

Significant year-round effect of small mixotrophic flagellates on bacterioplankton in an oligotrophic coastal system

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Abstract

The seasonal variation in the grazing effect of mixotrophic flagellates on bacterioplankton was assessed during an annual cycle in an oligotrophic coastal station in the northwest Mediterranean Sea. Ingestion rates of fluorescently labeled bacteria were estimated for different size categories of phytoflagellates (PF) and heterotrophic flagellates (HF) in short-term experiments and compared with long-term grazing estimates and published empirical models. The mixotrophic flagellates included haptophyte-like cells, cryptophytes, and dinoflagellates. The group-specific grazing rates (SGR) averaged 1.1 (3–5 μm PF), 1.3 (5–20 μm PF), 4.0 (<5 μm HF), and 15.4 bacteria individual⁻¹ h⁻¹ (5–20 μm HF). Lower SGR but higher abundances of PF resulted in an average mixotroph contribution of 50% to the total flagellate grazing. Remarkably, the effect was relatively high all through the year (35–65%). Regardless of the presence of chloroplasts, flagellates <5 μm in size accounted, on average, for about 80% of total flagellate bacterivory and ingested a large percentage of their cell carbon per day from bacteria. Soluble reactive phosphorus concentration was negatively correlated with the ingestion rate of both groups of PF, suggesting that mixotrophic flagellates would be using their phagotrophic capability to obtain phosphorus when this nutrient is limiting. HF grazing activity showed a marked seasonality, with grazing being higher during the warmer seasons, and clearance rates were positively correlated with water temperature. Total bacterivory accounted for most of the bacterial production. Short-term and long-term bacterivory measurements were highly correlated, confirming that the smallest flagellates were the main causative agent of bacterial loss. The bacterivory values were also well correlated to a published empirical model that considers HF as the only bacterivorous. However, this model underestimated (up to 50%) total flagellate grazing during periods of high effect of mixotrophic flagellates.

Many flagellated protists in marine and freshwater environments are capable of a mixotrophic mode of nutrition. Mixotrophy, defined here as the combination of photosynthesis and particle grazing (*sensu* Sanders 1991), has been observed in most phytoflagellated groups: chrysophytes (Bird and Kalff 1986), dinoflagellates (Stoecker et al. 1997), cryptophytes (Urabe et al. 2000), dictyochophytes (Nygaard and Tobiesen 1993), euglenophytes (Epstein and Shiaris 1992), and haptophytes (Skovgaard et al. 2003). Although the ability to ingest particulate material by unicellular algae had been described almost one century ago (Biecheler 1936), the potential significance of this behavior for the elemental flow through the microbial food web was recognized only during the last two decades (Sanders 1991). The fact that many algae are not strictly primary producers but part of their carbon

comes from the ingestion of picoplankton has important implications for the carbon flux within the microbial food web. Since nanoplankton is mainly grazed by zooplankton, mixotrophy would strengthen the link between the microbial and the classical aquatic food webs, increasing the trophic efficiency of the microbial loop (Sanders 1991).

Mixotrophic flagellates can be found in marine and freshwater systems at about 10² cell mL⁻¹ to 10³ cell mL⁻¹ (Sanders 1991). This suggests that they might contribute significantly to total bacterivory. In fact, they have been observed as major picoplankton grazers in a variety of environments. Havskum and Riemann (1996) calculated that mixotrophs were responsible for up to 86% of the total bacterial grazing in some coastal environments, whereas Safi and Hall (1999) estimated a contribution of 55% in the subtropical convergence east of New Zealand. In some freshwater lakes mixotrophs may remove more bacteria than the crustacean, rotifer, and ciliate communities combined (Bird and Kalff 1986). Berninger et al. (1992) showed that mixotrophic flagellates in an ice-covered lake could account for up to 88% of the total observed uptake of bacteria-sized particles. As an extreme, in some high mountain lakes (Medina-Sánchez et al. 2004), and in some ponds during the cold seasons (Hitchman and Jones 2000), the entire measured bacterivory has been assigned to plastidic flagellates.

In spite of this, models predicting grazing interactions within the microbial food web commonly consider heterotrophic nanoflagellates as the main grazers on bacterio-

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plankton (e.g., Vaqué et al. 1994; Legendre and Rivkin 2005), with exceptions, as mixotrophs were included in some food web models as potential grazers on picoplankton (Baretta-Bekker et al. 1998; Jost et al. 2004). Results of some of them indicate that a significant amount of bacterial carbon should flow toward higher trophic levels through the mixotrophic component (Baretta-Bekker et al. 1998). To incorporate mixotrophy in the models, it is necessary that a coherent data body exists with precise estimations of their grazing effect on bacterioplankton as compared to that of strictly heterotrophs and with evaluation of seasonal or trophic (e.g., oligotrophic–eutrophic) patterns in the contribution of mixotrophs to picoplankton grazing in different environments.

In contrast to the relatively large number of studies carried out with cultured mixotrophic protists, there are comparatively few estimations of the actual grazing effects of natural mixotrophic flagellate assemblages on bacterioplankton, discriminating the grazing attributable to mixotrophs from that due to strict heterotrophs (e.g., Bennett et al. 1990; Havskum and Riemann 1996; Hitchman and Jones 2000). Moreover, the marine surveys have been completed in relatively short periods, not considering the influence of variables that change at the seasonal scale, such as light, temperature, and nutrient concentrations, which could be also accompanied by changes in protist assemblage composition. Mixotrophic flagellates vary widely in their photosynthetic and feeding capabilities (Jones 1994), therefore the contribution of mixotrophic algal phagotrophy to the control of bacterial biomass would be highly variable, depending on the dominating algal species and the prevailing environmental conditions. Lake studies showed strong seasonal differences in the relative effect of mixotrophic flagellates (Sanders et al. 1989; Hitchman and Jones 2000) that have been associated with changes in species composition throughout the phytoplanktonic annual succession. In contrast, seasonality of mixotrophic effect has not yet been addressed in marine systems.

Additionally, seasonality might also affect underwater light conditions and nutrient concentrations, which can drive changes in the specific grazing rates of mixotrophs and their effect on bacterioplankton. Mixotrophic capabilities can be considered a successful strategy when resources are limiting and particularly in oligotrophic environments where inorganic nutrients usually limit the strictly autotrophic growth. Many experiments with cultured mixotrophic flagellates support this statement (Nygaard and Tobiesen 1993; Rothhaupt 1996), which predicts that an inverse relationship should be expected between dissolved inorganic nutrient concentrations and the phagotrophic activity of mixotrophs in oligotrophic systems.

We determined the significance of mixotrophic flagellates as grazers of bacterioplankton in an oligotrophic coastal system by comparing the rates of bacteria ingestion by autotrophic flagellates to the ingestion by strictly heterotrophic organisms, and determining whether this grazing effect varies throughout the year. We also attempted to identify the main factors that regulate the phagotrophic activity of these algae in this environment by

relating nutrient availability and mixotrophic activity. This was done with grazing experiments using fluorescently labeled bacteria (FLB) during an annual cycle in the coastal northwest Mediterranean Sea.

Material and methods

Study area and sampling—Samples were taken from the Microbial Observatory of Blanes Bay on the Catalan coast (NW Mediterranean 41°40'N, 2°48'E). The sampling site is placed at about 800 m offshore and has a sandy bottom at around 20-m depth. Extensive previous information exists about the microbial ecology and biogeochemistry of this site (e.g., del Giorgio et al. 1996; Schauer et al. 2003). Surveys were performed monthly between March 2003 and March 2004. Water temperature was measured with a thermometer at the surface and every 5-m depth, and samples were collected with a Niskin bottle. Depth profiles of underwater irradiance were obtained with a Li-Cor sensor (Li-193S). Secchi disc depth was also measured on every sampling date. Surface-water samples to estimate pico- and nanoplankton abundance, bacterial production, nutrients, and chlorophyll *a* (Chl *a*) concentrations were collected with a basket, filtered in situ through a 200- μm net, and transported to the laboratory in plastic carboys in the dark. Samples were always taken at approximately 11:00 h. Processing of the samples started 1.5 h after collection.

*Chl *a* and nutrient concentrations*—Chl *a* concentration was determined fluorimetrically according to Yentsch and Menzel (1963). One hundred fifty milliliters of sample were filtered through Whatman GF/F filters (25 mm). Pigments were extracted in 6 mL 90% acetone during 24 h in the dark. Soluble reactive phosphorus (SRP), nitrate, nitrite, ammonia, and silica concentrations were measured according to Grasshoff et al. (1983) on unfiltered frozen samples.

