Succession of bacterivorous protists on laboratory-made marine snow

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Abstract. Colonization and succession over time by bacterivorous protists on laboratory-made marine snow were analysed in five assays during 1994. Marine snow was made from natural seawater using rolling tanks. In all experiments, the macroaggregates were stable in size and consistency after the fourth day, and the colonization and succession processes were similar. Newly formed macroaggregates became colonized by heterotrophic nanoflagellates on the fourth day, most of them kinetoplastids (*Bodo designis* and *Rhynchomonas nasuta*) and bicosoecids (*Pseudobodo tremulans* and *Bicosoeca* sp.). Sarcodines and ciliates appeared 1 day later. Among the former, the most abundant genus was *Vannella* sp., while scuticociliates (*Uronema marinum*) and hypotrichs (*Euplotes vannus* and *Aspidisca steini*) were the most abundant ciliates. Most of the species observed in the study were more common to benthic habitats than to pelagic ones. The planktonic existence of the genera *Bodo*, *Rhynchomonas*, *Bicosoeca*, *Euplotes* and *Aspidisca* depends on the presence of surfaces because they are poor swimmers or immotile, and *Pseudobodo* and *Vannella* need attachment for feeding. The only pelagic protist observed was *Uronema*, probably because its opportunistic behaviour leads it to exploit enriched environments such as marine snow. Flagellate and ciliate abundances in laboratory-made macroaggregates were much higher than in surrounding water, which indicates that marine snow represents an enhanced habitat for protist growth.

Introduction

Much of the suspended organic and inorganic matter in the ocean exists as aggregates which range from a few micrometres to many centimetres in size. Those particles >500 μm in length (macroaggregates) were defined as marine snow by Alldredge and Silver (1988).

Macroaggregates are ubiquitous in marine waters. They are sites enriched in nutrients, and particularly in carbon, which enhances the colonization by microorganisms and their subsequent growth: macroaggregates are characterized by containing bacterial numbers and biomass two or three orders of magnitude higher than those found in surrounding water (Caron *et al.*, 1982; Müller-Niklas *et al.*, 1994). Therefore, marine snow plays an important role in the flow of energy through microbial communities as well as in the vertical transport of some elements in the ocean (Caron, 1991).

There are few studies on microbial communities associated with marine snow, probably because macroaggregates are fragile and commonly broken up by routine methods of collection and subsequent handling. Much of the information about marine snow has resulted from *in situ* observations and collections by divers, but its biological study has mainly focused on the bacterial community (Alldredge and Youngbluth, 1985; Alldredge *et al.*, 1986; Herndl, 1988; Azam *et al.*, 1993). The importance of protists in relation to macroaggregates remains almost unknown, although they are supposed to play an important role in the

transformation and decomposition of marine snow (Lochte, 1991; Turley, 1991). Bacterivorous protists, when feeding on attached bacteria, are probably a major controlling factor for the bacterial assemblages of this habitat, and thereby may indirectly control the remineralization rates of nutrients. On the other hand, they seem to contribute to the break-up of macroaggregates by mechanical disruption and direct consumption of particulate material (Pomeroy and Deibel, 1980; Biddanda and Pomeroy, 1988; Lochte, 1991).

In order to obtain information about the role of protists inhabiting marine snow, their characterization is a prior requirement. In the exhaustive work carried out by Patterson *et al.* (1993), the occurrence of 40 species of heterotrophic flagellates associated with detritus was described. However, the information available on this topic still remains contradictory. According to some authors (Caron *et al.*, 1982; Patterson and Fenchel, 1990; Caron, 1991), many of the species observed are 'particle-associated' forms and poorly adapted to planktonic existence, like benthic species. On the contrary, other authors (Fenchel, 1982) have observed the same species in aggregates and surrounding water.

The aim of this work was to recognize the most abundant bacterivorous protists inhabiting laboratory-made macroaggregates, describing their spatial location on them, and to analyse the appearance and succession in time of the main groups of protists on this artificial marine snow. To achieve this, we used an experimental design in order to obtain marine snow similar to that found in natural environments.

