Vol. 54: 209–219, 1989 | MARINE ECOLOGY PROGRESS SERIES
Mar. Ecol. Prog. Ser. | Published June 22

Simultaneous measurement of bacterioplankton production and protozoan bacterivory in estuarine water

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ABSTRACT: Simultaneous measurements were made of bacterioplankton productivity ([^{3}H]thymidine assay) and of bacterial mortality due to protozoan grazing (measured via uptake of fluorescently labeled bacterioplankton, FLB). Water samples were taken from a salt marsh tidal creek and from an estuarine sound near Sapelo Island, Georgia, USA at low tide over a 2 wk period in late summer. In control experiments performed to test the extent of selectivity of estuarine bacterivorous protozoa for or against FLB compared to natural bacterioplankton, we found no evidence for consistent discrimination. Rates of bacterial production and of protozoan bacterivory were greater in the tidal creek than in the open sound. Ciliates were responsible for the largest fraction of total protozoan consumption of bacteria in tidal creek water, and colorless flagellates in open estuary water. Bacterial production and protozoan bacterivory were not always in balance in individual samples, with the largest discrepancies in the open estuary. Estimated bacterivory was, on average, 80 % of bacterial production in the tidal creek and 50 % of production in the open estuary. Explanations for the measured shortfall in bacterial mortality include methodological problems with the assays used or alternate fates of bacterial production besides protozoan grazing.

INTRODUCTION

According to current theory, the relatively low and uniform cell concentrations of bacterioplankton in natural waters are a result of rapid predation by phagotrophic protozoa (Haas & Webb 1979, Fenchel 1982, Azam et al. 1983, Ducklow 1983, Sieburth 1984). Thus, bacterioplankton production should, on average, be balanced by bacterial mortality due to protozoan grazing. If this theory is correct, then by simultaneously measuring both parameters for a sufficient number of discrete samples taken at a particular sampling site, or within an individual water mass, the mean value for bacterial cell production and that for protozoan consumption of bacteria should be equivalent. Here we report the results of a 2 wk sampling program in the waters of a meso-eutrophic salt marsh estuary in late summer which was designed to test this idea.

The first problem in carrying out our study was to decide upon optimal techniques to measure the 2 parameters. The standard method for assaying bacterial production in natural waters is the tritiated thymidine (Tdr) uptake method (Fuhrman & Azam 1982, Riemann et al. 1982, Moriarty 1986). Although the validity of several of the basic assumptions of the Tdr technique are currently in question (Douglas et al. 1987, Hollibaugh 1988, Pedrós-Alió & Newell 1989), there is no better alternative at present. Moreover, the calibration factor for conversion of Tdr uptake into bacterial cell production has been empirically determined in our estuarine waters in several past investigations during late summer (Newell & Fallon 1982, Fallon et al. 1986, Riemann et al. 1987), thus we felt the method would yield reasonable results in this system.

There is at present no technique for determining rates of protozoan bacterivory which is as widely accepted as the Tdr uptake method. Methods most frequently used involve monitoring either rates of change in abundance of bacteria or bacterial analogues (e. g. *Escherichia coli* minicells) over time periods of 4 to 24 h, usually after manipulations to decrease protozoan grazing or bacterial production (Landry et al. 1984, Wright & Coffin 1984, Sherr et al. 1986a, Wikner et al. 1986), or rates of incorporation of bacterial analogues (fluorescent microspheres, bacteria stained with a fluorescent dye, or radiolabeled bacteria) by protozoa

0171-8630/89/0054/0209/$ 03.00

(Børsheim 1984, Lessard & Swift 1985, McManus & Fuhrman 1986, 1988a, b, Sherr et al. 1987).

In order to compare rates of protozoan bacterivory with rates of bacterial production estimated by the Tdr uptake method, the bacterial analogue uptake methods are preferable for 2 reasons: pre-assay manipulation of the water sample is kept to an absolute minimum; and incubation times for both the Tdr uptake and bacterial analogue uptake assays are comparable, >30 min, and are short relative to the population dynamics of bacteria and phagotrophic protozoa in situ. Because some protozoa show much lower rates of ingestion of inert microspheres compared to their uptake rates of bacteria (Pace & Bailiff 1987, Sherr et al. 1987, Nygaard et al. 1988), we used fluorescently labeled bacteria (FLB) (Sherr et al. 1987) prepared from natural bacterioplankton assemblages as the bacterial analogue for the protozoan grazing experiments. We also ran control experiments (1) to evaluate the effectiveness of several fixatives in preventing egestion of food vacuole contents by flagellates (Sieracki et al. 1987) and (2) to determine whether there is significant selection by estuarine protozoa for or against FLB compared to their uptake of live bacteria.

METHODS

Preparation of fluorescently labeled bacteria (FLB). Two separate batches of FLB were prepared from natural bacterioplankton in estuarine water collected at each of the 2 sampling sites (see below) at the beginning of the study. Bacterioplankton were concentrated from 20 l of each of the water samples after prefiltration through 0.8 µm Nuclepore membrane filters, using an Amicon hollow fiber filter apparatus, 0.1 µm pore size, and then heat-killed and stained with the fluorescent dye 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein (DTAF) as described by Sherr et al. (1987). The FLB were stored frozen in 2 ml aliquots, and thawed and briefly sonicated with three 1 s bursts at 30 W power level immediately prior to use. Since we have found that repeated cycles of freezing and sonication can lead to fragmentation of FLB, we recommend that FLB be stored frozen in small aliquots and checked for decrease in average cell size after 2 or 3 thawing-sonication cycles.

