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Clearance rates of < 6 μm fluorescently labeled algae (FLA) by estuarine protozoa: potential grazing impact of flagellates and ciliates

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ABSTRACT: Microzooplankton, numerically dominated by phagotrophic ciliates and flagellates, can have a significant grazing impact on phytoplankton, especially on ultraplanktonic (< 5 μm) algal cells, which are responsible for a large part of primary production in many pelagic systems. However, there are few analyses of actual in situ clearance rates of this size class of phytoplankton by natural protozoan assemblages. We estimated clearance rates of < 6 μm sized algal prey by heterotrophic flagellates and ciliates present in tidal creek water in a salt marsh estuary during the months of December 1989 to March 1990 via their rates of uptake of fluorescently labeled algae (FLA). Three types of FLA, ranging in size from 1.9 to 5.4 μm, were made from cultures of *Nannochloris atomis, Chlorella capsulata,* and *Thalassiosira pseudonana.* Both ciliates and flagellates ingested the FLA at rates greater than they ingested similarly sized fluorescent microspheres. Flagellates 5 to 10 μm in size predominantly ingested the 2 μm FLA, while oval cells 10 to 15 μm in size, likely athecate dinoflagellates, were important consumers of 3.4 and 5.4 μm FLA. Estuarine ciliates, mostly choreotrichs 15 to 60 μm in size, ingested all types of FLA, but with lower clearance rates for the 2 μm FLA than for larger sized FLA. Clearance rates determined for flagellates ranged from 0.004 to 0.83 μl $cell^{-1}$ h^{-1}, and for ciliates from 0.24 to 8.3 μl $cell^{-1}$ h^{-1}. Estimated daily clearance rate by algivorous protozoa over the 4 mo period averaged 45 % of the water volume based on uptake of the 2 μm sized FLA, and 107 % of the water volume based on uptake of the larger sized FLA. The average grazing impact of flagellates was about 33 % that of ciliates for 2 μm sized prey, and 50 and 85 % that of ciliates for 5.4 and 3.4 μm sized prey respectively. Two experiments were carried out to determine the functional feeding response of algivorous flagellates and ciliates over an order of magnitude range in concentration of each FLA type. The 2 groups of protozoa had differing clearance-rate and FLA-ingestion-rate responses; clearance rates generally decreased with increase in FLA concentration. The results support the ideas that (1) total microzooplankton herbivory is a combination of *partial* grazing pressures by individual species on different size classes of phytoplankton, and (2) the importance of algivory by < 20 μm sized phagotrophic flagellates should be considered in future studies.

INTRODUCTION

A large part of phytoplankton standing stock biomass and productivity in the sea is due to ultraphytoplankton, i.e. phototrophic cells between 0.5 and 5 μm in size (Murphy & Haugen 1984, Stockner & Antia 1986, Li & Wood 1988). Since most metazooplankton, e.g. micro-crustaceans, cannot effectively ingest cells < 3 to 5 μm in size (Bartram 1980, Sherr et al. 1986b, Turner et al. 1988), phagotrophic protozoa are presumed to be the major consumers of ultraphytoplankton. Laboratory studies of the grazing capabilities of pelagic ciliates, including tintinnids and aloricate species, have shown that ciliates are adapted to graze 2 to 10 μm sized algal cells at high rates (Heinbokel 1978, Heinbokel & Beers 1979, Rassoulzadegan 1982, Gifford 1985, Verity 1985, 1988, Jonsson 1986). More recently, laboratory experiments have also demonstrated that colorless flagellates, including *Paraphysomonas* spp., *Pseudobodo* sp., and *Oxyrrhis marina*, are also capable of ingesting and supporting rapid rates of growth on a variety of algae (Goldman & Caron 1985, Parslow et al. 1986, Suttle et al. 1986, Goldman et al. 1989).

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So far there is very little field data on the actual grazing impact of in situ protozooplankton on phytoplankton. Most studies to date have focused on pelagic ciliates, and the majority of these have been laboratory determinations of the ingestion rates and food selectivity of individual species of ciliates (Heinbokel 1978, Verity 1985, 1988, Jonsson 1986, Stoecker 1986). Reports of tintinnid consumption of phytoplankton production in coastal waters range from 4 to 60 % (Sherr et al. 1986b). Microzooplankton, which is often reported as being numerically dominated by aloricate ciliates, has been estimated to graze between 10 and 80 % of primary production in various marine environments (Beers & Stewart 1971, Takahaski & Hoskins 1978, Capriulo & Carpenter 1980, Burkill et al. 1987, Rassoulzadegan et al. 1988). Evidence for the importance of heterotrophic dinoflagellates as grazers of algae in marine systems is also accumulating (Smetacek 1981, Lessard & Swift 1985, Buck et al. 1990).

Here we report estimates of clearance rates of 1.9 to 5.4 µm sized algal cells by phagotrophic ciliates and flagellates in estuarine water, based on protozoan uptake of added fluorescently labeled algae (FLA) made from 3 species of cultured phytoplankton. Our results provide data about which components of the protozoan assemblage are potential consumers of phytoplankton in this size range, on the cell-specific clearance rates of small algae by in situ protozoa, and on the relative importance of phagotrophic flagellates versus ciliates as grazers of < 6 µm phytoplankton cells.

MATERIALS AND METHODS

Preparation of FLA. We followed the procedure of Rublee & Gallegos (1989), adapted from the method we had previous described for preparing fluorescently labeled bacteria (FLB) (Sherr et al. 1987), to make FLA from marine clonal algal cultures. Axenic cultures of the marine algae *Nannochloris atomis* (clone GSBNANNO), *Chlorella capsulata* (clone UTEX LB2074), and *Thalassiosira pseudonana* (clone NEPCC58) were obtained from the Provasoli-Guillard Marine Phytoplankton collection. Each algal species was grown up to a moderately dense culture (10^6 to 10^8 cells ml^{-1}), harvested via centrifugation, and heat-killed and stained with the fluorescent dye 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF) (Rublee & Gallegos 1989). The FLA preparations were divided into 1 ml aliquots and stored at −20 °C, in 10 % dimethyl sulfoxide (DMSO, to minimize cell damage due to freezing) in the pyrophosphate buffer. The ability to store the FLA preparations for months in this way greatly simplifies the use of FLA, since new preparations do not have to be made at the beginning of each experimental series. At the beginning of an experiment, stock solutions of FLA were thawed and diluted 1:10 with pyrophosphate buffer to make a working stock. Immediately before adding FLA, the working stock was sonicated with two or three 1 s bursts at a 30 W power level to disperse clumps of FLA.

The lengths and widths of 50 cells from each preparation of FLA were determined at 2000 × in order to compute the average biovolume of each type of FLA, using the equation for a prolate spheroid to estimate individual cell volumes.