Preparation of tracer—FLB were prepared from a *Brevundimonas diminuta* (syn. *Pseudomonas diminuta*) strain obtained from the Spanish Type Culture Collection (Burjassot, València). *B. diminuta* has already been used several times to prepare FLB (e.g., Vázquez-Domínguez et al. 1999) because of their small size (0.065 μm^3) comparable to that of the indigenous bacterial populations. FLB were produced by scraping cells from agar plates, suspending them in carbonate-bicarbonate buffer ($\text{CO}_3\text{Na}_2\text{-HCO}_3\text{Na}$ pH 9.5), and staining cells by incubating the suspension with 100 $\mu\text{g mL}^{-1}$ of 5-([4,6 dichlorotriazin-2-yl]-amino)-fluorescein (DTAF) for 2 h in a water bath at 60°C (Sherr et al. 1987). Stained cells were rinsed with filtered (<0.2 μm) carbonate-bicarbonate buffer, resuspended, and centrifuged 5 times (10 min, 10,000 rpm) to prevent the transfer of leftover dye to the natural samples. The cell suspensions were kept frozen (−20°C) until use. Before addition to the experimental containers, the FLB working solution was thawed and gently sonicated for three 10-s rounds with the microtip at 35% of power output to prevent cell clustering (Dynatech sonic dismembrator, Model 300).

Bacteria and FLB abundance—Preliminary results applying catalyzed reporter deposition-fluorescence in situ hybridization (Pernthaler et al. 2002) with oligonucleotide probes (Eury806, Cren554) targeting Archaea revealed that this group of prokaryotes rarely exceed 7% of the 4,6-diamidino-2-phenylindole (DAPI) counts (Alonso-Sáez, unpubl. data). Consequently, because Archaea are a very small fraction of heterotrophic prokaryotes, we use the term bacteria throughout the text.

Bacteria and FLB were counted by flow cytometry. A 1.8-mL aliquot was preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), left in the dark for 10 min at room temperature, frozen in liquid nitrogen, and then stored at -80°C . Aliquots were divided in two subsamples for counts of bacteria and FLB. To count bacteria, 400 μL were stained with a diluted SYTO-13 (Molecular Probes) stock (10:1) at 2.5 $\mu\text{mol L}^{-1}$ final concentration, left for about 10 min in the dark to complete the staining, and run in a flow cytometry. We used a FACSCalibur (Becton Dickinson) flow cytometer equipped with a 15-mW argon-ion laser (488 nm emission). Between 20,000 and 100,000 events, usually 50,000 events were acquired for each subsample. Fluorescent beads (1- μm Fluoresbrite carboxylate microspheres, Polysciences) were added at a known density as an internal standard. The bead standard concentration was determined by epifluorescence microscopy. Bacteria were detected by their signature in a plot of side scatter (SSC) versus FL1 (green fluorescence). Photosynthetic picoplankton were excluded from the bacterial counts by a plot of FL1 versus FL3. In this graph, beads fall in a line, bacteria in another line, and noise in a third (respectively, with more FL3 than FL1). Picocyanobacteria fall between noise and bacteria. Data analysis was done with the program Paint-A-Gate (Becton Dickinson); this method was discussed in Gasol and del Giorgio (2000). Bacterial concentrations were checked against DAPI-epifluorescence counts (Porter and Feig 1980).

Bacterial cell size was estimated using the relationship between the average bacterial size (obtained by image analysis of DAPI preparations following common procedures) and the average fluorescence of the SYTO-13-stained sample relative to beads (Gasol and del Giorgio 2000).

$$\mu\text{m}^3 \text{ cell}^{-1} = 0.0075 + 0.11 (\text{FL1 bacteria}/\text{FL1 beads}) \quad (1)$$

Bacterial biomass was calculated by using the carbon-to-volume relationship derived by Norland (1993) from the data of Simon and Azam (1989).

$$\text{pg C cell}^{-1} = 0.12 \text{ pg } (\mu\text{m}^3 \text{ cell}^{-1})^{0.7} \quad (2)$$

For FLB we used the same procedure as for bacteria, but without the addition of SYTO-13. FLB were easily identified in plots of SSC versus FL1 (Vázquez-Domínguez et al. 1999; Gasol and del Giorgio 2000).

Bacterial production—Heterotrophic bacterial production (BP) was estimated from the rate of protein synthesis determined by the incorporation of ^3H leucine into

trichloroacetic acid (TCA)-insoluble macromolecular material. Tritiated leucine was added at a saturating concentration (40 nmol L^{-1}) to four experimental replicates of 1.2 mL. Duplicate controls were used by adding 120 μL 50% TCA before the isotope addition. The Eppendorf vials were incubated in a temperature-controlled chamber during the 1–2 h at the in situ temperature. The incorporation was stopped by adding 120 μL of cold 50% TCA to the vials, which were subsequently frozen at -20°C before processing with the centrifuge tubes method of Smith and Azam (1992). Finally, 1 mL of cocktail was added to the vials; they were processed after 24–48 h with a Beckman scintillation counter with counting corrected for quenching. BP was calculated by multiplying leucine incorporation by the standard conversion factor (CF) of 3.1 kg C mol Leu $^{-1}$ and by empirical estimations of the CF determined at every sampling date (Alonso-Sáez, L., unpubl. data) using integrative (Riemann et al. 1987) and cumulative (Bjørnsen and Kuparinen 1991) methods.

Short-term grazing experiments—Estimates of grazing rates on bacteria by flagellates were determined by ingestion of FLB. Water samples were collected in plastic carboys, transported to the lab in the dark, and gently filtered through a 100- μm net mesh by an inverse filtration to reduce the presence of large predators (e.g., copepods, large ciliates) in the sample. Filtrated water was placed in plastic carboys into a culture chamber at in situ temperature conditions until experiments started. A fixed light intensity of 200 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ was used in all the experiments because of the difficulty in mimicking the same light measured in situ; mean light intensity in the water column ranged from 22 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ to 713 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ at the time of sampling (average = 275 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, SE = 66, $n = 11$). Experiments running in triplicate started within 3–4 h after collecting the water. Depending on the initial bacterial concentration, between 0.5 mL and 1.5 mL of FLB working solution were added to 4.5 liters of filtrated water to get about 10–30% of natural bacteria concentrations. The 4.5 liters were gently mixed and spread in equal volume in three 2-liter Nalgene bottles. Samples to estimate bacteria and FLB abundances were taken immediately after the addition of FLB (time zero, T0).

Samples to estimate ingestion of FLB by flagellates were taken at T0 and after 40 min of incubation (T40). One hundred twenty milliliters of sample from each replicate bottle were preserved with 120 mL of cold glutaraldehyde 4% (2% final concentration). Between 70 mL and 100 mL of fixed water were filtered through a 0.6- μm polycarbonate black filter (Poretics) and stained with DAPI (Porter and Feig 1980) to a final concentration of 5 mg mL $^{-1}$. Flagellates <5 μm in size were enumerated on this filter, and ingested FLB were counted at the same time. The remaining volume (ca. 120–140 mL) was filtered through a 3- μm polycarbonate black filter (Poretics) to enumerate organisms >5 μm in size. Protist abundance and ingested FLB were determined by epifluorescence (Nikon Optiphot) microscopy. Flagellates showing plastidic structures were considered phototrophic forms (phytoflagellates [PF]),

whereas colorless flagellates were counted as heterotrophic (heterotrophic flagellates [HF]). Sometimes, we observed HF with ingested pigmented prey. These “false” PF were distinguished by either the presence of another nucleus, the integrity of plastids, and/or the general morphology of both prey and predator. PF were divided into three size categories: $<3 \mu\text{m}$, $3\text{--}5 \mu\text{m}$, and $5\text{--}20 \mu\text{m}$. Because few HF were $<3 \mu\text{m}$, only two categories were considered among them: $<5 \mu\text{m}$ and $5\text{--}20 \mu\text{m}$.

