New tracer to estimate community predation rates of phagotrophic protists

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ABSTRACT: Predation of eukaryotic microbes on prokaryotes is one of the most important trophic interactions on Earth, representing a major mortality term and shaping morphology and composition of prokaryotic communities. Here we introduce and validate a new tracer to determine predation rates on prokaryotes. Minicells of *Escherichia coli* marked with a bright green fluorescent protein (GFP) vector have many operational advantages over previously used prey analogs such as fluorescently labeled bacteria. GFP-minicells are similar in size to naturally occurring bacteria from a variety of environments including the oligotrophic open ocean and the deep sea. They are relatively stable against microbial and light degradation, are easy to grow and process, and can be produced inexpensively in large numbers. No chemical alteration of the particle surface due to heat killing and staining is involved. Grazing coefficients were compared between GFP-minicells and other GFP-modified bacteria, as well as 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF)-stained cells. The grazing coefficients obtained from the removal of GFP-minicells compared favorably with estimates from tracer-independent estimates of grazing in the same experiments. Experiments with GFP-minicells resulted in community grazing coefficients similar to those reported for many different marine environments and those derived using various methods.

KEY WORDS: Bacteria · Prokaryotes · Mortality · Predation · Protist feeding · Eukaryotic microbes · Microbial predators · Size selectivity

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INTRODUCTION

Feeding by protists is an important and in some cases the most important loss term of prokaryotes in marine environments. A wide range of protists are phagotrophic including picoeukaryotes, heterotrophic nanoflagellates, and ciliates; even many autotrophs can supplement their nutrition by feeding on prokaryotes (Pernthaler 2005, Massana et al. 2009, Anderson et al. 2012, Hartmann et al. 2013). Many methods have been used to estimate community grazing rates including changes in prey communities after manipulation such as filtration and dilution (Landry & Hassett 1982, Landry et al. 1995) and the use of prey analogs, which can either be followed into the predator cells (Sherr et al. 1987) or for which their removal can be monitored (Vaque et al. 1994,

2008, Vazquez-Dominguez et al. 1999). The most frequently employed methods introduce some type of prey analog consisting of either fluorescent latex beads (Børsheim 1984), 5-(4,6-dichlorotriazinyl)amino-fluorescein (DTAF)-stained natural prokaryotes (Sherr et al. 1987), or DTAF-stained cultures (e.g. *Brevundimonas diminuta*, *Halomonas halodurans*) (Sanders et al. 2000, Vaque et al. 2001, Moorthi et al. 2009). None of these tracers seem to be ideal because cultured prokaryotes are either considerably larger than cells that occur naturally, especially by comparison to those prokaryotes found in the largest of marine environments (i.e. the subtropical gyres and the deep sea), or they have been significantly chemically modified from live cells due to heat denaturation and the introduction of chemical stains. For instance, some evidence suggests that DTAF-stained cells are se-

lected against by some organisms compared to green fluorescent protein (GFP)-expressing prey analogs (Fu et al. 2003).

Green and red fluorescent protein labeled cells have since increased in popularity (Parry et al. 2001, Ishii et al. 2002, Fu et al. 2003, Worden et al. 2006, Tuorto 2008), but these tracers have not been tested thoroughly for their validity as prey analogs, and most GFP-labeled cultured organisms that have been used in feeding experiments were much larger than cells found in natural prokaryote communities. Here we improve on this concept of a GFP-labeled tracer by using a more suitable cell size. We introduced a GFP-vector into a minicell-producing clone of *Escherichia coli* (Adler et al. 1967, Wikner et al. 1986, Pace et al. 1990) and evaluate this new tracer in detail by comparison with DTAF-stained cells, various other species transformed with a GFP vector, tracer-independent perturbation experiments, and data from previously published experiments.

MATERIALS AND METHODS

Grazing experiments

Prey analogs. The *Escherichia coli* minicell strain (Adler et al. 1967) χ1488 (CGSC #6556) was obtained from the Coli Genetic Stock Center (Yale University), grown on Luria Bertani (LB) broth (Lennox, Fisher Scientific), and transformed with the pGreenTIR vector (National Institute of Genetics, Mishima, Japan, Miller & Lindow 1997) that is paired with an ampicillin resistance vector. The strain was transformed following the heat shock protocol in Sambrook & Russell (2001). Detailed culture and transformation protocols can be found in the Supplement, available at www.int-res.com/articles/suppl/m524p055_supp.pdf. The new strain was deposited at the Coli Genetic Stock Center (Yale University), from where an inoculum can be obtained (CGSC #14165; designation: chi1488/pGreenTIR).

Other tracer particles included GFP-modified *Pseudomonas putida* (mutant *rpoS*⁻) (Maki et al. 2009), donated by Kam Leung (Lakehead University, Thunder Bay, Canada). A culture of GFP-modified *Brevundimonas diminuta* (Griffiths et al. 2000) was donated by the laboratory of Dr. Peter Andrew (Leicester University). A culture of the same GFP-*B. diminuta* strain was stained with DTAF (Sherr et al. 1987). Detailed culture and staining protocols can be found in the Supplement.

Natural community predators. For 3 experiments (October 18, 2010; October 25, 2010; and December 6, 2010), protists from the Elizabeth River were used as predators. The Elizabeth River at the Norfolk location is a brackish urban estuary that feeds into the James River and subsequently into the southern Chesapeake Bay. For the August 2011 experiment, the water sample came from the Virginia Beach Fishing Pier (14th Street at the oceanfront, Virginia Beach, VA, USA). Salinity and temperature were measured with a temperature and salinity probe (Model 30, YSI). Sediment was allowed to settle overnight prior to the experiment.

Protist culture predators. *Cafeteria roenbergensis* and *Neobodo designis* (formerly *Bodo designis*, see Moreira et al. 2004) were obtained from the culture collection of the Marine Biology Laboratory at the University of Copenhagen. The stock cultures were maintained in rice grain cultures in 10 ml well plates with ~¼ of a rice grain added to each well and kept in the dark at 9 to 10°C and at a salinity of 35. For the grazing experiments, *C. roenbergensis* and *N. designis* were grown to high abundances in 2% marine broth (Marine Broth 2216, BD Difco) in filtered (Whatman GF/F filter) and autoclaved Instant Ocean water with a salinity of 34 in 250 ml Erlenmeyer flasks with moderate shaking at 22°C.

Grazing experiment setup. Aliquoted prey analogs were thawed, and samples of predators, natural bacteria, and tracers were counted to determine their respective abundances. For controls, water samples were filtered through a 0.8 μm polycarbonate filter (Millipore type ATTP) with a vacuum pump pressure not exceeding 200 mbar. All experiments were run in triplicate for both controls and treatments. Four experiments (samples from October 18, 2010; October 25, 2010; December 2010; and experiments with cultures) were conducted using scintillation vials filled to 21 ml (i.e. 24 vials in total for each experiment). These sample vials were turned over on a timer once every 2 h to prevent settling. Continuous rotation of the incubation vessels was avoided to prevent aggregate formation. The August 22, 2011, experiment was conducted in six 1 l polycarbonate bottles filled to 400 ml, and water was subsampled from the bottles in a time series. Tracer particles were added at approximately 20% of the natural bacteria abundances (Sherr & Sherr 1993). Samples were covered in aluminum foil and kept in a dark room at approximately 22°C for the duration of the experiment.

