Limnol. Oceanogr., 47(1), 2002, 142–150

Grazing rates of bacterivorous protists inhabiting diverse marine planktonic microenvironments

Itxaso Artolozaga, María Valcárcel, Begoña Ayo, Ainhoa Latatu, and Juan Iriberri[1]
Departamento de Inmunología, Microbiología y Parasitología, Facultad de Ciencias, Universidad del País Vasco, Apartado 644, E-48080 Bilbao, Spain

Abstract

Bacterivory on aggregates and bulk seawater by three nanoflagellates and a microciliate was investigated. A new approach to measure bacterivory on the attached bacterial community is proposed. Macroaggregates containing attached fluorescently labeled bacteria (FLB) were obtained by adding labeled planktonic bacteria to rolling tanks filled with natural seawater, and they were used to measure protistan grazing rates on the bacteria attached to aggregates. Protistan grazing on free-suspended bacteria was measured according to the monodispersed FLB uptake technique. The four protists showed low grazing rates when feeding on free-suspended bacteria at the low densities usually found in seawater. Surprisingly, the analyzed protists also showed low grazing rates when feeding on aggregates despite the very high bacterial densities inhabiting them. The analysis of the influence of prey density on bacterial grazing rates suggests that feeding on bacteria attached to aggregates involves a relevant additional effort when compared to feeding on free-suspended bacteria. The paradox between these results and the very high abundance of protistan communities usually found in aggregates is discussed attending to the view of aggregates as biological reactors. Therefore, marine macroaggregates can be considered as microniches able to support the maintenance of a protistan community without food limitation, as well as to attract protistan colonizers by generating a water layer enriched in organic matter and bacteria.

The ubiquitous existence in marine systems of detrital particles, such as macroscopic aggregates or marine snow, has been widely documented (Silver et al. 1978; Alldredge and Silver 1988; Herndl 1995). Despite their diverse origins and components, once suspended particles are produced, they create a spatial heterogeneity, not only in the distribution of organic matter (Iriberri and Herndl 1995) but also in the abundance and specific composition of the heterotrophic microbial communities (Biddle and Fletcher 1995). Thus, bacterial and protistan densities in macroaggregates can exceed those of their free-suspended counterparts by two, three, or even as much as four orders of magnitude (Prézelin and Alldredge 1983; Alldredge et al. 1986; Caron et al. 1986; Turley and Mackie 1994; Artolozaga et al. 1997, 2000). These organic conglomerates constitute particular microenvironments where a differentiated bacterial community carries out high metabolic activities (Azam et al. 1993; Unanue et al. 1998*b*). The protistan inhabitants of particles also constitute a characteristic (Caron 1991) and presumably very active community because of the very high prey density available to them.

Despite the relevance of marine aggregates, our knowledge of the grazing activities of bacterivorous protists on particle-associated bacteria and the subsequent channeling of carbon and energy to higher trophic levels is still limited. This is probably due to the methodological problems inherent in the handling of seawater samples containing macroaggregates. These structures are very fragile (Alldredge and Silver 1988) and must be sampled using nondisturbing devices (Trent et al. 1978). In situ sampling by SCUBA divers may be the best option but it is of little use when large amounts of aggregates are required. In view of these problems several different methods have been used in order to create aggregates in the laboratory. Following the experimental design of Shanks and Edmonson (1989), Unanue et al. (1998*a*) succeeded in the formation of macroaggregates from natural seawater using microcosms (rolling tanks). These laboratory-made aggregates are suitable models to analyze, comparatively with the liquid phase, the abundance, activity, succession in time, and spatial distribution of the microbial assemblages (Artolozaga et al. 1997, 2000; Agis et al. 1998; Unanue et al. 1998*a*,*b*).

Agis et al. (1998) and Unanue et al. (1998*b*), working with phytoplankton derived macroaggregates, showed that these particles contained very high densities (3–4 orders of magnitude higher than those in seawater) of large and firmly attached bacteria, which exhibited high hydrolytic activities and not so high monomer uptake rates. Therefore, the aggregates should be considered as biological reactors that release low molecular weight nutrients (Smith et al. 1992; Grossart and Simon 1998; Unanue et al. 1998*b*); those nutrients should result in a high density of metabolically active bacteria in the seawater close to the aggregate. Artolozaga et al. (2000), working with macroaggregates made from natural seawater, showed that when the aggregates appear, the components of the protistan communities choose the most appropriate environment, aggregate, seawater or seawater layer surrounding the aggregate, and position themselves accordingly in the newly created spatial heterogeneity. The choice is mainly made on the basis of their feeding mech-

[1] To whom correspondence should be addressed (oipirraj@lg.ehu.es).

Acknowledgments

Two anonymous reviewers are thanked for critically reading the manuscript.

This work was supported by grants to A.L. and J.I. from the University of the Basque Country (UPV 093.310-EB 033/95), and to J.I. from the Spanish Government (MEC PB97-0635) and the Basque Government (PIGV-98-0025).

anisms and ability to swim, to move over surfaces, and to remain attached to surfaces.

Although protistan grazing on free-suspended bacteria has been widely documented over the last decades (e.g., Sherr et al 1989; Sanders et al. 1992), data about grazing rates on attached bacteria are scarce. Caron (1987) estimated the grazing on attached bacteria by heterotrophic microflagellates by comparing densities of bacteria attached to chitin particles in the presence and absence of protozoan grazing. Albright et al. (1987) used stained bacteria attached to alginate beads to quantify estuarine ciliate ingestion rates. Other authors (Hondeveld et al. 1992; Epstein 1997) calculated ingestion rates of protists in marine sediments by adding suspended fluorescently labeled bacteria (FLB) to sediments. Starink et al. (1994, 1996) developed a new method and measured benthic heterotrophic protistan grazing from stained sediments containing attached FLB.

In this study rolling tanks were used to generate organic macroaggregates in which the grazing capacity of several protists on particle-associated bacteria was measured. A preliminary objective was to modify the monodispersed FLB uptake technique described by Sherr et al. (1987) for making it effective for the measurement of grazing rates by protists on bacteria firmly attached to organic macroaggregates. In order to evaluate the relevance of grazing on particles, a comparative study was performed by measuring grazing on free-suspended bacteria at the low densities usually found in seawater as well as at the higher densities that it should be expected for the seawater layer surrounding the aggregates.

Materials and methods

Field collection—Seawater was collected in the coastal waters of the Bay of Biscay, 43°24.5′N; 3°2.7′W, (Northern Spain) at 2-m depth. Experiments were carried out between September 1997 and March 1999. Samples were taken using a Niskin bottle (PWS Hydro-Bios) and processed in the laboratory within 6 h of collection.