Grazing rates of bacteria were determined from FLB uptake by the different flagellate groups. Ingestion rates (FLB individual $^{-1}$ h $^{-1}$) were calculated from the total number of ingested FLB at T40 after subtracting the value from T0 inspections. This incubation time was chosen after a time series of up to 90 min (samples taken every 15 min), performed during the first experiment, showed that ingestion rates reached a plateau by 45 min. In each case, the 95% confidence intervals of T0 and T40 estimates were calculated to test the significance of the ingestion rates. Confidence intervals were calculated as follows:

$$\begin{aligned} P_{40} - 1.96[P_{40}(1 - P_{40})/n_{40}]^{1/2} > \\ P_0 + 1.96[P_0(1 - P_0)/n_0]^{1/2} \end{aligned} \quad (3)$$

where P is the number of FLB inside a cell, and n is the number of cells inspected at T40 and T0, respectively. Uptake was considered significant when the 95% confidence intervals of T0 and T40 estimates did not overlap (Rice 1988, cited in Havskum and Riemann 1996). Uptake results were significant in all cases except for $<3\text{-}\mu\text{m}$ PF. A mean of 100 cells per group of PF and 50 cells of HF were inspected in each filter (three replicates and two times), with at least 30 ingested FLB per group. Thus, a total of about 200–400 HF and 500–800 PF of each category were examined for FLB ingestion in each experiment. Clearance rates (nL ind. $^{-1}$ h $^{-1}$) were calculated by dividing the ingestion rate by the FLB concentration. Specific grazing rates (bact. ind. $^{-1}$ h $^{-1}$) for each group were estimated by multiplying the corresponding clearance rate by the bacterial concentration, assuming that native bacteria and FLB were grazed upon at the same rates. The grazing effect (bact. mL $^{-1}$ h $^{-1}$) of each flagellate group on bacterioplankton was estimated by multiplying the specific grazing rate by the flagellate abundance (ind. mL $^{-1}$). Bacterial turnover rates (% d $^{-1}$) were estimated by expressing the grazing effect (bact. mL $^{-1}$ d $^{-1}$) as a percentage of the corresponding bacterial abundance (bact. mL $^{-1}$). The specific ingestion rate, in percentage of the cell carbon per day (% d $^{-1}$), was calculated for each bacterivory group by dividing the respective specific grazing rates expressed in biomass of bacteria ($\mu\text{g C}$ bact. ind. $^{-1}$ d $^{-1}$) by the mean flagellate biomass ($\mu\text{g C}$ ind. $^{-1}$). Mean flagellate biovolumes were estimated for each size-group category by measuring the equivalent spherical diameter of about 150 individuals every month throughout the study period and by approximating each cell to a sphere. The mean cell volume of each group was converted to carbon assuming a conversion factor of $0.22 \text{ pg C } \mu\text{m}^{-3}$ (Børsheim and Bratbak 1987).

Long-term grazing experiments—Total bacterivory attributable to organisms smaller than $100 \mu\text{m}$ was estimated by the disappearance of FLB according to Vázquez-Domínguez et al. (1999). Experiments were run in the same bottle types and under the same conditions as for short-term experiments. In this case, however, the bottles were kept in the culture chamber for 48 h, and the photoperiod was adjusted in each case (Fig. 1). Bacteria and FLB abundances were counted immediately after the addition of FLB (T0) and at least at 24 h and at 48 h. Additional time counts (8 h, 16 h, etc.) were usually done in most of the samples to track the evolution of bacteria and tracer populations during the experiment. Two control bottles were also run to test for nonpredatory disappearance of FLB (e.g., fluorescence losses or FLB attachment on the bottle wall). Controls were established with $0.2\text{-}\mu\text{m}$ filtered sample water, amended with FLB, and maintained at the same conditions described above.

Calculations of total consumed bacteria are obtained following the mathematical model of Salat and Marrasé (1994). The grazing rate (g) was computed first as:

$$g = -(1/t) \ln (F_t/F_0) \quad (4)$$

where t is the incubation time; F_t the abundance of FLB at final time, and F_0 the abundance of FLB at initial time (FLB mL $^{-1}$).

Next, net bacterial growth rate (a) was calculated as:

$$a = (1/t) \ln (N_t/N_0) \quad (5)$$

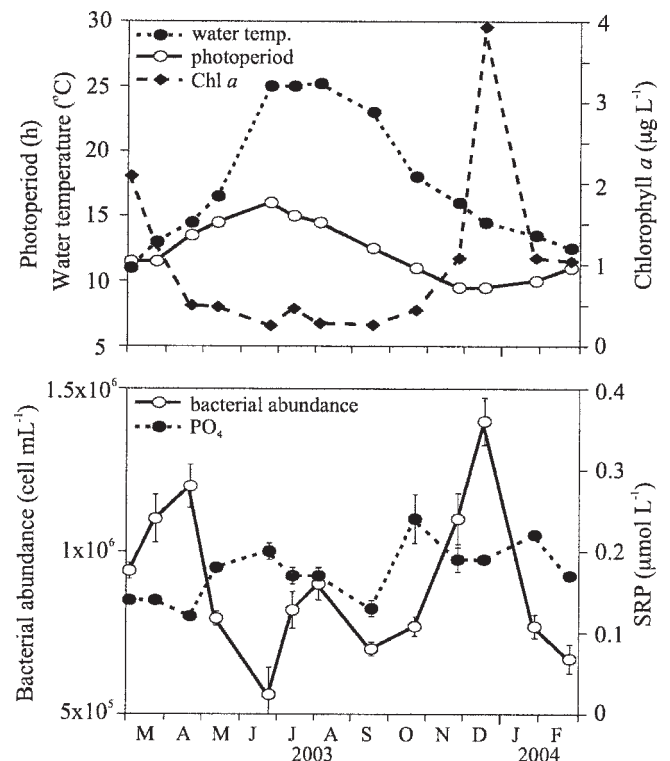


Fig. 1. Seasonal variation in water temperature, photoperiod (hours of light), Chl a concentration, SRP, and bacterial abundance from March 2002 to March 2003. Bars represent SE.

where t is the incubation time, and N_t and N_0 are the natural bacteria abundance at the end and beginning of the experiment, respectively (bact. mL⁻¹).

The decrease of FLB in control bottles was checked in every experiment and corrected, if necessary, by calculating

$$g_C = -(1/t) \ln (cF_t/cF_0) \quad (6)$$

where g_C is the apparent grazing rate in the control bottles (d⁻¹), t is the incubation time, cF_t is the abundance of FLB at final time, and cF_0 is the abundance of FLB at the initial time in the control (FLB mL⁻¹). This correction is a modification of the former model proposed by Salat and Marrasé (1994)

$$G = [(g - g_C)/a](N_t - N_0) \quad (7)$$

where G is the total grazing (bact. mL⁻¹). We divided by t to obtain the total grazing rate (bact. mL⁻¹ d⁻¹).

Model to estimate community grazing rates—We compared our estimations with the linear model (Model 1) proposed by Vaqué et al. (1994) that predicts total grazing rate (bact. mL⁻¹ h⁻¹)

$$\log GT = -3.21 + 0.99 \log HF + 0.028 T + 0.55 \log N \quad (8)$$

where GT is the community grazing rate, HF is the heterotrophic flagellate abundance (flag. mL⁻¹), T is the water temperature, and N is the bacterial abundance (bact. mL⁻¹).

Statistics analysis—We used Pearson's correlations coefficients to test for the significance of variable association.

Results

Surface-water temperature in Blanes Bay during the period studied fluctuated between 11°C and 25°C (Fig. 1). Bottom temperatures did not differ from the surface except from June and July, when water at 20-m depth was up to 5°C colder. Secchi depth ranged between 3.5 m and 20 m (average = 12 m, SE = 1.7, $n = 13$), and, except in a few cases, the photic layer always comprised the entire water column as observed in the measured light profiles. Annual mean light intensity in the mixed layer was, on average, 275 $\mu\text{mol m}^{-2} \text{s}^{-1}$. SRP concentration was low throughout the study period, varying between 0.12 $\mu\text{mol L}^{-1}$ and 0.24 $\mu\text{mol L}^{-1}$ (Fig. 1). Dissolved inorganic nitrogen (DIN) ranged between 0.21 $\mu\text{mol L}^{-1}$ and 8.58 $\mu\text{mol L}^{-1}$, and silica varied between 0.8 $\mu\text{mol L}^{-1}$ and 7.6 $\mu\text{mol L}^{-1}$, with a maxima in winter and minimal values in summer for both nutrients (data not shown). Bacterial abundance was within the range 5.6×10^5 – 1.4×10^6 cell mL⁻¹, with maximum concentrations at the beginning of winter and at the beginning of spring, in coincidence with the peaks of Chl *a* (Fig. 1). Except for the exceptionally high value measured in July (28.9 $\mu\text{g C L}^{-1} \text{d}^{-1}$ or 14.4 $\mu\text{g C L}^{-1} \text{d}^{-1}$,

using a standard or an empirical CF, respectively), BP ranged from 0.4 $\mu\text{g C L}^{-1} \text{d}^{-1}$ to 8 $\mu\text{g C L}^{-1} \text{d}^{-1}$, with a mean value of 2.6 $\mu\text{g C L}^{-1} \text{d}^{-1}$ (1.8 $\mu\text{g C L}^{-1} \text{d}^{-1}$ using the empirical CFs).