Sample processing. For the October 18, 2010, experiment, samples were taken at 3 time points (0, 48, and 72 h for GFP-minicells and GFP-parental

cells; 0, 48, and 120 h for GFP-*P. putida*). For the October 25, 2010, experiment and later, samples were collected at 4 time points (0, 24, 48, and 72 h). For all experiments (except for August 2011), an entire vial was sacrificed for each time point and replicate and then fixed in 2% formaldehyde. For the August 2011 experiment, a 20 ml subsample was taken from each 1 l bottle and then fixed in 2% formaldehyde. Samples were filtered onto 0.2 μm polycarbonate filters (Millipore GTTP) and washed twice with both 1× phosphate-buffered saline and Barnstead ultrapure water, respectively. For the natural communities, 7 ml were filtered for enumeration of predators, and 5 ml were filtered for the enumeration of prokaryotes. Because of higher concentrations in the cultures, 3 ml were filtered for predators, and 1 ml was filtered for prokaryotes. After drying, filters were embedded in Vectashield H-1200, an antifadent that includes 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories) as a counterstain. Slides were stored at −20°C in the dark until they were counted (within a week of the experiment). An Olympus BX 50 epifluorescence microscope equipped with a DAPI cube (U-MWU2, Olympus America) and a fluorescein isothiocyanate (FITC) cube (#31001, Chroma Technology) was used. Between 30 and 50 fields were counted for each replicate. Ambient bacteria were counted in the DAPI channel, while tracer particles were counted in the FITC channel.

Sample analysis. For the determination of bacterial volumes, images were taken with a high-resolution Olympus DP-70 digital camera (Olympus America). Bacterial length and width were measured manually using Image J (National Institutes of Health, Bethesda, MD, USA) and calibrated using a stage micrometer. Volumes were determined using the following equation (Loferer-Krössbacher et al. 1998):

$$V = \left[\left(\tfrac{1}{4} \pi w^2 \right) (l - w) \right] + \tfrac{1}{6} \pi w^3 \qquad (1)$$

where w is the width and l is the length of the cell. This formula is equally useful for cocci and bacilli as $l - w$ approaches 0 for cocci. Between 100 and 500 cells in at least 20 images were measured in total for each cell type. For comparison, deep-sea (>1000 m) samples obtained during a research expedition to the tropical and subtropical Atlantic were also analyzed. For details, see Morgan-Smith et al. (2013).

The instantaneous grazing coefficient (g') was defined as the observed changes of prokaryotic numbers corrected for any changes that occurred at the absence of predators in the control treatments (0.8 μm filtrate). Changes in prey abundance were

assumed to follow exponential decay or growth models (Eq. 2). Slopes in the grazing and control chambers (i.e. the instantaneous grazing and growth coefficients g and k) were calculated after natural log transformation of the prey or tracer concentrations using Eq. (2):

$$\ln C_t = \ln C_0 - g \cdot t \ \text{ or } \ \ln C_t = \ln C_0 - k \cdot t \qquad (2)$$

where C_t and C_0 are the concentration of prey at time t and at time 0, respectively; g and k are the instantaneous coefficients of change in cell numbers in the grazing and control vessels, respectively; and t is the elapsed time. Instantaneous grazing coefficients were then calculated using:

$$g' = g - k \qquad (3)$$

where each g and k retains its natural sign of the net change depending on the outcome of the experiment. For instance, if there were losses of prey in the control vessels, then k was negative, resulting in an overall positive term that would correct the observed grazing coefficient. This gross grazing coefficient should not be confused with the observed net growth coefficient termed $(g - k)$ in dilution experiments (Landry & Hassett 1982).

ANCOVA was employed to test for homogeneity of slopes to determine whether the changes in cell numbers over time were significantly different between grazing and control vessels. ANCOVA requires that the changes in the natural log-transformed prey numbers were linear with time. To determine if there was a systematic violation of this linearity assumption, we calculated the rate constants (g and k) based on different time intervals (24, 48, and 72 h) using Eq. (4):

$$g \text{ or } k = 1/t \times \ln(C_t/C_0) \qquad (4)$$

where g (h^{-1}) is the change of particle concentration (tracer or prey) in the treatments (whole water), k (h^{-1}) is the change of tracer or prey concentration in the controls (0.8 μm filtrate), t is the time interval (24, 48, or 72 h), C_t is the tracer particle concentration at t, and C_0 is the initial tracer concentration. In this case, g and k were calculated for each vessel for each of the time intervals. An average of k of the triplicate controls was then subtracted from each of the g to arrive at the gross grazing coefficient g' (h^{-1}).

The difference in changes in the natural bacteria abundances in the grazing and control treatments represents a trophic cascade/perturbation-type experimental design (Wright & Coffin 1984, Caron 2001). Since the predators were removed in the 0.8 μm filtrate controls, changes in prokaryote numbers reflected natural growth in the absence of predators

(see 'Discussion' for limitations of this approach). It follows that g' (h^{-1}) can then be calculated in the same fashion as for the tracer experiments.

Comparison with literature values from marine surface environments

For comparison of grazing loss terms across different marine environments, we chose studies that provided sufficient information to calculate the instantaneous grazing coefficients (g') directly. This is not an exhaustive, but rather a representative, list of marine surface water environments. We assumed that the data were all corrected for controls, although this was not apparent in all cases. In cases in which only ranges were available, the arithmetic mean between the 2 end points was calculated.

RESULTS

Cell morphologies and sizes

Fig. 1 shows *Escherichia coli* GFP-minicells and parental cells. The GFP-labeled cells displayed a very strong bright green color that separated from the background better than the DTAF-stained cells. Bleaching during counting was minimal even when many cells had to be counted per field. In our experience, DTAF-stained cells bleached faster than GFP-
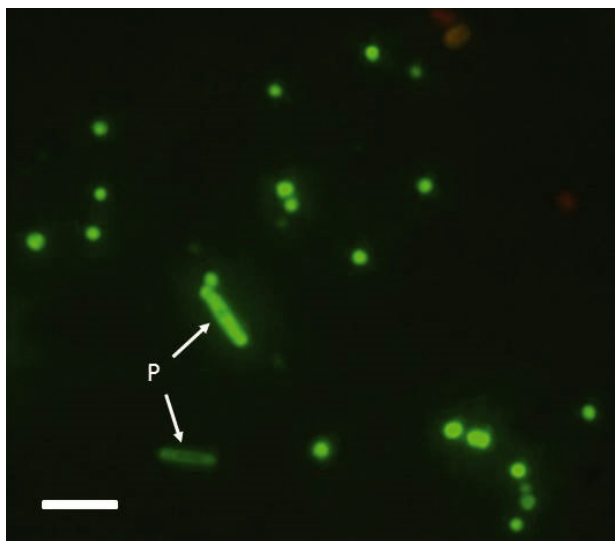
Fig. 1. Photomicrograph of minicells with 2 parental cells (P) of the new green fluorescent protein (GFP)-transformed minicell strain of *Escherichia coli* (deposited at Coli Genetic Stock Center, CGSC #14165). Scale bar = 3 μm

minicells. The dispersion shown in Fig. 1 is typical, and clumps as we frequently observed with DTAF cells are rare. GFP-minicells were similar to cocci and only slightly smaller than bacilli in environmental samples, whereas all other tracer particles used here (*Brevundimonas diminuta*, *E. coli* parental cells, and *Pseudomonas putida*) were substantially larger than environmental prokaryotes (Figs. 2 & 3). The distribution of cell shapes was 53.45% (±4.88 SD) cocci, 45.94% (±4.8 SD) bacilli, and 0.60% (±0.39 SD) vibrios in the Elizabeth River samples and 48.59% (±5.25 SD) cocci, 48.95% (±4.98 SD) bacilli, and 2.46% (±0.34 SD) vibrios in the Virginia Beach samples (Fig. 3).