Bacterivorous protists—Four species of bacterivorous protists were analyzed: three nanoflagellates, *Bodo designis, Jakoba libera* and *Rhynchomonas nasuta,* and the scuticociliate *Uronema marinum.* Except for *J. libera* (CCAP 1954/1), all of them were isolated from the sampling site. *B. designis* and *R. nasuta* are known to feed on attached bacteria (Fenchel 1991; Sleigh 1991), while *J. libera* mainly feeds on suspended bacteria (Patterson et al. 1993). The microciliate has been described as a good swimmer, which feeds on suspended bacteria using a filtering mechanism (Turley et al. 1986; Sleigh 1991), but it is also often associated to particles (Sieburth 1984).

In order to obtain a dense protistan suspension for grazing experiments, the analyzed protists were grown on bacterized 0.03% (*J. libera* and *U. marinum*) or 0.05% (*B. designis* and *R. nasuta*) cereal leaves extract at 20°C and 100 rpm in the dark until the cultures reached the stationary growth (1 week approx.).

Microbial counts—Free-suspended bacterial counts were made on 1-ml water subsamples fixed with formalin (2% v/v final concentration). Natural bacterial abundance was measured by acridine orange epifluorescence direct counting (Hobbie et al. 1977). Appropriate volumes of the fixed subsamples were stained with acridine orange (0.01% w/v final concentration) for 2 min and filtered onto 0.2-μm pore-size black polycarbonate filters. A similar process was used for counting free monodispersed FLB, but without AO staining. The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. The filters were examined under a Nikon epifluorescence microscope equipped with blue light at a magnification of $\times$1000. Bacteria or FLB present in at least 30 randomly selected fields, with 20–30 bacteria per field, were counted.

In order to know the percentage of FLB relative to the total number of bacteria attached to the aggregates, formalin fixed (*see above*) subsamples containing 7 μl of aggregates and 1 ml of sterile (0.2-μm pore-size filtered) seawater (Unanue et al. 1998*a*) were counted at the beginning ($t = 0$ min) of the grazing experiments. For detaching bacteria from the aggregate, the fixed subsamples were treated with sterile (0.2-μm pore-size filtered) tetrasodium pyrophosphate (10 mM final concentration), kept for 1 h at room temperature in an orbital shaker (12 rpm), cooled, and sonicated (100 w) for 3 min in 50% cycles of 3 seg (Velji and Albright 1986). Appropriate volumes of these subsamples were stained with 4′,6-diamidino-2-phenylindole (DAPI) (0.2 μg ml^{-1}) for 15 min and filtered onto 0.2-μm pore-size black polycarbonate filters (Porter and Feig 1980). The wet filters were placed on microscope slides and mounted in low-fluorescence immersion oil. The filters were examined under a Nikon epifluorescence microscope equipped with UV and blue light at a magnification of $\times$1000. Natural bacteria (observed under UV light) and FLB (observed under blue light) present in at least 30 randomly selected fields, with 20–30 bacteria per field, were counted.

In the case of the free-swimming protistan counts, 5-ml water subsamples were fixed with alkaline Lugol (0.5% v/v final concentration) formalin (3% v/v final concentration) (Sherr et al. 1988). For counting aggregate-attached protists, 14-μl aggregate subsamples were taken (Artolozaga et al. 2000), placed in vials containing 10 ml of sterile (0.2-μm pore-size filtered) seawater, and preserved with Lugol formalin as above. Appropriate volumes of preserved water and aggregate subsamples were DAPI stained as above and filtered onto 0.8-μm (for flagellate counts) or 3.0-μm (for ciliate counts) pore-size black polycarbonate filters. The filters were examined under UV light at a magnification of $\times$1000 for flagellate counts and $\times$200 and $\times$1000 for ciliate counts. At least 100 flagellates were counted for each subsample, while the entire filter surface was screened for ciliate counts.

Preparation of FLB-containing macroaggregates—The system used for the formation of aggregates from natural seawater was designed according to that of Shanks and Edmonson (1989) but with some modifications that allowed the handling of aggregates containing firmly attached FLB. Eleven liters of natural seawater from the sampling station were poured into 13-liter polypropylene cylindrical rolling tanks (34 cm diameter $\times$ 14 cm) and 7×10^8–1×10^9 FLB were added. The FLB were prepared from natural bacterio-

plankton collected in the sampling station, following the protocol of Sherr et al. (1987). The tanks were maintained in the dark at room temperature with a very slow rotation of 2.5 rpm and were opened every day, in order to prevent anoxic conditions developing during the experiments. In the first 4–8 h natural bacteria, FLB, dissolved and colloidal organic matter, and the microparticles present in the seawater agglutinated and the FLB-containing visible macroaggregates appeared. The mean size of these newly formed aggregates was 1.4 mm, ranging from 0.2 to 8.7 mm, and they maintained stable for at least 72 h. The tanks were rolled for 24–48 h, and during this time the consistency and the degree of protistan colonization of the aggregates were tested.

After 24–48 h, when the ingestion experiments started, the consistence of the aggregates, as well as their size, was high enough to allow their manipulation without breakage or loss of material, while the colonization by the natural community of protists was scarce. The FLB-containing aggregates were carefully collected using a sterile glass pipette with a flexible rubber tube at one end. Then, the aggregates were maintained for 1 min in a Petri dish filled with sterile (0.2-μm pore-size filtered) seawater in order to gently wash the nonattached bacteria out of them. Subsamples were taken to enumerate the attached FLB and the natural microbial communities associated to aggregates (*see microbial counts section*).

Protistan grazing on aggregate-attached bacteria—A total of 14 experiments were carried out, three each with *B. designis* and *J. libera,* and four each with *R. nasuta* and *U. marinum.*

After washing, the FLB-containing aggregates were carefully and equidistantly (3 cm approx.) placed in a Petri dish (140 mm in diameter), which was previously filled with a dense suspension of the protist to be assayed (*see bacterivorous protists section*). Since a previous study (Artolozaga et al. 2000) had shown that these protists are able to choose between different microhabitats in heterogeneous samples, the aggregates were left to stand for 3 min in order to give the protists a chance to choose among remaining habitats in the suspension or coming near and attaching to the aggregates. At this time ($t = 0$ min) the grazing experiment started. Duplicate 7-μl washed aggregate subsamples were taken at intervals of 2–5 min for 15–30 min, placed in vials that contained 10 ml of sterile (0.2-μm pore-size filtered) seawater, and preserved as described in the microbial counts section. These fixed subsamples were disrupted and stained with DAPI as above and examined under an epifluorescence microscope. Two different researchers counted the FLB inside 100 nanoflagellates or 30 ciliates by duplicate for each subsampling time, and the differences between the results were statistically tested by the Mann Whitney test.

The grazing rates of the protists on aggregate-attached bacteria were calculated from the grazing rates shown for FLB and assuming that natural bacteria were proportionally consumed:

$$\mathrm{GR} = \mathrm{GR}_{\mathrm{FLB}} \frac{B + \mathrm{FLB}}{\mathrm{FLB}}$$

where GR is the protistan grazing rate (bacteria protist^{-1} h^{-1}), $\mathrm{GR}_{\mathrm{FLB}}$ is the protistan grazing rate on FLB (FLB protist^{-1} h^{-1}), B is the density of natural bacteria in the aggregates (bacteria ml^{-1}), and FLB is the density of FLB in the aggregates (FLB ml^{-1}).