Method

Field collection

Marine snow was made from natural seawater collected in the coastal waters of the Bay of Biscay (43°24.5′N; 3°2.7′W, 2 m depth) on five different dates during 1994: three experiments were carried out between June and September (mean seawater temperature 19°C) and two experiments between February and April (mean seawater temperature 9°C). Samples were taken using a Niskin bottle (PWS Hydro-Bios) and processed in the laboratory within 2 h after collection.

Marine snow formation

The lab system used for the formation of marine snow was designed according to Shanks and Edmonson (1989) (Figure 1a).

This roller system consisted of a metallic frame on which two parallel rubber bars rested, connected to a variable-speed motor. Propylene cylindrical tanks (34 cm diameter × 14 cm) were filled with 11 l of seawater from the sampling location and set on this system. These tanks were not completely filled in order to avoid anoxic conditions during the experiments. The size of the aggregates depended on the rotation speed; marine snow stable in size (5–6 mm) and consistency was obtained at 2.5 r.p.m. In addition, at this speed air bubbles which could contribute to the disruption of macroaggregates did not form inside the tank. The experiments were carried out at *in situ* temperature.
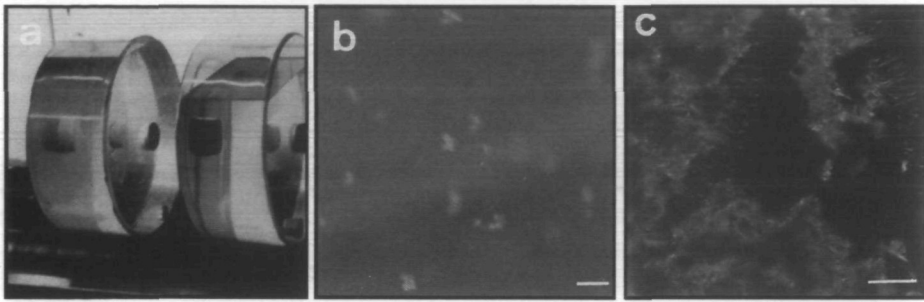
Fig. 1. Laboratory-made marine snow: (**a**) system used for its formation; (**b**) macroscopic and (**c**) microscopic observations. Scale bars (b) 2.5 mm and (c) 10 μm.

Protistan observation and enumeration

As soon as the macroaggregates were formed, subsamples were taken at 12 h intervals in order to observe and identify the bacterivorous protists associated with them. Sampling was carried out as long as there was marine snow, over a 10 day period. At each sampling interval, three macroaggregates were taken using a sterile flexible rubber tube. They were transferred to a 1 ml Sedgewick–Rafter chamber and were observed by light microscopy at a magnification from ×200 to ×1000 (Nikon Diaphot-TMD and Optiphot, both equipped with Nomarsky DIC system). This marine snow sample had to be left for several minutes for protists to settle. In order to observe protists contained inside the macroaggregates, we had to disrupt them. Because of their fragile nature, gentle shaking with a sterile tip was sufficient to break them without affecting the motility and behaviour of protists. Protists associated with marine snow were identified on the basis of their morphological and behavioural features as described in the literature (see below Table I).

In order to compare the protistan abundance on marine snow relative to that in surrounding water, enrichment factors (EF) were calculated. These factors represent the ratio between the number of protists in 1 ml of aggregate and the number of protists in 1 ml of surrounding water. Subsamples of aggregate and

Table I. Protists observed in the microcosm throughout the decomposition process of the macroaggregates, making reference to the appearance day and the description used in their identification

