

# Laboratory-made particles as a useful approach to analyse microbial processes in marine macroaggregates

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## Abstract

Two kinds of particles were created in the laboratory in rolling tanks to simulate natural macroaggregates. Laboratory-made particles type 1 were formed under sterile conditions from a mixture of cells and extracellular products of four diatom species in artificial seawater, and inoculated with a marine microbial assemblage. Laboratory-made particles type 2 were created directly from natural seawater. These particles were characterised in terms of maximum length, volume and abundance. Chemical composition (carbohydrates, amino acids and total organic carbon) and bacterial and flagellate abundances were measured in the particles and ambient water. We found that both kinds of laboratory-made particles were similar in terms of size, chemical composition and microbial abundance. Moreover, they resembled natural marine aggregates in size and volume. However, laboratory-made particles showed higher concentrations of carbohydrates, amino acids and total organic carbon as well as higher microbial abundance when compared to natural macroaggregates. This difference can be explained by the sampling method, since natural aggregates are frequently collected in the sea with syringes including ambient water, and consequently diluted, whereas in this study the laboratory-made particles were carefully collected without ambient water. Thus, both kinds of laboratory-made particles might be a good alternative for the analysis of microbial processes in marine macroaggregates. Advantages and disadvantages of these two types of laboratory-made particles are discussed. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

*Keywords:* Macroaggregate; Laboratory-made particle; Bacterium; Flagellate; Sea

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## 1. Introduction

Macroscopic amorphous aggregates (>0.5 mm), usually called ‘marine snow’, are ubiquitous in ma-

rine environments [1,2]. They are mainly of phytoplankton origin and may develop from two different processes: (i) senescent diatom cells become sticky, aggregate and eventually sink rapidly and (ii) coagulation of colloidal organic matter released by phytoplankton forms transparent exopolysaccharide particles (TEP) in the micrometre size range, which

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aggregate under favourable turbulent conditions [3]. These marine aggregates are enriched in both nutrients and microorganisms (phytoplankton, cyanobacteria, bacteria and protists) [4,5] and play an important role in the organic matter flux from the surface of the ocean to the sea bed [6,7].

The existence of marine snow was not proved until direct underwater observations were performed, because marine snow is very fragile and is normally broken up by routine methods of collection [4]. In sampling devices large particles may be lost because they tend to settle in the non-turbulent water inside the water bottle and sink below the spigot [8], whereas particles that are still suspended may break apart when they pass through the spigot [9]. Particles obtained by pumps are often fragmented in passage through the hoses and pump [10]. Recently, the particles were being collected by scuba divers, but this technique is quite time-consuming if large amounts of aggregates are required. Moreover, transport and storage of water samples containing the particles also result in disaggregation of aggregates [4].

Because of these constraints, the development of an experimental design to create aggregates in the laboratory under controlled conditions could be very helpful. This way, it would be possible to analyse not only the formation of the aggregates, but also the complex interactions between particulate matter and microbial communities. There have been some previous attempts to form macroaggregates in the laboratory. Krank and Milligan [11] made macroscopic aggregates from sediment (a deposit of glacial marine clay) and organic matter derived from fish and plankton ground in a blender. The aggregates formed in Biddanda's experiments [12] were made by growing bacteria on dissolved organic matter from ground seaweeds. Shanks and Edmonson [13] made aggregates from unfiltered seawater in rolling tanks. We analyse two kinds of laboratory-made particles, laboratory-made particles type 1 (LMP-1) from cells and extracellular products of four species of diatoms, and laboratory-made particles type 2 (LMP-2) from unfiltered seawater following the experimental design of Shanks and Edmonson [13]. The physical, chemical and microbiological characteristics of these artificial particles were compared with those of natural macroaggregates described in the literature.

