

Measuring the grazing losses of picoplankton: methodological improvements in the use of fluorescently labeled tracers combined with flow cytometry

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ABSTRACT: Fluorescently labeled tracers (FLT) are often used to estimate the loss rates of picoplankton to grazers. These tracers are commonly enumerated by epifluorescence microscopy, although flow cytometry is a viable alternative in the detection of FITC (fluorescein-5-isothiocyanate)- or DTAF (5-([4,6-dichlorotriazin-2-yl]amino)-fluorescein)-stained bacterial tracers. However, the bacterivory measured with FLT has hardly been applied to routine monitoring of oceanic waters, partly because of the time-consuming preparation of the tracers and other problems associated with the long-term incubations needed to generate detectable rates of tracer change. In addition, these long-term incubations make samples especially sensitive to the unwanted addition of nutrients carried over with the tracers. Here we present some experiments designed to ease the estimation of grazing rates on bacteria with this technique. Two bacterial strains and 2 fluorescent dyes were tested: *Escherichia coli* minicells (0.065 μm^3) and *Pseudomonas diminuta* (0.064 μm^3), stained with DTAF or with FITC. In addition, instead of the common use of pyrophosphate buffer during the staining protocol, the use of carbonate-bicarbonate buffer and cells scraped directly from solid media is suggested to avoid the problems associated with phosphorus enrichment of the sample that at times can occur in oligotrophic water samples. The FITC- or DTAF-stained tracers can be observed with either epifluorescence microscopy or flow cytometry. However, FITC- or DTAF-stained *P. diminuta* were more easily resolved with the flow cytometer than stained minicells. Flow cytometric detection of *P. diminuta* tracers, prepared in bicarbonate-buffer and stained with FITC, is a fast protocol for the estimation of the grazing loss rates of bacteria in oceanic environments.

KEY WORDS: Bacterial grazing rates · Flagellates · Flow cytometry · *Pseudomonas diminuta*

INTRODUCTION

Although quantification of protistan grazing is essential to understand the carbon flows within microbial food webs (e.g. Sanders et al. 1992), direct measurements of *in situ* grazing rates are relatively scarce in the literature. This is partly because measurements of grazing rates of protists on bacterial populations are time-consuming and tedious to perform. Among the different methods that researchers have used to determine the protistan grazing rates on bacteria, the uptake rates of fluorescently labeled tracers (FLT) are some of the most frequently used (see Landry 1994 for

a review of methods). Beads, natural or cultured bacteria or *Escherichia coli* minicells have been used as bacterial tracers (Cynar & Sieburth 1985, Pace & Bailiff 1987, Sherr et al. 1987, Bjørnsen 1988, Sanders et al. 1989, Pace et al. 1990, Christoffersen et al. 1997). In some of these measurements, the grazing rates are evaluated by the accumulation of labeled tracers inside the protozoan food vacuoles in short-term incubations (minutes to hours), whereas in others it is the disappearance of tracers over time that is measured in long-term incubations (often >24 h).

One of the most time-consuming steps before grazing rate determinations with FLT can be performed is the preparation of tracers from bacterial particles. These labeled tracers can be derived from different

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types of cells. Some studies used concentrates of natural bacterioplankton (Sherr et al. 1987, González et al. 1990, Epstein & Rossel 1995), although this procedure requires the concentration of the bacteria from several liters of water. Pace et al. (1990) described a method for obtaining small minicells derived from a culture of a specific strain of *Escherichia coli*. However, the whole protocol required 2 to 5 d preparation. In some cases FLT can be derived from cultured bacteria, but then the average size is much larger (often $>1 \mu\text{m}^3$) than that of native planktonic bacteria (usually below $0.06 \mu\text{m}^3$).

Before determinations can start, and as soon as enough tracer cells have been obtained, the tracer cells have to be stained with a fluorescent dye, such as DTAF (5-([4,6-dichlorotriazin-2-yl]amino)-fluorescein) or FITC (fluorescein-5-isothiocyanate). For short-term uptake experiments, Monger & Landry (1992) introduced the use of FITC-stained *Pseudomonas diminuta*. This bacterial strain has an average cellular volume (ca $0.064 \mu\text{m}^3$) similar to that of natural bacterioplankton from oligotrophic environments and can be harvested and stained within several hours. In their experiments, Monger & Landry determined the uptake of labeled bacteria by a culture of heterotrophic nanoflagellates with the help of flow cytometry, which improved the precision of the measurement while significantly reducing the analysis time as compared to epifluorescence microscopy determinations. In that experiment, these authors used flow cytometry to measure the increase in flagellate fluorescence caused by the ingested tracer cells. However, due to the low concentration of nanoflagellates commonly found in oligotrophic seawater, the use of the Monger & Landry procedure in these waters is not possible using flow cytometry. Epifluorescence microscopy must be used instead, and this is another time-consuming procedure. For example, if 60% of the protozoan population has ingested 1 tracer cell (e.g. Monger & Landry 1992) and 10 ml of sample have been filtered with a concentration of 500 heterotrophic nanoflagellates ml^{-1} , more than 1000 observation fields must be inspected in each sample in order to enumerate at least 60 protists with 1 ingested tracer cell (with a typical microscope conversion factor of 50 000).

As a consequence of the difficulties in determining bacterial uptake rates in oligotrophic systems using short-term incubations, minicells and long-term incubations have been used in some studies to determine the disappearance rate of the tracers over time and from them extrapolate grazing on bacteria (Pace et al. 1990, Vaqué & Pace 1992, Vaqué et al. 1992). This method produces results comparable to the short-term determinations (see review of data in Vaqué et al. 1994).