Protist	Appearance day	References
Aspidisca steini Buddenbrock, 1920	6/7	Carey, 1992
Bicosoeca maris Picken, 1941	4	Throndsen, 1993
Bodo designis Skuja, 1948	4	Patterson *et al.*, 1993
Euplotes vannus (Müller, 1786) Minkjewicz, 1901	6/7	Carey, 1992
Pseudobodo tremulans Griessmann, 1913	4	Patterson *et al.*, 1993 Throndsen, 1993
Rhynchomonas nasuta (Stokes, 1888) Klebs, 1893	4	Patterson *et al.*, 1993 Throndsen, 1993
Uronema marinum Dujardin, 1841	6/7	Carey, 1992
Vannella sp. Bovee, 1965	6/7	Page, 1983 Page, 1988

surrounding water were taken and fixed with alkaline Lugol (0.5% v/v final concentration)–formalin (3% v/v final concentration) (Sherr *et al.*, 1988) at 24 h intervals. This was carried out in two experiments: one in February, the colder ecosystem situation (see above); the other in September, the warmer ecosystem situation. The flagellate and ciliate protists contained in preserved subsamples were enumerated by epifluorescence microscopy after DAPI staining (Porter and Feig, 1980) with a final stain concentration of 0.2 µg ml^{-1}.

Isolation and maintenance of protists

The most representative protists growing on the macroaggregates were isolated by micropipetting individual cells with bacteria originally in the seawater using a microinjector, IM-188 Narishige. These cells were inoculated into 10 ml culture wells containing 0.01% cereal leaf infusion medium. These cultures were maintained at 15°C in the dark and transferred to fresh medium every 3 weeks.

Results

Marine snow formation

Marine snow formation followed a similar trend in the five assays performed. In the first 24 h of tank rotation, small visible particles were observed, which adhered together to make aggregates stable in size (5–6 mm) and consistency from Day 4 until Day 8 (Figure 1b). Then, they began to disaggregate, resulting in smaller particles which showed less consistency and higher fragility. This might be due to the disruption and degradation effects of microbial communities.

Microscopic observations of these macroaggregates showed that they were composed of diatoms, diatom frustules, macroalgae, faecal pellets and the remains of dead organisms coming from the original seawater (Figure 1c).

Protistan succession on marine snow

In all experiments, protistan succession on marine snow was similar. Newly formed aggregates (Day 4), containing high numbers of bacterial prey, became rapidly colonized by small flagellates (Figure 2a). Most of these were
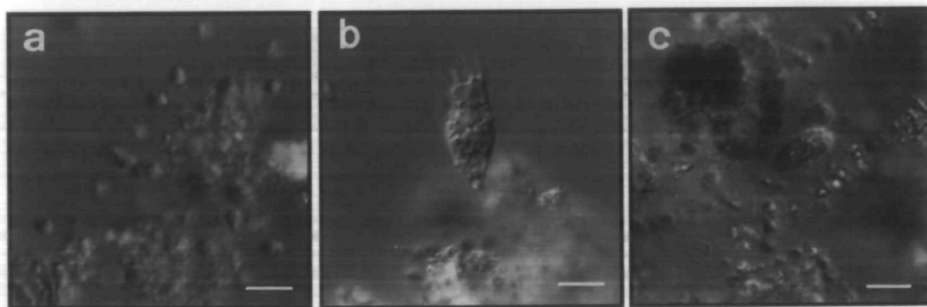

Fig. 2. Macroaggregate colonization by protists: (**a**) flagellates, (**b**) sarcodine (amoeba) and (**c**) ciliate. Scale bars 20 µm.

characterized as bodonids, because of their characteristic movement of smooth gliding in close contact with the substrate, and as bicosoecids owing to their attachment to the substrate with the shorter contractile flagellum or with a lorica.

Later (Days 6 and 7), the first sarcodines (Figure 2b) and ciliates (Figure 2c) were detected. It was very difficult to detect sarcodines because of their slow movement and little contrast with the marine snow. Thus, amoebae could only be detected when they left the macroaggregate and settled on the Sedgewick–Rafter chamber, and Heliozoa and Radiolaria were observed after disrupting the macroaggregate. Most of the sarcodines were naked amoebae distinguishable by the pseudopodial morphology of the locomotive and floating forms.

Among the ciliates, the most numerous were ovoid cells with holotric ciliature and rapid movement, identified as scuticociliates, and ovoid cells with ventral cirri used for movement over the substrate and an adoral zone of membranelles (AZM), identified as hypotrichs.