Protozoan algivory experiments. We carried out experiments to determine rates of clearance of FLA by protozooplankton in water of a tidal creek in the Duplin River estuary, Sapelo Island, Georgia, USA, over a 4 mo period in winter 1989–90. In one of the experiments, we compared uptake of FLB and FLA with uptake of similarly sized fluorescent microspheres by both flagellates and ciliates. In the other experiments, we analysed uptake of various types of FLA. In two of these experiments, we also determined protozoan clearance rates as a function of concentration of FLA made from *Nannochloris atomis, Chlorella capsulata*, and *Thalassiosira pseudonana.*

FLA uptake experiments followed procedures developed for analysis of FLB uptake by pelagic protozoa (Sherr et al. 1989). Water samples were collected at slack low or high tide from the surface water of a tidal creek in the upper Duplin River. Samples were returned to the lab, gently screened through 20 µm Nitex netting to remove macrozooplankton, and 200 ml aliquots poured into Whirl-pak bags which had been previously soaked in 10 % HCl and copiously rinsed. The bags were put into 1 l plastic beakers half full of the estuarine water sample and then placed in an incubator at in situ temperature. After 30 to 60 min to allow the protozoa to recover from handling shock, aliquots of FLA were gently stirred into the samples. Subsamples (20 ml) were taken every 5 to 10 min for periods of 20 to 30 min and preserved with sequential addition of alkaline Lugol solution (0.5 % final concentration), borate buffered formalin (3 % final concentration), and a drop of 3 % sodium thiosulfate to clear remaining discoloration from the Lugol (Sherr et al. 1989). This procedure minimizes egestion of food vacuole contents by flagellates, and in our experience preserves the shape of flagellates and ciliates better than does formalin preservation alone (Sherr et al. 1988, Sherr et al. 1989). Samples were stored in glass vials at 5 °C in the dark until microscopic analysis could be carried out, normally within a few days. There was no apparent fading of FLA fluorescence in the stored samples.

Aliquots (10 ml) of the preserved samples were stained with diamidinophenylindole (DAPI) (Porter &

Feig 1980), filtered onto 0.8 μm Nuclepore-black polycarbonate filters, and inspected via epifluorescence microscopy (Sherr et al. 1987) in order to determine protozoan abundance and average number of FLA cell^{-1} at each time point. Ca 20 to 50 ciliates, and 50 to 100 flagellates, were inspected for FLB ingestion in each sample. FLA uptake rates were calculated for ciliates and flagellates from the change in average no. FLA cell^{-1} with time over the linear portion of the uptake curve via linear regression analysis (Sherr et al. 1989). FLA were enumerated on the same filters at 1250 × to determine FLA concentration in each sample. From this information, per-cell hourly clearance rates were calculated for flagellates and ciliates (Sherr et al. 1989). From our previous work using FLB, we have determined that the overall coefficient of variation, as determined by propagation of indeterminant error, is about 50 to 60 % for such protozoan grazing rate experiments (Sherr et al. 1989). The per-cell clearance rates were multiplied by the empirically determined abundance of flagellates or ciliates in estuarine water, and by 24 h, to estimate daily clearance of FLA by the entire assemblages of flagellates and ciliates. Enumeration of in situ algal cells in the size range of 2 to 6 μm was also made for each sampling date.

In one experiment, a comparison was made of the clearance rate of 3 sizes of fluorescent microspheres (FM; Polysciences, Inc.) with the clearance rates of similarly sized fluorescently labeled prey (FLP): FLB (made from a culture of *Escherichia coli;* Gonzalez et al. 1990), FL-*Nannochloris,* and FL-*Chlorella.* Six 200 ml subsamples of 20 μm screened estuarine water were prepared as for the grazing experiments: to individual samples we added 1.1 μm FM or 1.1 μm FLB at a concentration of 3×10^5 ml^{-1}, 2.4 μm FM or 1.9 μm FL-*Nannochloris* at a concentration of 3×10^4 ml^{-1}, and 3.4 μm FM or 3.4 μm FL-*Chlorella* at a concentration of 1×10^4 ml^{-1}. After addition of FM and FLP, a time series of samples was taken from each bag, and the samples were preserved and analysed for protozoan clearance rates per hour as described above.

In 2 experiments, we investigated the functional feeding response (clearance rate and rate of prey ingestion) of phagotrophic flagellates and ciliates to variations in concentration of the 3 types of FLA. In the first experiment, the functional feeding response of assemblages of estuarine flagellates and ciliates was determined over a concentration range of 0.3 to 2.6 × 10^4 FLA ml^{-1} of FL-*Nannochloris* and of 0.14 to 1.2 × 10^4 ml^{-1} of FL-*Chlorella*. In the second experiment, the feeding response of in situ protozoa was determined over a concentration range of 0.11 to 1.5 × 10^4 ml^{-1} of FL-*Thalassiosira*. The experimental protocol and analysis of clearance rates were as described above.

RESULTS

Preparation of FLA

In general, we obtained good results with the procedure of Rublee & Gallegos (1989) for preparation of FLA. Both *Nannochloris atomis* and *Chlorella capsulata* stained brightly and were resistant to fragmentation during sonication. *Thalassiosira pseudonana* did not stain as well, perhaps owing to the higher lipid content which is characteristic of diatoms, since DTAF binds specifically to proteins. No chlorophyll *a* (chl *a*) autofluorescence was observed in the FLA. The average size of FLA made from the 3 species of algae was 1.9 μm (3.4 μm^3) for FL-*Nannochloris,* 3.4 μm (20 μm^3) for FL-*Chlorella* and 5.3 μm (80 μm^3) for FL-*Thalassiosira*. These sizes were somewhat smaller than those of the living algal cells. There was no apparent cell damage due to freezing and thawing for any of the FLA preparations stored at −20 °C in DMSO-pyrophosphate buffer solution.

Characterization of the microbial assemblage in sampled water during the study

During the study, the temperature of the sampled water varied between 12 °C in January and 19 °C in March. Numbers of 2 to 6 μm sized autotrophic cells in the water samples ranged from 360 to 3900 cells ml^{-1}. On 16 March, besides 2400 cells ml^{-1} of 2 to 6 μm sized algae, there was a bloom (2500 cells ml^{-1}) of a 7 to 9 μm long (100 to 170 μm^3) chlorophyte which appeared to be ingested by the same classes of protozoa which ingested FLA. Total phytoplankton standing stocks were not determined; however, from a previous study of primary production in the Duplin River, chl *a* concentrations during winter and spring are in the general range of 0.5 to 4 μg l^{-1} (Haines, Whitney & Pomeroy unpubl.).