The main goal of this paper is to describe several improvements in the procedure originally described by Sherr et al. (1987), and later modified by Landry et al. (1995), to determine the disappearance of fluorescent tracers by flow cytometry in long-term incubations. Sherr et al. (1987) used several DTAF-stained monospecific bacterial cultures, or bacterioplankton concentrates, to determine the ingestion of protists in short-term experiments with the aid of epifluorescence microscopy, whereas Landry et al. (1995) used the decrease of a DTAF-stained *Vibrio damsella* ($1.1 \mu\text{m}$ diameter), used as a *Synechococcus* tracer, over 24 h to derive the grazing rates of protists on cyanobacteria. To the best of our knowledge, however, the use of long-term incubations combined with flow cytometry to enumerate the tracer has not been used in field studies of grazing on heterotrophic bacteria.

Additionally, one of the factors to take into account in long-term incubations of oligotrophic water is the possible stimulation of bacterial growth with nutrients that are carried over with the tracer cells. Here we show that phosphorus-free FITC- or DTAF-stained *Pseudomonas diminuta* and incubations of up to 48 h, combined with flow cytometry, provide a useful protocol to estimate grazing rates on natural populations of heterotrophic bacteria.

In Mediterranean coastal seawater, several long-term (up to 48 h) experiments were done to further resolve: (1) which combination of tracer strain (*Pseudomonas diminuta* or minicells) and fluorescent dye (DTAF or FITC) provides the best resolution using flow cytometry; (2) the advantages associated with the use of cells scraped from solid media and carbonate-bicarbonate buffer in the staining protocol; (3) the accuracy of flow cytometric counts of the fluorescent tracers as compared to epifluorescence microscopy counts; and (4) whether the estimates of protist bacterial ingestion using either minicells or *P. diminuta* as tracers are comparable.

MATERIAL AND METHODS

Tracers. Two kinds of tracers were tested, a *Pseudomonas diminuta* strain obtained from the Spanish Type Culture Collection (Burjasot, Valencia), and minicells derived from the minicell-producing *Escherichia coli* strain X-1488, obtained from the *E. coli* Genetic Stock Center (Yale University). The 2 strains were maintained at 20°C in Luria-Bertani (LB) medium (Maniatis et al. 1982) agar plates and transferred to new plates every other week.

Pseudomonas diminuta tracers were produced by scraping cells from 2 wk old agar plates and suspending them in carbonate-bicarbonate buffer (CO_3Na_2 -

HCO₃Na pH 9.5; Robinson et al. 1994). Minicells were produced by inoculating the minicell strain in LB liquid medium and allowing it to grow overnight at 37°C. Minicells were separated from the larger *Escherichia coli* cells by sucrose-gradient centrifugation as described by Pace et al. (1990). The overnight culture was first centrifuged at 2000 rpm (409 × *g*, Sorval RC5C centrifuge, GSA rotor) for 5 min to remove debris and some larger cells. The supernatant containing a mixture of minicells and larger cells was then pelleted by centrifugation at 6000 rpm (3687 × *g*, Sorval RC5C centrifuge, GSA rotor) for 20 min. The pellet was suspended in 2 to 3 ml of buffered gelatin saline (BSG; 0.85% NaCl, 0.03% KH₂PO₄, 0.06% Na₂HPO₄, and 100 µg of gelatin ml⁻¹) and this cell suspension was layered over 30 ml of sterile 5 to 20% (wt/vol) sucrose gradients. Finally, the cells were centrifuged at 4000 rpm (3077 × *g*, Sorval RC5C centrifuge, HS-4 rotor) for 15 min, and bands of purified minicells were recovered from the sucrose gradients, pelleted by centrifugation and suspended in carbonate-bicarbonate buffer. The whole procedure took at least 2 d.

Staining procedure. To stain *Pseudomonas diminuta* or minicells with FITC, the cell suspension was heat-killed for 2 h in a water bath at 60°C and then 100 µg ml⁻¹ FITC were added, allowing the cells to stain for 2 h (Cantinieux et al. 1989). Minicells or *P. diminuta* were stained with DTAF by incubating the cell suspension with 100 µg ml⁻¹ DTAF for 2 h in a water bath at 60°C (Sherr et al. 1987). Stained cells were rinsed with filtered (<0.2 µm) seawater or carbonate-bicarbonate buffer, resuspended and centrifuged 3 to 5 times (10 min, 10 000 rpm [21 004 × *g*], IEC Micromax centrifuge, 851 rotor) to prevent the transfer of leftover dye to the natural samples. The cell suspensions were refrigerated (4°C) for use within hours, or frozen (-20°C) for later use. Before addition to the experimental containers, the tracers were thawed and gently sonicated (3 rounds of 10 s with the microtip at 30% of power output, Dynatech sonic dismembrator) to prevent cell clustering.

The size distribution of the stained tracers was measured with an image-analysis system (described in Massana et al. 1997). *Pseudomonas diminuta* scraped from agar plates are small bacillar cells of 0.064 µm³ volume (1.07 µm length, 0.29 µm width), whereas minicells are small spheres of 0.065 µm³ volume (0.5 µm in diameter).