The smallest size category of PF (cells <3 μm) was composed mostly of coccoid unicellular unidentified organisms, and only occasionally were flagellated forms observed within this group. These cells were very abundant during winter (abundances up to 2.4×10^4 cell mL⁻¹), but never ingested FLB at a significant grazing rate. Therefore, this group was not further considered here. The PF of the 3–5- μm fraction was mainly represented by mixotrophic haptophyte-like cells, whereas the 5–20- μm PF were dominated by 5- to 10- μm -sized flagellates, mostly haptophyte-like cells, dinoflagellates, and cryptophytes. These three groups of phytoflagellates actively ingested FLB. According to the presence of their indicator pigments determined by high-performance liquid chromatography, they did not display a clear seasonal pattern throughout the year (Latasa, pers. comm.). Notwithstanding some fluctuations, the three groups of algae, and particularly haptophytes, were present throughout the year.

Both PF and HF abundances were higher at the beginning of winter and at the beginning of spring (Fig. 2a,b). An increase in abundance of the smallest flagellates during the summer was also observed. Flagellates <5 μm in size were usually much more abundant than >5- μm flagellates, whereas plastidic flagellates usually reached higher densities than strictly heterotrophic ones. After 40 min of incubation, a mean of 18% of 3–5- μm PF and 11% of 5–20- μm PF were observed with ingested FLB. The specific grazing rates (SGRs) of 3–5- μm PF varied between 0.2 bact. ind.⁻¹ h⁻¹ and 2.1 bact. ind.⁻¹ h⁻¹, whereas for the 5–20- μm PF, SGRs fluctuated between 0.5 bact. ind.⁻¹ h⁻¹ and 3.6 bact. ind.⁻¹ h⁻¹ (Fig. 2c). Seasonal patterns were not evident for PF. Contrarily, the HF presented a noticeable seasonality, with higher grazing activity during the warmer months (Fig. 2d). In August, the SGR of <5- μm HF reached 4 bact. ind.⁻¹ h⁻¹, and exactly the same pattern was observed for 5–20- μm HF, although the grazing rate reached by this fraction was significantly higher (15.4 bact. ind.⁻¹ h⁻¹). SGRs of both HF size-categories were significantly correlated ($r = 0.93$; $p < 0.001$). The clearance rates (CRs) followed in all cases exactly the same trend observed for the SGRs (data not shown), with higher values for HF than PF groups. The CR for <5- μm and 5–20- μm HFs averaged 2.2 nL ind.⁻¹ h⁻¹ and 6.1 nL ind.⁻¹ h⁻¹, respectively, in contrast to 1.2 nL ind.⁻¹ h⁻¹ (3–5- μm PF) and 1.5 nL ind.⁻¹ h⁻¹ (5–20- μm PF). The specific ingestion rate in % of cell carbon per day was always higher for the smaller fractions, especially the heterotrophic ones (graph not shown). It ranged between 11% d⁻¹ and 59% d⁻¹ for <5- μm HF (avg. 29% d⁻¹), 1% d⁻¹ and 13% d⁻¹ for 5–20- μm HF (avg. 3.6% d⁻¹), 2% d⁻¹ and 21% d⁻¹ for 3–5- μm PF (avg. 10% d⁻¹), and 0.4% d⁻¹ and 3% d⁻¹ for 5–20- μm PF (avg. 0.9% d⁻¹).

As for the grazing effect, the four groups showed the same three peaks: one during the summer and two more at the beginning of winter and at the beginning of spring (Fig. 2e and 2f). The smallest flagellates (<5 μm) always

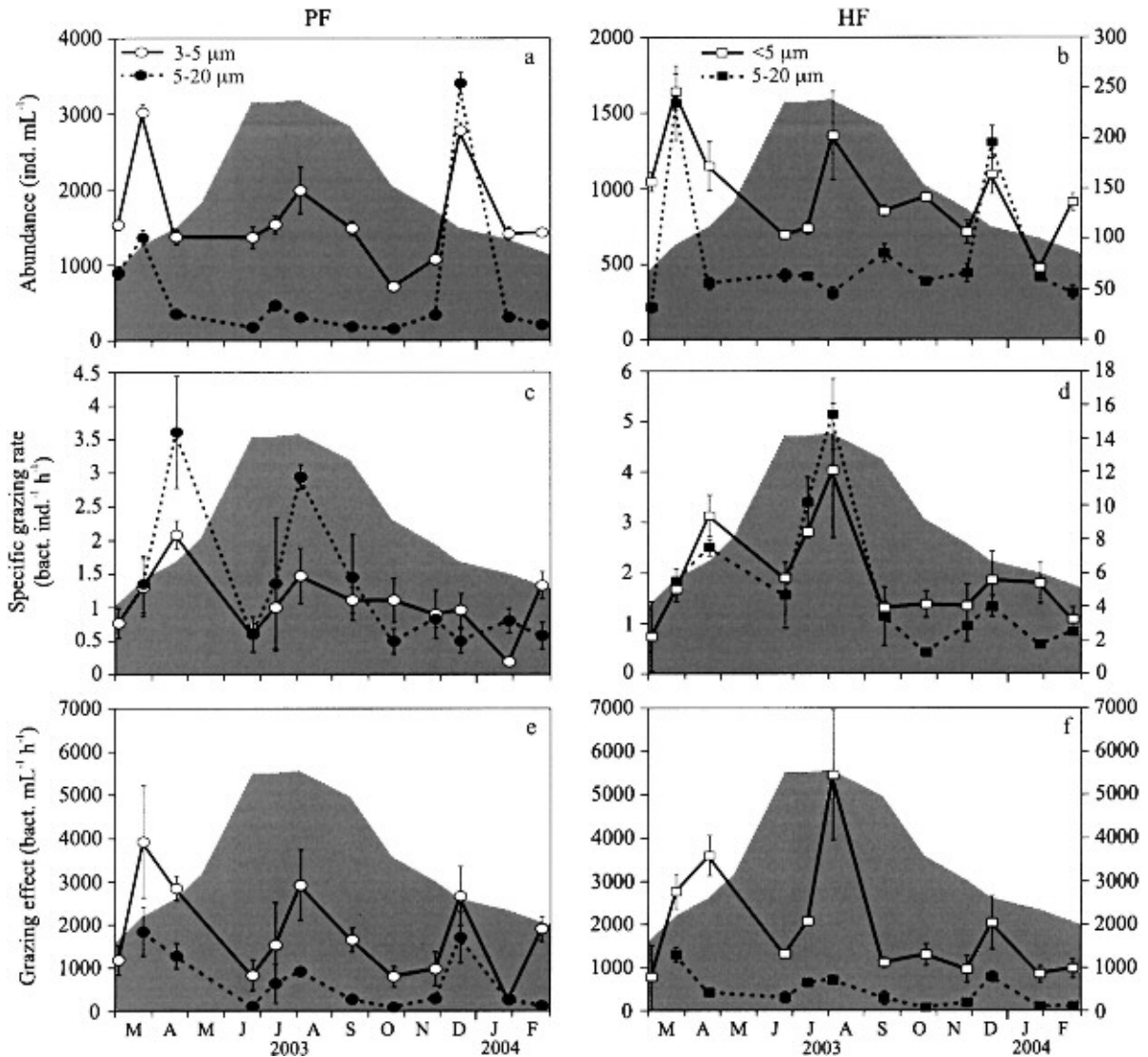


Fig. 2. Temporal fluctuation in abundance (a, b), specific grazing rate (c, d), and grazing effect (e, f) of the four flagellate categories analyzed. Bars represent SE. Grey shaded area represents temperature as shown in Fig. 1. Secondary y-axis in panels b, d, and f correspond to HF 5–20 μm .

had a greater effect on bacterioplankton than the larger ones. Averaging the data obtained in the annual survey, the SGRs of PF were considerably lower than those of HF (Fig. 3). However, flagellate abundances presented the opposite trend, thus resulting in very similar grazing effects of the groups (Fig. 3). The grazing effect on bacteria due to total PF was relatively high throughout the study period, accounting for 35–65% of the measured total flagellate bacterivory (Fig. 4a). PF 3–5 μm in size were responsible for 19–61% of the bacterivory, 5–20- μm PF for 4–24%, <5- μm HF for 28–58%, and 5–20- μm HF for 3–13%. On average, 50% of the total flagellate bacterivory was attributable to mixotrophic organisms (Fig. 4b). The smallest flagellates were the major grazers throughout the year. Regardless of the presence of chloroplast, flagellates <5 μm in size accounted for ca. 80% of the total bacterivory measured by FLB ingestion (Fig. 4b).