Phagotrophic flagellates and ciliates were abundant in all samples. Ciliates up to 60 μm in length passed through the 20 μm mesh screen. Some fraction of larger sized ciliates may have been lost during the screening process, though in our previous work in the Duplin River, ciliates larger than about 30 to 40 μm are typically a small fraction of the total ciliate assemblage (Sherr et al. 1986a, Gonzalez et al. 1990). Although the assemblage of colorless flagellates was numerically dominated by < 5 μm sized cells, 5 to 20 μm flagellates were always present at concentrations of 10's to 100's per ml. In December, a 6 to 9 μm (size of fixed cells) irregularly spherical nonpigmented cell was common (500 to 1200 ml^{-1}) in tidal creek water. During most of the 4 mo study, 10 to 15 μm colorless oval cells were also present (20 to

200 ml^{-1}). The phagotrophic ciliate assemblage (2 to 20 cells ml^{-1}) was dominated by a variety of morphological types of choreotrichous ciliates. Most of the ciliates appeared to be aloricate, in the genera *Strobilidium* and *Strombidium*, as previously reported for these waters (Gonzalez et al. 1990). A large fraction of ciliates in each sample were mixotrophic, i.e. contained sequestered chloroplasts (Stoecker et al. 1989). The mixotrophic ciliate *Laboea strobilia* was incidentally noted in some samples in March, but was not abundant. The autotrophic ciliate *Mesodinium* spp. was present in most samples, but was never observed to ingest FLA and was not included in the data.

Comparison of clearance rates of FM and FLA by estuarine protozoa

FM as well as nonfluorescent latex beads have been used to determine clearance rates of differently sized particles for both ciliates (Fenchel 1980, Borsheim 1984, Jonsson 1986) and flagellates (McManus & Fuhrman 1986); however, subsequent studies comparing protozoan uptake of inert particles and similarly sized prey organisms have generally found lower clearance rates for inert particles (Stoecker et al. 1986, Sherr et al. 1987, Stoecker 1988). We compared the uptake of 3 different size categories of FM and FLP. For the purpose of this study, only larger (> 5 µm) flagellates were inspected, since lower rates of uptake of FM vs FLB have already been reported for bacterivorous flagellates in these waters (Sherr et al. 1987). The results (Table 1) indicate that use of FM yields lower clearance rates for both > 5 µm flagellates and ciliates. Only incidental uptake of FM was observed in > 5 µm flagellates, resulting in undetectable clearance rates, while clearance rates of 0.02 to 0.29 µl $cell^{-1}$ h^{-1} were obtained using FLP (Table 1). For ciliates, measured clearance rates using FM were about half the rates obtained using the same concentration of similarly sized FLP (Table 1).

Table 1. Comparison of clearance rates of fluorescent microspheres (FM) and of fluorescently labeled prey (FLP) by assemblages of flagellates and ciliates in estuarine water. 1.1 µm FM and *Escherichia coli* added at 3×10^5 ml^{-1}; 2.4 µm FM and FL-*Nannochloris* added at 3×10^4 ml^{-1}; 3.4 µm FM and FL-*Chlorella* added at 1×10^4 ml^{-1}. n: negligible

Particle type	Particle size (µm)	Clearance rate (µl $cell^{-1}$ h^{-1})	
		> 5 µm Flagellates	Ciliates
FM			
	1.1	n	0.82
	2.4	n	0.33
	3.4	n	0.86
FLP			
E. coli	1.1	0.02	0.17
Nannochloris aromis	1.9	0.11	0.67
Chlorella capsulata	3.4	0.29	1.8

Clearance rates of estuarine flagellates and ciliates measured using the 3 types of FLA

Clearance rates calculated for in situ phagotrophic flagellates and ciliates, both on an hourly per-cell basis and as daily clearance of the water volume by the entire assemblage of flagellates or ciliates, are presented in Tables 2, 3 & 4 for FL-*Nannochloris*, FL-*Chlorella*, and FL-*Thalassiosira* respectively. Actual concentrations of added FLA in the experiments ranged from 0.67 to 5.8×10^4 ml^{-1} for FL-*Nannochloris*, 0.25 to 1.5×10^4 ml^{-1} for FL-*Chlorella*, and 0.11 to 3.1×10^4 ml^{-1} for FL-*Thalassiosira*. Rates of clearance of FLA ranged from negligible to 0.02–0.83 µl $cell^{-1}$ h^{-1} for > 5 µm flagellates, and from 0.24 to 8.3 µl $cell^{-1}$ h^{-1} for ciliates (Tables 2 to 4). Rates of clearance were lowest, and frequency of negligible uptake by flagellates greatest, for FL-*Nannochloris* (Table 2), compared to results for the other 2 types of FLA (Tables 3 & 4). The daily clearance rates determined for the assemblages of FLA-ingesting flagellates and ciliates in the water were a function both of average per-cell clearance rate and of cell abundances. Estimated daily clearance varied from negligible to between 1 and 160 % of the water volume per day for flagellate assemblages, and from 2 to 330 % of the water volume per day for ciliate assemblages (Tables 2 to 4). The average flagellate assemblage clearance rate estimated for the 3 types of FLA varied from 0.11 ± 0.16 to 0.56 ± 0.50 d^{-1}; the average ciliate assemblage clearance rate varied from 0.34 ± 0.60 to 0.65 ± 0.93 d^{-1} (Tables 2 to 4).

Flagellates < 5 µm in size did not take up any of the 3 types of FLA. In our previous studies, the majority of FLB-ingesting flagellates have been < 5 µm in size. Most of the flagellates ingesting the 2 µm FLA were cells 5 to 10 µm in size, and the majority of these appeared to be irregularly shaped cells with no observable flagella. These cells were less common in the latter part of the study, which accounts for the negligible grazing rates on FL-*Nannochloris* by flagellates for most of the later sampling dates. The oval, 10 to 15 µm cells only rarely took up FL-*Nannochloris*, but did ingest the other 2 FLA at respectable rates. Fig. 1 shows 2 of the 10 to 15 µm oval cells with ingested FL-*Chlorella*.