Grazing experiments. Using natural plankton communities from Blanes bay (NW Mediterranean), two 48 h grazing experiments were carried out in November and December 1997. Subsurface seawater was collected from the bay at a station 12 m deep (41° 39.90' N, 2° 48.03' E), about half a mile offshore. Both experiments were run in the laboratory in a constant

temperature chamber (ca 19°C, similar to *in situ* water temperature) with a 12 h light:12 h dark period. Sub-samples were withdrawn after 0, 24 and 48 h to estimate abundance of HB (heterotrophic bacteria), HNF (heterotrophic nanoflagellates) and FLT.

In November (19 to 21 November 1997), two 1.5 l seawater samples were 50 µm filtered (to avoid larger ciliates and nauplii), and FITC-stained *Pseudomonas diminuta* were added to a concentration of 10.1% that of natural bacteria. The abundance of the *P. diminuta* tracer was obtained from epifluorescence microscopy and flow cytometry, whereas HB and HNF were counted by epifluorescence microscopy.

In December (12 to 14 December 1997), 6 l of water were 50 µm filtered. Two 1.5 l replicates received FITC-stained *Pseudomonas diminuta* to a concentration of 18.6% that of natural bacterial abundance, while another 2 replicates received DTAF-stained minicells to a concentration of 14.1% that of natural bacterial abundance. FLT, HB and HNF abundances were obtained by epifluorescence microscopy.

In these experiments, specific grazing rates (d⁻¹) and community grazing rates (HB ml⁻¹ d⁻¹) were calculated from the change in tracer and bacterial abundance over time following the model of Salat & Marrasé (1994). From community grazing rates, with the average abundance of nanoflagellates during the incubation, nanoflagellate ingestion rates were calculated assuming that nanoflagellate grazing was the only factor responsible for the disappearance of bacterial tracers.

Disappearance of FLT not associated with grazing. Seawater samples from 2 NW Mediterranean locations were used to test for non-predatory mortality of bacteria. In one experiment two 1.5 l samples from the Blanes harbor (19 to 21 November 1997) were 0.8 µm filtered, amended with FITC-stained *Pseudomonas diminuta*, and maintained at ambient temperature (ca 20°C). In the other experiment (3 to 5 June 1998), water from a 2 mile (3.2 km) offshore location near Masnou was 0.2 µm filtered and samples (125 ml) were incubated with DTAF-stained *P. diminuta*. Twenty-ml samples were withdrawn at 0 and 48 h and processed by epifluorescence microscopy to obtain FLT abundance.

Additionally, to determine the effect of freezing cycles on preserved samples, 45 Mediterranean seawater samples containing DTAF-stained *Pseudomonas diminuta* (frozen at -70°C) were thawed and FLT abundance was measured by flow cytometry. Once counted, samples were frozen a second time (-70°C), thawed and counted once again. Finally, 11 of these 45 samples were frozen (-70°C), thawed and counted a third time.

Inorganic phosphorus addition. Two experiments were done to know if leftovers of inorganic phosphorus

were added with FLT to the experimental containers. NW Mediterranean seawater from Masnou was amended with tracers and dissolved inorganic nutrients were analyzed against non-amended seawater controls.

Before the first experiment began, the *Pseudomonas diminuta* cells were grown overnight (at 30°C) in a liquid LB culture. Thereafter, 2 ml of this culture was withdrawn, centrifuged (10 min, 10 000 rpm [21 004 × *g*], IEC Micromax centrifuge, 851 rotor) and suspended in filtered (0.2 µm) Masnou seawater. Then, the cells were stained with DTAF as described above. Once stained, the cells were rinsed 5 times with filtered (0.2 µm) Masnou seawater and resuspended in the same seawater. In this experiment, 2 l of Masnou seawater were amended with *P. diminuta*. One liter of this seawater sample was amended with 10 ml of FLT suspension (for a final concentration of 3.0 10⁷ FLT ml⁻¹), whereas the second 1 l sample served as control without tracer addition.

In the second experiment, *Pseudomonas diminuta* was obtained from a 2 wk old agar plate, suspended in carbonate-bicarbonate buffer and stained with DTAF as described above. The stained cells were rinsed 5 times with filtered (0.2 µm) seawater and suspended in the same seawater. Two 60 ml seawater samples were amended with 1.7 ml of FLT suspension (for a final concentration of 3.0 10⁷ FLT ml⁻¹), and a third sample served as a control without tracer addition.

From every experiment and sample, subsamples were withdrawn for FLT counts, which were performed with epifluorescence microscopy, and for nutrient analyses (20 ml), which were performed with an Evolution II autoanalyzer according to standard methods (Hansen & Grasshoff 1983).

Counting by epifluorescence microscopy. Thirty-ml samples were collected and preserved with glutaraldehyde (1% final concentration). Subsamples of 5 ml for HB, or 20 ml for HNF, were filtered through 0.2 or 0.6 µm pore size black polycarbonate filters to collect the different microorganisms. Subsamples were stained with DAPI (4'-6-diamidino-2-phenylindole) at 5 µg ml⁻¹ (final concentration), as recommended by Sieracki et al. (1985), following the technique of Porter & Feig (1980). HB and HNF abundances were obtained at 1250× magnification (Nikon Labophot 2) under UV excitation. *Pseudomonas diminuta* and minicells were counted using an optical filter set specific for yellow-green fluorescence (485 ± 11 nm excitation and 530 ± 15 nm emission wavelength, 505 nm dichroic mirror). At least 200 cells were counted in 20 random fields to enumerate HB and FLT (Kirchman et al. 1992), whereas from 20 to >100 cells were counted surveying three 10 mm strips to enumerate HNF.