In order to ascertain the degree of colonization of the aggregates by the assayed protists, a Colonization Index (CI) was calculated as the quotient between the number of protists in 7 μl of aggregate and the number of protists in 7 μl of the suspension. This was measured at the beginning ($t = 0$ min) of the ingestion experiments. CI values higher than 1 indicated a positive tendency to approach and colonize the aggregate by the protist.

Protistan grazing on free-suspended bacteria—Grazing experiments on free-suspended bacteria were carried out at two different prey density conditions: at the low densities usually found in seawater and at the higher densities that should be expected for the seawater layer surrounding the aggregates.

These sets of grazing experiments were performed using different approaches. For the low prey density experiments we used the same microcosms (rolling tanks without FLB addition) in which aggregates were made. Once the aggregates were formed, they were left to settle and the bulk water was used. The bacterial density was in the range 10^5–10^6 bacteria ml^{-1}. A total of 12 experiments, three with each protist, were carried out.

For the high prey density experiments it was necessary to enrich the seawater collected in the sampling station. Prey densities in the range 10^7–10^8 bacteria ml^{-1} were obtained. The natural seawater was gravity filtered through a 0.45-μm pore-size filter and cultured with 0.06% cereal leaves extract for 1 week at 100 rpm, in the dark and at 17°C, before starting the grazing experiments. A total of 21 experiments were carried out, eight with *B. designis*, three with *J. libera*, seven with *R. nasuta*, and three with *U. marinum.*

Protistan grazing rates on free-suspended bacteria under these two conditions (low and high prey densities) were measured following the protocol of Sherr et al. (1987). The monodispersed FLB used were prepared from natural bacterioplankton collected in the sampling station.

The grazing rates of the protists on free-suspended bacteria were calculated from the FLB grazing rates, as previously described.

Results

The technique used to measure protistan grazing rates on aggregate-attached bacteria—The characteristics of the aggregates developed in the 14 microcosm experiments are shown in Table 1. Natural bacterial communities colonized all of them. Attached bacterial abundance in aggregates ranged between 2.0×10^8 and 21.0×10^8 bacteria per ml of aggregate, with a mean value of 6.2×10^8 bacteria per ml of aggregate. The same amount of FLB was added to all the microcosms, but different percentages of FLB with respect to the total bacteria were found in each microcosm at the beginning of the ingestion experiments. These percent-

Table 1. Characterization of the aggregates used in the determinations of grazing rates on aggregate-attached bacteria by the nanoflagellates *Bodo designis, Jakoba libera,* and *Rhynchomonas nasuta* and the microciliate *Uronema marinum.*

Protist	Experiment	Assayed protistan density (protists ml^{-1})*	Colonization index (C.I.)	Attached bacterial density (bacteria ml^{-1})*	Percentage of attached FLB (%)
B. designis	I	1.4×10^6	1.6	4.1×10^8	24.6
	II	1.3×10^6	0.7	20.0×10^8	2.0
	III	3.6×10^6	3.3	2.2×10^8	28.0
J. libera	I	0.4×10^6	3.5	4.7×10^8	7.9
	II	0.5×10^6	4.2	4.8×10^8	8.0
	III	1.1×10^6	6.5	2.0×10^8	19.6
R. nasuta	I	0.4×10^6	30.4	3.2×10^8	27.3
	II	0.7×10^6	5.2	2.4×10^8	31.6
	III	2.1×10^6	4.4	21.0×10^8	3.0
	IV	1.5×10^6	3.4	2.3×10^8	29.0
U. marinum	I	0.9×10^3	7.6	4.2×10^8	30.7
	II	2.9×10^3	26.1	2.6×10^8	65.0
	III	0.4×10^3	0.1	2.4×10^8	14.0
	IV	0.3×10^3	0.2	2.7×10^8	56.0

* Microbial densities given as microorganisms per milliliter of aggregate.

ages ranged between 2.1% and 65.0%, although in 75% of the experiments it fell between 10.0% and 30.0%.

The aggregates were also colonized by protists that came from the seawater. The natural protistan densities in the aggregates were monitored during their formation, and the experiments were started before these densities were too high: the communities of natural nanoflagellates ranged between 0.3×10^5 and 6.9×10^5 protists per ml of aggregate, while the communities of natural ciliates ranged between 0.7×10^2 and 10.0×10^2 protists per ml of aggregate.

When the aggregates were carefully placed in a dense suspension of the assayed protists, they were colonized densely and quickly (*see Table 1*). In fact, the values of the colonization index show that the nanoflagellates actively moved toward and inhabited the aggregates, with the one exception of the experiment II carried out with *B. designis.* The three nanoflagellates, *B. designis, J. libera,* and *R. nasuta* became the most abundant protistan population in the aggregate, since their densities (mean value 1.3×10^6 protists per ml of aggregate) were one order of magnitude higher than those of natural nanoflagellates.

Regarding the microciliate *U. marinum,* four experiments were carried out, although the aggregates were only colonized in experiments I and II. In these two experiments the observed densities of *U. marinum* were also one order of magnitude higher than the density of the natural community of ciliates. In experiments III and IV the colonization index was too low to allow an accurate estimate of protistan grazing rates.

The regression lines of bacterial uptake versus time corresponding to the 12 experiments carried out with aggregates are shown in Fig. 1. A linear increase ($R^2 > 0.95$, $p < 0.05$) on the plots of number of bacteria ingested per protist versus time was observed during the first 10–15 min of the incubation period in all experiments.