Ciliates ingested FLA in all of the experiments. For the 10 sampling dates (3 Jan 1990 to 7 Mar 1990) on

Table 2. Clearance rates, per cell and per assemblage, of FL-*Nannochloris* (FL-N) by estuarine flagellates and ciliates. n: negligible

Date	FL-N Concentration ($\times 10^4$ ml^{-1})	Per-cell rates (µl cell^{-1} h^{-1})		Per-assemblage rates (ml ml^{-1} d^{-1})	
		Flagellates	Ciliates	Flagellates	Ciliates
1989					
6 Dec	1.3	0.017	0.59	0.29	0.06
7 Dec	4.8	0.015	0.77	0.36	0.11
11 Dec	4.6	0.007	0.75	0.22	0.24
11 Dec	4.8	n	0.44	n	0.08
12 Dec	5.8	n	0.45	n	0.23
1990					
3 Jan	3.6	0.11	0.67	0.41	0.18
5 Jan	4.0	n	1.8	n	0.27
9 Jan	3.8	0.004	0.240	0.01	0.04
10 Jan	4.2	0.012	0.25	0.44	0.02
11 Jan	4.1	n	0.84	n	0.08
18 Jan	1.9	n	1.9	n	0.80
30 Jan	0.83	n	6.2	n	2.5
8 Feb	0.67	n	2.6	n	0.29
5 Mar	1.25	n	1.0	n	0.09
7 Mar	1.15	n	2.4	n	0.22
Average daily clearance				0.11 ± 0.16	0.35 ± 0.60

Table 3. Clearance rates, per cell and per assemblage, of FL-*Chlorella* (FL-C) by estuarine flagellates and ciliates. n: negligible

Date (1990)	FL-C Concentration ($\times 10^4$ ml^{-1})	Per-cell rates (µl cell^{-1} h^{-1})		Per-assemblage rates (ml ml^{-1} d^{-1})	
		Flagellates	Ciliates	Flagellates	Ciliates
3 Jan	1.0	0.29	1.8	1.1	0.20
5 Jan	1.0	0.30	3.0	0.09	0.43
9 Jan	1.2	0.16	1.3	0.56	0.11
10 Jan	1.5	0.42	1.2	1.6	0.11
11 Jan	1.1	0.32	2.1	0.93	0.21
18 Jan	0.60	n	2.9	n	1.2
30 Jan	0.42	n	8.3	n	3.3
8 Feb	0.25	0.83	5.0	0.40	0.29
5 Mar	0.46	0.19	3.7	0.22	0.33
7 Mar	0.57	0.25	3.8	0.72	0.36
Average daily clearance				0.56 ± 0.50	0.65 ± 0.93

Table 4. FL-*Thalassiosira*. Clearance rates, per cell and per assemblage, of FL-*Thalassiosira* (FL-T) by estuarine flagellates and ciliates

Date (1990)	FL-T Concentration ($\times 10^4$ ml^{-1})	Per-cell rates (µl cell^{-1} h^{-1})		Per-assemblage rates (ml ml^{-1} d^{-1})	
		Flagellates	Ciliates	Flagellates	Ciliates
9 Mar	3.1	0.09	1.8	0.05	0.37
9 Mar	0.46	0.25	4.6	0.14	0.82
16 Mar	0.11	0.26	1.7	0.17	0.32
20 Mar	0.28	0.14	1.1	0.63	0.40
Average daily clearance				0.25 ± 0.22	0.48 ± 0.20

which clearance rates of both FL-*Nannochloris* and FL-*Chlorella* were obtained (Tables 2 & 3), the average ciliate clearance rate of FL-*Nannochloris*, 1.8 ± 1.7 µl cell^{-1} h^{-1}, was ca half of the average ciliate clearance rate of FL-*Chlorella*, 3.3 ± 2.0 µl cell^{-1} h^{-1}. The average ciliate clearance rate of FL-*Thalassiosira* for the 4 dates measured (Table 4) was 2.3 ± 1.3 µl cell^{-1} h^{-1}. Variations in clearance rate were also influenced by

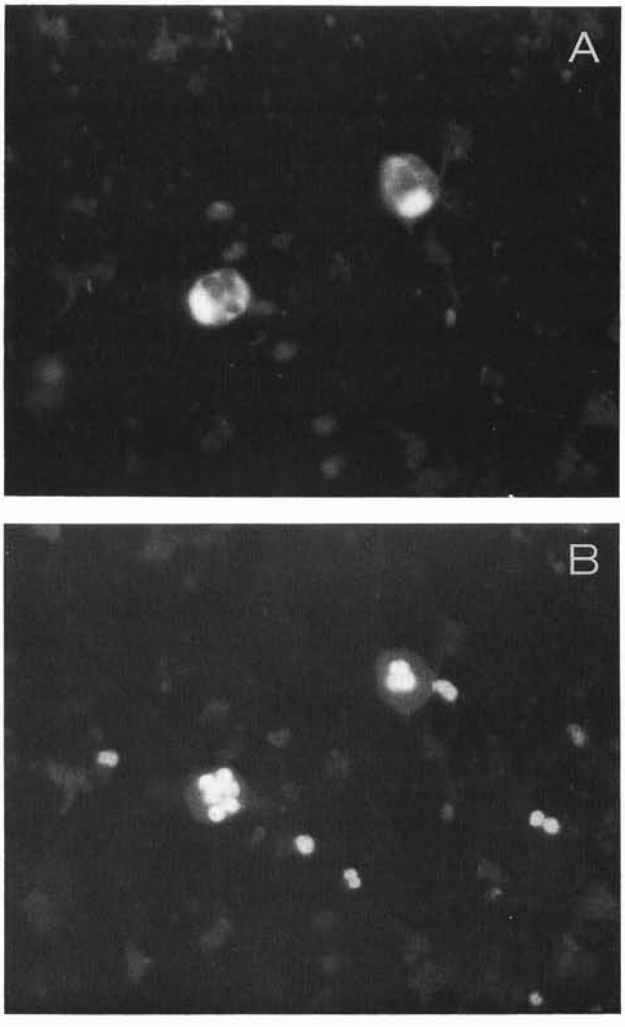

Fig. 1. Micrographs of two 12 μm long heterotrophic flagellates in water of a salt marsh tidal creek, which had ingested 3.4 μm FL-*Chlorella*. (A) Flagellate cells visualized by DAPI staining, using UV filter set; (B) DTAF-stained FLA in the flagellates' food vacuoles, visualized using blue light filter set. Note that the ingested FLA appear brighter than the non-ingested FLA scattered around the flagellates

the composition of the ciliate assemblage, since smaller ciliates typically clear water at lower rates than do larger ciliates (Fenchel 1980, Jonsson 1986). For example, the ciliate assemblage in tidal creek water on 18 and 30 January was dominated by an average of 17 cells ml^{-1} of a 50 to 60 μm long (1.8 to 3.8 × 10^4 μm^3, fixed volume) mixotrophic *Strombidium* sp., which had high rates of clearance for both FL-*Nannochloris* and FL-*Chlorella*. This bloom of ciliates resulted in the highest calculated daily clearance rates for both types of FLA (Tables 2 & 3). However, it should be noted that there was about a 3-fold difference in per-cell clearance rate determined for this *Strombidium* sp. on the 2 dates. On the other sampling dates, the ciliate assemblage consisted of a variety of cell types and sizes.