Counting by flow cytometry. Two ml of sample were preserved, with 200 µl of 10% paraformaldehyde (1% final concentration) and stored between 0 and 4°C. Subsamples (200 µl) were withdrawn for analysis and processed in a FACS Calibur (Becton Dickinson) flow cytometer within 24 h of collection. The flow cytometer was equipped with a 15 mW Argon-ion laser (488 nm emission). Fluorescent beads (1 µm, Fluoresbrite carboxylate microspheres, Polysciences Inc., Warrington, PA) were added at a known density as internal standard. The bead standard concentration was determined by epifluorescence microscopy. DTAF and FITC fluorescence could be easily identified in plots of SSC (side scatter) versus green fluorescence (525 nm). Ten thousand events were acquired at low flow rate (ca 16 µl min⁻¹) in every sample. Data acquisition and analysis were performed with specific flow cytometry software (Becton Dickinson).

Microscopy versus flow cytometry. In 1998, 27 Mediterranean seawater samples containing DTAF-stained *Pseudomonas diminuta* were counted with flow cytometry and epifluorescence microscopy. FLT abundance was log transformed and methods of counting were compared.

RESULTS AND DISCUSSION

Staining

FITC-stained or DTAF-stained *Pseudomonas diminuta* had noticeably different fluorescence when observed in epifluorescence microscopy under blue light excitation. FITC cells appeared green, whereas DTAF cells were more yellowish and strongly stained throughout.

In the flow cytometer, there were no differences between the signal of the FITC- or the DTAF-stained minicells when added to natural seawater. They were also difficult to discriminate from the noise in the FL1 (green fluorescence) versus SSC (side scatter) scatterplot (Fig. 1A). On the contrary, DTAF-stained *Pseudomonas diminuta* added to natural seawater were clearly resolved (Fig. 1B). In addition, FITC-stained *P. diminuta* had nearly 2-fold higher fluorescence than DTAF-stained tracers (Fig. 1C) with identical instrument settings.

The specifications for both dyes in solution are almost identical, in terms of excitation and emission maximum wavelengths (Haughland 1996), but there are some differences. At 60°C and pH > 9, DTAF stains polysaccharides and proteins whereas FITC stains only proteins (Schumann & Rentsch 1998). Additionally, the FITC quantum yield (the number of photons produced per photon absorbed) is higher than that of the DTAF.

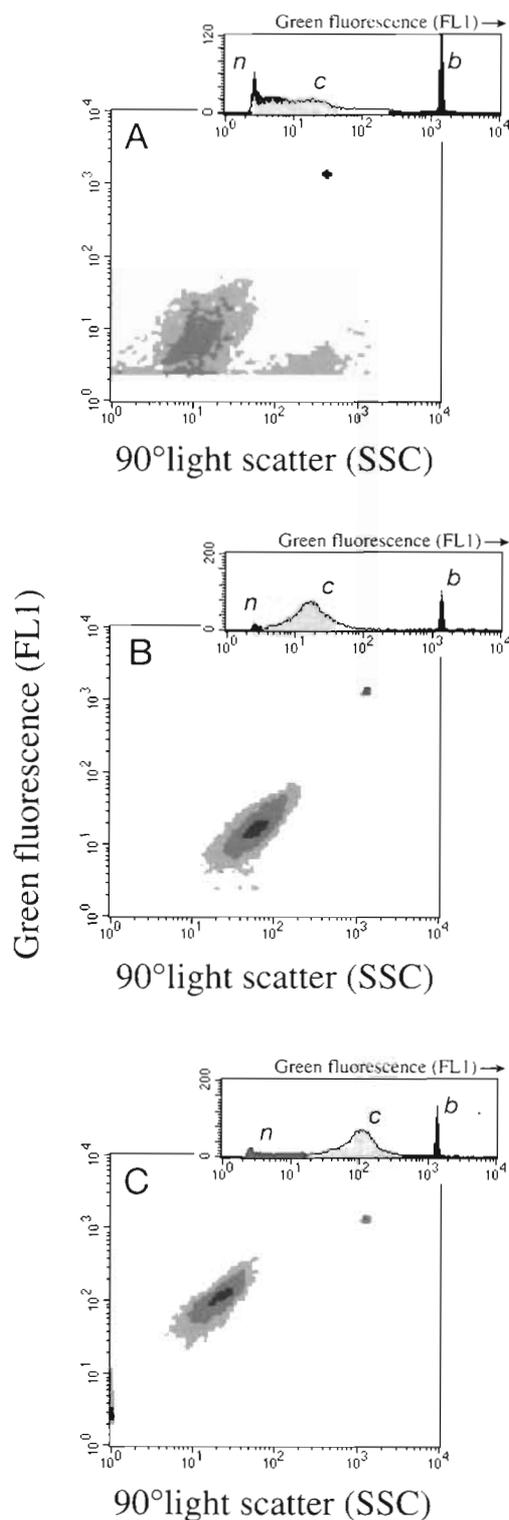


Fig. 1. Flow cytometric acquisition plots of *Escherichia coli* minicells and *Pseudomonas diminuta*. Green fluorescence (FL1) versus 90° light scatter (SSC) of (A) DTAF-stained minicells in seawater, (B) DTAF-stained *P. diminuta* in seawater and (C) FITC-stained *P. diminuta* in seawater. (n: noise, c: cells; b: beads)

These parameters, which apply to the dyes in solution, can be altered as a consequence of the actual binding, and thus, it is not surprising that the 2 dyes produce somewhat distinct staining. Differences between epifluorescence microscopy and flow cytometry may be explained because of the very narrow window for excitation wavelengths that the flow cytometry uses as compared to epifluorescence microscopy. Although the dyes have almost identical excitation and emission maxima, they have broad excitation and emission spectra that can account for some of the differences between both techniques.