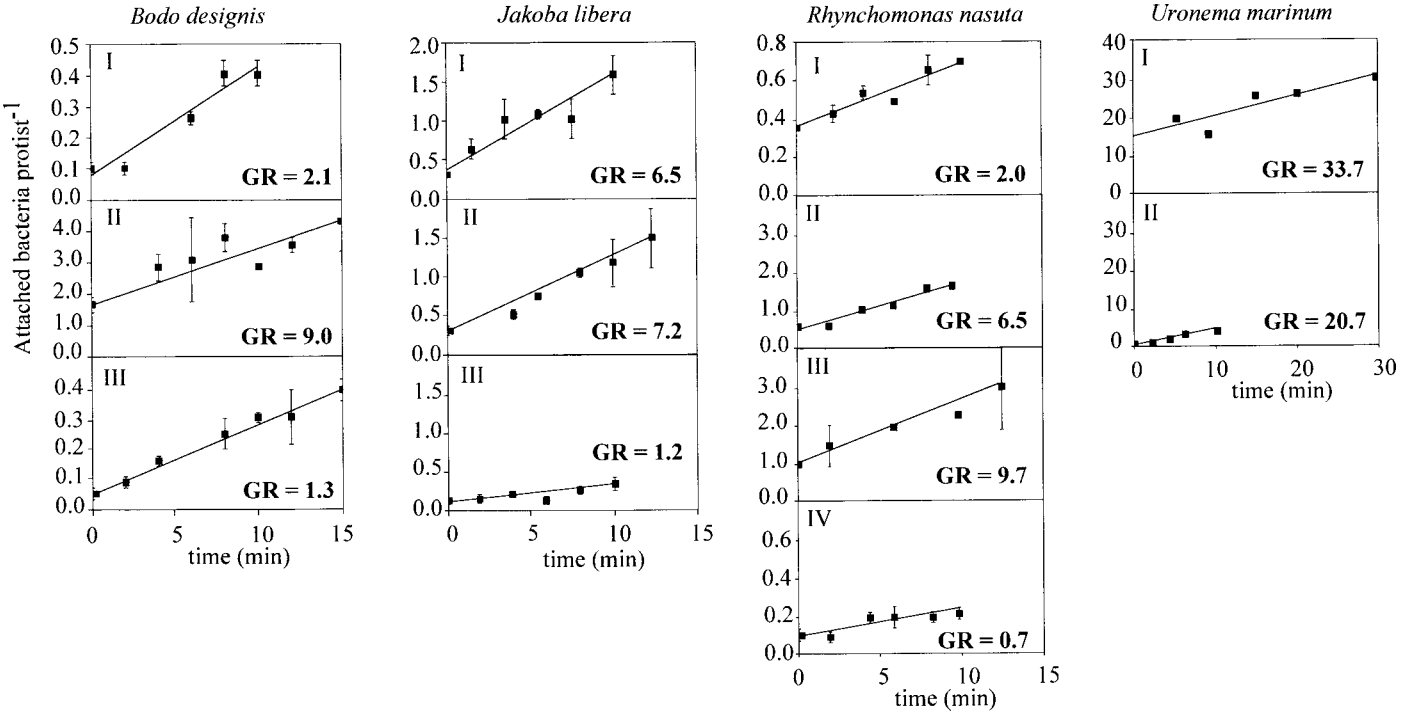

Fig. 1. Plots of bacterial ingestion per protist versus time in the experiments carried out in aggregates. GR is the grazing rate (bacteria $protist^{-1}$ h^{-1}), and it is given by the slope of the plot. Notice the different scales used for the ingestion of attached bacteria per protist.

Table 2. Grazing rates shown by the four protists on bacteria attached to aggregates and on free-suspended bacteria in the water. Data are given as mean value (range).

	Aggregate		Water: High prey density		Water: Low prey density	
Protist	Grazing rate (bacteria protist^{-1} h^{-1})	*n*	Grazing rate (bacteria protist^{-1} h^{-1})	*n*	Grazing rate (bacteria protist^{-1} h^{-1})	*n*
B. designis	4.1 (1.3–9.0)	3	9.1 (3.8–15.9)	8	1.3 (0.2–2.9)	3
J. libera	5.0 (1.2–7.2)	3	29.7 (14.5–41.0)	3	6.5 (4.2–10.0)	3
R. nasuta	4.7 (0.7–9.7)	4	10.3 (4.9–18.7)	7	1.0 (0.4–1.5)	3
U. marinum	27.2 (20.7–33.7)	2	382 (262–592)	3	33.3 (26.1–46.4)	3

The detection and enumeration of the ingested FLB inside the digestive vacuoles of the assayed protists is a major factor affecting the precision of this technique. We performed a statistical analysis to detect any bias leading to different results when different researchers processed samples. There were no significant differences between these counts (Mann Whitney test, $p > 0.05$).

Protistan grazing rates on bacteria in different microenvironments—The protistan grazing rates in the 45 experiments were obtained from FLB grazing rates. In all of them we obtained statistically significant regression lines ($p < 0.05$) of bacterial ingestion per protist versus time. The grazing rates of each protist in the different conditions are shown in Table 2. These results indicate that (1) the three nanoflagellates and the microciliate were able to feed on both attached and free-suspended bacteria and (2) the location of the protist in the spatially heterogeneous habitat affects the protistan grazing rates.

In this respect, it is noticeable that the protistan grazing rates on attached bacteria were not different (Mann Whitney test, $p > 0.05$) from those found on free-suspended bacteria at the low prey densities usually found in seawater: the mean values for the nanoflagellates were 4.6 bacteria protist^{-1} h^{-1} (range 0.7–9.7 bacteria protist^{-1} h^{-1}) when they grazed on aggregates and 2.9 bacteria protist^{-1} h^{-1} (range 0.2–10.0 bacteria protist^{-1} h^{-1}) when they grazed on free-suspended bacteria in low prey density conditions. The microciliate grazed 27.2 bacteria protist^{-1} h^{-1} (mean value) in aggregates and 33.3 bacteria protist^{-1} h^{-1} (mean value) in low prey density conditions.

As prey density is known to be a key parameter influencing protistan grazing rates, it was not surprising to find the highest grazing rates associated with higher bacterial densities in the case of free-suspended bacteria: the mean value observed for the three nanoflagellates was 13 bacteria protist^{-1} h^{-1} (range 3.8–41.0 bacteria protist^{-1} h^{-1}), and the ciliate was able to prey 382 bacteria protist^{-1} h^{-1} (range 262–592 bacteria protist^{-1} h^{-1}).

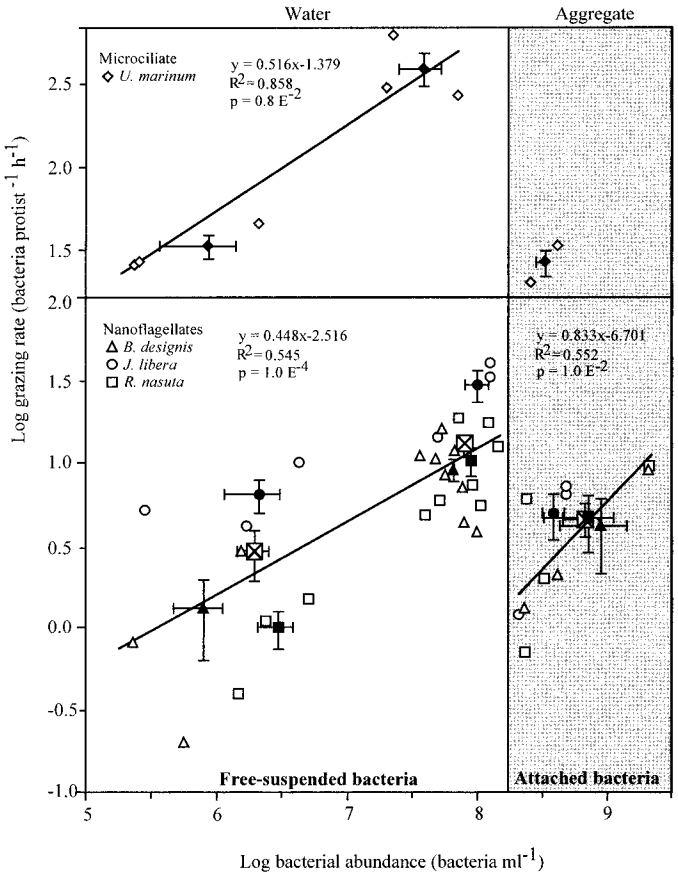

Fig. 2. Grazing rates by the microciliate *U. marinum* and the nanoflagellates *B. designis*, *J. libera*, and *R. nasuta* on free-suspended bacteria in water at low and high prey density conditions and on aggregate-attached bacteria. Open symbols are the results of each experiment; closed symbols indicate the mean grazing rate by each protist under the three environmental conditions; and the crossed squares indicate the mean grazing rates by the three nanoflagellates in each condition.