Change in clearance rates of estuarine flagellates and ciliates in response to variation in FLA concentration

In order to determine how sensitive protozoan clearance rates were to abundance of added FLA, we carried out 2 experiments using tidal creek water in which we varied over 1 order of magnitude the concentration of FLA added. In the first experiment, on 8 February, FL-*Nannochloris* was added at 0.3 to 2.6 × 10^4 FLA ml^{-1} and FL-*Chlorella* at 0.14 to 1.2 × 10^4 FLA ml^{-1}. The natural abundance of 2 to 5 μm algae in the water was 400 cells ml^{-1}. The assemblage of >5 μm flagellates was dominated by the 10 to 15 μm oval cell at a concentration of 20 ml^{-1}. The ciliate assemblage was composed of the 50 to 60 μm mixotrophic *Strombidium* sp. which had been dominant on the previous 2 sampling dates, along with a tintinnid and a 20 to 30 μm diam spherical *Strobilidium* sp., with a total ciliate abundance of 2.4 cells ml^{-1}. The second experiment was carried out on 16 March to assess clearance rates as a function of concentration of FL-*Thalassiosira*, over the range of 0.1 to 1.5 × 10^4 FLA ml^{-1}. The algivorous protozoa in this sample were the 10 to 15 μm oval flagellates, 27 cells ml^{-1}, and a mixed assemblage of choreotrichs, 8 cells ml^{-1}. In the first experiment, we obtained data on the 2 types of FLA for the ciliates, but only on FL-*Chlorella* for the flagellates, as the flagellates did not take up FL-*Nannochloris*.

Clearance rates varied for both the ciliates and the flagellates over the range of FLA concentrations (Fig. 2A, B). Clearance rates increased as prey concentration decreased, until at some critical level of prey density the clearance rates showed a marked decrease (Fig. 2A, B). The variation in clearance rates was especially dramatic for the flagellates, ranging from 0.2 to 0.8 μl $cell^{-1}$ h^{-1} (Fig. 2B). The particle uptake rate also changed with changing prey density (Fig. 2C). For the ciliates, there was a decrease in rate of FLA uptake as the FLA concentration decreased, while the flagellates appeared to show saturated feeding until a critical FLA density of ca 2500 ml^{-1}, after which the uptake rate of FLA $cell^{-1}$ h^{-1} decreased (Fig. 2C). Where the concentration ranges of the 2 FLA overlapped, both clearance rates and particle uptake rates were lower for the ciliates grazing FL-*Nannochloris* than for those grazing FL-*Chlorella* (Fig. 2A, C).

In the second experiment, ciliate clearance rates varied between 1.5 and 2.1 (average 1.8 ± 0.2) μl $cell^{-1}$ h^{-1} over the range of FL-*Thalassiosira* added (Fig. 3A). Flagellate clearance rates were constant at 0.1 μl $cell^{-1}$ h^{-1} at the 3 highest concentrations of FLA, then increased to 0.26 μl $cell^{-1}$ h^{-1} at the lowest concentrations (Fig. 3A). The particle uptake rates of both ciliates and flagellates decreased with decreasing FLA concentration, with no evidence of saturated feeding over the range of FLA added (Fig. 3B).

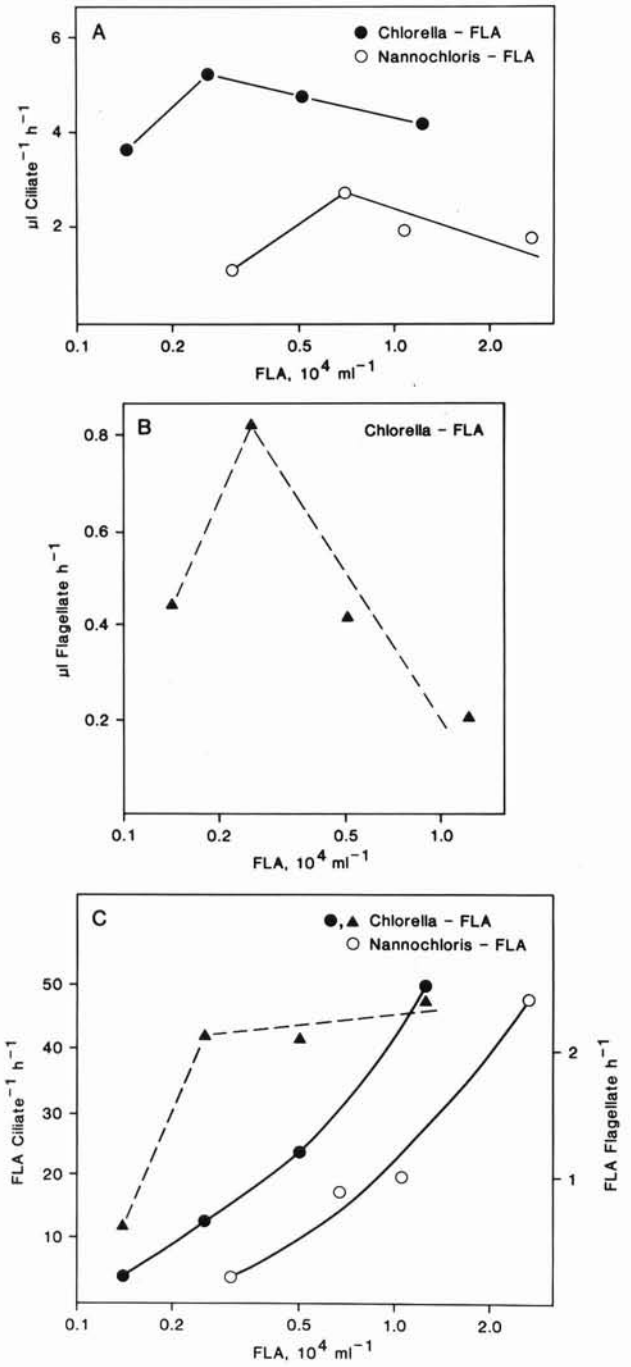

Fig. 2. Functional feeding responses of estuarine protozoa. (A) Clearance rates of ciliates as a function of the concentration of FL-*Chlorella* and FL-*Nannochloris*. (B) Clearance rates of 10 to 15 µm long heterotrophic flagellates as a function of FL-*Chlorella* concentration. (C) FLA ingestion rates by algivorous ciliates (circles) and flagellates (triangles) as a function of concentration of FL-*Chlorella* or FL-*Nannochloris* (note different scales for ciliates and flagellates)

Examination of the relation of ciliate clearance rates as a function of the concentration of FL-*Nannochloris* and FL-*Chlorella* added for the routine experiments