Grazing experiments

The October 18, October 25, and December 6 (2010) samples had salinities and temperatures, respectively, of 18.2 and 18°C, 20.1 and 17°C, and 20.2 and 7°C. The August 2011 sample had a salinity of 28 and a temperature of 23°C. An example of 1 experimental time course (August 2011) is shown in Fig. 4, comparing a treatment and a control. The results of all grazing experiments are summarized in Table 1, including the tests for homogeneity of slopes (interaction term in ANCOVA) between individual controls and treatments. No grazing was observed in experiments with *Neobodo designis* where none of the tracer particles were accepted as food (i.e. slopes between controls and grazing containers were not significantly different according to an ANCOVA homogeneity of slopes test, Table 1). DTAF-*B. diminuta* was not significantly removed by the natural population in the Elizabeth River, and removal of GFP-*B. diminuta* was not significant in the *Cafeteria roenbergensis* culture (Table 1). All other experiments showed significant grazing (Table 1).
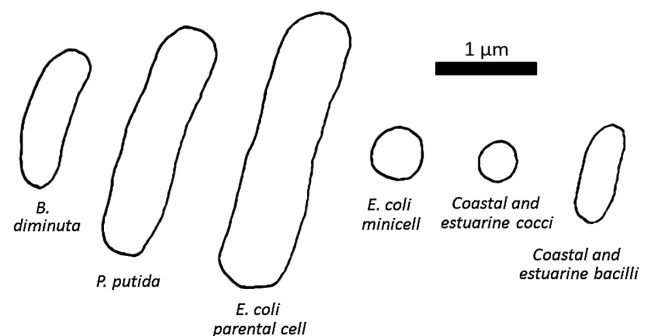

Fig. 2. Shapes and sizes of average prey types used in this study. Length and widths of the cells were based on the average lengths and widths from Fig. 3
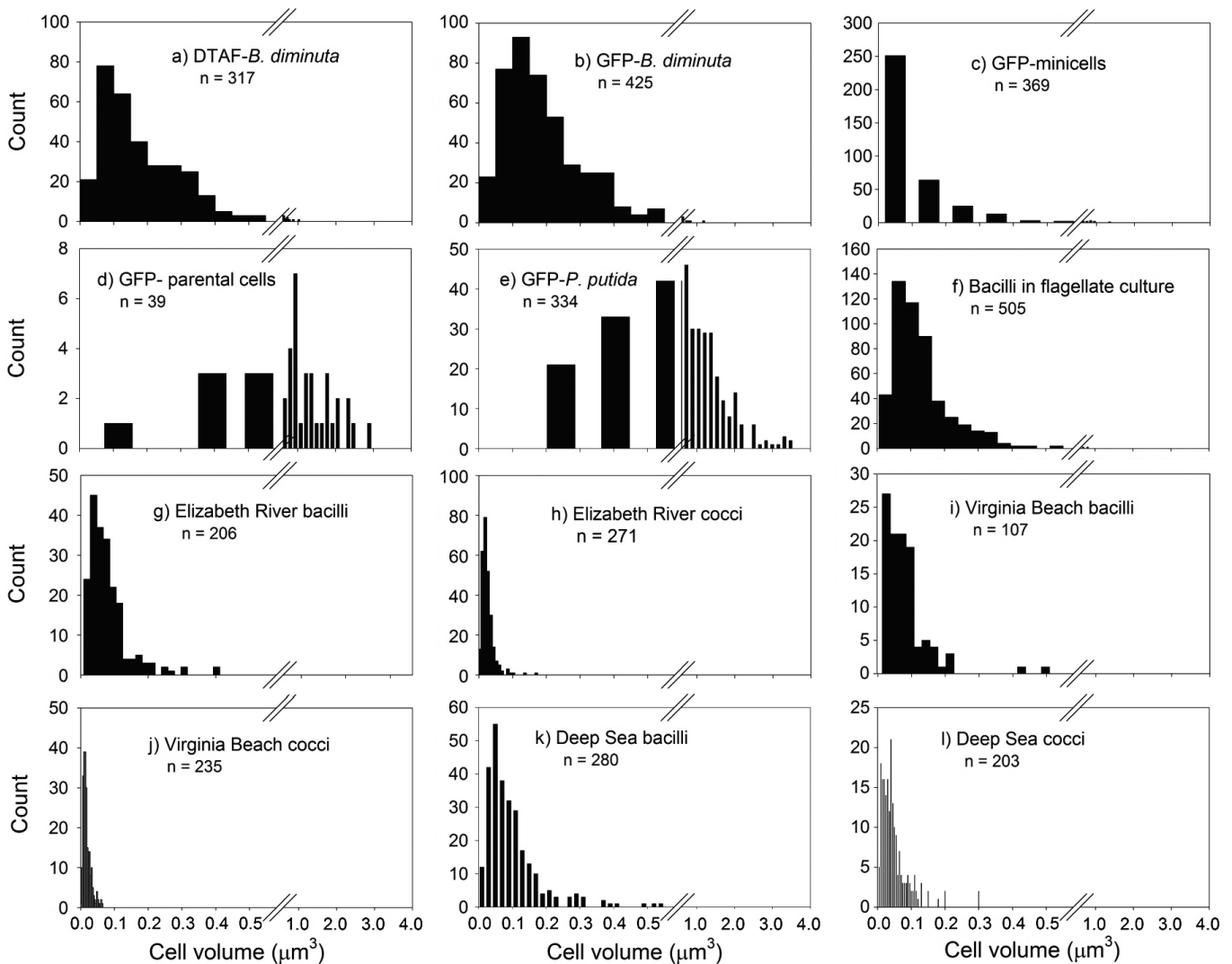
Fig. 3. Frequency distribution of cell volumes of (a–e) various tracers used in this study compared to (f) 1 example of bacilli in a culture of *Cafeteria roenbergensis*; (g–j) naturally occurring bacilli and cocci in Elizabeth River and Virginia Beach; (k–l) deep-sea prokaryotes collected in the deep North Atlantic (>1000 m). *B. diminuta*: *Brevundimonas diminuta*; DTAF: 5-(4,6-dichlorotri-azinyl)aminofluorescein; GFP: green fluorescent protein; n: number of measurements; *P. putida*: *Pseudomonas putida*

Pairwise comparisons between types of tracer particles

Despite their different sizes, grazing between GFP-minicells and GFP-parental cells was not significantly different in the *Cafeteria* cultures, in the Virginia Beach water, or in the Elizabeth River water on December 6, 2010 (Table 2). Grazing, however, was significantly different between GFP-minicells and GFP-parental cells in the experiments with Elizabeth River water on October 18 and 25, 2010 (Table 2). The strongest difference was observed in the October 18 Elizabeth River water (g' = 0.0146 for minicells and 0.0286 for parental cells). This preference for the larger size also extended to *P. putida*, with a g' of −0.0275 (Table 2). *C. roenbergensis* had a preference for *B. diminuta* labeled with DTAF over those labeled with GFP (Table 2). By contrast, DTAF-*B. diminuta* was not consumed by a natural community in the Elizabeth River, while GFP-*B. diminuta* was removed significantly, albeit at lower rates than those observed for GFP-minicells and parental cells (Table 2). The ratio of g' for *P. putida* over g' for GFP-minicells was the highest of all ratios, with a value of 1.88 (Table 2). On average, in the 9 comparisons of GFP-minicells with other tracers, the ratio (g'_2/g'_1) was 1.24 (SD = 0.30, n = 9) (Table 2).