Experiments to evaluate effects of fixation on retention of FLB. Three different fixation methods were compared with respect to 2 parameters of flagellate FLB-uptake: FLB cell^{-1} and % of cells containing FLB. The parameters were also determined for live flagellates which were immobilized by addition of 0.5 % final volume $NiSO_4$. The fixation methods were: (1) addition of concentrated, tetrasodium borate-saturated formalin to the sample for a final concentration of 2 % formalin; (2) addition of 4 % ice-cold glutaraldehye volume/volume with sample for a final concentration of 2 % glutaraldehyde (R. Sanders pers. comm.); and (3) addition of alkaline Lugol solution (10 g iodine, 20 g potassium iodide, 10 g sodium acetate in 140 ml distilled water) for a final concentration of 0.5 %, followed by immediate addition of borate-buffered formalin for a final concentration of 3 %, which hardens the cells and clears the Lugol coloration (R. Rassoulzadegan pers. comm.).

Three different flagellate cultures were used to test the effect of the fixation methods on the FLB-uptake parameters: (1) a 3 to 4 µm diameter choanoflagellate species isolated from estuarine water and maintained on wheat-grain culture; (2) *Ochromonas* sp., clone IC_1 from the Provasoli-Guillard Culture Center, Boothbay Harbor, Maine, USA, a pigmented phagotroph maintained non-axenically on inorganic medium at low light; and (3) a mixed species assemblage of estuarine flagellates grown up in 5 µm screened water for 2 d after amendment with 1 mg l^{-1} of yeast extract to promote growth of bacterioplankton.

Two bacterial strains were isolated to make FLB for the experiments; one was obtained from tidal creek water, and the second one was isolated from actively growing *Ochromonas* cultures. Both bacterial strains were short rods. We grew up each isolate overnight on nutrient broth, and after harvesting and washing via centrifugation, prepared FLB from each (Sherr et al. 1987).

One experiment was carried out for each of the 3 types of flagellates. The tidal creek isolate-FLB were added to 100 ml of the choanoflagellate and mixed flagellate cultures, and the *Ochromonas* isolate-FLB to 100 ml of the *Ochromonas* culture, to yield a concentration of about 10^6 FLB ml^{-1}. After 1 h of incubation with FLB, 10 ml aliquots of the culture were fixed by each of the 3 methods described above. An additional aliquot of culture was gently poured into a clean 10 ml settling chamber to which 0.5 ml of 10 % $NiSO_4$ solution had been added. After 10 min, flagellates in the live, $NiSO_4$-treated sample were examined for ingested FLB at 1000× using a Nikon inverted microscope outfitted for epifluorescence microscopy. Flagellates were located by transmitted light, which was sufficiently dim that fluorescence of FLB could be detected. Five ml aliquots of the fixed samples were stained with diamidinophenylindole (DAPI) (Porter & Feig 1980), filtered onto 0.8 µm Nuclepore-black filters, and examined at 1250× as described by Sherr et al. (1987). For each treatment, 50 flagellates were examined for ingested FLB.

FLB selectivity experiments. Since the primary assumption of the FLB uptake assay is that protozoa do

not ingest added FLB either more or less readily than they do in situ bacterioplankton, we monitored the long-term disappearance of both FLB and live bacteria in the presence of protozoan grazers in 4 experiments. FLB were prepared from a natural bacterioplankton assemblage grown up in 0.6 µm screened water amended with 1 mg l^{-1} yeast extract. A mixed species assemblage of bacterivorous flagellates was obtained by incubating 5 µm screened estuarine water enriched with boiled wheat grains. We also isolated and cultured a 20 to 25 µm diameter bacterivorous choreotrich, tentatively identified as *Strobilidium conicum* (Maeda 1986). Bacterivorous flagellates were also present in the ciliate culture.

Aliquots (200 ml) of flagellate or ciliate culture were placed in duplicate 400 ml acid-soaked Whirl-pak bags. A combination of 200 mg l^{-1} vancomycin and 1 mg l^{-1} penicillin were added to inhibit growth of live bacteria (Sherr et al. 1986a). Tracer quantities of the cultured FLB were added, and the bags were incubated in the dark at 20 °C. Decreases in concentration of FLB and of live bacteria were monitored over periods of 18 to 24 h. FLB concentrations were determined by filtering 0.5 to 1 ml subsamples onto unstained 0.2 µm membrane filters. Subsamples of 100 ml were filtered onto 0.2 µm Nuclepore-black filters for enumeration of total bacteria by the acridine orange direct count (AODC) method of Hobbie et al. (1977). At the start of the experiments, concentrations of protozoa were determined as described in Sherr et al. (1987).

Field study. ***Sampling sites:*** Samples of water were collected at slack low tide for 10 d over a 2 wk period in September 1987, at 2 separate sites in the estuary adjacent to Sapelo Island, Georgia, USA. The first site was located in Post Office Creek, a tertiary tidal creek of the Duplin River Estuary, which is a tidal embayment draining a 1100 ha *Spartina alterniflora* salt marsh (Pomeroy & Wiegert 1981, Imberger at al. 1983). The second site was in the mouth of Doboy Sound, at the southern end of Sapelo Island. Previous studies at both locations have shown that during summer, the phagotrophic protozoan assemblage is dominated by small aloricate ciliates in tidal creek waters, and by colorless flagellates in waters of the open estuary (Sherr et al. 1984, Sherr & Sherr 1987). Water was collected into 4 l plastic jugs at a depth of about 10 cm below the surface. The jugs were capped and kept at in situ temperature in the dark until the water was processed in the laboratory, less than 1 h from time of sampling. Temperature and salinity (refractometer) were measured at each station when samples were taken.