Protistan identification

The most representative protists associated with marine snow were identified according to their morphological characteristics and behavioural features as observed by light microscopy (Table I).

Aspidisca steini Buddenbrock, 1920 (Figure 3a) was a small, ovoid hypotrich ciliate, dorso-ventrally flattened, ~35 μm long and 20 μm wide. It presented a characteristic AZM divided into two parts and a small number of strongly developed cirri on which it moved.

Bicosoeca sp. James-Clark, 1866 (Figure 3b) was a bicosoecid biflagellated protist. The cells were spherical to ellipsoidal, ~10 μm long and attached to a lorica. Flagella were inserted next to a peristomal structure that resembles a protruding lip. The long flagellum was extended and held in a slight curve in front of the cell. The other flagellum curved backwards to attach to the base of the lorica. When the cell was disturbed, the shorter flagellum contracted and the cell was withdrawn to the bottom of the lorica, while the long flagellum was rolled up into a tight coil at the anterior end of the cell. On the basis of these characteristics, it was tentatively identified as *Bicosoeca maris* Picken, 1941.

Bodo designis Skuja, 1948 (Figure 3c) was a small, oval bodonid flagellate, ~10 μm long, with two flagella differing in size and movement that were inserted into the anterior part of the cell. The shorter anterior flagellum usually beat actively, while the longer recurrent one was directed backwards making contact with the substrate. It was a poor swimmer, but was capable of movement on surfaces due to the activity of the anterior flagellum which swept from side to side and moved the cell forwards with a jerky motion.

Euplotes vannus (Müller, 1786) Minkjewicz, 1901 (Figure 3d) was an ovoid hypotrich ciliate, dorso-ventrally flattened with the body curved slightly towards the right, <60 μm long and ~35 μm wide. It presented ventral cirri, which allowed its easy movement over surfaces, and AZM to create water currents under the cell towards the cytostome.
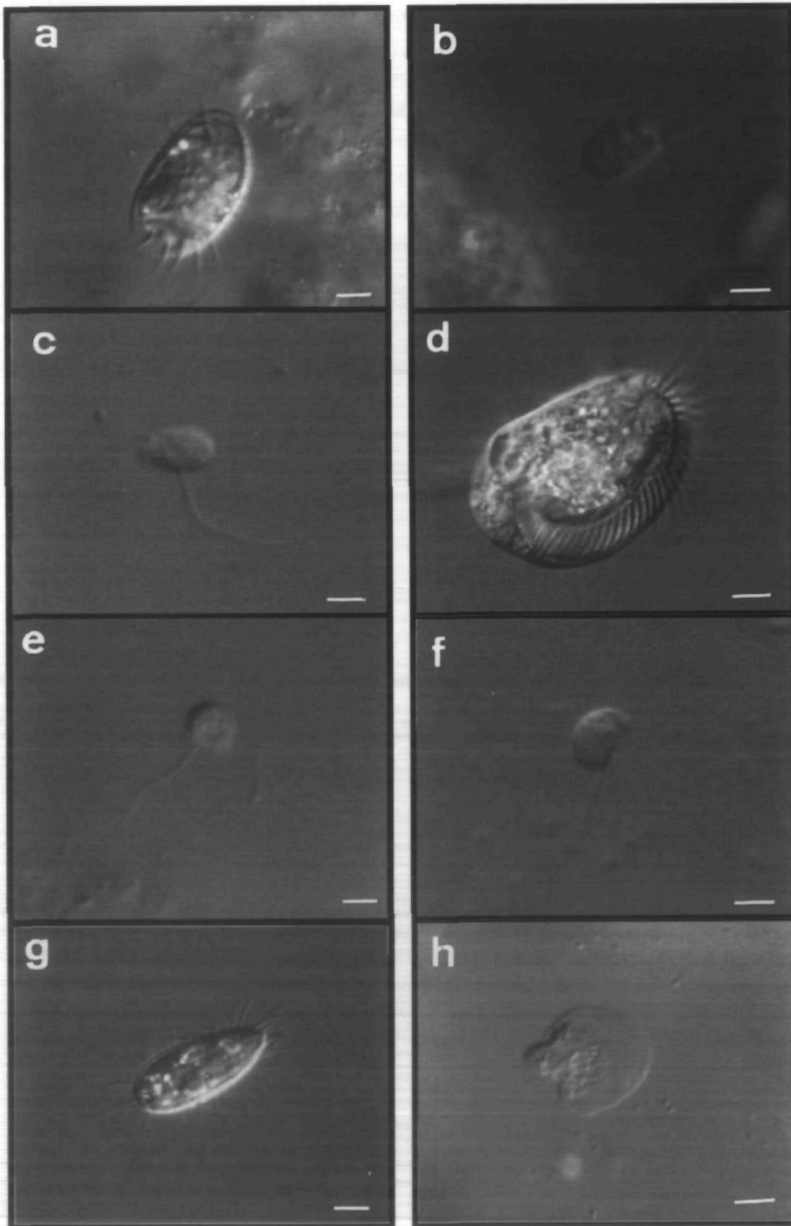
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Fig. 3. The most representative protists associated with marine snow: (**a**) *Aspidisca steini*; (**b**) *Bicosoeca* sp.; (**c**) *Bodo designis*; (**d**) *Euplotes vannus*; (**e**) *Pseudobodo tremulans*; (**f**) *Rhynchomonas nasuta*; (**g**) *Uronema marinum*; (**h**)*Vannella* sp. Scale bars (b,c,e,f) 5 µm, (a, d, g, h) 15 µm.