Ingestion rates of FLB by ciliates were also calculated in our experiments every time an individual appeared in the filters while counting flagellates. However, because of the low number of specimens enumerated, our estimates are not accurate. Thus, they should be considered as a rough estimation of their effect on bacterioplankton. Taking into account these restrictions, SGRs were significantly higher than those measured for flagellates (10–305 bact. ciliate⁻¹ h⁻¹). However, owing to their low abundance (0.2–3.5 ciliates mL⁻¹, Vázquez-Domínguez, pers. comm.), ciliates would account, on average, for a relative low percentage of the total grazing (3.5%). The highest rate was estimated in summer (200–670 bact. mL⁻¹ h⁻¹).

The SGRs of 3–5- μm and 5–20- μm PFs were negatively correlated ($p < 0.05$) with the SRP concentration, whereas HF did not show any relationship with any nutrient (Fig. 5). CRs were also inversely related with SRP

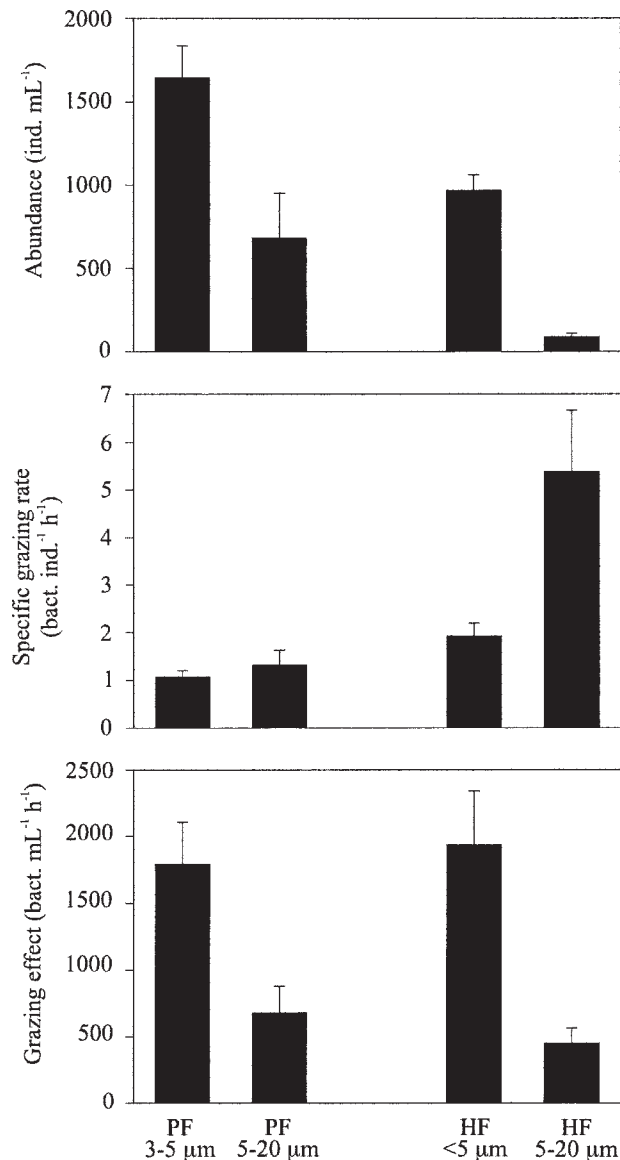


Fig. 3. Mean annual values in abundance, SGR, and grazing effect of the four flagellate categories analyzed. Bars represent SE.

concentration, although with lower significance. The CRs of <5- μm and 5-20- μm HFs were strongly correlated ($p < 0.01$) with water temperature ($r = 0.79$ and 0.74 respectively, graph not shown). SGRs of both HF size fractions were also positively related with temperature although correlations were slightly less significant. As for PF, neither CR nor SGR were at all correlated with temperature.

We compared our short-term estimations of grazing rates to two other estimations of total grazing, one measured from the long-term disappearance of FLBs, and the other modeled following the equations published in the literature (Fig. 6a). Total grazing assessed by FLB disappearance varied between $0.7 \mu\text{g C L}^{-1} \text{d}^{-1}$ and $4.7 \mu\text{g C L}^{-1} \text{d}^{-1}$ (with a value of $10.1 \mu\text{g C L}^{-1} \text{d}^{-1}$ in July). Applying Eq. 8 (Model 1 by Vaqué et al. 1994) grazing

fluctuated between $0.6 \mu\text{g C L}^{-1} \text{d}^{-1}$ and $3.7 \mu\text{g C L}^{-1} \text{d}^{-1}$. Apart from July, the three approaches followed approximately the same trend, with maximum bacterivory during summer, at the beginning of winter, and at the beginning of spring (Fig. 6a). Remarkably, the absolute numbers of the three grazing estimations were in the same range. In fact, all of them were highly correlated ($p < 0.01$). Eq. 8, which uses bacterial abundance, temperature, and HF abundance, adjusted quite well with the measured total flagellate bacterivory assessed by ingestion of FLB (Fig. 6b), although it tended to underestimate grazing in some samples, which coincided with periods of high PF effect. Total grazing from FLB disappearance, which includes flagellate but also small ciliates, fit reasonably well with flagellate bacterivory estimated by FLB ingestion, except for the July event. On average, total grazing consumed 84% of BP (139% using the empirical CFs, Fig. 7). Bacterial turnover rates caused by total grazing (assessed by FLB disappearance) reached $61\% \text{d}^{-1}$ in July, although it usually ranged between $4\% \text{d}^{-1}$ and $26\% \text{d}^{-1}$ (avg. $10\% \text{d}^{-1}$), with almost the same pattern observed in Fig. 6a.

Discussion

The present study shows that mixotrophic algae contribute significantly to total flagellate bacterivory in this marine oligotrophic coastal system and that their grazing effect was relatively high throughout the year. Our data also indicate that <5- μm flagellates accounted for most of the flagellate bacterivory. A negative relationship between the PF ingestion rate and SRP concentration suggests that mixotrophic flagellates could be using their phagotrophic capability to obtain phosphorus when this nutrient is limiting. HF grazing activity showed a marked seasonality related to water temperature. Furthermore, the comparison of short-term measurements against long-term experiments and an empirical model developed to predict community grazing estimations also points to the importance of the smallest flagellates as the main cause of bacteria losses in this system. This comparison also gives confidence to our estimates of bacterivory.

Bacterial losses and production—BP and total grazing assessed by FLB disappearance were in the same range and were similar to values found in other oligotrophic marine environments (Vaqué et al. 1994; del Giorgio et al. 1996). Bacterial turnover rates were also within the range usually measured in the sea (Ichinotsuka et al. 2006). Thus, bacterial losses attributable to total grazing commonly accounted for most BP, indicating that grazing would be the main loss process of bacteria in this system. A positive relationship between the processes of bacterial growth and losses to grazing is usually observed in most aquatic systems (Sanders et al. 1989; 1992). Virus-induced mortality has also been proposed as an important mechanism regulating bacterial abundance in the oceans (see review by Suttle 2005). However, results obtained by Weinbauer and Peduzzi (1995) and Guixa-Boixereu et al. (1999) indicate that bacterial mortality caused by viral lysis does

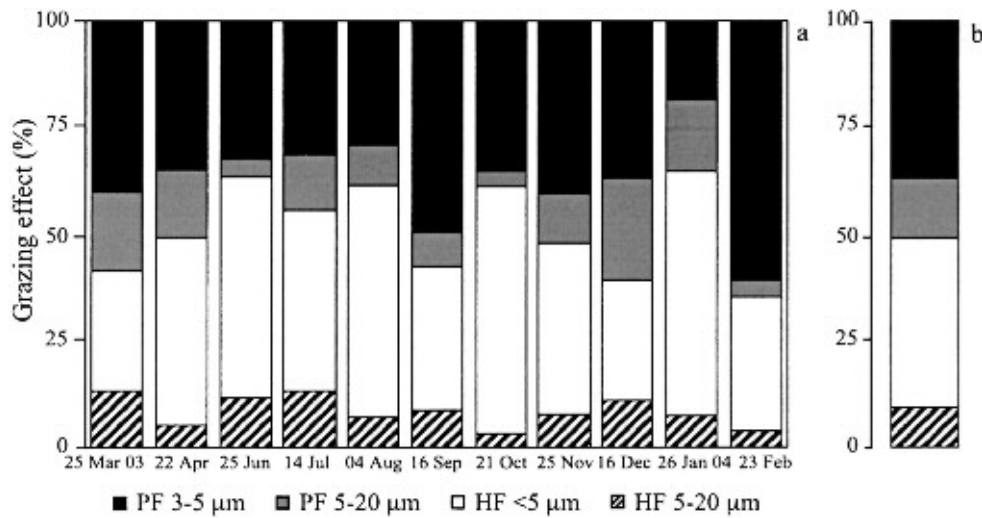


Fig. 4. The proportion of total flagellate bacterivory, expressed as a percentage, contributed by each of the four groups of flagellates (a) throughout the year and (b) as average annual values.

not seem to be very relevant in the oligotrophic northwestern Mediterranean.