Bacterial production and abundance: We followed the experimental protocol of Riemann et al. (1987) for determining cell production of bacterioplankton based on incorporation of [^{3}H]thymidine. Three 10 ml subsamples of water from each of the 2 stations were incubated with 20 n*M* of [methyl-^{3}H]thymidine (Tdr, ICN Radiochemicals) for 30 min. One subsample from each set received 0.5 ml of 30 %, borate-buffered formalin before addition of the Tdr to serve as a killed control. Incubations were stopped by addition of 0.5 ml of 30 % buffered formalin, and each sample was filtered onto a 0.45 µm Gelman GN6 membrane filter and washed with 5 aliquots of ice-cold 5 % TCA. Each filter was placed in a glass vial, dissolved in 1 ml of ethylacetate, and 10 ml of Aquasol was added as a scintillation cocktail. In order to convert Tdr uptake to cell production, we used the conversion factor, 1.1×10^{18} cells mol^{-1} thymidine incorporated into TCA precipitate (SE $= 0.5 \times 10^{18}$, $n = 63$) reported by Riemann et al. (1987), based in part on samples collected in waters around Sapelo Island. Total bacterial abundances were determined using 4 separate 100 to 300 µl aliquots of each water sample via the AODC method.

Protozoan grazing and abundance: Bacterial grazing experiments were run as follows: premeasured aliquots of estuarine water were poured into acid-soaked and rinsed 400 ml Whirl-pak bags and the bags were placed in several hundred ml of estuarine water in a 1 l plastic beaker. The bags were then placed in the dark in an incubator set at in situ temperature for 30 min to allow the protozoa to recover from handling shock. We had previously found a 10-fold difference in average clearance rate for bacterivorous flagellates and ciliates in these waters (2 to 10 nl $cell^{-1}$ h^{-1} for flagellates, 50 to 300 nl $cell^{-1}$ h^{-1} for ciliates; Sherr et al. 1986a, 1987, Sherr & Sherr 1987). Therefore we determined flagellate and ciliate FLB uptake rates in separate treatments in which we added tidal creek or open estuary FLB to equal either 30 %, for flagellates, or 5 %, for ciliates, of the total bacterial standing stock.

Duplicate bags were set up for each treatment, thus there were 4 bags for each time for each water sample. Owing to a lower total amount of FLB made from open estuarine bacterioplankton compared to the amount of FLB made from tidal creek bacterioplankton, quantities of water used in the experiments varied: for the open estuary site, 50 ml and 100 ml subsamples were used for flagellate and ciliate grazing experiments, respectively; and for the tidal creek site, 200 ml subsamples were used for all bags. Incubations were run in the dark at in situ temperature, averaged between the 2 sites, which were never more than 2 °C different.

Ten ml subsamples for protozoan enumeration and FLB ingestion determinations were taken at 0, 10 and 20 min after addition of FLB and fixed by adding 0.5 % final volume alkaline Lugol solution, immediately followed by 3 % final volume borate-buffered formalin to preserve the cells and decolorize the Lugol solution. This fixation treatment greatly reduces dissolution of

Table 1 Comparison of number of FLB cell^{-1} (mean ± 1 standard deviation) and of % of cells containing FLB determined for bacterivorous flagellates examined either live after treatment with $NiSO_4$ or fixed using 3 different methods. 50 flagellates were examined for each measurement

Fixative	Choanoflagellate sp.		*Ochromonas* sp.		Mixed flagellates	
	FLB cell^{-1}	% with FLB	FLB cell^{-1}	% with FLB	FLB cell^{-1}	% with FLB
Live, $NiSO_4$	3.3 ± 1.6	97	3.4 ± 2.2	82	–	–
Formalin	4.0 ± 2.8	88	0.8 ± 1.9	15	3.9 ± 6.6	36
Glutaraldehyde	3.7 ± 1.9	94	2.4 ± 3.3	61	7.5 ± 7.3	74
Lugol + formalin	4.5 ±2.4	94	3.3 ± 2.9	80	7.6 ± 8.0	68

naked ciliates in samples preserved with formalin alone (Sherr et al. 1989), as well as preventing flagellate egestion of food vacuole contents (Table 1).

Five ml aliquots of the preserved samples were stained with DAPI, filtered onto 0.8 µm Nuclepore filters, and inspected via epifluorescene microscopy (Sherr et al. 1987) in order to determine total protozoan counts and average number of FLB per cell at each time point. A total of 20 to 50 ciliates or flagellates were inspected for FLB ingestion in each sample. FLB uptake rates were calculated for ciliates and flagellates from the change in average number of FLB cell^{-1} with time using simple regression analysis (Statpak Onevreg program; Northwest Analytical Inc., Portland, Oregon USA). Subsamples of 0.5 to 1.0 ml were also filtered onto 0.2 µm Nuclepore membrane filters to determine the average number of FLB ml^{-1}. From this information, per cell clearance rates were calculated for flagellates and ciliates, as described in Sherr et al. (1987), and multiplied by the in situ abundance of flagellates and ciliates to estimate total protozoan grazing.

