdot$ h^{-1}, depending on the type of prey (Jeong and Latz 1994, Buskey 1997). *Gymnodinium* sp. fed *Synechococcus* sp. exhibited a maximum carbon-specific grazing rate of 0.41 h^{-1} (Strom 1991). Comparable grazing rates have been reported only for *Oxyrrhis marina*, which ingested as many as 28 *Amphidinium carterae* cells $\cdot$ d^{-1} (Jeong et al. 2001), and a *Crypthecodinium*-like peduncle-dependent heterotrophic dinoflagellate, which exhibited a maximum grazing rate of 20 prey cells $\cdot$ d^{-1} (Ucko et al. 1997).

Prey thresholds for grazing have been observed for other dinoflagellates. *Gymnodinium* sp. exhibited a threshold prey concentration of 17 to 19.8 μg C $\cdot$ L^{-1} (Strom 1991, Jakobsen and Hansen 1997), equivalent to 350–410 Rhod cells $\cdot$ mL^{-1}. Higher grazing threshold concentrations (50–1250 μg C $\cdot$ L^{-1}) have been documented for other dinoflagellates and protozoan grazers (Strom 1991). In contrast, no threshold prey concentration (or prey-to-predator ratio) was apparent for *P. piscicida*. This apparent lack of prey threshold might allow *P. piscicida* to deplete the prey efficiently and hence account for the demise of *Rhodomonas* sp. in the LT Batch cultures, which is reminiscent of an "imprudent" type of grazer (Slobodkin 1974).

In the natural estuarine environment where *P. piscicida* may co-occur with potential prey algae as well as competing micrograzers, prey depletion as observed in the simplistic culture systems used in this study may not occur. However, the results from this study suggest that *P. piscicida* is a voracious grazer, which can exert considerable impact on prey populations (Lessard 1991, Jacobson 1999, Jeong 1999, Stoecker 1999). The influence of predators on the dynamics of their prey has been documented for *Polykrikos kofoidii* preying on *Gymnodinium catenatum* (Matsuyama et al. 1999) and *Protoperidinium* spp. grazing on other dinoflagellates and diatoms (Jeong and Latz 1994, Buskey 1997). However, the significance of *P. piscicida* as a grazer in natural ecosystem needs to be further assessed with

thorough studies of its *in situ* grazing rates and abundances.

Growth and cell cycle characteristics as a potential survival strategy. A population maximum of *P. piscicida* of 1.4×10^5 cells $\cdot$ mL^{-1} was predicted by the Monod function. However, some batch cultures in our laboratory reached cell concentrations as high as 2×10^5 cells $\cdot$ mL^{-1} (data not shown). The maximum observed (1.4 d^{-1}) and predicted (1.76 d^{-1}) growth rates for *P. piscicida* are also high compared with other dinoflagellates. Although *Oxyrrhis marina* can grow at a similar rate (1.17 d^{-1}; Jeong et al. 2001), a rate of 0.9 d^{-1} observed for *Polykrikos kofoidii* was considered unusually high for dinoflagellates (Matsuoka et al. 2000). The maximum growth rate ranged from 0.31 to 0.84 d^{-1} in *Gymnodinium* spp. (Bjørnsen and Kuparinen 1991, Strom 1991) and from 0.35 to 0.48 d^{-1} in *Protoperidinium* spp. (Jeong and Latz 1994, Naustvoll 2000). *Diplopsalis lenticula* grows at rates up to 0.25 d^{-1} (Naustvoll 1998). In concert with its high grazing potential, the high growth potential of *P. piscicida* might provide a competitive advantage by allowing cells to exploit pulses of prey efficiently to achieve large population increases within a short time period.

The cell cycle arrest of *P. piscicida* at both G1 and G2 + M phases after a period of prey deficiency is uncommon among eukaryotic microalgae (Vaulot 1995) but has also been observed in *Oxyrrhis marina* (Whiteley et al. 1993). The cell cycle of photosynthetic dinoflagellates and other eukaryotic phytoplankton tends to be arrested at G1 in response to nutrient deficiency (Vaulot 1995) with few exceptions (e.g. G2-arrest in silicate-starved diatoms; Brzezinski et al. 1990). Whether the difference is related to trophic mode remains to be investigated, but the arrest of the *P. piscicida* cell cycle at both G1 and G2 + M suggests that growth in these stages probably relies on an external source of carbon or energy. *Pfiesteria piscicida* cultures starved for 9 days reentered the S phase and resumed cell division quickly (within 1 day) after prey was resupplied, suggesting that these organisms may be able to survive short periods of starvation while arrested in the cell cycle. Arrest at both G1 and G2 + M phases under prey depletion and rapid resumption of cell division upon prey replenishment might provide survival advantage in an environment where prey supply is discrete. Rapid *P. piscicida* population response to supply and removal of fish and other prey observed earlier (Burkholder and Glasgow 1997) might be associated with the cell cycle behavior noted here.

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