However, as we did not know whether prey density could affect the grazing rates in the same way when the prey are free suspended and when they are attached, we plotted the grazing rates obtained versus the bacterial prey densities (*see Fig. 2*). To perform this analysis, it was distinguished how the prey was found: attached in the experiments carried out in aggregates and free suspended in those carried out at the low densities usually found in seawater as well as at the higher densities that it should be expected for the seawater layer surrounding the aggregates. We found a close and statistically significant ($p < 0.05$) positive relationship between (log) grazing rate and (log) bacterial density in all cases. However, the values of the slopes indicated that this relationship was different when bacterial prey were free suspended or attached to aggregates. The slopes of (log) grazing rates versus (log) suspended bacterial prey density for the microciliate and the nanoflagellates were very similar (0.51 and 0.45, respectively), which indicates that prey density affects the grazing rates of these different protists in the same

way. On the other hand, the high value of the slope of (log) grazing rates versus (log) attached bacterial prey density detected for the nanoflagellates (0.83) indicates that prey density has more influence on grazing rates when bacteria are attached than when they are free suspended.

If we consider prey density alone, it is noticeable that the four protists showed their highest grazing rates when feeding in high densities of free-suspended bacteria, although the bacterial densities were much lower than those found in aggregates.

Discussion

The technique used to measure grazing rates on aggregate-attached bacteria—The proposed methodological approach relies on the formation of FLB-containing macroscopic aggregates from natural seawater using rolling tanks. The grazing of FLB by single species of bacterivorous protists through time was used to measure the protistan grazing rates on the attached bacterial community. We will now discuss some of the most important features of the technique.

An important issue is with the kind of experimental aggregates used. Unanue et al. (1998*a*) analyzed the characteristics of aggregates made in a similar way than those used in this study and showed that the densities of bacteria and flagellates as well as the DOC concentration per ml of aggregate were in the range detected in the few studies focused on natural aggregates. Since only natural seawater was used in their formation, these aggregates offer a realistic view of the aquatic system, and therefore their composition and structure will depend on the amount and quality of the particles, their abundance, and the structure of the microbial communities in the water samples.

This natural variability was reflected in the presence of attached FLB in the aggregates at the beginning of the ingestion assays: in spite of adding the same amount of tracers (7×10^8–1×10^9 FLB) per microcosms in all the experiments, the relative percentage of attached FLB with respect to the total number of bacteria in the aggregates was different. However, it did not influence the determination of the protistan grazing rates since in 75.0% of the experiments the percentage of FLB was between 10.0% and 30.0% (*see Table 1*). Moreover, the lowest values were not low enough to make the FLB observation inside the protist difficult, and the highest values were not high enough to change the ingestion rates significantly as a result of an increase in prey availability.

The accuracy of the determination of grazing rates on attached bacteria using this approach depends upon the bacteria ingested by the protists being truly aggregate associated (firmly attached or embedded into the polymeric matrix) and not suspended in the interstitial water of the aggregates. For this reason the technique includes a step in which the nonattached bacteria are ''gently washed'' from the interstitial spaces of the aggregates. Thus, when the aggregates are added to the protistan suspension, they contain mainly attached bacteria.

The use of FLB as bacterial surrogates has received much attention, and several studies have focused on determining the nature of the protistan discrimination between the natural bacteria and their heat-killed counterparts (González et al. 1993). Although a certain degree of discrimination has been found, mainly due to differences in bacterial surface characteristics and motility, this discrimination is probably lower than in those cases in which nonbacterial tracers (alginate beads, microspheres) were used (reviewed in Landry 1994). In the particular case of the use of attached FLB, these two factors should be minimized with reference to the special characteristics of the aggregates. Bacterial attachment processes under natural conditions may be active, by means of fimbria or the production of polymeric material by living bacteria, or passive, by adsorption, which would be the attachment mechanism for the FLB. In case that this passive mechanism would result in a strength of attachment weaker than that of living bacteria, an overestimation of grazing rates on attached bacteria should be considered. However, during the process of aggregate formation, natural bacteria produce copious amounts of attachment material, which allows not only the attachment of the producer bacteria but also the embedding of planktonic particles (Heissenberger et al. 1996). In this work these particles were material present in the natural seawater, such as bacterioplankton, remains of dead organisms, and the added FLB. The strength of the attachment of all these particles remains unknown, but since natural attached bacteria and FLB are mixed and trapped in these aggregates, great differences should not be expected. Regarding motility, the attachment restricts the capacity of natural bacteria to move freely into the particles, and therefore this factor is not to be considered important in discriminating between living bacteria and FLB.

Finally, this technique is useful to measure bacterial ingestion rates by those protists that tend to associate to the aggregates. In order to quantify this tendency, we estimated a colonization index (CI), which was the quotient of the densities of the protists in the aggregate versus the liquid phase. On that score, we found two different responses, depending on the kind of analyzed protist: in all cases except one, the CIs for the nanoflagellates were >1, which indicates a positive effort of approaching and colonization and shows that the aggregates were attractive for these nanoflagellates. However, this result is not surprising because the three analyzed nanoflagellates have been frequently described in the literature as able to feed on attached bacteria (Fenchel 1991). Regarding the ciliate, *U. marinum* is a good swimmer and does not present any kind of morphological structure for attaching to the aggregate, although it is often associated to particles (Sieburth 1984). From the four experiments carried out with this protist, only two showed CI > 1. It may be possible that in these two cases the composition of the aggregates attracted the ciliate, which swam toward and remained near them. Such a kind of chemotactic mechanism has already been reported for some heterotrophic protists (Sibbald et al. 1987; Blackburn and Fenchel 1999; Fenchel and Blackburn 1999).

To summarize, we propose a methodology that is effective in measuring the grazing on attached bacteria by bacterivorous protists in aggregates. Although it is tedious and requires careful manipulation and observation, it appears to be

suitable and reliable for analyzing protistan bacterivory processes on aggregates at the species level.