RESULTS

Effect of fixation on retention of FLB by flagellates

Two of the fixation methods, ice-cold glutaraldehyde and Lugol plus formalin, yielded values for both parameters of FLB-uptake comparable to those obtained for live flagellates for both the choanoflagellate sp. and the *Ochromonas* sp. (Table 1). Choanoflagellates fixed with formalin alone also had comparable values for FLB cell^{-1} and % of cells with ingested FLB, but these parameters were lower, than with other treatments, for formalin-fixed *Ochromonas* sp. (Table 1). For the mixed flagellates, we could not find enough $NiSO_4$-treated cells to obtain data for live cells, however the FLB-uptake parameters were similar for both glutaraldehyde and Lugol plus formalin fixed samples, while the values for FLB cell^{-1} and % with FLB were much lower in samples fixed with formalin alone (Table 1). Since there was little difference among treatments for the choanoflagellate sp., but marked differences between formalin fixation and other treatments for the *Ochromonas* sp. and mixed flagellates, it is apparent that the effect of fixation on retention of food vacuole contents varies with flagellate species, Both the ice-cold glutaraldehyde and Lugol plus formalin fixation methods gave good results for all 3 flagellate cultures.

FLB selectivity experiments

The initial concentrations of live bacteria, FLB, and protozoa for each of the experiments are presented in Table 2. Both bacterial and protozoan abundances were comparable to those previously measured in our local estuarine waters (Sherr et al. 1984, 1986a). In three of the experiments, the percent decrease in both types of FLB was about the same as the decrease in growth-inhibited live bacteria (Table 2). In the other

Table 2. Long-term (18 to 25 h) percent decrease in numbers of live bacteria and of FLB $[(T_o-T_f)/T_o \times 100]$ in protozoan cultures treated with vancomycin and penicillin to inhibit growth of live bacteria. A and B represent values of duplicate bags

Expt	Abundance of:				% decrease in bacterial numbers			
	Bacteria (10^6 ml^{-1})		Flagellates	Ciliates	Live		FLB	
	Live	FLB	(10^3 ml^{-1})	(ml^{-1})	A	B	A	B
1	11.2	2.8	31	–	51	52	30	28
2	16.4	1.4	6.3	–	37	38	41	51
3	5.5	0.6	1.3	83	36	41	31	54
4	6.7	1.5	2.0	90	39	44	48	49

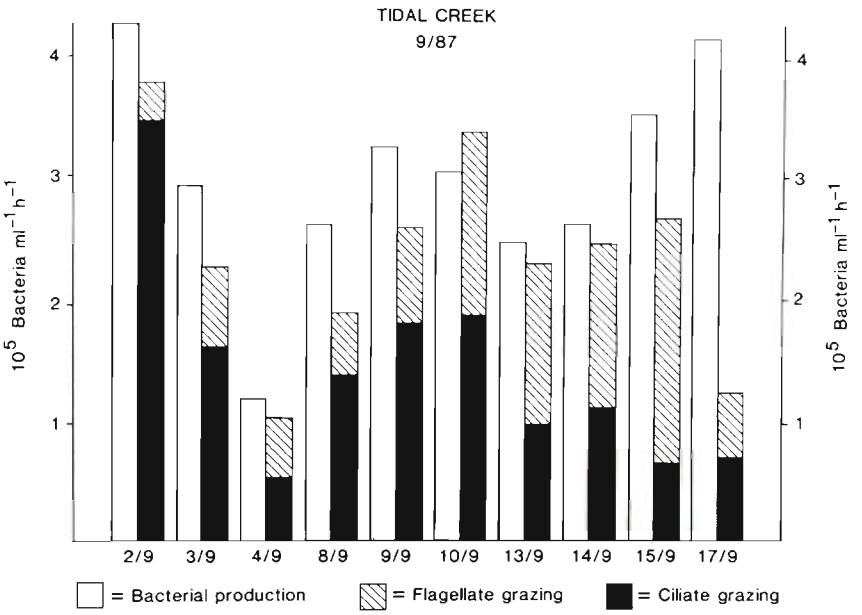

Fig. 1. Comparison of bacterial production and bacterial mortality due to protozoan grazing measured simultaneously in tidal creek water for 10 discrete sampling dates from 2 to 17 September 1987. Parameters are expressed as 10^5 bacteria produced or consumed $ml^{-1} h^{-1}$. Stippled bars represent bacterial production; total protozoan bacterivory is divided into ciliate bacterivory (dark portion of bar) and flagellate bacterivory (hatched portion of bar)

experiment (Expt 1), in which the only protozoan grazers were flagellates, the percent FLB decrease was only about 60 % of the observed percent decrease in the live, growth-inhibited bacteria.

Field study: comparison of bacterial production and protozoan bacterivory

Temperature (tidal creek: 26.3 to 31.0 °C, open estuary: 28.0 to 30.0 °C) and salinity (tidal creek: 18.3 to 20.1 ‰, open estuary: 23.5 to 29.5 ‰) were fairly constant at each station during the 10 d sampling period. No major rainstorms, which can significantly alter both parameters, as well as bacterial production, especially in marsh tidal creeks (Imberger et al. 1983, Sherr et al. 1986b, Pedrós-Alió & Newell 1989) occurred during this time.