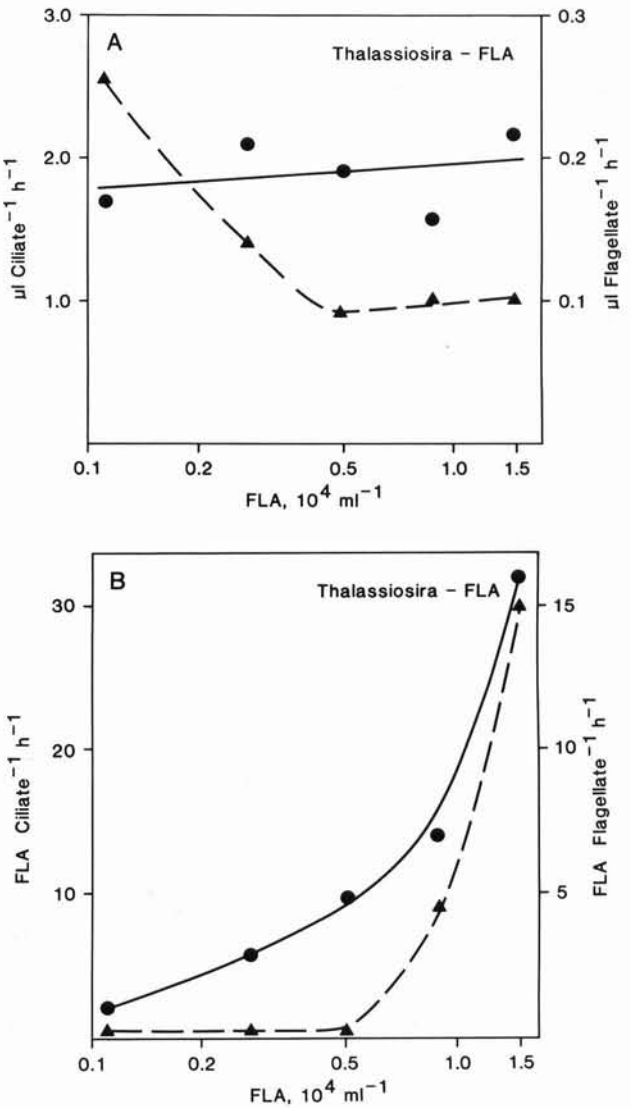

Fig. 3. Feeding responses of estuarine ciliates (●) and 10 to 15 µm flagellates (▲) as a function of concentration of FL-*Thalassiosira*. (A) Clearance rates; (B) FLA ingestion rates. (Note different scales for ciliates and flagellates)

carried out during the study (Fig. 4A, B) also indicated a general decrease in clearance rate with increase in prey concentration. However, there was no strong relation between 10 to 15 µm flagellate clearance rate and concentration of FL-*Chlorella* for the routine experiments (Fig. 4C).

DISCUSSION

Use of FLA in estimating rates of protozoan algivory in situ

Although in theory live algae are already naturally fluorescently tagged with chl *a* autofluorescene, there

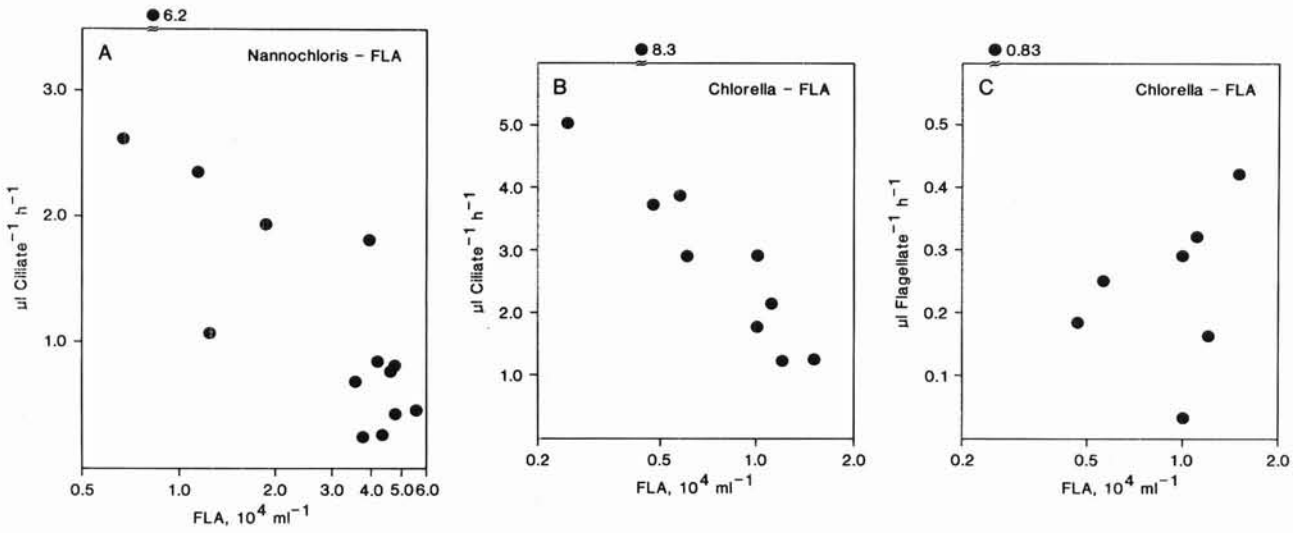

Fig. 4. Relation of clearance rate to concentration of FLA added during the 4 mo of routine experiments. (A) Ciliate clearance rates vs FL-*Nannochloris* concentration. (B) Ciliate clearance rates vs FL-*Chlorella* concentration. (C) Flagellate (10 to 15 µm) clearance rates vs FL-*Chlorella* concentration

are several advantages to using FLA instead of live algae in studies of in situ protozoan algivory. FLA can be used off-the-shelf, whereas algal cultures have to be maintained and grown up to suitable densities prior to an experiment. Based on our past experience with FLB (Sherr et al. 1988), the DTAF fluorescence of ingested FLA should be visible in protozoan food vacuoles until the algal cell is mostly digested, while chl *a* autofluorescence may rapidly degrade after an algal cell is ingested (Sieracki et al. 1987). FLA can be easily recognized as a tracer, while added live algae may be confused with phytoplankton already present in the sample. Finally, a large percent of pelagic ciliates contain highly autofluorescent sequestered chloroplasts (Stoecker et al. 1989). We have found that the green-yellow fluorescence of DTAF-stained FLA is easily visible in mixotrophic ciliates, but the chl *a* autofluorescence of live algae would be difficult to discern in such cells.

The 3 types of FLA used in this study were readily ingested by a variety of pelagic ciliates and flagellates present in estuarine water samples taken over a 4 mo period. The lack of uptake of FM by >5 µm flagellates, and the lower clearance rates of FM compared to those of FLP (Table 1), indicate that FLA are superior to FM as an analogue for live phytoplankton. However, protozoa can show differential uptake of live compared to dead prey cells (Stoecker 1988). A comparison of clearance rates of FLA and live phytoplankton remains to be done, although such a study will have to overcome the problems with the use of live algal prey discussed above. Higher rates of FLA uptake were obtained with FL-*Chlorella* and FL-*Thalassiosira* than with FL-*Nannochloris*. The smaller cell size of FL-*Nannochloris* may have contributed to lower clearance rates by species of flagellates and ciliates which had a larger optimum prey size (Jonsson 1986, Goldman & Dennett 1990).