Protistan grazing rates on bacteria attached to aggregates versus free-suspended bacteria—Several studies have shown very clearly the preference of nanoflagellates and ciliates to graze on large and active bacterial prey (Davis and Sieburth 1984; Gasol et al. 1995; del Giorgio et al. 1996). Moreover, the ingestion rates are usually closely related to the prey density (Peters 1994; Iriberri et al. 1995). Thus, higher protistan grazing rates could be expected on bacteria attached to aggregates than on free-suspended bacteria because this microhabitat is densely colonized by attached bacteria that are frequently larger and more active than their free-suspended counterparts (Iriberri et al. 1987; Simon et al. 1990).

However, in the case of the nanoflagellates and as shown in Fig. 2, these protists showed very low grazing rates in the aggregates, despite the high bacterial densities found in them. The mean values for the experiments carried out in aggregates and at the low prey densities usually found in seawater were no different, which is highly relevant mainly because these nanoflagellates are described as particle associated bacterivores (Patterson et al. 1993) and thought to be able to feed efficiently on attached bacteria (Caron 1987; Sibbald and Albright 1988; Fenchel 1991). The ingestion rates shown by *U. marinum* in aggregates were too low for supporting the growth of this protist (Iriberri et al. 1995). It seems that this microciliate was unable to take advantage of the very high prey densities of the aggregates, probably due to the impossibility of capturing attached bacteria.

The grazing rates on attached bacteria observed in this study fell well within the range of values given by other authors. Starink et al. (1996), working on sediments, used FLB attached to the particles as bacterial surrogates to detect nanoflagellate grazing rates and obtained values ranging between 3.8 and 64.2 bacteria protist^{-1} h^{-1}. Albright et al. (1987) also used bacteria truly attached to stained microspheres, and they detected ciliate grazing rates ranging between 14 and 334 bacteria protist^{-1} h^{-1} for natural assemblages of ciliates and a grazing rate value of 28 bacteria protist^{-1} h^{-1} for a marine species of *Uronema.* There are other studies about protistan grazing on particles, but comparisons are of little use since most of them used FLB not truly attached to the particles but dispersed and added to them, and therefore the amount of effort required for the protists to detach the bacterial prey from the particles was not reflected in their results. Hence, grazing rates ranging from 2.5 to 72 bacteria protist^{-1} h^{-1} for flagellates (Epstein and Shiaris 1992; Hondeveld et al. 1992; Epstein 1997) and from 37 to 525 bacteria protist^{-1} h^{-1} for ciliates (Epstein and Shiaris 1992; Epstein 1997; Kemp 1988) have been reported.

Indeed, it does seem that feeding on attached bacteria involves an effort for the studied protists. The application of the regression equation between grazing rate and prey density obtained for free-suspended bacterial prey to the mean bacterial densities found in the aggregates (6.65×10^8 bacteria ml^{-1}) gives theoretical grazing rate values of 30.2 bacteria protist^{-1} h^{-1} for the nanoflagellates and 1,349 bacteria protist^{-1} h^{-1} for the microciliate. These theoretical values are much higher than the experimental values observed, 4.6 bacteria protist^{-1} h^{-1} for the nanoflagellates and 27.2 bacteria protist^{-1} h^{-1} for the microciliate. This difference indicates that these protists graze attached bacteria much slower than free-suspended bacteria, seven times in the case of the nanoflagellates and 50 times in that of the microciliate. In case that the grazing rates on attached bacteria were overestimated, as it has been previously discussed, this difference would be even higher. Therefore, attachment could confer grazing resistance to bacteria since they become less edible to the bacterivorous protists. This resistance may be a consequence of a spatial refuge depending on the origin and physical structure of the particles and the location of the bacteria within the particle (Jürgens and Gude 1994).

However, despite the low grazing rates shown by the protists on bacteria attached to aggregates at an individual level, the fact still remains that the community of protists inhabiting these particles is very dense and usually 2–4 orders of magnitude higher than the protistan planktonic community (Prézelin and Alldredge 1983; Caron et al. 1986; Turley and Mackie 1994). Several things may cause this. First, it may be caused by considering the total amount of bacterial prey available in the aggregates, which are also several orders higher than in the bulk water. Although the grazing rates shown by the protists were very similar when feeding on aggregates and on the low densities of suspended bacteria usually found in seawater, the growth of the protistan attached communities would not be food limited as seems to happen with their free-swimming counterparts. The energetic cost associated with moving in order to find bacterial prey should be much lower on these densely colonized aggregates than in the bulk water, and it might compensate for the effort that the protists must make in order to detach the bacterial prey. In addition, the abundance in aggregates of other kinds of food like macromolecules and dissolved organic matter should be taken into account because they can also be used by phagotrophic protists (Tranvik et al. 1993). Second, it may be caused if the aggregates are considered as dynamic microhabitats where the hydrolytic activities of the attached bacterial community generate and release organic material into the surrounding water layers. In that case, the growth of a dense and active subcommunity of free-suspended bacteria all around the aggregates should be expected (Cho and Azam 1988; Smith et al. 1992; Grossart and Simon 1998; Unanue et al. 1998*b*). The colonization of the aggregates would be increased by those planktonic protists able to detect and come nearer their surroundings where bacteria would be growing quickly. This kind of protistan chemosensory motile behavior has been recently described by Blackburn and Fenchel (1999) and Fenchel and Blackburn (1999) and seems to play a relevant role in the search for food patches in aquatic systems (Antipa et al. 1983; Sibbald et al. 1987).

Finally, the relevance of those different microhabitats generated in the spatially heterogeneous aquatic systems is made clear when a comparison between the potential channeling of bacterial biomass in bulk water versus the aggregates is performed at a community level. Our results indicated that free-swimming and particle-associated heterotrophic nanoflagellates showed similar grazing rates on an individual ba-

sis, but the densities of these two communities in natural ecosystems are usually very different. The abundance of free-swimming flagellates has been reported to be relatively low and similar in diverse aquatic marine systems, ranging from 1×10^2 to 13×10^2 HNF ml^{-1} (Davis et al. 1985; Fuhrman et al. 1989; Caron et al. 1995, 1999; Artolozaga et al. 2000), while the abundance of flagellates attached to aggregates is much higher and more variable, reaching values as high as 1.3×10^5 and 330×10^5 HNF ml^{-1} (Caron et al. 1986; Turley and Mackie 1994). Therefore, the bacterial biomass canalized through the community of flagellates associated to aggregates may be even three orders of magnitude higher than that canalized by the free-swimming community of flagellates. These data may reflect the potential ecological significance of the aggregates in the transfer of matter in the aquatic systems.

References

AGIS, M., M. UNANUE, J. IRIBERRI, AND G. J. HERNDL. 1998. Bacterial colonization and ectoenzymatic activity in phytoplankton-derived model particles. Part. II. Cleavage and uptake of carbohydrates. Microb. Ecol. **36:** 66–74.