| Date | Environment | Tracer | Grazing | | | | Predator abund. |
			Equation: $\ln C =$	n	r^2	p	
Oct 18, 2010	Elizabeth River	GFPmini	$11.59(0.25) - 0.0146(0.0049) \cdot t$	9	0.874	0.0002	7060
Oct 18, 2010	Elizabeth River	GFP-Pp	$13.32(0.78) - 0.0402(0.0104) \cdot t$	9	0.922	<0.0001	8371
Oct 18, 2010	Elizabeth River	GFPparental	$9.23(0.61) - 0.0286(0.0121) \cdot t$	9	0.816	0.00	7060
Oct 25, 2010	Elizabeth River	GFPmini	$12.46(0.40) - 0.0338(0.0090) \cdot t$	12	0.875	<0.0001	3553
Oct 25, 2010	Elizabeth River	GFP-Pp	$12.82(0.26) - 0.0438(0.0057) \cdot t$	12	0.967	<0.0001	5180
Oct 25, 2010	Elizabeth River	GFPparental	$10.13(0.46) - 0.0582(0.0102) \cdot t$	12	0.942	<0.0001	3553
Dec 6, 2010	Elizabeth River	GFPmini	$12.66(0.40) - 0.0171(0.0089) \cdot t$	12	0.647	0.0016	7678
Dec 6, 2010	Elizabeth River	GFPparental	$10.37(0.67) - 0.0272(0.0149) \cdot t$	12	0.623	0.0023	7678
Dec 6, 2010	Elizabeth River	DTAF-Bd	$12.58(0.27) - 0.0045(0.0060) \cdot t$	12	0.216	0.1284	8228
Dec 6, 2010	Elizabeth River	GFP-Bd	$12.01(0.21) - 0.0114(0.0047) \cdot t$	12	0.743	0.0003	6842
Jan 18, 2011	*Neobodo designis*	GFPmini	$12.44(0.29) - 0.0020(0.0066) \cdot t$	12	0.045	0.5097	16 707
Jan 18, 2011	*N. designis*	GFPparental	$9.69(0.99) - 0.0049(0.0221) \cdot t$	12	0.024	0.6317	16 707
Jan 18, 2011	*N. designis*	DTAF-Bd	$12.87(0.16) - 0.0068(0.0034) \cdot t$	12	0.660	0.0013	8431
Jan 18, 2011	*N. designis*	GFP-Bd	$12.14(0.18) - 0.0001(0.0040) \cdot t$	12	<0.001	0.9545	14 885
Jan 18, 2011	*Cafeteria roenbergensis*	GFPmini	$12.27(0.24) - 0.0137(0.0054) \cdot t$	12	0.766	0.0002	48 658
Jan 18, 2011	*C. roenbergensis*	GFPparental	$9.27(0.29) - 0.0138(0.0065) \cdot t$	12	0.694	0.0008	48 658
Jan 18, 2011	*C. roenbergensis*	DTAF-Bd	$12.82(0.19) - 0.0203(0.0043) \cdot t$	12	0.916	<0.0001	49 606
Jan 18, 2011	*C. roenbergensis*	GFP-Bd	$11.67(0.28) - 0.0041(0.0063) \cdot t$	12	0.174	0.1773	46 699
Aug 2011	Virginia Beach	GFPmini	$12.78(0.40) - 0.0344(0.0090) \cdot t$	12	0.879	<0.0001	9858
Aug 2011	Virginia Beach	GFPparental	$9.72(0.78) - 0.0461(0.0174) \cdot t$	12	0.777	0.0002	9858

Effect of incubation time

The time interval of the incubation (i.e. 24, 48, or 72 h) had no effect on the estimates of g' based on a 1-way ANOVA, with the result $F = 1.14$, p = 0.3277 (3 groups, n = 51). Overall, the residual pattern was heteroscedastic, with the 2 shorter time intervals (24 and 48 h) yielding less precision and a higher standard deviation of the g' estimates (SD = 0.0150 and SD = 0.0116, respectively) than the 72 h time intervals (SD = 0.0088).
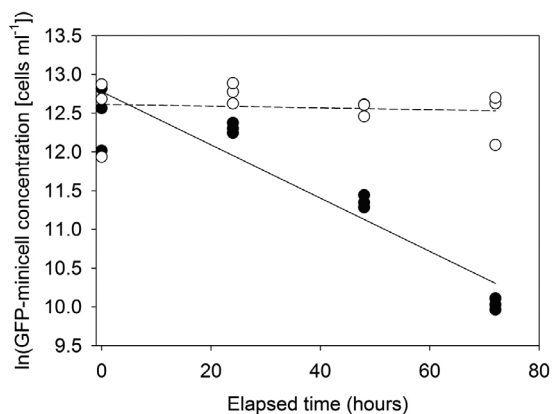

Fig. 4. Example of a tracer-removal experiment with natural seawater (Virginia Beach, August 2011) and using green fluorescent protein (GFP)-minicells as prey. Solid circles: untreated seawater (linear regression: $y = 12.78 - 0.034x$, n = 12, $r^2 = 0.897$, p < 0.0001); open circles: 0.8 μm filtered controls. No significant change in GFP-minicells was detected in the controls over 3 d (linear regression: $y = 12.61 - 0.0012x$, n = 12, $r^2 = 0.013$, p = 0.727)

Dynamics of natural bacteria in grazing experiments

Table 3 shows the results of tracer-independent estimates of grazing by comparing the growth trajectories of prokaryotes in the treatments (*g*) and predator-free controls (*k*). Grazing was calculated in the same way as shown above ($g - k$) (Table 3). A linear regression between the GFP-minicell experiments and the tracer-independent approach yielded a significant slope of 0.74 (Fig. 5). The tracer-independent method yielded grazing coefficients that were 38 to 47% lower than those obtained from GFP-minicell counts.