To enhance the binding of FITC to the cell surface, Monger & Landry (1992) amended the *Pseudomonas diminuta* cell suspension with 40 $\mu\text{g ml}^{-1}$ dithioerythritol and let the cells stain overnight at 37°C. However, no difference in fluorescence yield was observed when this procedure was used (data not shown). Furthermore, cellular volume in overnight flasks tended to increase during the incubations, while the cells produced from 2 wk old agar plates had a more standardized volume, closer to that of planktonic bacteria. We, thus, decided to avoid any manipulation of the cultures before staining.

Based on the easier visualization of the cells, and the more constant cellular volume, we recommend the use of *Pseudomonas diminuta* recovered from agar plates. If only flow cytometry has to be used, FITC-stained cells give a higher fluorescence signal than DTAF-stained cells. If both flow cytometry and epifluorescence microscopy has to be used (e.g. when HNF-specific grazing rates are to be estimated), DTAF-stained cells are better overall.

Some questions associated with the use of fluorescent labeled tracers

When FLT are used for measuring grazing rates, one possible problem is the unwanted addition of nutrients carried over with the tracers. This can be particularly important when (as recommended by Sherr et al. 1987) pyrophosphate buffer is used and the measurement is done in oligotrophic waters, where nutrients are scarce and P is likely to be limiting (e.g. Cotner et al. 1997, Thingstad et al. 1998). Our data show that, when tracers are obtained from liquid culture, it is very difficult to eliminate excess nutrients. Even after having washed the tracers 5 times with filtered (0.2 μm) seawater (instead of the recommended pyrophosphate solution), its addition still produced a 50-fold increase in P concentration as compared to P concentration in seawater alone (Fig. 2). However, when tracers were obtained from a 2 wk old agar plate and carbonate-bicarbonate buffer was used, the concentration of inor-

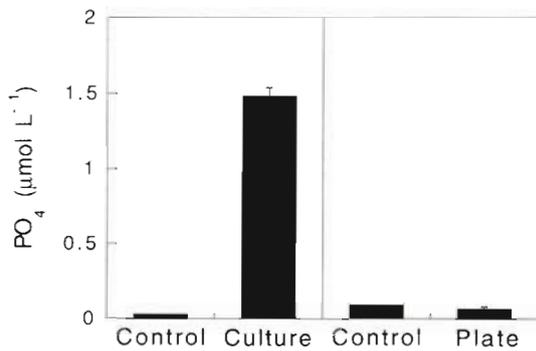


Fig. 2. Contamination of seawater with inorganic phosphorus carried with the addition of fluorescent labeled tracers (DTAF-stained *Pseudomonas diminuta*). Control: seawater samples without tracer addition; Culture: tracers obtained from liquid culture; Plate: tracers scraped from agar plates and rinsed with carbonate-bicarbonate buffer

ganic phosphorus was not different from the control seawater samples.

Another possible problem in grazing determinations is the disappearance of the tracers over time owing to factors external to protozoan grazing. Light-degradation, cellular lysis, free-enzyme attacks, etc. are likely causes for this disappearance. When FLT were added to 0.2 or 0.8 µm filtered seawater (thus effectively eliminating most bacterial predators but including these other factors), no differences were found in the abundance of FITC-stained ($n = 2$, Student's t -test = 0.49, $p = 0.66$) or DTAF-stained ($n = 2$, Student's t -test = 1.91, $p = 0.19$) *Pseudomonas diminuta* between 0 and 48 h (Fig. 3). Thus, at least for the time periods of this study, the disappearance of FLT could not be attributed to causes other than protist grazing.

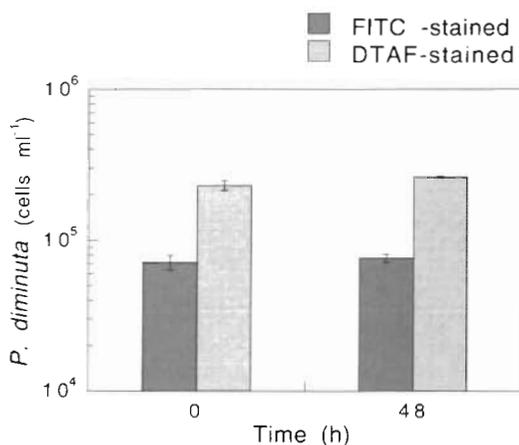


Fig. 3. Variation of the concentration of FITC- or DTAF-stained *Pseudomonas diminuta* after 48 h of incubation in filtered (<0.2 µm) Mediterranean seawater

In addition, care has to be taken with refreezing samples for flow cytometric analysis, since the freezing and thawing process generates disappearance of tracer cells. When the samples were thawed for a second time, the abundance of FLT was on average 56% ($n = 45$) of the number counted in the initial subsamples. Finally, when they underwent a third freezing and thawing cycle, abundances went down to a 27% average ($n = 11$). The freezing and thawing process possibly produce some leakage of bound dye into the liquid phase. This process has to be taken into consideration so that either samples are counted only once after thawing them for the first time or appropriate corrections are applied when counting refrozen samples.