Comparison of estimated rates of bacterioplankton production and of protozoan grazing on bacteria for each discrete sampling date are shown in Fig. 1 and 2. Total protozoan bacterivory is divided into ciliate grazing and flagellate grazing. The average coefficient of variation for duplicate estimates of ciliate and flagellate

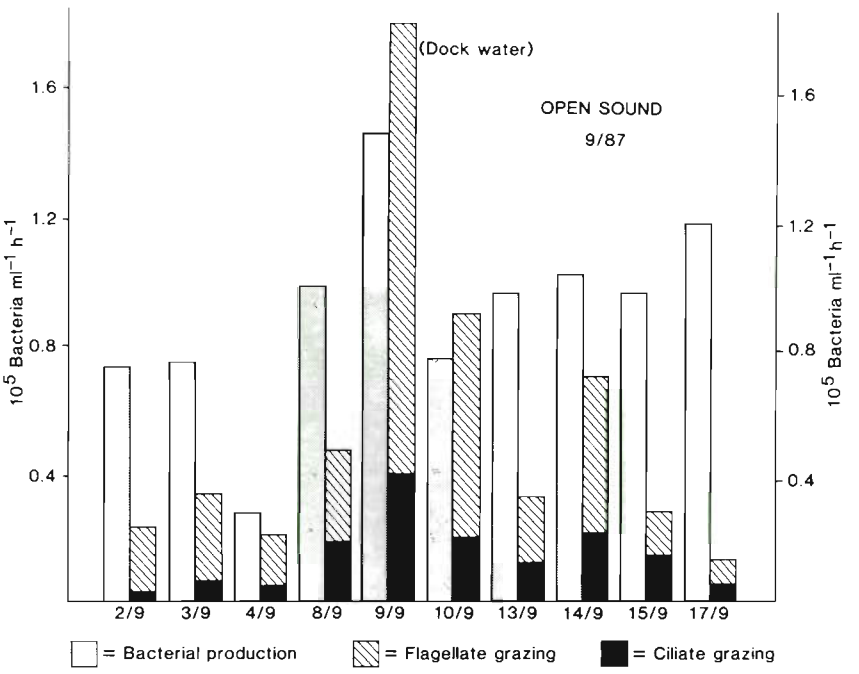

Fig. 2. Comparison of bacterial production and bacterial mortality due to protozoan grazing measured simultaneously in open sound water for 10 discrete sampling dates from 2 to 17 September 1987. Parameters are expressed as 10^5 bacteria produced or consumed $ml^{-1} h^{-1}$. Stippled bars represent bacterial production; total protozoan bacterivory is divided into ciliate bacterivory (dark portion of bar) and flagellate bacterivory (hatched portion of bar)

Table 3. Comparison of bacterial standing stocks and productivity with protozoan bacterivory in estuarine water over a 2 wk period in late summer. Mean value ± 1 standard deviation. Number of samples in parentheses

Parameter	Open sound (9)	Tidal creek (10)
Bacteria, 10^6 cells ml^{-1}	5.3 ± 0.9	12.8 ± 1.9
Bacterial production,		
10^5 cells ml^{-1} h^{-1}	0.9 ± 0.4	3.1 ± 1.5
(10^6 cells ml^{-1} d^{-1})	(2.1 ± 1.0)	(7.5 ± 3.6)
Total protozoan bacterivory,		
10^5 cells ml^{-1} h^{-1}	0.4 ± 0.25	2.4 ± 1.0
(10^6 cells ml^{-1} d^{-1})	(1.0 ± 0.6)	(5.8 ± 2.3)
	50 % of production	80 % of production

clearance rates was 13 % for tidal creek data and 19 % for open estuary data, and was 37 % for bacterial production data at both sites.

In the tidal creek (Fig. 1), except for the last sampling date, protozoan grazing was roughly equivalent to bacterial productivity, and ciliate grazing dominated total protozoan bacterivory. In contrast, in the open sound (Fig. 2) (excluding 9 September when the sample was taken at the dock due to boat failure) bacterial productivity was generally higher than protozoan grazing, and flagellates were the dominant bacterivores. Average values for the 2 estuarine sites (Table 3) showed that bacterial standing stocks, bacterial production, and total bacterial mortality due to protozoan grazing were all higher in the tidal creek than in the open sound. In the tidal creek, protozoan grazing averaged 80 % of bacterial production, while in the open sound, it averaged only 50 % of production (Table 3). Comparisons of average abundances of flagellates and ciliates, and of per cell clearance rates, in the open sound and tidal creek (Table 4) indicated that the lower total protozoan grazing at the former site was a result both of smaller standing stocks of protozoa as well as of lower grazing rates.

In order to address the question of the relative precision of the 2 parameters, we calculated overall coefficients of variation (CV) for both measurements via propagation of indeterminant error (Day & Underwood 1974, Pace & Bailiff 1987) using the equations:

$$CV_{bp} = (CV_b^2 + CV_{Tdr}^2 + CV_{cf}^2 + CV_{sv}^2)^{0.5} \quad (1)$$

$$CV_{pg} = (CV_f^2 + CV_c^2 + CV_{fcl}^2 + CV_{ccl}^2 + CV_{FLB}^2 + CV_b^2 + CV_{sv}^2)^{0.5} \quad (2)$$

where Eq. (1) defines the composite CV for the derived value of bacterial production (CV_{bp}), and Eq. (2) the composite CV of the derived value of protozoan grazing (CV_{pg}); CV_x = coefficient of variation for the mean value of bacterial numbers (b), Tdr incorporation rates (Tdr), conversion factor for Tdr uptake:bacterial cell production (cf), sampling variability (sv), flagellate numbers (f), ciliate numbers (c), flagellate clearance rate (fcl), ciliate clearance rate (ccl), and FLB numbers (FLB).