Pseudobodo tremulans Griessmann, 1913 (Figure 3e) was a spherical bicosoecid flagellate, ~10 µm long. It presented two unequal antero-laterally inserted flagella. The shorter contractile flagellum was posteriorly directed with the tip

Table II. Enrichment factors of flagellate and ciliate protists in the two ecosystem situations studied

	Time (days)						
	4	5	6	7	8	9	10
Flagellates							
Cold situation	7130	3560	35 000	3130	990	630	190
Warm situation	670	12 500	7560	7610	10 200	2540	340
Ciliates							
Cold situation	–	54 800	47 600	–	1600	670	150
Warm situation	2970	–	330	570	480	160	80

attached to the substrate and in swimming cells it was trailing. The long flagellum was oriented forwards in free-swimming cells and beat as a whip in sessile cells.

Rhynchomonas nasuta (Stokes, 1888) Klebs, 1893 (Figure 3f) was an ellipsoidal bodonid flagellate <10 μm long. It was distinguished by the presence of a distinctive anterior proboscis. Only one posteriorly oriented flagellum was evident, which maintained contact with the surface. This protist was also a poor swimmer, moving forwards very slowly with a jerky motion of the proboscis.

Uronema marinum Dujardin, 1841 (Figure 3g) was an ovoid, small scuticociliate, 30 μm long and 10 μm wide. It had holotric ciliature, except at the unciliated anterior edge, and a single longer caudal cilium. A small, not prominent, oral membrane was located at the middle of the cell body. It moved rapidly in all directions.

Vannella sp. Bovee, 1965 (not isolated) (Figure 3h) was a flattened, fan-shaped amoeba with a broad, distinctive hyaline cap forming the anterior of the cell. The size was variable between 20 and 50 μm. It did not show pseudopodia except in the floating forms. It moved its body over the substrate as a single mass.

Protistan enumeration

The enrichment factors of flagellate and ciliate protists at the two ecosystem situations are shown in Table II. Protists were always much more abundant on the aggregates than in the surrounding water (EF > 1). Enrichment factors were much higher at the beginning of the experiments than at the end, except for flagellates in the warm situation, in which the values were less variable over time.