The last factor studied here was the choice of counting method. Tracer counts using flow cytometry and epifluorescence microscopy were similar ($n = 27$, $r^2 = 0.87$, $p < 0.001$). The relation between the log-transformed abundance of DTAF-stained *Pseudomonas diminuta* counted with flow cytometry (FCM) and epifluorescence microscopy (EPI) was: $\log_{\text{FCM}} = 0.23 (\pm 0.32) + 0.95 (\pm 0.07)\log_{\text{EPI}}$, indicating that there were no significant differences associated with the counting method (slope not significantly different from 1 and intercept not significantly different from 0).

Estimation of grazing losses and final considerations

In the experiment performed in November 1997 (Fig. 4), mean (\pm SE) bacterial abundance increased nearly 2-fold after 48 h, from $7.88 (\pm 0.06) \times 10^5$ to $1.35 (\pm 0.15) \times 10^6$ HB ml⁻¹. In the same period, HNF had increased nearly 3-fold, from $236 (\pm 19)$ to $728 (\pm 242)$ HNF ml⁻¹. Simultaneously, *Pseudomonas diminuta* tracers were added at an initial mean density of $7.35 (\pm 0.34) \times 10^4$ FLT ml⁻¹ determined by epifluorescence microscopy, or $7.93 (\pm 0.68) \times 10^4$ FLT ml⁻¹ determined by flow cytometry, and the differences between methods were not significant (Student's t -test = 0.78, $p = 0.53$). Moreover, the losses of *P. diminuta* tracers determined at 24 and 48 h by epifluorescence microscopy and flow cytometry were also similar. Grazing rates derived from the change in abundance of *P. diminuta* determined by flow cytometry, $1.04 (\pm 0.34) \times 10^5$ HB ml⁻¹ d⁻¹, did not differ significantly (Student's t -test = 0.34, $p = 0.74$) from those obtained by epifluorescence microscopy, $1.31 (\pm 0.69) \times 10^5$ HB ml⁻¹ d⁻¹, but had smaller standard errors (Fig. 5).

In December 1997, the replicates containing minicells or *Pseudomonas diminuta* showed a similar evolution of bacterial abundance with time (Fig. 6A,B). Mean bacterial abundance increased nearly 2-fold after 48 h in the replicates containing minicells, from $1.07 (\pm 0.09) \times 10^6$ HB ml⁻¹ to $1.82 (\pm 0.11) \times 10^6$ HB ml⁻¹,

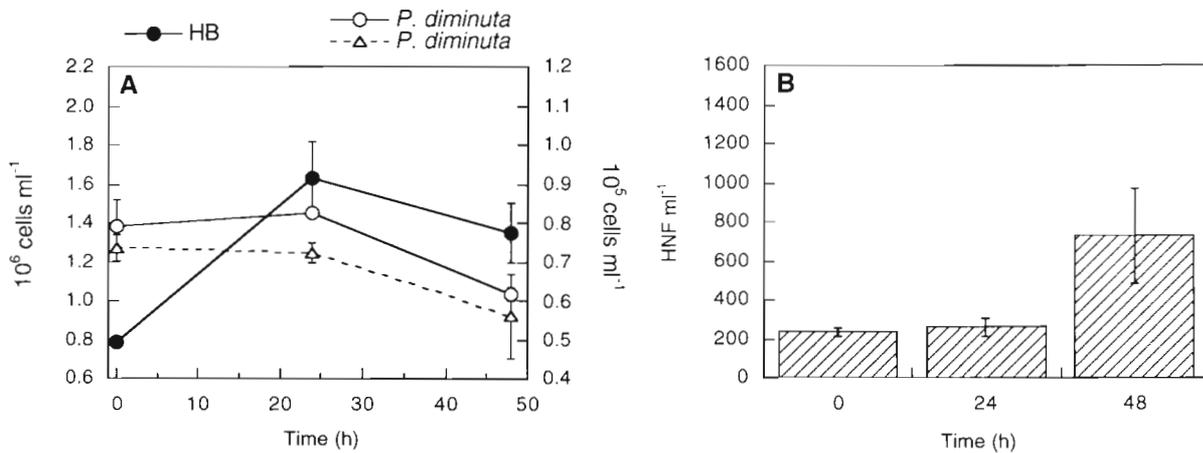


Fig. 4. Disappearance with time of FITC-stained *Pseudo-monas diminuta* as counted by flow cytometry (Δ) or by epifluorescence microscopy (\circ) in a typical determination of bacterial loss rates. Evolution of the concentration of (A) heterotrophic bacteria (HB) and (B) heterotrophic nanoflagellates (HNF), both counted by epifluorescence microscopy

or in those containing *P. diminuta*, from $1.05 (\pm 0.03) \times 10^6 \text{ HB ml}^{-1}$ to $1.77 (\pm 0.10) \times 10^6 \text{ HB ml}^{-1}$. Compared to the previous experiment there was higher initial abundance of HNF, $926 (\pm 83) \text{ HNF ml}^{-1}$ in the replicates with minicells or $1143 (\pm 223) \text{ HNF ml}^{-1}$ in the replicates with *P. diminuta*. However, no protist growth was observed at 24 or 48 h (Fig. 6C,D). The added concentration of *P. diminuta*, $1.96 (\pm 0.10) \times 10^5 \text{ FLT ml}^{-1}$, was slightly higher (Student's *t*-test = 3.76, $p = 0.06$) than the added amount of minicells, $1.51 (\pm 0.06) \times 10^5 \text{ FLT ml}^{-1}$, (Fig. 6A,B), whereas no significant differences in the number of ingested bacteria, calculated with *P. diminuta*, $3.41 (\pm 0.82) \times 10^5 \text{ HB ml}^{-1} \text{ d}^{-1}$, or minicells,

$4.06 (\pm 0.63) \times 10^5 \text{ HB ml}^{-1} \text{ d}^{-1}$, were detected (Fig. 5, Student's *t*-test = 0.32, $p = 0.55$).