The total grazing estimated by the disappearance of FLB, which includes the effect of flagellates and also small ciliates, fit well with the flagellate bacterivory estimated by FLB ingestion, which includes only flagellates. This suggests that flagellates, either heterotrophic or mixotrophic, would be the main grazers of bacteria in this environment. However, as it has been observed before, ciliates could also be important grazers of bacterioplankton (Sherr and Sherr 1987; Sanders et al. 1989; Epstein and Shiaris 1992). Our rough estimation of ciliate SGRs are in the same range as previous estimations performed by Sherr

et al. (1989) in the Bay of Villefranche, located also in the northwestern Mediterranean Sea. However, their low abundance meant that ciliates accounted for a relatively low proportion of total grazing. The highest effect was estimated in summer, and this could explain the inconsistency between the grazing rates obtained by ingestion and FLB disappearance in July. Apart from this experiment, ciliates seem to have a minor global effect on bacterioplankton in our study site. These results reinforce the idea that flagellates are the main grazers on bacteria here.

The empirical Model 1 proposed by Vaqué et al. (1994), which predicts grazing of HF on bacteria from prey abundance, HF abundance, and water temperature, ad-

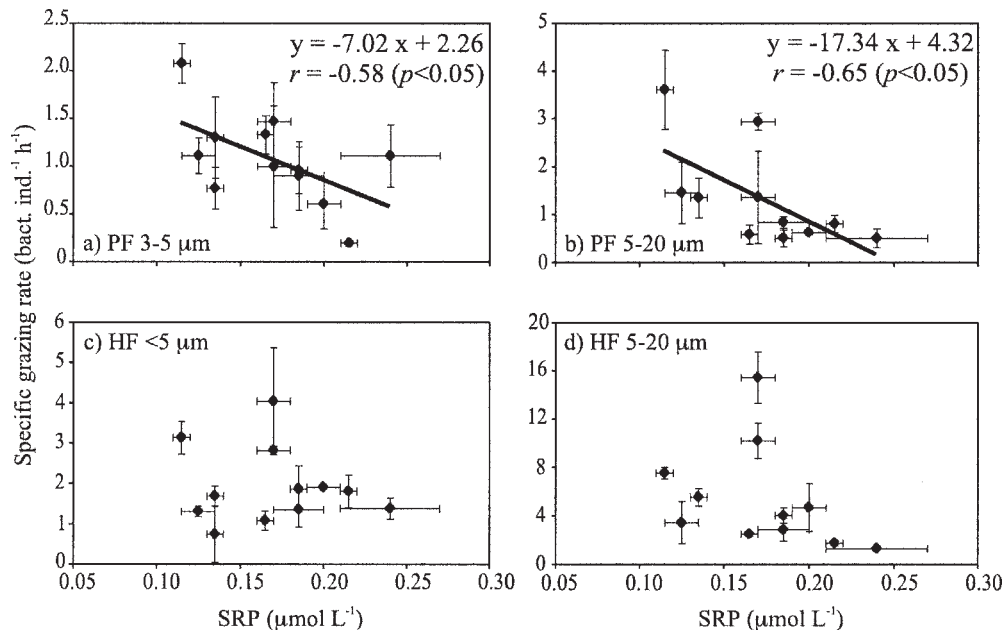


Fig. 5. (a-d) Relationship between the SGR of each flagellate category and SRP concentration. When significant, regression equation and Pearson's correlation coefficient are shown. Bars represent SE.

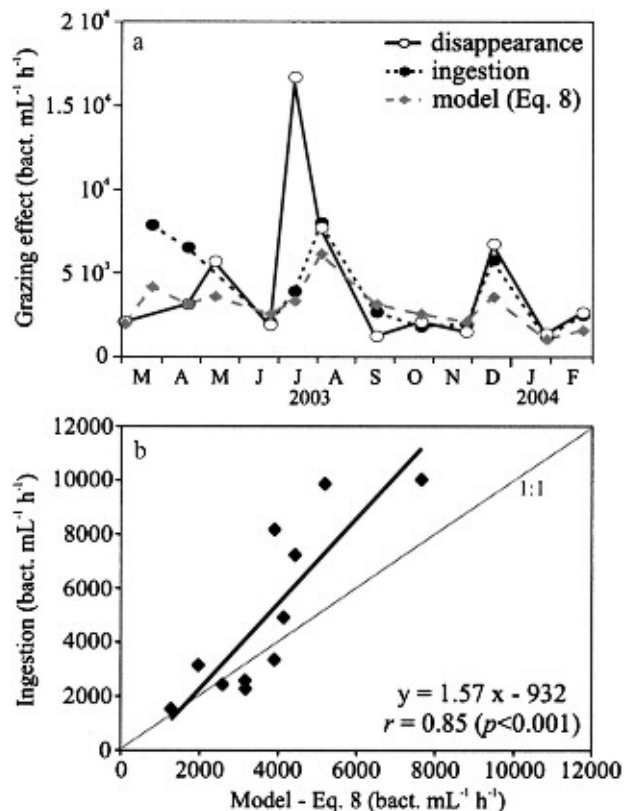


Fig. 6. (a) Grazing effect on bacterioplankton assessed by FLB disappearance, FLB ingestion, and applying the Model 1 (Eq. 8) proposed by Vaqué et al. (1994) based on temperature, bacterial abundance, and HF abundance. (b) Relationship between total flagellate bacterivory estimated by ingestion of FLB and using Eq. 8. Dashed line indicates 1 : 1 relationship.

justed fairly well to our data despite the fact that the model does not consider mixotrophic flagellates. Both estimations were significantly correlated ($r = 0.85$; $p < 0.001$), but the slope of the regression notably differs from the 1 : 1 line (Fig. 6b). Thus, the model underestimates total flagellate grazing by up to 50% during some periods. In particular, the four points that clearly separated from the 1 : 1 line, corresponding to the experiments carried out in summer, at the beginning of winter, and at the beginning of spring, all coincided with periods of high grazing effect of mixotrophic flagellates.

Relative grazing effect of the different flagellate types—The SGRs and CRs of HF estimated in the present survey are in the same range of those reported in previous studies (Epstein and Shiaris 1992; Hall et al. 1993; Safi and Hall 1999). Despite a lower grazing rate per individual, a high number of $<5\text{-}\mu\text{m}$ flagellates resulted in a significantly higher grazing effect than $5\text{--}20\text{-}\mu\text{m}$ HF. Exactly the same was observed for PF, although with lower grazing rates. Very similar grazing rate values have been reported in different marine systems dominated by haptophytes (Havskum and Riemann 1996; Christaki et al. 1999; Safi and Hall 1999). Interestingly, regardless of the presence of chloro-

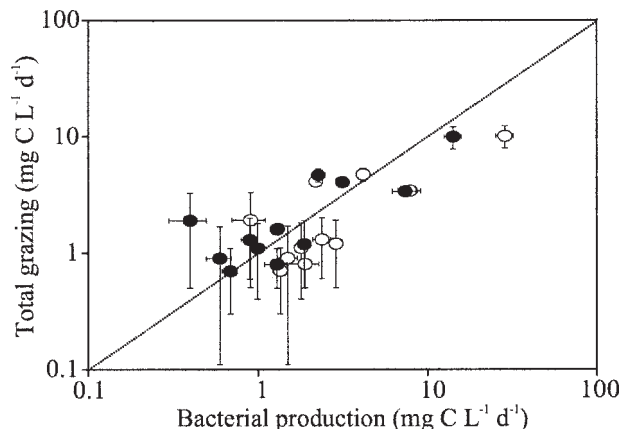


Fig. 7. Relationship between total bacterivory assessed by FLB disappearance and bacterial production estimated with a standard conversion factor (open circles) or with empirical CFs (solid circles). Dashed line indicates 1 : 1 relationship.

plast, 80% of the total measured bacterivory in Blanes Bay was attributable to flagellates $<5\text{-}\mu\text{m}$ in size, conforming to the view that the smallest flagellates are primarily bacterivorous and larger organisms prefer larger particles (Sherr and Sherr 1991, 2002). Accordingly, the ratio of acquired carbon through ingested bacteria to flagellate biomass was significantly higher for small flagellates than for larger ones. Although bacteria probably do not completely fulfill the carbon demand of $<5\text{-}\mu\text{m}$ flagellates (since photosynthetic picoplankton are present in this environment and could also be considered potential prey), at least a substantial proportion of their carbon came from bacterial ingestion. HF $<5\text{-}\mu\text{m}$ in size obtained about 29% d^{-1} of their body carbon by ingesting bacteria, whereas mixotrophic flagellates that also could photosynthesize had a mean specific ingestion rate of 10% d^{-1} . The specific ingestion rates of $>5\text{-}\mu\text{m}$ flagellates were considerably lower, suggesting that they would obtain most of their carbon demand feeding on larger prey. In agreement with this view, Havskum and Hansen (1997) found a considerable grazing effect of $10\text{--}20\text{-}\mu\text{m}$ phagotrophic protists on nanoplankton, but not on picoplankton. These results point to the importance of the smallest flagellates in the flow of material (carbon and nutrients) throughout the microbial food web of oligotrophic marine sites.