Comparison with literature values from marine surface environments

A representative list of literature values for which the calculation of grazing coefficients (*g'*) was possible is shown in Table 4. The mean values from 4 experiments with GFP-minicells (0.268 d^{-1}, SD = 0.088) fall close to the means of published data (Fig. 6). The literature values from ingestion of fluorescent beads or fluorescently labeled bacteria (FLB) yielded a mean of 0.428 d^{-1} (SD = 0.506). Eleven experiments based on the removal of FLB resulted in a mean of g' of 0.359 d^{-1} (SD = 0.165). Sixteen experiments with radiolabeled minicells by Wikner et al. (1986, 1990) performed in a wide variety of environments (mean = 0.833 d^{-1}, SD = 0.979) were plotted separately (Fig. 6). Very few dilution experiments were available for heterotrophic bacteria in marine

Control Equation: lnC =	n	r^2	p	Predator abund.	ANCOVA F, p
11.60(0.24) + 0.0031(0.0051)·t	8	0.263	0.1933	16	35.09, <0.0001
12.90(0.70) − 0.0127(0.0105)·t	8	0.591	0.0257	509	19.43, 0.0007
9.48(0.38) − 0.0123(0.0082)·t	8	0.693	0.0103	16	6.64, 0.023
12.31(0.30) − 0.0053(0.0066)·t	12	0.244	0.1024	112	32.26, <0.0001
12.31(0.37) − 0.0068(0.0083)·t	12	0.246	0.1012	121	66.68, <0.0001
9.79(0.24) − 0.0122(0.0052)·t	12	0.728	0.0004	112	80.44, <0.0001
12.50(0.21) + 0.0007(0.0046)·t	12	0.012	0.7369	17	15.68, 0.0008
9.92(0.20) + 0.0024(0.0045)·t	12	0.130	0.2506	17	17.98, 0.0004
12.41(0.19) + 0.0023(0.0041)·t	12	0.137	0.2372	29	4.32, 0.051
11.51(0.24) + 0.0054(0.0053)·t	12	0.345	0.0448	19	28.02, <0.0001
12.27(0.17) + 0.0050(0.0038)·t	12	0.465	0.0145	249	4.27, 0.0519
9.31(0.30) + 0.0048(0.0066)·t	12	0.205	0.1396	249	0.87, 0.3613
12.73(0.38) − 0.0150(0.0085)·t	12	0.605	0.0029	71	3.93, 0.0613
12.14(0.20) − 0.0053(0.0044)·t	12	0.425	0.0217	71	3.84, 0.0642
12.08(0.14) + 0.0023(0.0031)·t	12	0.221	0.1234	202	33.58, <0.0001
9.26(0.33) + 0.0048(0.0073)·t	12	0.178	0.1721	202	18.16, 0.0004
12.76(0.19) + 0.0039(0.0043)·t	12	0.291	0.0705	165	35.87, <0.0001
11.93(0.10) + 0.0008(0.0023)·t	12	0.057	0.4566	99	2.65, 0.1191
12.61(0.32) − 0.0012(0.0072)·t	12	0.013	0.7265	55	41.19, <0.0001
9.28(0.66) − 0.0003(0.0148)·t	12	<0.001	0.9611	55	19.94, 0.0002

Table 1. Summary of regressions of bacterivory experiments with various tracers for treatments (grazing) and 0.8 µm filtered controls. The equations are linear regressions based on natural log-transformed tracer abundance (lnC, cells ml^{-1}) versus time (t, h). Values in brackets are the ±95% confidence intervals for the parameter estimates (i.e. intercept and slope). The last column shows the F and p values for the homogeneity of slopes test of an ANCOVA (i.e. whether the slopes were significantly different between treatment and control chambers). GFPmini: green fluorescent protein (GFP)-minicells; GFP-Pp: GFP-*Pseudomonas putida*; GFP-parental: GFP-parental cells; DTAF-Bd: 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF)-stained *Brevundimonas diminuta*; GFP-Bd: GFP-*B. diminuta*. Predator abundance (cells ml^{-1}) is based on the geometric mean of predator abundances for all time periods for each grazing experiment

and estuarine surface water (for the 4 studies we found, mean = 0.447 d^{-1}, SD = 0.301) (Fig. 6). The medians of all studies were closer to each other than the average, ranging from 0.198 (FLB ingestion) to 0.449 (dilution experiment), with a median of 0.242 in the GFP-minicell experiments (Fig. 6). A Hochberg GT2 test that controls for the type I error rate in multiple pairwise comparisons (Sokal & Rohlf 1981) revealed no significant differences (at α = 0.05) in the means of any of the methods used, despite the variety of environmental conditions.

DISCUSSION

Prey size

Minicells are attractive model particles because their sizes closely match those of marine bacteria (Figs. 2 & 3), and size plays a significant role in the selection process by protists (Gonzalez et al.1990, Šimek & Chrzanowski 1992). It has been well documented that larger bacteria are preferentially preyed on than smaller bacteria in a variety of aquatic envi-

Table 2. Pairwise comparison between various tracers used in this study (homogeneity of slopes, ANCOVA). The instantaneous grazing coefficients (g', h^{-1}) are based on $g - k$ (Table 1). Experiments for *Neobodo designis* are not shown because grazing rates were not significant in that culture (Table 1). Where grazing was 0 in at least 1 tracer, ratios could not be calculated (n/a). GFPmini: green fluorescent protein (GFP)-minicells; GFP-Pp: GFP-*Pseudomonas putida*; GFPparental: GFP-parental cells; DTAF-Bd: 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF)-stained *Brevundimonas diminuta*; GFP-Bd: GFP-*B. diminuta*

Date	Environment	Tracer 1	g'_1 (tracer 1) h^{-1}	Tracer 2	g'_2 (tracer 2) h^{-1}	Ratio g'_2/g'_1	n	ANCOVA F, p
Oct 18, 2010	Elizabeth River	GFPmini	−0.0146	GFPparental	−0.0163	1.12	15	6.44, 0.0237
Oct 18, 2010	Elizabeth River	GFPmini	−0.0146	GFP-Pp	−0.0275	1.88	15	16.81, 0.0011
Oct 25, 2010	Elizabeth River	GFPmini	−0.0338	GFPparental	−0.0460	1.36	21	16.08, 0.0007
Oct 25, 2010	Elizabeth River	GFPmini	−0.0338	GFP-Pp	−0.0438	1.30	21	4.38, 0.0493
Dec 6, 2010	Elizabeth River	GFPmini	−0.0178	GFPparental	−0.0272	1	21	1.65, 0.2137
Dec 6, 2010	Elizabeth River	GFPmini	−0.0178	DTAF-Bd	0	n/a	21	6.89, 0.0163
Dec 6, 2010	Elizabeth River	GFPmini	−0.0178	GFP-Bd	−0.0114	1	21	1.58, 0.2230
Dec 6, 2010	Elizabeth River	GFPparental	−0.0272	GFP-Bd	−0.0114	0.42	21	9.9, 0.0051
Dec 6, 2010	Elizabeth River	DTAF-Bd	0	GFP-Bd	−0.0144	n/a	21	8.02, 0.0103
Jan 18, 2011	*Cafeteria roenbergensis*	GFPmini	−0.0137	GFPparental	−0.0138	1	21	<0.01, 0.9851
Jan 18, 2011	*C. roenbergensis*	GFPmini	−0.0137	DTAF-Bd	−0.0203	1.48	21	4.45, 0.0476
Jan 18, 2011	*C. roenbergensis*	GFPparental	−0.0138	DTAF-Bd	−0.0203	1.47	21	3.41, 0.0797
Jan 18, 2011	*C. roenbergensis*	DTAF-Bd	−0.0203	GFP-Bd	0	n/a	21	22.35, 0.0001
Aug 2011	Virginia Beach	GFPmini	−0.0332	GFPparental	−0.0458	1	21	1.77, 0.1983