## 2. Materials and methods

### 2.1. Laboratory-made particles type 1 (LMP-1) formation

Seven experiments were carried out in 1994 and 1995 to create phytoplankton-derived model particles. A mixture of four species of diatoms (*Skeletonema costatum*, CCAP 1077/1B, *Navicula hansenii*, CCAP 1050/8, *Chaetoceros muelleri*, CCAP 1010/3 and *Nitzschia epithemoides*, CCAP 1052/18) commonly found in marine aggregates [14,15] and in the Bay of Biscay [16] was used to obtain phytoplankton-derived model particles. Batch cultures were grown on Guillard's medium for diatoms (f/2+Si) [17] at 15°C on a lab shaker (100 rpm), with aeration and light (125  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 16 h light-8 h dark). Growth was characterised in terms of algal abundance (samples were filtered on 0.2  $\mu\text{m}$  pore-size polycarbonate black filters and analysed under epifluorescence microscopy at a magnification of  $\times 1250$ ) and dissolved organic carbon (DOC) (TOC analyser Shimadzu TOC-5000/Shimadzu ASI-5000). The highest algal abundances and DOC concentrations were observed at 25–30 days after inoculation, when the cultures were harvested. Cultures were centrifuged at 2100 $\times g$  for 20 min in order to separate the particulate material (algal cells) from the dissolved material (extracellular products). The pellets were resuspended in sterile artificial seawater and the supernatant (extracellular products) was filtered onto 0.2  $\mu\text{m}$  pore-size cellulose acetate filters in order to eliminate any particulate material potentially present in the supernatant. Both fractions were autoclaved (115°C, 20 min) and algal abundance and DOC concentration measured in the resuspended pellets and the supernatant, respectively. Algal cells and extracellular products were stored at  $-20^\circ\text{C}$  until used for experiments.

Five-litre bottles were filled with sterile artificial seawater (Sigma) and a mixture of algal cells and extracellular products of the four diatom species. The cells and extracellular products were mixed so that the contribution of each species to the final algal abundance and DOC concentration in the mixture was the same. The optimum conditions to obtain a high number of uniform particles were  $10^9$  algae  $\text{l}^{-1}$ , 5 mg DOC  $\text{l}^{-1}$  and a rotation velocity of 2.5 rpm.