Grazing effect of mixotrophic flagellates—Despite lower grazing rates per individual, higher numbers of PF meant that, on average, they accounted for 50% of the total measured grazing rate. These results are relatively in accordance with those obtained by other authors in oligotrophic areas of the sea, although a general trend cannot be established with the few data that have been published to date (Table 1). In the Sargasso Sea, Arenovski et al. (1995) found on two occasions as much as 50% of phototrophic nanoplankton with ingested fluorescent tracers of bacterivory after 24 h of incubation. Sanders et al. (2000) reported a maximum of 18% of PF with ingested tracers in the same environment and between $<2\%$ to 38% of PF with ingested FLB in Georges Bank. Unfortunately,

Table 1. Percentage of total protist bacterivory accounted for by mixotrophic flagellates in different environments.*

System	Trophic status†	Season	Tracer	Dominating mixotrophic taxa	% bacterivory by mixotrophs	Reference
Marine						
Southwest coast of New Zealand, 10 m	Oligotrophic	Winter	FLM	‡	40%	Hall et al. 1993
Aegean Sea, E Mediterranean Sea, 1–100 m	Oligotrophic	Spring	FLM	Hapto 2–4 µm	5%	Christaki et al. 1999
Aegean Sea, E Mediterranean Sea, 1–100 m	Oligotrophic	Summer	FLM	PF 8–10 µm	5%	Christaki et al. 1999
New Zealand, Subtropical Convergence, around DCM	Oligomesotrophic	Summer	FLBc	Hapto, Dino	55%	Safi and Hall 1999
New Zealand, Subtropical Convergence, around DCM	Oligomesotrophic	Summer	FLM	Hapto, Dino	40%	Safi and Hall 1999
Bay of Aarhus, Denmark, surface	Mesotrophic	Spring	FLBn	Hapto, Dictyo	86%	Havskum and Riemann 1996
Bay of Aarhus, Denmark, below the pycnocline	Mesotrophic	Spring	FLBn	Hapto, Dictyo	19%	Havskum and Riemann 1996
Boston Harbor, surface	Mesotrophic?	Autumn	FLBc	Eugleno, Crypto, Chryso	38%	Epstein and Shiaris 1992
Coast of Norway, 0–4 m	Mesotrophic	§	FLB‡	Hapto	0–60%	Nygaard and Tobiesen 1993
Norwegian fjord, surface	Mesotrophic	Summer	FLBc	Dictyo, Hapto, Dino	0%	Havskum and Hansen 1997
Black Sea, mixing layer	Oligo to eutrophic	Summer	FLBn	Dino	14%	Bouvier et al. 1998
Blanes Bay, NW Mediterranean Sea, surface	Oligotrophic	Annual	FLBc	Hapto, Crypto, Dino	35–65%	This study
Freshwater						
High mountain lakes, Sierra Nevada, 0–5 m	Oligotrophic	Summer	RLB	Chryso	~100%	Medina-Sánchez et al. 2004
Ace lake, saline and meromictic, Vestfold Hills, Antarctica, 0–8 m	Oligotrophic	Summer	FLBc	Prasinophyte	14–56%	Bell and Laybourn-Parry 2003
Perennially ice-cover lakes, Dry Valleys, Antarctica, 6–12 m	Oligotrophic	Summer	FLBc	Crypto	10–66%	Roberts and Laybourn-Parry 1999
Svalbard Archipelago, Arctic lakes, 0–8 m	Oligotrophic	Summer	FLBc	Chryso	0–14%	Laybourn-Parry and Marshall 2003
Lake Örträsket, brown water lake, Sweden, epilimnion	Oligotrophic	Summer	FLM	Chryso	36–86%	Isaksson et al. 1999
Lake Biwa, day-night, epilimnion	Oligotrophic	Summer	FLM	Crypto	0–30%	Urabe et al. 2000
Lake Giles, ice-covered, 0–3 m	Oligotrophic	Winter	FLBc	‡	20%	Berninger et al. 1992
Lake Memphremagog, 3–5 m	Mesotrophic	Summer	FLM	Chryso	75%	Bird and Kalf 1986
Lake Lacawac, ice-covered, 0–3 m	Mesotrophic	Winter	FLBc	Chryso	69–88%	Berninger et al. 1992
Lake Waynewood, ice-covered, 0–3 m	Eutrophic	Winter	FLBc	‡	5%	Berninger et al. 1992
Lake Anney, Apls, epilimnion	Oligotrophic	Annual	FMP	Chryso, Crypto	19–100%	Domaizon et al. 2003
Ace and Highway lakes, Vestfold Hills, Antarctica, 0–8 m	Oligomesotrophic	Annual	FLBc	Prasinophyte, Crypto	0–71%	Laybourn-Parry et al. 2005
Lake Oglethorpe, 0–6 m	Eutrophic	Annual	FMP	Chryso	2–79%	Sanders et al. 1989
Lake Oglethorpe, 0–6 m	Eutrophic	Annual	FMP	Chryso	1–60%	Bennett et al. 1990
Lily pond, surface	Eutrophic	Annual	FLBc	Chryso, Dino	0–100%	Hitchman and Jones 2000

* FLBn, fluorescently labeled bacteria prepared with natural bacteria; FLBc, FLB prepared with a bacterial culture; FLM, fluorescently labeled micellites; FMP, fluorescently microparticles; RLB, radiolabeled bacteria. Chryso, Chrysophyceae; Crypto, Cryptophyceae; Dictyo, Dictyochophyceae; Dino, Dinophyceae; Eugleno, Euglenophyceae; Hapto, Haptophyceae.

† Trophic status was determined on the basis of nutrient concentration, chlorophyll *a*, or both bacterial abundance provided by the authors.

‡ Not specified.

§ Grazing rates were measured during a bloom of *Prymnesium parvum* in 1989.

|| Mixotrophic ciliates represented 95% of the mixotrophic biomass.

the grazing effect could not be estimated in either of these two studies. In our short-term experiments, after 40 min of incubation a mean of 18% of <5- μ m PF and 11% of 5–20- μ m PF were observed with ingested FLB.

Mixotrophic flagellates accounted for a significant proportion of the total flagellate grazing in a number of environments, including also oligotrophic lakes (Table 1). Bird and Kalff (1986), for example, calculated that during a bloom of *Dinobryon* (Chrysophyte) this phytoflagellate alone accounted for 55% of the total loss of bacterial production. Studies carried out in more eutrophic waters are scarce. In the Black Sea, for instance, the feeding activity by mixotrophic organisms contributed to 14% of the ingestion of bacteria (Bouvier et al. 1998). However, in this case chloroplasts-bearing ciliates dominated the mixotrophic community, whereas plastidic dinoflagellates represented only a small proportion of the mixotrophic community. In addition, the experiments were run in dark conditions, which may have reduced the phagotrophic activity of light-dependent mixotrophic flagellates, as it has been observed in some dinoflagellates (Stoecker et al. 1997). Sanders et al. (1989) recorded in a eutrophic lake during a winter bloom of mixotrophic chrysophytes that up to 79% of the total bacterivory was attributable to these algae, and Berninger et al. (1992), comparing lakes of different trophic status, found bacterivory by mixotrophs to be most important in mesotrophic rather than in oligotrophic or eutrophic lakes. However the fact that in this study only one experiment was run in the oligotrophic and the eutrophic lakes precludes a firm conclusion. Interestingly, in the dynamic simulation models developed by Baretta-Bekker et al. (1998), mixotrophs accounted for ca. 40% of the total grazing on bacteria regardless of the treatment (inorganic nutrient enriched and unenriched enclosures), indicating that the rate of phagotrophy in mixotrophs was not controlled by nutrient levels in the model. In this model, light was the factor that controlled the mixotrophic mode of nutrition.