Table 3. Summary of regression trends with live prokaryotes for unfiltered and 0.8 µm filtered water in the same vessels as the tracer experiments were performed. These represent perturbation experiments where the main effect is seen in the 0.8 µm filtered controls in which the prokaryotes grew in the absence of predators. The equations are linear regressions based on natural log-transformed natural prokaryote abundance (lnC, cells ml^{-1}) versus time (t, h)

| Date | Environment | ——— Grazing (unfiltered) ——— | | | | ——— Control (0.8 µm filtered) ——— | | | |
		Equation: lnC =	n	r^2	p	Equation: lnC =	n	r^2	p
Oct 18, 2010	Elizabeth River	13.54 + 0.0022·t	9	0.075	0.4758	13.14 + 0.0144·t	8	0.821	0.0019
Oct 18, 2010	Elizabeth River	14.38 – 0.0081·t	9	0.486	0.037	14.59 – 0.0013·t	8	0.061	0.556
Oct 25, 2010	Elizabeth River	13.65 – 0.0059·t	12	0.165	0.1900	13.19 + 0.0119·t	12	0.801	0.0001
Oct 25, 2010	Elizabeth River	14.12 – 0.0142·t	12	0.865	<0.0001	13.99 + 0.0090·t	12	0.478	0.0128
Dec 6, 2010	Elizabeth River	13.60 + 0.0032·t	12	0.140	0.2316	13.29 + 0.0121·t	12	0.718	0.0005
Dec 6, 2010	Elizabeth River	13.67 + 0.0028·t	12	0.109	0.296	13.48 + 0.0033·t	12	0.185	0.163
Dec 6, 2010	Elizabeth River	13.39 – 0.0022·t	12	0.061	0.437	13.09 + 0.0035·t	12	0.109	0.295
Jan 18, 2011	*Cafeteria roenbergensis*	15.71 – 0.0036·t	12	0.450	0.0169	14.88 + 0.0089·t	12	0.812	0.0001
Jan 18, 2011	*C. roenbergensis*	15.64 – 0.0016·t	12	0.110	0.292	14.91 + 0.0007·t	12	0.013	0.726
Jan 18, 2011	*C. roenbergensis*	15.67 – 0.0040·t	12	0.247	0.100	15.02 + 0.0004·t	12	0.004	0.837
Jan 18, 2011	*Neobodo designis*	16.54 – 0.0018·t	12	0.018	0.677	16.20 – 0.0039·t	12	0.370	0.036
Jan 18, 2011	*N. designis*	16.63 – 0.0038·t	12	0.176	0.175	15.79 + 0.0018·t	12	0.142	0.227
Jan 18, 2011	*N. designis*	16.50 – 0.0014·t	12	0.131	0.248	16.13 – 0.0046·t	12	0.263	0.088
Aug 2011	Virginia Beach	14.244 – 0.0074·t	12	0.416	0.0235	14.00 + 0.0094·t	12	0.486	0.0117

ronments (e.g. Chrzanowski & Šimek 1990, Gonzalez et al. 1990, Monger & Landry 1991, 1992, Kinner et al. 1998). We can assume that *Escherichia coli* parental cells were identical to their minicells in all other respects (surface properties, nutritional value) except for size; therefore, the effect of size can be isolated from other factors using this model particle. Other artificial prey items usually are much larger or at
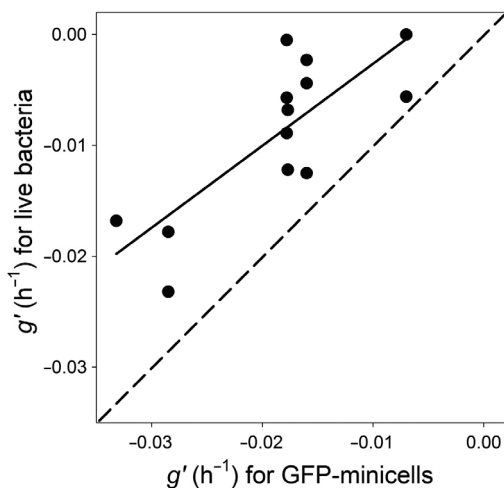
least at the very upper prey size spectrum of natural prey. Monger & Landry (1992) introduced *Brevundimonas diminuta* (formerly *Pseudomonas diminuta*) as a useful tracer. *B. diminuta*, its name notwithstanding, is still larger than the average naturally occur-

Fig. 5. Gross grazing coefficient g' ($g - k$) corrected for changes in the control treatments for green fluorescent protein (GFP)-minicells and naturally occurring bacteria. Two positive values for g' (minus grazing) in *Neobodo designis* were set to 0, as no grazing took place in these cases. The linear regression is $y = 0.00048 + 0.739x$, r^2 = 0.660, F = 23.30, p = 0.0004, n = 14. The dashed line depicts the 1:1 ratio between the 2 estimates

Fig. 6. Boxplots of instantaneous grazing coefficients (g') from this study (n = 5; environmental data only, GFP-mini) and from the literature shown in Table 4. FLB ingest: values based on ingestion of fluorescently labeled bacteria or beads (n = 5); FLB rem: values based on the removal of fluorescently labeled bacteria (n = 11); Radiol minicells: estimates based on the removal of radioactively labeled minicells (n = 18). Dilution method (n = 4). Solid lines represent medians, dashed lines represent means, edges of boxes represent 25th and 75th percentiles, and whiskers represent 10th and 90th percentiles. The means among techniques were not significantly different at α = 0.05 according to a Hochberg GT2 multiple comparison test

Table 4. Literature values used in Fig. 6. Grazing coefficients (g') represent community loss terms of the prokaryote community. Mini: minicells; FLB: fluorescently labeled bacteria; Natural: natural bacteria; I: ingestion of tracers; R: removal of tracer; D: dilution experiment