ALBRIGHT, L. J., E. B. SHERR, B. F. SHERR, AND R. D. FALLON. 1987. Grazing of ciliated protozoa on free and particle-attached bacteria. Mar. Ecol. Prog. Ser. **38:** 125–129.

ALLDREDGE, A. L., J. J. COLE, AND D. A. CARON. 1986. Production of heterotrophic bacteria inhabiting macroscopic aggregates (marine snow) from surface water. Limnol. Oceanogr. **31:** 68–78.

———, AND M. W. SILVER. 1988. Characteristics, dynamics and significance of marine snow. Prog. Oceanogr. **20:** 41–82.

ANTIPA, G. A., K. MARTIN, AND M. T. RINTZ. 1983. A note on the possible ecological significance of chemotaxis in certain ciliated protozoa. J. Protozool. **30:** 55–57.

ARTOLOZAGA, I., B. AYO, A. LATATU, I. AZÚA, M. UNANUE, AND J. IRIBERRI. 2000. Spatial distribution of protists in the presence of macroaggregates in a marine system. FEMS Microbiol. Ecol. **33:** 191–196.

———, E. SANTAMARÍA, A. LÓPEZ, B. AYO, AND J. IRIBERRI. 1997. Succession of bacterivorous protists on laboratory-made marine snow. J. Plankton Res. **19:** 1429–1440.

AZAM, F., J. MARTÍNEZ, AND D. C. SMITH. 1993. Bacteria-organic matter coupling on marine aggregates, p. 410–411. *In* R. Guerrero and C. Pedrós-Alió [ed.], Trends in microbial ecology. Spanish Society for Microbiology.

BIDDLE, K. D., AND M. FLETCHER. 1995. Comparison of free-living and particle associated bacterial communities in the Chesapeake Bay by stable low-molecular weight RNA analysis. Appl. Environ. Microbiol. **61:** 944–952.

BLACKBURN, N., AND T. FENCHEL. 1999. Modelling of microscale patch encounter by chemotactic protozoa. Protist **150:** 337–343.

CARON, D. A. 1987. Grazing of attached bacteria by heterotrophic microflagellates. Microbiol. Ecol. **13:** 203–218.

———. 1991. Heterotrophic flagellates associated with sedimenting detritus, p. 77–92. *In* D. J. Patterson and J. Larsen [eds.], The biology of free-living heterotrophic flagellates. The Systematics Association Special Volume, 45. Clarendon.

———, AND OTHERS. 1995. The contribution of microorganisms to particulate carbon and nitrogen in surface waters of the Sargasso Sea near Bermuda. Deep-Sea Res. I **42:** 943–972.

———, P. G. DAVIS, L. P. MADIN, AND J. MCN. SIEBURTH. 1986. Enrichment of microbial populations in macroaggregates (marine snow) from surface waters of the North Atlantic. J. Mar. Res. **44:** 543–565.

———, E. R. PEELE, E. L. LIM, AND M. R. DENNET. 1999. Picoplankton and nanoplankton and their coupling in surface waters of the Sargasso Sea South of Bermuda. Limnol. Oceanogr. **44:** 259–272.

CHO, B. C., AND F. AZAM. 1988. Major role of bacteria in biogeochemical fluxes in the ocean's interior. Nature **332:** 441–443.

DAVIS, P. G., D. A. CARON, P. W. JOHNSON, AND J. MCN. SIEBURTH. 1985. Phototrophic and apochlorotic components of pikoplankton and nanoplankton in the North Atlantic: Geographic, vertical, seasonal and diel distributions. Mar. Ecol. Prog. Ser. **21:** 15–26.

———, AND J. MCN. SIEBURTH. 1984. Estuarine and oceanic microflagellate predation of actively growing bacteria: Estimation by frequency of dividing-divided bacteria. Mar. Ecol. Prog. Ser. **19:** 237–246.

DEL GIORGIO, P. A., J. M. GASOL, D. VAQUÉ, P. M. MURA, S. AGUSTÍ, AND C. M. DUARTE. 1996. Bacterioplankton community structure: Protist control net production and the proportion of active bacteria in a coastal marine community. Limnol. Oceanogr. **41:** 1169–1179.

EPSTEIN, S. S. 1997. Microbial food webs in marine sediments. I. Trophic interactions and grazing rates in two tidal flat communities. Microb. Ecol. **34:** 188–198.

———, AND M. P. SHIARIS. 1992. The rates of microbenthos- and meiobenthos-bacterivory in a temperate muddy tidal flat community. Appl. Environ. Microbiol. **58:** 2426–2431.

FENCHEL, T. 1991. Flagellate design and function, p. 7–19. *In* D. J. Patterson and J. Larsen [eds.], The biology of free-living heterotrophic flagellates. The Systematics Association Special Volume, 45. Clarendon Press.

———, AND N. BLACKBURN. 1999. Motile chemosensory behaviour of phagotrophic protists: Mechanisms for and efficiency in congregating at food patches. Protist **150:** 325–336.

FUHRMAN, J. A., T. D. SLEETER, C. A. CARLSON, AND L. M. PROCTOR. 1989. Dominance of bacterial biomass in the Sargasso Sea and its ecological implications. Mar. Ecol. Prog. Ser. **57:** 207–217.

GASOL, J. M., P. A. DEL GIORGIO, R. MASSANA, AND C. M. DUARTE. 1995. Active versus inactive bacteria: Size-dependence in a coastal marine plankton community. Mar. Ecol. Prog. Ser. **128:** 91–97.

GONZÁLEZ, J. M., E. B. SHERR, AND B. F. SHERR. 1993. Differential feeding by marine flagellates on growing versus starving, and on motile versus nonmotile, bacterial prey. Mar. Ecol. Prog. Ser. **102:** 257–267.

GROSSART, H.-P., AND M. SIMON. 1998. Bacterial colonization and microbial decomposition of limnetic organic aggregates (lake snow). Aquat. Microb. Ecol. **15:** 127–140.

HEISSENBERGER, A., G. G. LEPPARD, AND G. J. HERNDL. 1996. Relationship between the intracellular integrity and the morphology of the capsular envelope in attached and free-living marine bacteria. Appl. Environ. Microbiol. **62:** 4521–4528.

HERNDL, G. J. 1995. Microbial dynamics in marine snow, p. 81–105. *In* S. Floderus, A.-S. Heiskanen, M. Olesen, and P. Wassmann [eds.], Sediment trap studies in the nordic countries. NurmiPrint.

HOBBIE, J. E., R. J. DALEY, AND S. JASPER. 1977. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:** 1225–1228.

HONDEVELD, B. J. M., R. P. M. BAK, AND F. C. VAN DUYL. 1992. Bacterivory by heterotrophic nanoflagellates in marine sediments measured by uptake of fluorescently labeled bacteria. Mar. Ecol. Prog. Ser. **89:** 63–71.