The overall CV for bacterial production (Eq. 1) was 50 % for open sound data and 48 % for tidal creek data; for protozoan grazing (Eq. 2) the overall CV was 62.4 % for open sound data and 48 % for tidal creek data. The largest sources for error were due to sampling variability and to the conversion factor used to calculate bacterial production. From these CV's we calculated 95 % confidence limits for the means of the 2 parameters (Students *t*-test; Sokal & Rohlf 1981). For the open sound, the 95 % limits were 0.6 to 1.2×10^5 cells ml^{-1} h^{-1} for bacterial production and 0.3 to 0.6×10^5 cells ml^{-1} h^{-1} for protozoan grazing. For the tidal creek, the 95 % limits were 2.0 to 4.2 cells ml^{-1} h^{-1} for production and 1.6 to 3.2 cells ml^{-1} h^{-1} for grazing. Since the 95 % confidence limits of the 2 parameters did not overlap for the open estuary data, it appears that the difference between our estimates of bacterial production and bacterial mortality due to protozoan grazing in the open estuary was in fact significant.

DISCUSSION

Validation of the FLB method

Three potential problems with the FLB-uptake assay have been recently proposed: (1) possible rapid digestion of FLB by protozoa during the linear portion of the FLB uptake curve (McManus & Fuhrman 1986,

Table 4. Standing stocks and bacterial clearance rates determined for populations of colorless flagellates and of ciliates in estuarine water over a 2 wk period on late summer. Mean value ± 1 standard deviation. Number of samples in parentheses

Parameter	Open sound (9)	Tidal creek (10)
Flagellates, cells ml^{-1}	2070 ± 600	2850 ± 1230
Flagellate clearance rate, nl $cell^{-1}$ h^{-1}	1.65 ± 1.20	2.70 ± 1.10
Ciliates, cells ml^{-1}	14.0 ± 4.6	71.0 ± 46.0
Ciliate clearance rate, nl $cell^{-1}$ h^{-1}	138 ± 44	168 ± 68

Sieracki et al. 1987); (2) flagellate egestion of food vacuoles upon preservation (Sieracki et al. 1987); and (3) protozoan discrimination against heat-killed FLB in favor of live bacteria (Landry et al. 1987). As described below, the first problem has already been addressed prior to this study (Sherr et al. 1988). However, it was evident that an evaluation of both fixation effects and of possible selection for or against our heat-killed FLB by protozoa was in order before we could judge the validity of our protozoan bacterivory estimates.

(1) Possible rapid/random digestion of FLB in protozoan food vacuoles (McManus & Fuhrman 1986, Sieracki et al. 1987), which would lead to underestimation of rates of bacterial uptake: We examined this idea in a laboratory study in which the rates of digestion of ingested FLB by phagotrophic protozoa were determined at temperatures ranging from 12 to 22 °C (Sherr et al. 1988). There was an initial processing period, lasting from 20 to 90 min, of FLB in protozoan food vacuoles, before bacterial digestion proceeded to the point at which FLB were no longer identifiable. Results indicated that linear rates of FLB uptake could be obtained during the initial processing period, without concern about random disappearance of FLB due to digestion during this time.

(2) Preservation-induced egestion of contents of food vacuoles in flagellates (Sieracki et al. 1987): Sieracki et al. proposed using Van der Veer's fixative, which contains tannic acid, acrolein, and glutaraldehyde. However, in addition to the hazards of working with acrolein (the active ingredient of tear gas, and potentially explosive when in concentrated form), we found that the tannic acid formed a precipitate upon standing with seawater, which meant that the preservative must be prefiltered before each use. F. Rassoulzadegan (pers. comm.) proposed a simpler method involving quick fixation with alkaline Lugol solution, followed by preservation with formalin which also decolorized the Lugol. The experiments we carried out comparing rates of flagellate ingestion of FLB by live cells versus cells fixed with various preservatives showed that the Lugol-formalin method was superior to formalin alone, and gave comparable results to FLB-uptake by live cells (Table 1). If the formalin is added immediately after addition of the Lugol solution, chlorophyll *a* autofluorescence is not markedly affected. The decolorization of the Lugol by formalin is occasionally inhibited by compounds present in the sample, e. g. vancomycin plus penicillin added to inhibit bacterial growth. In such cases, a drop of 3 % sodium thiosulfate solution (Pomroy 1984) in a 10 ml sample will clear the Lugol color (L. Carlough pers. comm.).

(3) Selectivity by protozoa either against or for added FLB in comparison to their uptake of in situ bacterioplankton: this potential problem was also addressed in this study. In our selectivity experiments, we found that the long-term proportional disappearance of FLB was comparable to that of live bacteria in the presence of bacterivorous flagellates and ciliates (Table 2). More such studies will be required to completely address the question of food selectivity by protozoa in natural waters; however these initial results are evidence that the FLB uptake method is not in general compromised by protozoan discrimination.

Comparison of bacterial production and protozoan bacterivory in estuarine water

The purpose of the present study was to make simultaneous measures of both bacterial production and of bacterial mortality due to protozoan grazing for a number of samples from the same water mass to test the idea that instantaneous protozoan grazing is the major control on bacterial growth in natural waters. From previous work (Imberger et al. 1983), and from our salinity and temperature data, we are fairly confident that the low tide water at the 2 sites we sampled, Duplin River tidal creek and mouth of Doboy Sound, represented reasonably coherent water masses during the 2 wk study period. There were also consistent differences between the 2 sites with respect to characteristics of the microbial assemblage: bacterial standing stock and productivity, abundance of ciliates, and total protozoan grazing (Figs. 1 and 2; Tables 3 and 4).