Location	Prey	Method	g' (h^{-1})	Average g' (d^{-1})	How g' was calculated	Reference
Korea	FLB	I	0.055	1.320	Available in reference	Choi et al. (1995)
Atlantic Sector Southern Ocean	FLB	I	0.0042–0.0083	0.150	Table 3	Vaque et al. (2008) table (original Becquevort 1997)
Antarctic Peninsula	FLB	I	0.0021–0.0083	0.125	Table 3	Vaque et al. (2008) table (original Bird & Karl 1999)
Masa Bay Korea	FLB	I		0.198	7 data points from Table 1[a]	Choi et al. (2003)
McMurdo Sound	Beads	I	0.0083–0.0208	0.349	Table 3	Vaque et al. (2008) table (original Moisan et al. 1991, Putt et al. 1991)
Plankton Japanese lagoon	FLB	R	0.002–0.03	0.384	Table 3	Sime-Ngando et al. (1999)
Ice-Brine Japanese lagoon	FLB	R	0.003–0.039	0.504	Table 3	Sime-Ngando et al. (1999)
Mediterranean Sea	FLB	R	0.004–0.0194	0.281	Calculated from Table 1: Grz/Bac abun/24	Vaque et al. (2001)
Antarctic Waters	FLB	R	0.01125	0.270	Available in reference	Vaque et al. (2004)
Georges Bank autumn	FLB	R	0.0110	0.264	Tables 1 & 2	Sanders et al. (2000)
Georges Bank summer	FLB	R	0.0060	0.144	Tables 1 & 2	Sanders et al. (2000)
Sargasso Sea summer	FLB	R	0.0050	0.120	Tables 1 & 2	Sanders et al. (2000)
Canadian Arctic Franklin Bay	FLB	R	0.01670	0.401	Table 3	Vaque et al. (2008) table
Santa Monica, CA	FLB	R	0.018–0.034	0.612	Ranges from Table 1	Fuhrman & Noble (1995)
Harbor N of Barcelona	FLB	R	0.015–0.035	0.597	10 values from Fig. 3C	Guixa-Boixereu et al. (1999)
Canary Current	FLB	R	0.0036–0.0382	0.375	Table 1: grazing rate (G)/ bacterial conc (BA)	Boras et al. (2010)
Antarctic Peninsula	Mini	R	0.0021–0.0333	0.425	Table 3	Vaque et al. (2008) table (original Vaque et al. 2002a)
Antarctic Peninsula	Mini	R	0.0004–0.0083	0.104	Table 3	Vaque et al. (2008) table (original Vaque et al. 2002b)
Baltic Sea	Mini	R	0.0681	1.634	Calculated from Table 1[b]	Wikner et al. (1986)
Mediterranean Sea	Mini	R	0.0232	0.558	Calculated from Table 1	Wikner et al. (1986)
Baltic Sea	Mini	R	0.0076	0.184	Calculated from Table 1	Wikner et al. (1986)
Bothnian Sea	Mini	R	0.0089	0.214	Calculated from Table 1	Wikner et al. (1986)
Baltic Sea	Mini	R	0.1484	3.562	Calculated from Table 1	Wikner et al. (1986)
Baltic Sea	Mini	R	0.0229	0.549	Calculated from Table 1	Wikner et al. (1986)
Baltic Sea	Mini	R	0.0198	0.475	Calculated from Table 1	Wikner et al. (1986)
Mediterranean Sea	Mini	R	0.0211	0.507	Calculated from Table 1	Wikner et al. (1986)
Bothnian Sea	Mini	R	0.0375	0.900	Calculated from Table 1	Wikner et al. (1986)
Bothnian Sea	Mini	R	0.0103	0.247	Calculated from Table 1	Wikner et al. (1986)
Baltic Sea	Mini	R	0.0051	0.124	Calculated from Table 1	Wikner et al. (1986)
Mediterranean Sea	Mini	R	0.0269	0.647	Calculated from Table 1	Wikner et al. (1986)
Baltic Sea	Mini	R	0.0663	1.592	Calculated from Table 1	Wikner et al. (1986)
Baltic Sea	Mini	R	0.1189	2.853	Calculated from Table 1	Wikner et al. (1986)
Bothnian Sea	Mini	R	0.0085	0.203	Calculated from Table 1	Wikner et al. (1986)
Bothnian Sea	Mini	R	0.0090	0.216	Table 2	Wikner et al. (1990)
Hokkaido Japan	Natural	D	0.0021–0.0054	0.090	Ranges from abstract (grazing of protists)	Taira et al. (2009)
Barbados, Caribbean Sea	Natural	D	0.01–0.052	0.546	11 values from Table 3	Choi et al. (2001)
Tomales and Suisun bays, CA	Natural	D	0.012–0.034	0.351	8 values from Table 4 (bacterioplankton)[c]	Murrell & Hollibaugh (1998)
Kaneohe Bay, HI	Natural	D	0.021–0.046	0.800	2 values from Table 2[d]	Landry et al. (1984)

[a]Values from oxygenated environments only; [b]Calculated by dividing predation rates by the sum of bacterial and grazer concentrations; [c]Where grazing rates were nonsignificant, g' was set to 0; [d]Heterotrophic bacteria only

ring prey, even in the eutrophic environments of estuarine and coastal areas of the North Atlantic (Figs. 2 & 3). Size reduction to a length of 1 μm and a width of 0.3 μm was previously achieved by starvation (e.g. Karayanni et al. 2008), but this adds a time-consuming step. Another solution to provide a natural prey size spectrum for the protists is to concentrate naturally occurring bacteria (Sherr et al. 1989). However, by heat killing and staining these cells with DTAF, we observed much nonspecific staining and clumping of cells. Minicells, though, have a size distribution that matches natural bacteria for most marine systems, especially oligotrophic and deep-sea systems. They remain surprisingly mono-dispersed even after the several rounds of centrifugation needed to clean bacteria from the LB growth medium.

Despite the robust and significant preference of protists for larger prey cells shown in the literature and our experiments, the difference was not very large, even in the most extreme circumstances of size differences (maximum ratio of minicells versus *P. putida* only yielded a factor of 1.88). This is very much in line with the results of Monger & Landry (1991), who demonstrated that the ratio of clearance rates of large versus small particles only ranged from 1.1 to 1.3 for mixed natural protist communities. This small range in ratios seems to reflect the fact that every prokaryotic size category has its own specialized predators, ranging from the smallest picoeukaryotes to ciliates.

Incubation times

The method presented here shares the disadvantage of long incubation times with other tracer experiments. While our analysis showed no systematic changes in grazing coefficients over various incubation time intervals, the predator and prey communities certainly change over time when kept in an incubation vessel over several days. Changes in bacterial transcriptomics, abundance, and composition are almost immediate, and changes in flagellate abundance and diversity can be seen within 1 to 5 d depending on the type of nutrients introduced (Stewart et al. 2012, del Campo et al. 2013). Rates estimated based on the accumulation of tracer particles in food vacuoles are based on much shorter time periods, thus avoiding drastic changes in the microbial communities during incubation. However, there are other problems associated with short incubations. Protists may display stress responses to their most

recently changed environment, which may provide low estimates of actual feeding rates, or gorge feeding may occur immediately after introduction of the tracer, thus overestimating average feeding rates.

Flagellate cultures

While the main focus of this study was community grazing of naturally occurring microbes, it was interesting to find differences between 2 cultures of heterotrophic flagellates. While *Cafeteria roenbergensis* accepted various tracers (DTAF-stained and GFP-labeled cells), *Neobodo designis* did not. Feeding rates of *N. designis* were below detection in all treatments except for *B. diminuta* stained with DTAF, and even in that case, the observed rates were extremely low, given a thriving culture of *N. designis* and cell abundances 2 to 4 times those of natural predator populations (Table 1). This means that either *N. designis* greatly selects against all types of tracer particles we offered or, more likely, this flagellate restricts its predation to attached bacteria, largely ignoring particles that are offered in suspension. This is not a trivial problem because kinetoplastids have been identified as particle affiliated before (Artolozaga et al. 2000, Boenigk & Arndt 2002), and other methods need to be considered when feeding rates on particle surfaces are to be determined.

Prey selection other than size

In any tracer study, there is concern whether the tracers are selected for or against in comparison to natural prey. Various studies have shown that some protists select against fluorescent latex beads, dead prey, and prey that has been modified chemically (e.g. fluorescent beads or DTAF staining; e.g. Nygaard et al. 1988, Landry et al. 1991, Jürgens & De Mott 1995, Fu et al. 2003). In our experiments with a natural community, GFP-*B. diminuta* was significantly preferred over DTAF-*B. diminuta* (Table 2). This would agree with a previous study in which GFP-labeled cells were preferred over DTAF-stained cells by a factor of 1.42 (Fu et al. 2003). However, the grazing experiments with *C. roenbergensis* and *B. diminuta* showed the opposite result. A few studies reported feeding rates on natural prey assemblages that were 10 to 20 times higher than those based on prey analogs (Landry et al. 1991, Monger & Landry 1992). Based on immunofluorescent staining of specific bacterial strains after uptake into food vacuoles, Christof-

fersen et al. (1997) concluded that protist grazers select against fluorescently labeled cells. Heat killing of prey may denature some of the surface molecules that may be used for detection by predators, and thus tracer particles may be selected against (Ishii et al. 2002). Yet some studies showed no difference between heat-killed *E. coli* and live *E. coli* (Arana et al. 2003), and others demonstrated that feeding rates on fluorescently labeled bacteria were sufficiently high to conclude that these labeled bacteria are representative of naturally occurring prey (Chrzanowski & Šimek 1990). Some of the selectivity may not be constant and may be modulated by differences in prey abundance, as shown in Jürgens & De Mott (1995) and Dolan & Šimek (1998). Recently, it has been shown for a freshwater system that different types of bacteria represent different food quality that can influence the growth of flagellates (Šimek et al. 2013).