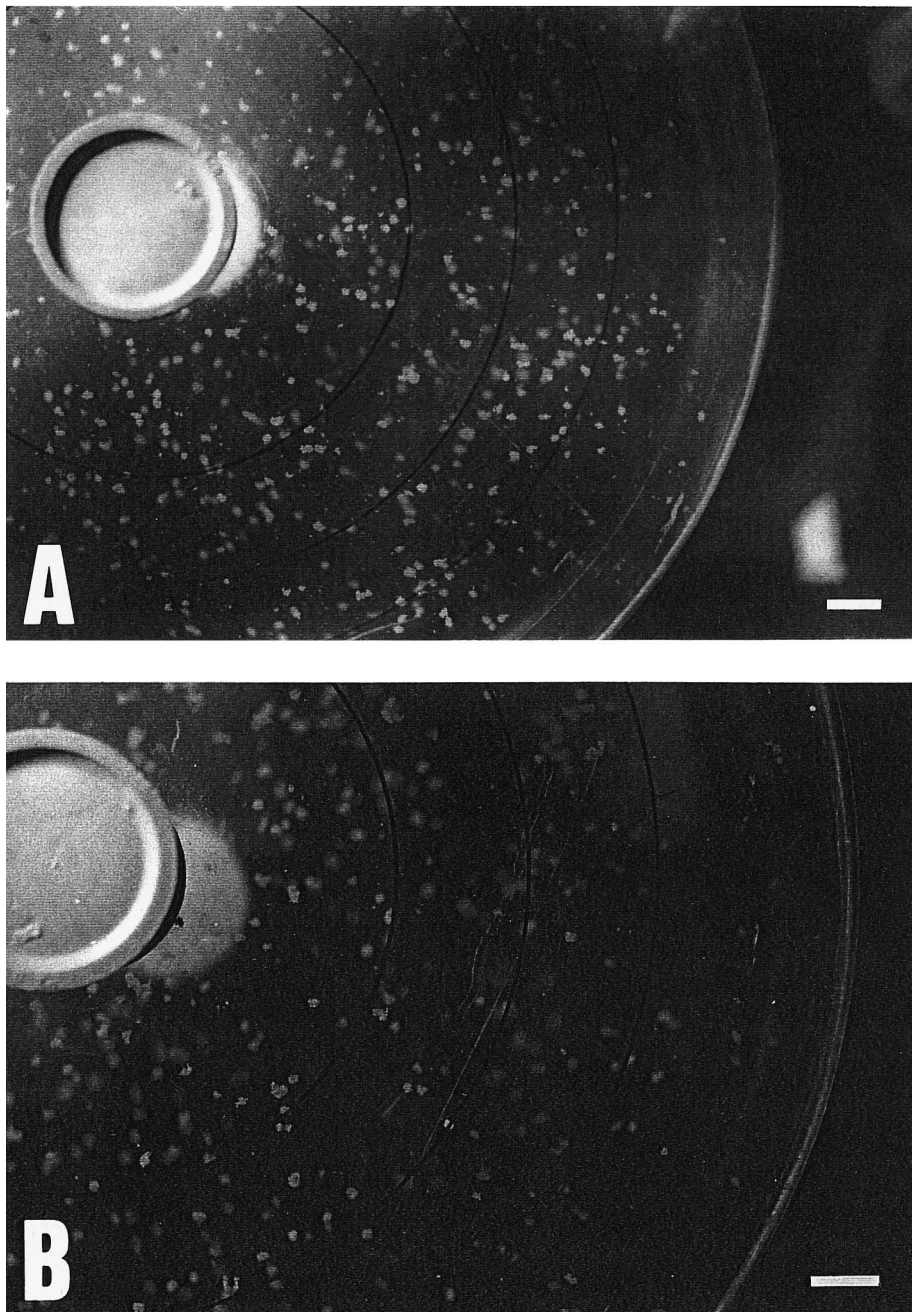


Fig. 1. Photographs of (A) laboratory-made particles type 1 (LMP-1) and (B) laboratory-made particles type 2 (LMP-2) formed in rolling tanks in the laboratory (bars represent 1 cm).

The bottles were autoclaved and placed on the roller table. After 2–3 days, the roller table was stopped,

the particles allowed to sediment and the supernatant was removed. Then, the sterile particles were

carefully transferred from the bottles to polypropylene cylindrical tanks containing 13 l of sterile artificial seawater using a glass pipette with a plastic tube 0.5 cm in diameter and 15 cm long at one end. The final DOC concentration was adjusted at the beginning of the experiments to the levels observed in waters of the Bay of Biscay (1.5–2 mg C l<sup>-1</sup>) with algal extracellular products. Afterwards, the tanks rolled (2.5 rpm) for 12 h to allow the flux to stabilise and the particles to become uniform in size (Fig. 1A).

After the formation of the phytoplankton-derived model particles, they were inoculated with a microbial assemblage obtained by tangential-flow filtration (100 000 Da, nominal molecular mass limit, Millipore) from the sampling location. Bacterial abundance at the beginning of the experiments was adjusted to that found in natural seawater (10<sup>9</sup> bact l<sup>-1</sup>). Microcosms were incubated in the dark at room temperature while rolling at 2.5 rpm.

## 2.2. Laboratory-made particles type 2 (LMP-2) formation

Particles were formed from natural seawater collected from coastal waters of the Bay of Biscay, 43°24.5' N, 3°2.7' W (North Spain) at 3 m in depth. Seven experiments were carried out in 1996 and 1997. Samples were taken using a Niskin bottle (PWS Hydro-Bios) and processed in the laboratory within 2 h after collection.

Aggregates (Fig. 1B) were formed according to the experimental design proposed by Shanks and Edmonson [13]. Thirteen-litre polypropylene cylindrical tanks were filled with 12 l of seawater from the sampling location and placed on the roller table with a rotation velocity of 2.5 rpm in the dark at room temperature.

## 2.3. Analysis of laboratory-made particles and ambient water

Seven decomposition experiments were carried out, and samples of particles and ambient water were taken every 24 h over 300 h, long enough to characterise the particles and the ambient water as was determined in previous experiments [18,19]. The particle abundance was determined from photo-

graphs of the tanks which were digitised (Omnimedia Scanner XRS). The volume of the particles was calculated by measuring their length and width and assuming that the particles were ellipsoid in shape. Particles were carefully transferred from the tanks to Petri dishes by pipette with a plastic tube at the end. Precise volumes of particles without ambient water were taken from the Petri dishes with a digital micropipette, placing the tip directly on the particles.

The chemical composition of the particles (70 µl in 100 ml of deionised water) and ambient water (100 ml) was measured. The concentration of total organic carbon (TOC) was determined with a TOC analyser Shimadzu TOC-5000/Shimadzu ASI-5000. Total amino acid and carbohydrate concentrations were determined according to [20]. Regarding amino acid concentrations, this technique actually measures the concentration of primary amines and therefore the amino acid concentrations are overestimated since they include ammonium. Carbohydrate concentrations were measured by the MBTH method.

Bacterial abundances were determined in three replicates of particles (7 µl in 1 ml of sterile artificial seawater) and ambient water (10 ml) fixed with formalin (2% v/v final concentration). The samples of particles were sonicated (100 W, 6 pulses of 5 s) immediately before the staining to count the attached bacteria. Bacterial abundance was measured by acridine orange epifluorescence direct counting (AODC) [21]. Samples 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. The filters were examined under a Nikon epifluorescence microscope with blue light at a magnification of ×1250. Bacteria present in at least 30 randomly selected fields, with 20–30 bacteria per field, were counted.