The data obtained in the tidal creek water supported the concept of equivalency between bacterial production and protozoan grazing, as the average rate of grazing by ciliates and flagellates at this site was 80 % of production (Table 3). The 20 % shortfall can be accounted for solely by removal of bacteria from tidal creek water by ribbed mussels *Geukensia demissa* during the twice daily excursion of water onto the surrounding salt marsh. Wright et al. (1982) demonstrated in laboratory experiments that ribbed mussels can filter out bacteria from estuarine water, and S. Newell (pers. comm.) has estimated from experimental studies in salt marsh drained by the Duplin River system that ribbed mussels can remove 25 % of suspended bacterial standing stock per day from tidal water.

The tidal creek data also confirmed previous observations of the importance of ciliates as grazers of bacteria in creek water (Sherr et al. 1986b, Sherr & Sherr 1987). Ciliate grazing ranged from 30 to 93 %, and averaged 61 %, of total estimated protozoan bacterivory in creek water, primarily as a result of a higher standing stock of ciliates in the creek than in the open estuary (Table 4). Ciliates also were responsible for a significant fraction (36 %) of overall bacterivory in the open estuary.

The data from the open estuary, i. e. that total protozoan bacterivory averaged only 50 % of bacterial production, do not support the idea of equivalence of the 2 parameters. This result is in line with 2 previous studies on potential of protozoa to consume bacteria in these waters. Sherr et al. (1984) calculated, based on standing stocks of bacteria and colorless flagellates, a few determinations of flagellate growth rates, and previous estimates of bacterial production, that heterotrophic nanoplanktonic protozoa were cropping between 30 and 50 % of daily bacterial production in Georgia coastal waters. Subsequently, Sherr et al. (1986a) determined rates of protozoan bacterivory in the Duplin River Estuary via long-term (24 h) rates of disappearance of bacterioplankton whose growth was inhibited by vancomycin and penicillin. In that study, average protozoan grazing rates could account for only 40 to 45 % of previous estimates of bacterial production in the Duplin River.

The discrepancy between the estimates for bacterial production and for protozoan bacterivory found in the open estuary could be due to either methological problems with one or both techniques, or to other, unmeasured, sources of bacterial mortality, or to uncoupling of the 2 parameters during the time we sampled (see reviews by Pace 1988 and McManus & Fuhrman 1988a).

Problems with bacterial production estimates

The currently perceived problems with the Tdr uptake method are that not all bacteria incorporate thymidine, leading to an underestimate of production (Douglas et al. 1987, Pedrós-Alió & Newell 1989), or that thymidine is metabolized by the bacteria rather than being used exclusively for DNA synthesis, leading to overestimation of bacterial growth rates (Hollibaugh 1988). We felt that such error was minimized in this study due to the empirically derived conversion factor of bacterial cells produced:moles of Tdr incorporated determined for Georgia estuarine water during the summer (Riemann et al. 1987). In a previous study of bacterial production in the upper and lower Duplin Estuary, empirically determined conversion factors were 1.2 to 3.2×10^{18} cells per mole of thymidine incorporated (Newell et al. 1988), thus the average conversion factor of 1.1×10^{18} cells per mole Tdr, derived by Reimann et al. (1987) and used in this study, is at the low end of the range of Newell et al. (1988) and would be expected to yield a conservative estimate of bacterial production.

Based on an average biovolume of 0.05 μm^3 $cell^{-1}$ for estuarine bacterioplankton (Newell et al. 1988) and a conversion factor of 0.35 pgC μm^3 for bacteria Bjørnsen 1986, Lee & Fuhrman 1987), the mean bacterial production given in Table 3 is equivalent to 37 $\mu g\ C\ l^{-1}\ d^{-1}$ in the open estuary and 131 $\mu g\ C\ l^{-1}\ d^{-1}$ in the tidal creek. These values are above the average 26.4 $\mu g\ C\ l^{-1}\ d^{-1}$, but below the highest value of 153 $\mu g\ C\ l^{-1}\ d^{-1}$, reported by Cole et al. (1988) based on a summary of bacterial production estimates in pelagic systems. Although the bacterial production estimates are on the high side of values found in other systems, as might be expected for a productive estuary in summer, the average turnover time of the bacterioplankton assemblage, based on our production estimates, was on the order of 2 d for both sampling sites (Table 3). Previous work in these waters has suggested generation times of suspended bacteria of 1 to 2 d in nearshore coastal water, and of 1 d in tidal creeks, during summer (Newell & Christian 1981, Fallon et al. 1986).

Given the information discussed above, if the values for bacterial production obtained in this study were in error, they were probably underestimates. If bacterial production were actually higher, mortality due to protozoan grazing would be an even lower fraction of production than we have estimated.

Problems with protozoan bacterivory estimates

Even though 3 potential problems with respect to the FLB uptake method raised by other workers were addressed as discussed above, there are remaining possibilities for underestimation of protozoan bacterivory using this technique. The most likely is that there is some selection against FLB by some protozoa. For example, in our comparisons of long-term decrease of FLB with that of live bacteria (Table 2), we found in one experiment in which flagellates were the only bacterivores that FLB decrease was only about 60 % of the decrease of live bacteria. This suggests that some populations of bacterivorous flagellates (for instance contact-feeding or surface feeding species; Caron 1987) might show selection against FLB; thus FLB uptake would underestimate bacterivory in such cases. Since flagellate bacterivory composed a larger share of total protozoan bacterivory in the open estuary (from 49 to 95 %, average 64 %) compared to tidal creek water (from 7 to 70 %, average 39 %), protozoan bacterivory may have been more prone to underestimation at the former site.