There are many other factors that may influence selectivity for certain prokaryotes including stoichiometry of the prey (Gruber et al. 2009), motility (Monger & Landry 1992, Gonzalez et al. 1993), hydrophobicity (Monger et al. 1999, but see Matz & Jürgens 2001), and defense mechanisms (Hahn et al. 2000, Hahn & Hofle 2001, Matz et al. 2008, and reviews by Jürgens & Güde 1994, Pernthaler 2005), raising the question of how meaningful a single value of bacterivory is for complex communities of prokaryotes (Jürgens & Güde 1994). However, estimates based on the growth dynamics of natural bacteria were very similar to those of GFP-minicells (Fig. 5), which greatly increased our confidence in this particular tracer. Rates derived from these types of experiments are usually considered minimum estimates because there is no nutrient release for the prokaryotes through grazing (Sherr et al. 1988). Our data were consistent with this notion, as estimates in these perturbation experiments were lower than those using GFP-minicells as tracers (Fig. 5). It is thus highly encouraging that the discrepancy was relatively small overall (estimates diverged from 38 to 47% over the measured ranges of *g*, Fig. 5).

Convergence of estimates

We combined work on brackish and marine water samples based on the observation that brackish and marine waters are similar in terms of grazing coefficients (Sanders et al. 1992, Almeida et al. 2001). Variability between studies was greatly reduced by using only 1 average per study for a specific environment (Table 4, Fig. 6). In fact, the variability within a particular study usually exceeded the variability among studies.

Methods based on radiolabeling have been employed in the past both as uptake and removal experiments (Nygaard & Hessen 1990, Zubkov & Sleigh 1995, Zubkov et al. 1998). There are certainly problems with recycling of tracer, loss of tracer from the prey cells during the incubation (e.g. through respiration or through diffusion when cells are heat killed), and the fact that autotrophs also take up significant amounts of organic nutrients (Bronk 2002). Nevertheless, the dual labeling procedure proposed by Zubkov et al. (1998) gives an average grazing coefficient of 0.17 d^{-1} for the oligotrophic regions of the North and South Atlantic, which is also close to the other literature estimates (Fig. 6).

We separated the method by Wikner et al. (1986) because it is somewhat unique in its approach of using ^{35}S-methionine labeled *E. coli* minicells (Wikner et al. 1986, Wikner & Hagström 1988, 1991). While this method resulted in highly variable estimates, the median of all experiments combined again came close to other methods (Fig. 6).

Measurements of uptake of tracers into the food vacuoles over time (Sherr et al. 1987) have several advantages: they are more specific about the predator that consumes prokaryotes and, most importantly, incubation times are shorter. Despite the drastically different approaches, the estimated rate measurements were surprisingly close to other estimates such as inhibitor or dilution experiments, both here and in those reviewed in Vaque et al. (1994) (Fig. 6).

The dilution method (Landry & Hassett 1982, Landry et al. 1995) has most frequently and successfully been used for grazing on small autotrophs. However, in a few cases, it has been used for feeding on heterotrophic bacteria (e.g. Ducklow & Hill 1985, Tremaine & Mills 1987, Geider 1989, Choi et al. 2001, Fonda Umani et al. 2010), for which it received mixed reviews (e.g. Landry 1994, Vaque et al. 1994, Murrell & Hollibaugh 1998, York et al. 2011). We plotted data from a few published dilution experiments we found on heterotrophic bacteria in surface ocean environments (Landry et al. 1984, Murrell & Hollibaugh 1998, Choi et al. 2001, Taira et al. 2009), and again both medians and means were close to those from other techniques (Fig. 5).

Advantages of the GFP-minicell model

There are many advantages of GFP-minicells over other tracers: (1) Minicells are simple and inexpen-

sive to grow and separate well from parental cells.
(2) GFP-minicells already contain their own label,
without heat killing and the use of a fluorescent
stain. This is an advantage even over vital stains
(Epstein & Rossel 1995). (3) Minicells remain mono-
dispersed and do not clump even after repeated
centrifugation. The latter was especially apparent in
comparison with DTAF staining, which always pro-
duced a large number of clumps that needed to be
removed or broken up by sonication in additional
preparatory steps. (4) The GFP vector we inserted is
very bright and photostable in contrast to DTAF-
stained cells (Caron 2001). (5) Minicells keep well
on filters for many months when frozen at −20°C or
for years at −80°C. (6) There is no background fluo-
rescence because no dye, which can stain the filter
surface nonspecifically, is used. In the FITC channel
of the epifluorescence microscope, the background
is almost completely black, even when using un-
stained (and much cheaper) white polycarbonate fil-
ters (e.g. Millipore GTTP). (7) Grown in the same
universally available LB medium, conditions, and
harvesting times, one can assume that the biochem-
ical composition does not change much from experi-
ment to experiment, suggesting a highly repro-
ducible prey quality. (8) The size of the minicells is
much closer to the sizes of naturally occurring bac-
teria than other tracers including even the relatively
small *B. diminuta*. (9) Once ingested, the GFP-
labeled cells quickly disappear. We never encoun-
tered accumulation of GFP-minicells in any of the
predator cells. This is likely due to the loss of the
GFP signal in the acidic vacuoles (pH < 6) and
digestion of GFP by proteases (Parry et al. 2001). A
rapid disappearance upon ingestion has its advan-
tages, as GFP-minicells will not be overcounted ac-
cidentally by including those cells already ingested
by predatory cells.

Disappearance of the tracer once ingested could be
exploited in future studies. Power et al. (2006), for
instance, successfully used the loss of fluorescence of
GFP due to bacterivory by the ciliate *Tetrahymena
thermophile* in microtiter plates. Parry et al. (2001)
used a fluorometer instead of the epifluorescence
microscope to track the removal of GFP-labeled prey
analogs. These approaches save much counting time
and would allow for high throughput. Unfortunately,
the fluorometers and fluorescence plate readers we
had access to were not sensitive enough to accurately
measure the abundance of GFP-minicells at the con-
centrations at which we added them. Flow cytometry,
however, is an obvious choice for future experiments
to increase throughput.

CONCLUSIONS

An argument can be made that the shortcomings of
many methods and the many factors that influence
grazing rate measurements make any attempt to pro-
duce a single loss term for complex aquatic prokary-
otic communities futile. However, the convergence of
estimates from different methods, tracers, and envi-
ronments supports the notion that ecological factors
responsible for constraining the lower and upper lim-
its of grazing exist in mixed microbial communities.
Knowledge of these boundaries is valuable for the
parameterization of ecosystem and biogeochemical
flux models. The GFP-minicell model shown here fits
well within other estimates and, given its many pro-
cedural advantages, is a good candidate for use in
standardized grazing experiments across a wide
range of aquatic environments.

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