The relative contribution of mixotrophs to total bacterivory along a trophic spectrum remains unclear, and the advantage of being both autotrophic and heterotrophic in a eutrophic environment still does not have a comprehensible explanation. The apparent contradictions in the literature as regard to the role of mixotrophs in poor and replete nutrient conditions are perhaps because of the variety of nutritional strategies that exist within mixotrophic flagellates (Stoecker 1998). Several experimental evidences support the idea that the mixotrophic mode of nutrition is an adaptive strategy for growth in nutrient-poor systems (Nygaard and Tobiesen 1993; Rothhaupt 1996) and that they frequently dominate bacterivory in many oligotrophic environments (e.g., Bird and Kalff 1986; Roberts and Laybourn-Parry 1999; Domaizon et al. 2003).

Seasonality of the effect of mixotrophs—The relative effect exerted by mixotrophic flagellates on the bacterial community was rather constant throughout the year. It reached its maximum in late winter (65%), although it never fell below 35%. To our knowledge, this is the first

study that has examined seasonal variations in the relative grazing effect of pigmented and unpigmented flagellates in a marine system. However, some comparisons can be made with freshwater environments. In contrast to our results, the few seasonal studies carried out in lakes or ponds commonly observed strong differences throughout the year (e.g., Sanders et al. 1989; Hitchman and Jones 2000; Domaizon et al. 2003). These variations can be explained by significant changes in the abundance of the dominant mixotrophic groups throughout the annual succession. In these studies, chrysophytes (*Dinobryon*, *Ochromonas*, *Chrysostephanosphaera*), dinoflagellates (*Peridinium*, *Gymnodinium*), or cryptophytes (*Cryptomonas*) had a significant grazing effect mainly during winter and/or bloom events, whereas HF were usually the main grazers in summer. In Blanes Bay, HF also had their maximum grazing rates during summer, and from June to August they were responsible for 55–64% of the total flagellate grazing. Nevertheless, the effect exerted by mixotrophic flagellates still remained very high. As in many other marine environments (Table 1), in Blanes Bay mixotrophic phytoflagellates included haptophytes, cryptophytes, and dinoflagellates. Indicator pigments determined by high-performance liquid chromatography revealed that, even though some fluctuations were observed throughout the year, these three groups of algae, and particularly haptophytes, were always present in the Blanes Bay. This fact would partially explain the relatively constancy in grazing effect on the bacterioplankton throughout the study period in our system. Since the heterotrophic community of Blanes Bay, and likely also the autotrophic community, are limited by phosphorus for most of the year (Pinhassi et al. in press), this could also promote phagotrophy among mixotrophic flagellates (see below).

In culture studies, at least, two groups of algae, *Cryptomonas* spp. (Cryptophyte) and *Dinobryon* spp. (Chrysophyte), have been shown to exhibit significant diurnal differences in bacterial ingestion rates, with the highest rates measured in late afternoon (Urabe et al. 2000; Pålsson and Granéli 2003). Experimental evidences obtained in vitro with cultures of mixotrophic chrysophytes (*Dinobryon cylindricum*) and haptophytes (*Chrysochromulina ericina*) have also demonstrated that higher biomass, cell density, or growth rates are usually achieved in light conditions as compared to darkness (Caron et al. 1993; Hansen and Hjorth 2002). Surprisingly, similar results were recently obtained in cultured heterotrophic protists (Jakobsen and Strom 2004), with maximum grazing rates measured during the day. In light of these past studies, our results could not yield a global estimation of the grazing effect of mixotrophs because we did not perform measurements during a light : dark cycle. However, even being extremely conservative, and assuming no uptake of bacteria by mixotrophic flagellates during the night, and an invariant rate for HF, mixotrophs would still be responsible for at least 25% of the annual flagellate bacterivory.

Factors controlling the ingestion rates of the flagellates—PF did not show a seasonal pattern in feeding activity.

Also, we did not find any relationship between grazing rates and bacterial abundance. However, the significantly negative correlation between SRP concentration and SGR within both PF groups indicates a tendency toward a decrease of their ingestion rates at higher SRP concentrations.

One potential explanation for this negative correlation between available P concentration and group-specific grazing rates of pigmented flagellates could be the fact that PF groups include mixotrophic but also strictly phototrophic species. Hypothetically, the strictly autotrophs might increase in number at high SRP concentration, resulting in lower per-group ingestion rates. Similarly, mixotrophs having a competitive advantage at lower P concentrations might show the opposite pattern, accounting for a higher abundance when phosphorus is limiting. Thus, assuming that mixotrophs maintain the same SGR throughout the year, and the autotrophs:mixotrophs ratio increases or decreases in response to the SRP concentration, the SGR would increase or decrease when the percentage of strictly phototrophic species change in the samples. This hypothetical scenario would explain changes in SGR without changes in average ingestion rates of mixotrophic flagellates.

Another plausible explanation could be that mixotrophs increase their grazing rates when SRP decreases. Thus, mixotrophs would be using their phagotrophic capability to obtain phosphorus from an alternative source (particulate phosphorus) when this nutrient is scarce. The mixotrophic strategy may be successful when resources (e.g., light or nutrients) are limiting (Rothhaupt 1996). In Blanes Bay, light is unlikely to be a limiting factor for autotrophic organisms since the euphotic layer usually comprised the whole water column. Contrarily, Pinhassi et al. (in press) compiled several different evidences that suggest that P was the main limiting nutrient for bacteria, and also most likely for phytoplankton growth, during most of the year. Bacteria are superior to algae in the uptake of dissolved inorganic phosphorous (Currie and Kalff 1984) and can outcompete strictly autotrophic organisms under P-limiting conditions. Such a situation would be a clear advantage for mixotrophs, obtaining nutrients from the particulate fraction by ingesting bacteria.

The competition for essential nutrients (e.g., phosphorus) between mixotrophic and autotrophic organisms largely depends on the taxa involved. This comprises the affinity of each algal species for taking up inorganic nutrients and the extent to which phagotrophy is used by the mixotrophic flagellate to obtain mineral nutrients. As a general rule, the assimilation of nutrients in particulate form should be energetically more expensive than absorbing nutrients in the dissolved inorganic form (Stoecker 1998). The observed negative relationship between SRP and mixotrophic organisms grazing rate is probably due to this fact. Declines in the phagotrophic activity of mixotrophs when nutrients are plentiful have been reported for cultures of haptophytes *Chrysochromulina polylepis* (Nygaard and Tobiesen 1993) and *Prymnesium patelliferum* (Legrand et al. 2001), although unclear results were obtained by Nygaard and Tobiesen (1993) and Skovgaard

et al. (2003) for cultures of *Prymnesium parvum*. A negative relationship between ingestion rate and inorganic nutrient concentration was also observed in the dinoflagellates *Gyrodinium galateanum*, *Alexandrium tamarense* (Nygaard and Tobiesen 1993), and *Prorocentrum minimum* (Stoecker et al. 1997). Cryptophytes (*Cryptomonas* spp.) from Lake Biwa seemed also to obtain nitrogen and phosphorus from bacteria under conditions of nutrient depletion (Urabe et al. 2000). Nutrient enrichment experiments in the Sargasso (Arenovski et al. 1992) and Mediterranean Seas (Christaki et al. 1999), both oligotrophic areas, resulted in a marked decline in phagotrophically-active PF in treatments, including phosphorus addition. All of these evidences strongly support our results obtained with natural phytoflagellate assemblages, suggesting that the mixotrophic algae inhabiting this oligotrophic coastal system would use their feeding capability to supplement phosphorus at low SRP concentration, but they would take up phosphorus primarily from the dissolved pool at high SRP concentrations.

Water temperature and prey abundance are usually considered among the most important factors regulating HF phagotrophic activity (Choi 1994; Vaqué et al. 1994). SGRs in both HF size categories were significantly correlated, which suggests that the same environmental factor controls the feeding activity of both HF size categories. No relationships were observed between bacterial concentration and feeding activity. Conversely, CRs were significantly correlated with water temperature. Even though SGRs were slightly less significant, they showed the same positive relationship. A seasonal pattern in feeding activity of HF was evident in our study, reaching their maximum values during the warmest months. This agrees with the higher relative effect estimated during the spring-summer periods and with other seasonal surveys comparing mixotrophic and heterotrophic bacterivory carried out in lakes (Sanders et al. 1989; Bennett et al. 1990; Hitchman and Jones 2000).

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