Discussion

Marine snow is a potentially important component of the marine environment, whose study involves many difficulties because macroaggregates are usually extremely fragile and easily disrupted by sampling methods. Therefore, several attempts have been made to make marine snow in the laboratory (Kranck and Milligan, 1980; Biddanda, 1985; Davoll and Silver, 1986). In these model systems, homogeneous substrates were used to produce marine snow, e.g. abandoned larvacean houses (Davoll and Silver, 1986), phytoplankton (Biddanda and Pomeroy, 1988) or mucus from ctenophores (Caron *et al.*, 1986), but it is questionable whether they represent a general model of marine snow (Shanks and Edmonson, 1989).

In this work, we chose Shanks and Edmonson's biological model as the best lab system to make organic macroaggregates which resemble natural marine snow. Because these aggregates were formed from unfiltered seawater, they were composed of a mixture of different substrates, such as diatoms, diatom frustules, faecal pellets, remains of dead organisms, etc.

Microbial succession on the marine snow formed in this lab system was characterized by rapid bacterial growth followed by the appearance of nanoflagellates, ciliates and sarcodines. This pattern of colonization has previously been described by other authors (Pomeroy and Deibel, 1980; Pomeroy *et al.*, 1984; Davoll and Silver, 1986; Biddanda and Pomeroy, 1988; Caron, 1991).

Heterotrophic nanoflagellates were always the first protists to appear on macroaggregates. In the studies performed with mucus from ctenophores and appendicularian houses by Caron *et al.* (1986), initial colonization by flagellate protists on these particulate materials was also noted. This pioneering role may be due to the fact that flagellates occur at higher abundances than other protists in the seawater, as a consequence of their ability to grow and reproduce at the low food abundances found there (Caron, 1991). Ciliates appeared later in time, probably because they need high abundances of bacterial or flagellate prey. Therefore, they may play a pivotal role in structuring the detrital communities, because, as previously suggested by other authors (Silver *et al.*, 1984), ciliates can affect overall decomposition rates not only by consuming the abundant smaller protists, but also by grazing bacteria. Few studies are available on sarcodines associated with marine snow, mainly due to methodological and taxonomic problems. However, it is known that high concentrations of sarcodines, mainly naked amoebae, can occur on aggregates (Arndt, 1993).

Within marine snow, most protists we observed were specifically adapted to inhabit surfaces. These were the heterotrophic nanoflagellates *B.designis* and *R.nasuta*, the amoeba *Vannella* sp., and the ciliates *E.vannus* and *A.steini*. All of them grazed attached bacteria, but with different feeding mechanisms. Thus, *B.designis* and *R.nasuta* maintained contact with the substrate by means of their trailing flagellum and, for feeding, they swept the substrate with their anterior flagellum and proboscis, respectively. In 1987, Caron showed that *Bodo* sp. and *R.nasuta* moved and fed effectively when in contact with a surface, but were poor swimmers and had a low efficiency for feeding on suspended bacteria. On the other hand, *Vannella* sp. had the ability to penetrate into very small spaces of our laboratory-made marine snow whilst in a feeding sedentary form without pseudopodia, although it also appeared at last in a non-feeding floating form with pseudopodia. Similar behaviour has been observed in the benthic flagellate *Massisteria marina* which assumes a sedentary or a motile form depending on the environmental conditions (Patterson and Fenchel, 1990). Finally, the ciliates *E.vannus* and *A.steini* moved easily inside and on the macroaggregate surface using their cirri and created water currents with their AZM, removing attached bacteria. They filtered the suspended bacterial prey from these current waters.

The flagellates *P.tremulans* and *Bicosoeca* sp. and the ciliate *U.marinum* benefited from higher concentrations of suspended bacteria that developed near the marine snow. *Pseudobodo tremulans* was observed in the surrounding water, but

with transient attachments to the macroaggregate surface. It fed on suspended prey by directing its anterior flagellum into the water around it, using its posterior flagellum for attachment to the surface. *Bicosoeca* sp. was permanently attached to the particle surface by means of its lorica, feeding on suspended prey using its long anterior flagellum. *Uronema marinum* was always swimming in the surrounding water and showed a filter-feeding mechanism. As it is an opportunistic ciliate adapted to exploit transitory patches of high bacterial prey abundance (Fenchel, 1980; Sieburth, 1984), these enriched environments could be ideal sites for its growth and feeding.