In these experiments, there were differences in the total ingestion of HB (Student's *t*-test = 3.91, $p < 0.01$). However, these differences disappear when the number of bacteria ingested by protists is compared (Student's *t*-test = 1.31, $p = 0.20$). These results suggest that the differences found were due to different abundances of protists and not to the type of tracer used. Furthermore, the average number of bacteria ingested per nano-flagellate was $11.41 (\pm 2.6) \text{ HB HNF}^{-1} \text{ h}^{-1}$ when *Pseudomonas diminuta* had been used as tracer and $19.43 (\pm 4.5) \text{ HB HNF}^{-1} \text{ h}^{-1}$ when minicells had been used, and these results are not different between tracers (Student's *t*-test = 1.53, $p = 0.14$). Finally, the range of bacteria ingested per nanoflagellate was from 1 to $32 \text{ HB HNF}^{-1} \text{ h}^{-1}$, which is within the habitual values for HNF (Sherr et al. 1986, Weisse 1989, Weisse & Scheffel-Möser 1991, Vaqué et al. 1994).

A combination of flow cytometry and FLT has been used before to estimate grazing rates, but these studies involved: short-term experiments (e.g. 22 min in Monger & Landry 1992), the use of fluorescent latex microspheres as tracers (Gerritsen et al. 1987), or the use of bacterial strains with a size (diameter $1.1 \mu\text{m}$) larger than the average HB found normally in oligotrophic waters (Landry et al. 1995). In the present work we have presented a protocol that uses *Pseudomonas diminuta*, with an average cellular volume similar to that of natural bacteria, as FLT for bacterial loss rates to grazing determinations in long-term incubations. As noted by Landry (1994), the use of long-term experiments and flow cytometry allows the possibility of assessing the decline in labeled tracers even if they are in a very low concentration with respect to the natural

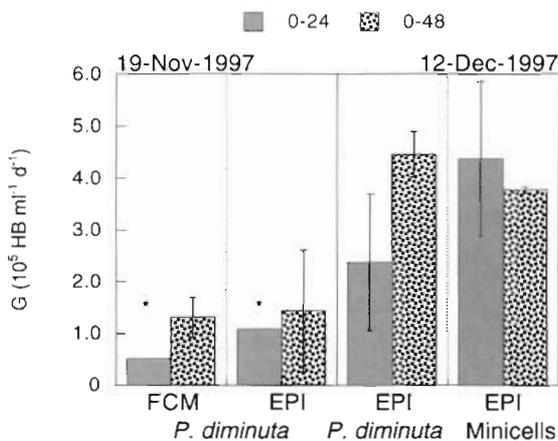


Fig. 5. Total number of grazed bacteria (G) between time 0 and 24 or 48 h as estimated from the disappearance of the bacterial tracers. Different counting methods (FCM: flow cytometry, EPI: epifluorescence microscopy) and different fluorescent labeled tracers (FLT) were used in the 2 experiments. *Grazing detected in 1 of the replicates only