In our previous studies using FLB to estimate clearance by in situ protozoa of bacterial-sized prey, we were able to add FLB at tracer concentrations of 5 to 30 % of the standing stock of bacterioplankton and get reasonable rates of FLB ingestion per cell over short time periods (Sherr et al. 1989). In contrast, adding such tracer concentrations of FLA is problematical, due to the disparity between the order-of-magnitude differences in bacterioplankton vs phytoplankton standing stocks and the differences in protozoan grazing rates on the 2 size classes of prey. Bacterial concentrations in our estuarine waters are on the order of 10^6 to 10^7 cells ml^{-1}, while concentrations of 2 to 6 µm algae measured in this study were about 4 orders of magnitude lower, i.e. 10^2 to 10^3 cells ml^{-1}. Previous estimates of clearance rates of FLB have been on the order of 10^{-3} µl cell^{-1} h^{-1} for <5 µm flagellates and 10^{-2} µl cell^{-1} h^{-1} for ciliates in these waters (Sherr et al. 1989). The clearance rates of FLA determined in this study were only about 2 orders of magnitude greater: 10^{-1} µl cell^{-1} h^{-1} for >5 µm flagellates and 10^0 µl cell^{-1} h^{-1} for ciliates.

As a practical example of the experimental problems with adding tracer amounts of FLA, suppose estuarine protozoa were exposed to 500 FL-*Chlorella* ml^{-1}, and that ciliates and >5 µm flagellates cleared at rates of 3.0 and 0.3 µl cell^{-1} h^{-1}, respectively. After 20 min, which was the usual time to leveling off of the uptake curve found in this study, the ciliates would have on average 0.5 FLA cell^{-1}, and the flagellates only 0.05 FLA cell^{-1}. The time necessary to examine sufficient numbers of cells to generate adequate data for statisti-

cal treatment is prohibitive at such low FLA incorporation rates. Only when the abundance of phytoplankton is greater than several thousand ml^{-1} in situ can rates of protozoan algivory be easily determined in short-term experiments via addition of tracer (< 50 % of the natural abundance) concentrations of FLA. Assessment of algivory by determining disappearance rates of low concentrations of added FLA over longer time periods (24 h or more) would still be possible, although substantial changes in the protozoan assemblage could occur during such incubations.

Even with this limitation, use of FLA to assess short-term clearance rates of algivorous protozoa can provide valuable information on which components of the in situ protozoan assemblage are capable of ingesting various-sized prey particles, and on the relative magnitudes of the clearance rates of various protozoa types.

Clearance rates of FLA by estuarine flagellates and ciliates

As noted above, the per-cell clearance rates calculated for the algivorous protozoa in this study were 1 to 2 orders of magnitude higher than rates previously determined for bacterivorous flagellates and ciliates in these waters (Sherr et al. 1989). The higher rates can be at least partly explained by the larger cell size of algivorous flagellates (5 to 15 µm) and ciliates (15 to 60 µm) compared to that of bacterivorous flagellates (< 5 µm) and ciliates (< 20 µm) found in our earlier studies (Sherr & Sherr 1987, Sherr et al. 1989, Gonzalez et al. 1990), and by the lower abundances of phytoplankton compared to those of bacterioplankton. The average cell size of flagellates ingesting 2 µm FL-*Nannochloris* was about 8 µm, and that of flagellates ingesting 3.5 and 5.3 µm FLA was about 13 µm. This suggests size-dependent resource partitioning among the flagellate assemblage, with a predator:prey size ratio of ca 3:1 to 4:1 for the algivorous protozoa, based on inspection of fixed cells. Goldman & Caron (1985) reported a predator:prey size ratio of ca 2:1 for live cultures of a microflagellate, *Paraphysomonas imperforata*, fed several different types of phytoplankton.

The pelagic ciliates observed in our experiments did not show any apparent resource partitioning; the same morphological types of ciliate in the assemblage ingested all 3 types of FLA. However, ciliate clearance rates were always lower for FL-*Nannochloris* than for FL-*Chlorella* and FL-*Thalassiosira*, which may be related to differences in feeding efficiency by algivorous ciliates grazing various sizes of prey (Jonsson 1986). This idea is supported by the observation that similar morphological types of 15 to 25 µm *Strobilidium* spp., which had been found to clear FLB at rates of 0.1 to 0.4 µl $cell^{-1}$ h^{-1} (Sherr & Sherr 1987, Gonzalez et al. 1990), cleared FLA at rates of 0.5 to 3.0 µl $cell^{-1}$ h^{-1}. Phagotrophic ciliates also show behavioral responses to different types of prey, resulting in varying rates of ingestion (Verity 1988). The lower grazing rates of ciliates on FM compared to FLP (Table 1) found in this study further indicate that grazing by ciliates can be influenced by factors other than prey size.

In carrying out the first set of FLA-uptake experi-

Table 5. Comparison of clearance rates by marine algivorous flagellates and ciliates reported in various studies, based on uptake of inert particles, living phytoplankton, or fluorescently labeled algae

Protozoan	Prey density (no. cells ml^{-1})	Clearance rate (µl $cell^{-1}$ h^{-1})	Source
Flagellates			
Paraphysomonas imperforata	$\sim 10^6$	0.005–0.013	Goldman & Caron (1985)
Oxyrrhis marina	$\sim 10^6$	0.002–0.015	Goldman et al. (1989)
Pseudobodo sp.	10^5–10^6	0.001–0.002	Parslow et al. (1986)
5–10 µm flagellates, Georgia estuary	10^4	0.02–0.15	This study
10–15 µm flagellates, Georgia estuary	10^3–10^4	0.03–0.83	This study
> 20 µm colorless dinoflagellates, North Atlantic	In situ phytoplankton	0.5–28.3	Lessard & Swift (1985)
Ciliates			
Lohmaniella spiralis	In situ phytoplankton	2.3–8.9	Rassoulzadegan (1982)
Lohmaniella spiralis	10^4–10^5	1.6–13.4	Jonsson (1986)
Strombidium reticulatum	10^4–10^5	1.1–3.1	Jonsson (1986)
Strombidium vestitum	10^4–10^5	0.1–0.5	Jonsson (1986)
Eutintinnus pectinis, Helicostomella subulata	10^3–10^5	0.5–5.0	Heinbokel (1978)
Tintinnopsis vasculum, Tintinnopsis acuminata	10^3–10^5	0.1–7.5	Verity (1985)
15–60 µm ciliates, Georgia estuary	10^3–10^4	0.2–8.3	This study
> 20 µm ciliates, North Atlantic	In situ phytoplankton	1–213	Lessard & Swift (1985)

ments, we added excess FLA compared to the concentrations (360 to 3900 cells ml^{-1}) of 2 to 5 µm phytoplankton in estuarine water during the study. FL-*Nannochloris* was added at ca 3×10^4 ml^{-1}, and FL-*Chlorella* at 1×10^4 ml^{-1}, which represented approximately equal prey densities by volume. In subsequent experiments, we added lower concentrations of FLA to approximate those concentrations which resulted in the highest clearance rates found in the 2 feeding response experiments. It is possible that the results obtained in these latter experiments could represent maximum clearance rates for the protozoa, and thereby overestimate the actual in situ clearance rates. The data presented here should be considered as a potential grazing capability of the protozoan assemblages examined.