IRIBERRI, J., B. AYO, E. SANTAMARÍA, I. BARCINA, AND L. EGEA.

1995. Influence of bacterial density and water temperature on the grazing activity of two freshwater ciliates. Freshw. Biol. **33:** 223–231.

———, AND G. J. HERNDL. 1995. Formation and microbial utilization of amorphous aggregates in the sea: Ecological significance. Microbiología SEM **11:** 309–322.

———, M. UNANUE, I. BARCINA, AND L. EGEA. 1987. Seasonal variation in population density and heterotrophic activity of attached and free-living bacteria in coastal waters. Appl. Environ. Microbiol. **53:** 2308–2314.

JÜRGENS, K., AND H. GUDE. 1994. The potential importance of grazing-resistant bacteria in planktonic systems. Mar. Ecol. Prog. Ser. **112:** 169–188.

KEMP, P. 1988. Bacterivory by benthic ciliates: Significance as a carbon source and impact on sediment bacteria. Mar. Ecol. Prog. Ser. **49:** 163–169.

LANDRY, M. R. 1994. Methods and controls for measuring the grazing of planktonic protists. Mar. Microb. Food Webs **8:** 37–57.

PATTERSON, D. J., K. NYGAARD, G. STEINBERG, AND C. M. TURLEY. 1993. Heterotrophic flagellates and other protists associated with oceanic detritus throughout the water column in the mid North Atlantic. J. Mar. Biol. Ass. U.K. **73:** 67–95.

PETERS, F. 1994. Prediction of planktonic protistan grazing rates. Limnol. Oceanogr. **39:** 195–206.

PORTER, K. G., AND Y. S. FEIG. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:** 943–948.

PRÉZELIN, B. B., AND A. L. ALLDREDGE. 1983. Primary production of marine snow during and after an upwelling event. Limnol. Oceanogr. **28:** 1156–1167.

SANDERS, R. W., D. A. CARON, AND U. G. BERNINGER. 1992. Relationships between bacteria and heterotrophic nanoplankton in marine and freshwaters: An inter-ecosystems comparison. Mar. Ecol. Prog. Ser. **86:** 1–14.

SHANKS, A. L., AND E. W. EDMONSON. 1989. Laboratory-made artificial marine snow: A biological model of the real thing. Mar. Biol. **101:** 463–470.

SHERR, B. F., E. B. SHERR, AND R. D. FALLON. 1987. Use of monodispersed, fluorescently labeled bacteria to estimate in situ protozoan bacterivory. Appl. Environ. Microbiol. **53:** 958–965.

———, ———, AND C. M. PEDRÓS-ALIO. 1989. Simultaneous measurement of bacterioplankton production and protozoan bacterivory in estuarine water. Mar. Ecol. Prog. Ser. **54:** 209–219.

———, ———, AND F. RASSOULZADEGAN. 1988. Rates of digestion of bacteria by marine phagotrophic protozoa: Temperature dependence. Appl. Environ. Microbiol. **54:** 1091–1095.

SIBBALD, M. J., AND L. J. ALBRIGHT. 1988. Aggregated and free bacteria as food sources for heterotrophic microflagellates. Appl. Environ. Microbiol. **54:** 613–616.

———, ———, AND P. R. SIBBALD. 1987. Chemosensory response of a heterotrophic microflagellate to bacteria and several nitrogen compounds. Mar. Ecol. Prog. Ser. **36:** 201–204.

SIEBURTH, J. MCN. 1984. Protozoan bacterivory in pelagic marine waters, p. 405–443. *In* J. E. Hobbie and F. J. LeB. Williams [eds.], Heterotrophic activity in the sea. Plenum.

SILVER, M. W., A. L. SHANKS, AND J. D. TRENT. 1978. Marine snow: Microplankton habitat and source of small-scale patchiness in pelagic populations. Science **201:** 371–373.

SIMON, M., A. L. ALLDREDGE, AND F. AZAM. 1990. Bacterial carbon dynamics on marine snow. Mar. Ecol. Prog. Ser. **65:** 205–211.

SLEIGH, M. A. 1991. A taxonomic review of heterotrophic protists important in marine ecology, p. 9–38. *In* P. C. Reid, C. M. Turley, and P. H. Burkill [eds.], Protozoa and their role in marine processes. Springer.

SMITH, D. C., M. SIMON, A. ALLDREDGE, AND F. AZAM. 1992. Intense hydrolytic activity on marine aggregates and implications for rapid particle dissolution. Nature **359:** 139–141.

STARINK, M., M. J. BÄR-GILISSEN, R. P. M. BAK, AND T. E. CAPPENBERG. 1996. Bacterivory by heterotrophic nanoflagellates and bacterial production in sediments of a freshwater littoral system. Limnol. Oceanogr. **41:** 62–69.

———, I. N. KRYLOVA, M. J. BÄR-GILISSEN, R. P. M. BAK, AND T. E. CAPPENBERG. 1994. Rates of benthic protozoan grazing on free and attached sediment bacteria measured with fluorescently stained sediment. Appl. Environ. Microbiol. **60:** 2259–2264.

TRANVIK, L., E. B. SHERR, AND B. F. SHERR. 1993. Uptake and utilization of ''colloidal DOM'' by heterotrophic flagellates in seawater. Mar. Ecol. Prog. Ser. **92:** 301–309.

TRENT, J. D., A. L. SHANKS, AND M. W. SILVER. 1978. In situ and laboratory measurements on macroscopic aggregates in Monterey Bay, California. Limnol. Oceanogr. **23:** 626–635.

TURLEY, C. M., AND P. J. MACKIE. 1994. Biogeochemical significance of attached and free-living bacteria in the flux of particles in the NE Atlantic Ocean. Mar. Ecol. Prog. Ser. **115:** 191–203.

———, R. C. NEWELL, AND D. B. ROBINS. 1986. Survival strategies of two small marine ciliates and their role in regulating bacterial community structure under experimental conditions. Mar. Ecol. Prog. Ser. **33:** 59–70.

UNANUE, M. A., I. AZÚA, J. M. ARRIETA, G. J. HERNDL, AND J. IRIBERRI. 1998*a*. Laboratory-made particles as a useful approach to analyse microbial processes in marine macroaggregates. FEMS Microbiol. Ecol. **26:** 325–334.

———, ———, ———, A. LABIRUA-ITURBURU, L. EGEA, AND J. IRIBERRI. 1998*b*. Bacterial colonization and ectoenzymatic activity in phytoplankton-derived model particles: Cleavage of peptides and uptake of amino acids. Microb. Ecol. **35:** 136–146.

VELJI, M. I., AND L. J. ALBRIGHT. 1986. Microscopic enumeration of attached marine bacteria of seawater, marine sediment, fecal matter, and kelp blade samples following pyrophosphate and ultrasound treatments. Can. J. Microbiol. **32:** 121–126.

Received: 2 January 2001
Accepted: 23 July 2001
Amended: 2 August 2001