Alternate possibilities for underestimation include failure to account for all sources of bacterial mortality due to protozoan grazing as well as to other factors, and uncoupling of protozoan grazing and bacterial production. These are discussed below.

Other sources of bacterial mortality

The FLB uptake method only determines bacterivory for that portion of the plankton which is examined, in

this case colorless flagellates and naked ciliates. Other protists, in particular pigmented flagellates (Estep et al. 1986, Bird & Kalff 1987, Porter 1988) and heterotrophic dinoflagellates (Lessard & Swift 1985) have been suggested as potential consumers of bacteria. We have not yet found significant uptake of FLB by either of these groups of protists in Georgia coastal waters. Based on results of other studies in coastal systems, metazooplankton do not appear to consume substantial amounts of suspended bacteria (King et al. 1980, Turner er al. 1988). We do not know the potential for removal of bacteria by benthic filters feeders in the open estuary, although we presume that removal of bacteria by benthos would be proportionally less at the open estuary site compared to the tidal creek site. The magnitude of bacterial cell loss due to sedimentation from the water column is unknown. The question of cell lysis, either spontaneous or due to predatory microbes such as *Bdellovibrio* sp. or bacteriophages remains controversial. Although Servais et al. (1985) did not find significant decrease in bacterial populations after phagotrophic protozoa had been eliminated via filtration, Proctor et al. (1988) have recently shown that virions present in seawater may cause significant bacterial mortality.

Uncoupling of bacterial production and protozoan bacterivory

Out-of-phase oscillations of bacterioplankton and heterotrophic protozoan standing stocks over periods of days to weeks, as expected for a predator-prey interaction, have been demonstrated for Georgia estuarine waters (Fallon et al. 1986, Sherr et al. 1986b) as well as for other systems (Fenchel 1982, Andersen & Sørensen 1986, Rassoulzadegan & Sheldon 1986). Large variations in bacterioplankton production rates have also been found over a period of a few days in a previous study of bacterial production in Georgia coastal waters (Newell et al. 1988). Therefore, we did not expect to find a close correspondence between the rate of bacterial production and of protozoan bacterivory in any one water sample.

We did expect to find an overall balance between the 2 parameters, which was not obtained in the open estuary. It is possible that bacterial production and protozoan grazing rates varied in different ways over a diurnal cycle, so that over the course of a day the 2 parameters would have been equivalent. Our samples were taken at various times during the daylight hours, from 07:00 to 21:00 h, and the data did not show any consistent trends in either parameter with respect to time of day. In the previous study of Newell et al. (1988), bacterial production was determined at slack low tide at 2 sites in the Duplin River during both night and day for a 17 d period; no differences in bacterial production were found for night and day samples. Lessard et al. (1987) have reported diel variations in protozoan bacterivory in the Chesapeake Bay; however they found high grazing during the day and low grazing at night. This information suggests that uncoupling of the 2 parameters due to diurnal variation is not likely to help explain the discrepancies between bacterial production and protozoan bacterivory found for the open estuary site.

Bacterial production measured via the Tdr method has been compared with bacterial mortality, estimated via various techniques, in several other coastal marine systems (Fuhrman & McManus 1984, Wikner et al. 1986, McManus & Fuhrman 1988b). Considerable variation in the ratio between bacterial cells produced and bacterial cells lost was found in individual samples in each of these studies. Fuhrman & McManus (1984) and Wikner et al. (1986) reported an overall equivalency between the 2 parameters. However, the estimates of bacterial cell loss in these 2 studies, in which disappearance of bacterial cells was followed over time, may have included sources of mortality other than protozoan grazing. Using the fluorescent microsphere uptake method. McManus & Fuhrman (1988b) determined that flagellate grazing could balance bacterial production in the winter, but was only about 23 % of bacterial production in the summer; they speculated that grazing by ciliates, which they did not measure, may also have been an important factor in bacterial mortality at that time of year.

CONCLUSIONS

Overall, the results of the present study indicated that protozoan bacterivory is the most significant source of bacterial mortality in Georgia coastal waters, which supports the findings of previous work in this and other systems (Fenchel 1982, Sieburth 1984, Wright & Coffin 1984, Sherr et al. 1986a, Wikner et al. 1986, McManus & Fuhrman 1988b). Our results also confirmed previous reports concerning the importance of bacterivory by small aloricate ciliates in estuarine water (Sherr et al. 1986c, Sherr & Sherr 1987). In the open estuary 36 %, and in the tidal creek 61 %, of total protozoan grazing was on average due to ciliates.

However, in this study protozoan bacterivory did not account for 100 % of bacterial production. If our bacterial production estimates were too low (Hollibaugh 1988, Pedrós- Alió & Newell 1989), then the shortfall in bacterial mortality would be even greater. It is apparent that in future research problems with both bacterial production and protozoan grazing assays must be

addressed, and alternate fates of bacterial production must be considered (see Pace 1988, McManus & Fuhrman 1988a).

Acknowledgements. We thank Lea Kneib and Eileen Hedick for preparation of the tables and figures, Dr Lynda Shapiro of the Bigelow Laboratory for Ocean Sciences for providing the *Ochromonas* culture, and anonymous revierwers for their comments. This work was supported by NSF Grant No. OCE-8700456 to B. and E. Sherr and by grants from the Sapelo Island Research Foundation. Contribution No. 632 of the University of Georgia Marine Institute.

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This article was submitted to the editor

Manuscript first received: September 28, 1988
Revised version accepted: April 6, 1989