In Table 5, the per-cell clearance rates for >5 µm flagellates and choreotrichous ciliates determined in the present study are compared to literature values for clearance rates of algivorous protozoa in the field and of monospecific cultures of flagellates and ciliates fed various species of algae or inert particles. Even though the experimental methodology and prey concentrations differed among the studies, the clearance rates for algivorous ciliates were fairly similar. For the flagellates, however, clearance rates for laboratory-cultured species fed high concentrations of algae were 1 to 2 orders of magnitude lower than for the flagellate clearance rates determined in this study (Table 5). Lessard & Swift (1985) reported a wide range in clearance rates, including some very high values, for >20 µm dinoflagellates and ciliates taken from open ocean water, via analysis of incorporation of ^{14}C-labeled in situ phytoplankton.

Estimates of the potential daily clearance by assemblages of algivorous flagellates and ciliates in 20 µm screened estuarine water ranged from 5 to 250 % (average of 45 %) for 2 µm sized cells and from 42 to 330 % (average of 107 %) for 3.4 and 5.3 µm sized cells (Tables 2 to 4). The average daily clearance by the flagellate assemblage was 33, 85 and 52 % of that by the ciliate assemblage for FL-*Nannochloris*, FL-*Chlorella*, and FL-*Thalassiosira*, respectively (Tables 2 to 4).

Variation in protozoan clearance rate in response to differences in FLA concentration

In order to determine how sensitive the clearance rates of in situ algivorous flagellates and ciliates were to variation in FLA concentration, we carried out experiments with each of the FLA used. The results (Figs. 2 & 3) indicated that the flagellates and ciliates had different feeding response functions over the same range of prey densities. Data from the routine grazing experiments also indicated a general decrease in ciliate clearance rates with increasing concentrations of FL-*Nannochloris* and FL-*Chlorella* (Fig. 4A, B). Such a relationship is expected, based on theory of predator-prey interactions for suspension feeding (Fenchel 1980, Gallegos 1989), as well as on the empirical results of numerous laboratory experiments with algivorous protozoa (e.g. Heinbokel 1978, Goldman & Caron 1985, Verity 1985, Jonsson 1986, Stoecker 1988). However, there was no strong relation between clearance rate of 10 to 15 µm flagellates and concentration of FL-*Chlorella* observed in the routine experiments (Fig. 4C).

Variability of protozoan grazing rate has implications for measurement of algivory by microzooplankton via the dilution method (Landry & Hassett 1982). As Gallegos (1989) has demonstrated, the assumption of linear grazing response by microzooplankton over the range of prey dilutions is often violated. However, nonlinearity in feeding response can be accounted for when the results of dilution experiments are interpreted, so long as the feeding response functions are defined (Gallegos 1989). Our results support the idea that total grazing pressure by microzooplankton on phytoplankton is composed of a number of different partial grazing pressures by individual species of algivorous organisms, each of which may have a different functional feeding response to changes in prey concentration. Thus, the overall functional response curve of microzooplankton feeding on in situ assemblages of phytoplankton would be a complex combination of the responses of various protozoans to specific prey.

CONCLUSIONS

The use of FLA may yield either underestimates or overestimates of the actual clearance rates by field assemblages of algivorous protozoa, for several reasons. FLA cannot always be added at concentrations less than those of in situ phytoplankton in experiments to estimate short-term clearance rates. Besides the possible lowering of protozoan grazing rates due to higher prey density, adding high FLA concentrations compared to in situ phytoplankton concentrations could also result in overestimation of protozoan grazing rate, to the extent that the protozoa had been feeding on suboptimal levels of prey or were below threshold concentrations of prey. FLA are not perfect analogues of living phytoplankton, which may result in protozoan selection for or against FLA. Experiments comparing protozoan clearance rates of FLA to those of similar live algal prey should be done in future work, although it may be difficult to carry out short-term uptake experiments with live prey when working with natural planktonic assemblages and/or mixotrophic ciliates. Despite the potential shortcomings of FLA, the clearance rates

determined in this study for estuarine flagellates and ciliates compared favorably to those reported for algivorous protozoa in other studies (Table 5).

Combining hourly clearance rates per individual flagellate and ciliate with measured abundances of protozoans in the water samples, we estimated that, on average, the assemblages of algivorous protozoa present in our experimental samples could potentially consume about 45 % of the standing stock of 2 µm sized cells per day, and about 107 % of the standing stock of 3 to 6 µm sized cells per day. The fairly low abundances of 2 to 6 µm phytoplankton cells observed in tidal creek water during this study may thus be a result, at least in part, of protozoan grazing pressure. [Since small autotrophs are a dominant food resource for the oysters and ribbed mussels common in southeastern salt marshes (Dame et al. 1980, Kemp et al. 1990), bivalve filtration is also a source of mortality for phytoplankton in tidal creek water.] The notion that small-sized microplanktonic grazers (mostly protozoans) can be major grazers of phytoplankton in coastal waters is supported by a recent report of diurnal variations in phytoplankton standing stock in a North Carolina estuary, in which the authors concluded that grazing by <75 µm sized organisms was responsible for the largest portion of the observed nightly decrease in chl *a* (Litaker et al. 1988).

Flagellates, particularly oval cells in the size range of 10 to 15 µm (Fig. 1), were responsible for a substantial part of the estimated protozoan grazing pressure on 2 to 6 µm phytoplankton. These oval cells are probably nonthecate dinoflagellates. A similarly sized algivorous dinoflagellate, *Gymnodinium* sp., has been isolated from the North Pacific (Strom & Frost 1990). Another algivorous dinoflagellate in this size range is the ubiquitous *Oxyrrhis marina* (Goldman et al. 1989). Larger phagotrophic dinoflagellates are also common in marine systems (Smetacek 1981, Lessard & Swift 1985, Buck et al. 1990). The results of our study confirm speculations that algivorous flagellates, as well as algivorous ciliates, are consumers of phytoplankton biomass in pelagic food webs (Goldman & Caron 1985, Sherr & Sherr 1988).

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