According to Davoll and Silver (1986), macroaggregates are environments in the pelagic zone that support populations with combined features to succeed on both the pelagic and benthic systems. However, most of the species observed in this study were more common to benthic habitats than to pelagic ones. The planktonic existence of species of *Bodo*, *Rhynchomonas* and *Bicosoeca* undoubtedly depends on the existence of surfaces in the plankton (Caron, 1991), and *Pseudobodo* also needs attachment for feeding. The existence of a floating and a feeding form in *Vannella* appears to be an adaptation to exploit widely spaced, enriched detrital macroaggregates (Patterson and Fenchel, 1990).

Two of the three genera of ciliate protists observed to be associated with laboratory-made marine snow, *Euplotes* and *Uronema*, have also been found in detrital aggregates (Caron *et al.*, 1982; Davoll and Silver, 1986), and they have very different survival and feeding strategies. *Euplotes* is a poor swimmer more typical of a benthic environment, while *Uronema* moves rapidly and is more common in pelagic systems. *Aspidisca* is a benthic protist, although it has also been observed in the water column, but always associated with surfaces (Lee *et al.*, 1985). This agrees with other studies (Caron *et al.*, 1982; Caron, 1991) which indicated that the macroaggregates host bacterivorous protists atypical of the planktonic environment, even in the case of the ciliate *Ovolimbus caronii* characteristic of the marine snow habitat (Turley, 1991).

In our case, we should take into account that the use of experimental enclosures and the consequent presence of a solid surface could have biased the protistan specific composition from a pelagic to a benthic one. However, we consider that the existence of macroaggregates as a relevant particulate phase in our microcosm should minimize this effect.

The importance of marine snow versus surrounding water as a factor that enhanced protistan growth has been reflected in the literature (Caron *et al.*, 1986; Alldredge and Silver, 1988; Turley and Mackie, 1994). It is difficult to quantify precisely the degree of concentration of protists inhabiting marine snow because macroaggregates typically are highly convoluted and contain certain amounts of water that cannot be excluded during collection (Alldredge and Cox, 1982; Karner and Herndl, 1992). We have observed a higher protistan abundance on laboratory-made macroaggregates with respect to the seawater: during our experiments, for flagellates the EF ranged from 200 to 35 000, while for ciliates they ranged from 80 to 55 000. The highest values corresponded to the experiment performed with colder seawater (in February), which suggests that the harder the trophic conditions are, the greater the importance of marine snow as

a site for protists becomes. A similar trend was observed by Caron (1991) on a larger scale, when studying a gradient from eutrophic to oligotrophic pelagic environments.

Moreover, the enrichment factors tended to become lower as the marine snow was getting older, due to the increase in protistan density in the surrounding water. This situation may reflect a release of organic nutrients from marine snow to surrounding water, and a subsequent increase in free-living bacterial abundance, as Herndl reported in 1988. Therefore, marine snow would enhance microbial growth not only in the particles, but also in the surrounding water.

As can be deduced from all these results, our artificial macroaggregates appear as stressed microhabitats enriched in nutrients, bacteria and, consequently, bacterivorous protists, both planktonic and benthic. The relevance of this view deals with two aspects. First, the significant disturbance of the functioning of the microbial loop in a situation of abundance of particulate material in seawater. Second, the relevance of these very high numbers of protists as a mechanism to transfer carbon and energy from microorganisms to higher trophic levels.

Although protistan predation upon free-living bacteria is widely documented (McManus and Fuhrman, 1986; Sherr and Sherr, 1987; González *et al.*, 1990; Barcina *et al.*, 1991), there are very few studies on grazing by protists on attached bacteria. In this work, the first step to obtain information about the role of protists inhabiting laboratory-made marine snow has been accomplished, by identifying and isolating the most representative protists associated with macroaggregates. These will be used in future investigations focused on protistan grazing on bacteria in marine snow.

Acknowledgements

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