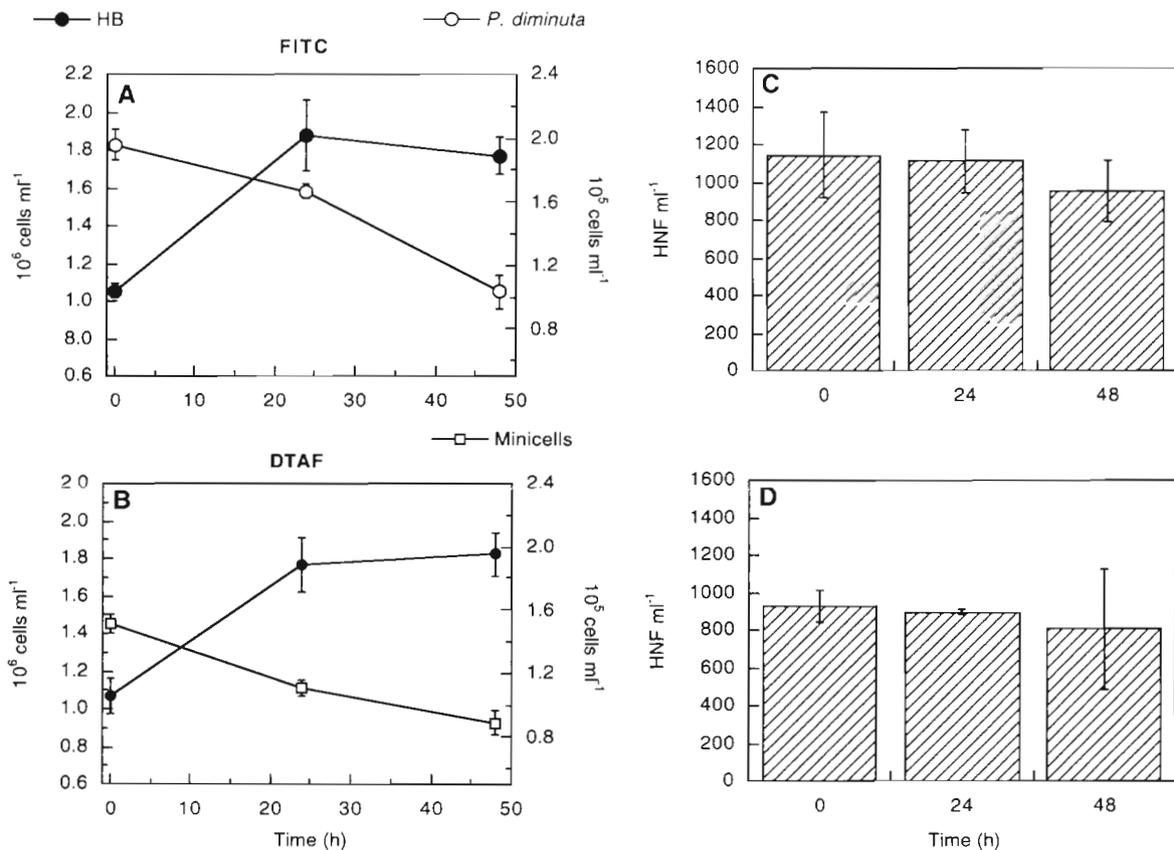


Fig. 6. Disappearance with time of (A) FITC-stained *Pseudomonas diminuta* and (B) DTAF-stained minicells. Evolution of the concentration of (A, B) bacteria (HB) and (C, D) heterotrophic nanoflagellates (HNF). All the microorganisms were enumerated with epifluorescence microscopy

population of heterotrophic bacteria. In oligotrophic environments, and especially during long-term incubations, it is necessary to be careful not to enrich the sample with carried-over nutrients. The use of bacteria grown on agar plates, and the use of the carbonate-bicarbonate buffer to prepare the cells, is a practical solution in those environments.

Some caution should be taken when using FLT. If bacterial size and bacterial specific activity influence the likelihood of a bacterium being predated, the use of an inactive tracer that has a fixed size can introduce measurement artifacts. Various studies have shown that larger bacterial sizes suffer higher grazing pressure by protozoa (Chrzanowski & Šimek 1990, González et al. 1990, Monger & Landry 1991, 1992), although it seems that size-selectivity might depend on the protists' species composition (Kinner et al. 1998). In any case, the use of a bacterial strain with a size which is at the upper limit of the size distribution of the oceanic bacteria that can usually be found in coastal Mediterranean waters (Massana et al. 1997, authors' unpubl. data) or in more oligotrophic places (Kirchman

et al. 1995, Carlson et al. 1996) would overestimate more than underestimate grazing rates. To solve the problem of the different size distribution of natural bacteria and added tracers, some authors have suggested concentrating the natural bacteria (e.g. Sherr et al. 1989) before staining. However, this is not a useful protocol when many determinations in widely different water masses must be performed in a short time: many liters of seawater must be concentrated before a large enough number of FLT can be obtained.

Furthermore, protozoa select their prey according to their activity (Landry et al. 1991, Epstein & Rossel 1995, Christoffersen et al. 1997) although probably depending on the relative amount of available food (e.g. Jürgens & DeMott 1995). Then the use of heat-killed tracers would underestimate grazing rates, thus affecting the measured rates in the other direction. The use of radioactivity-labeled tracers (Zubkov et al. 1998) could solve this problem, but this procedure involves size fractionation for separation of protozoa from their prey, which introduces other artifacts. Besides, the abundance of active bacteria is low in oligotrophic sys-

tems and tends to decrease with decreasing system productivity (del Giorgio & Scarborough 1995). Under such conditions, the calculation of grazing rates on active bacteria might overestimate the rates of total bacterivory. Finally, no difference has been observed between grazing rates determined in long-term experiments with inactive cells or metabolic bacterial inhibitors (Sherr et al. 1986). This might indicate that in this kind of experiment the activity of the tracers added would not influence the grazing rates obtained.

An additional problem is that related to the container volume. We assume that volumes larger than 1 l do not affect grazing rates on bacteria (e.g. Marrasé et al. 1992). However, when experiments are carried out inside containers, community activity and even composition might be altered (Ferguson et al. 1984, Lim et al. 1998), and this could certainly affect the grazing rates obtained. Future research should be addressed to better understand the effect of the container in the changes of community composition and the associated grazing rates.

In this study, the abundance of HB was determined by epifluorescence microscopy. However, total bacteria counts can be obtained by flow cytometry in the same aliquot where tracers are counted (staining with Syto13, SybrGreen or PicoGreen, e.g. Gasol & del Giorgio in press). The advantages of using flow cytometry instead of epifluorescence are well known: (1) small volumes (<1 ml) of sample are needed, compared to larger volumes required by epifluorescence microscopy (5 to 10 ml); (2) enumeration is faster and more precise in the cytometer than in epifluorescence microscopy; (3) in some cytometers automatic counting of samples is possible, thus enabling an increased sampling frequency and improving temporal resolution (e.g. Jacquet et al. 1998); (4) flow cytometry also involves lower costs per sample analyzed than epifluorescence microscopy (Gerritsen et al. 1987); and (5) the cytometer allows the use of biochemical probes for the direct determination of grazing measurements (E. Sintés & P. A. del Giorgio pers. comm.).

FLT (beads, natural bacteria, minicells) are commonly used to determine the losses of bacteria to grazers. Our results let us suggest that the flow cytometric analysis of the loss rates of FITC- or DTAF-stained *Pseudomonas diminuta*, scraped from agar plates and washed in carbonate-bicarbonate buffer, in long-term experiments is a fast and easy way of estimating the *in situ* loss rates of natural planktonic bacteria to grazers.

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