In some experiments flagellate abundance was also measured. Particles were colonised by flagellates after 3 days (LMP-1) or 4 days (LMP-2). Diamidinophenylindole (DAPI)-stained preparations for epifluorescence microscopy [22] were used for flagellate enumeration. Samples preserved in alkaline Lugol (0.5% v/v final concentration)-formalin (3% v/v final concentration) were stained with DAPI (0.2 µg ml<sup>-1</sup> final concentration) for 7 min and filtered onto 0.8 µm pore-size polycarbonate filters. The filters were examined through a Nikon epifluorescence micros-

cope with UV light at a magnification of  $\times 1250$ . At least 60 flagellates were counted from each sample.

Once the particles and the ambient water were characterised, the enrichment factors (EF) in the particles in relation to the ambient water were calculated by dividing the number or concentration observed in 1 ml of the particles by the corresponding value of the ambient water.

### 3. Results and discussion

We have used two experimental systems to obtain laboratory-made particles and try to simulate both the physical and chemical conditions leading to the formation of macroaggregates in the sea. LMP-1 were formed from diatom cultures resembling the situation of a phytoplankton bloom in the sea: namely, high abundance of particles [23], high concentration of DOC [24] and specific laminar flow conditions [25]. Diatom cells acted as aggregating nuclei and the extracellular products as agglutinative material. The number and size of particles formed following this experimental design depend on the

amount of particulate and dissolved material added to the tanks as well as the rotation speed. This approach allows the experimental conditions to be controlled, and therefore microcosms can be reproduced with the same number, size and quality of particles. The LMP-1 showed a maximum length of 0.9–10.2 mm, with a mean value of  $2.2 \pm 0.02$  mm (mean  $\pm$  standard error). Their volume ranged from 0.1 to  $25.0 \mu\text{l part}^{-1}$  with a mean value of  $3.0 \pm 0.06 \mu\text{l part}^{-1}$ . The abundance of these particles ranged from 50 to  $300 \text{ part l}^{-1}$ , with a mean value of  $185 \pm 15 \text{ part l}^{-1}$  and they represented between 0.01% and 0.13% of the total volume of the system (Table 1).

The strength of the LMP-1 noticeably increased when they were colonised by a natural bacterial community. During the first 12 h after inoculation the LMP-1 were extremely fragile making their collection difficult. After this period, model particles became more and more robust probably due to the colonisation of bacteria and the subsequent exopolymer synthesis. This observation agrees with those reported by other authors [35,36] who detected that bacteria decisively contributed to the aggregation

Table 1  
Volume, abundance and contribution of aggregates to the total volume of the system in this study and in marine systems

Aggregate volume (ml)	Aggregate abundance (agg $\text{l}^{-1}$ )	% of aggregates <sup>a</sup>	Reference
0.0001–0.0250 (0.0030) (75)	50–300 (185) (75)	0.01–0.13 (0.073) (75)	This study <sup>b</sup> LMP-1
0.0001–0.0830 (0.0013) (37)	na	na	This study <sup>b</sup> LMP-2
0.01–0.29	na	0.006–0.33	[26] <sup>c</sup>
0.003–2.24	1.9–28	0.01–0.67	[27] <sup>c</sup>
0.1–1.0	0.1–1.1	0.01–0.15	[28] <sup>c</sup>
0.034	79.4	0.27	[29]
1.0	1	0.1	[29] <sup>d</sup>
0.025–0.15	9.4–35	< 0.1	[30] <sup>c</sup>
0.002–0.29	2.8–5.6	0.001–0.1	[14] <sup>c</sup>
0.0012–0.019	na	na	[31] <sup>c</sup>
0.0001–0.0002	291–489	0.02–0.03	[32] <sup>c</sup>
0.0001–6	na	na	[33]
na	0.2–1.65	na	[34]
na	425–5300	na	[24] <sup>e</sup>

<sup>a</sup>Percentage of aggregates with respect to the total volume of the system.

<sup>b</sup>Variation range (mean value) (number of samples).

<sup>c</sup>See review of Alldredge and Silver [4].

<sup>d</sup>Data after an upwelling event.

<sup>e</sup>Data from an upwelling event.

na: data not available.

process by synthesising extracellular polymers which strengthen the links between the particles constituting the aggregate. However, the presence of active bacteria is not essential for aggregate formation as indicated also by other authors [35,37], because in this study the LMP-1 were formed under sterile conditions.

The second type of laboratory-made particles (LMP-2) were formed directly from unaltered seawater, and therefore their number and size varied considerably between experiments depending on the concentration and quality of the particulate and dissolved material in the seawater. The advantage of this approach is that particles formed in the rolling tanks are made of autochthonous material. Its disadvantage is the variability of the experimental conditions, since the particles and the ambient water, as well as the microbial community, change between experiments. In some experiments the number of particles obtained per tank was very low. In these experiments the particles from several tanks were carefully collected using a glass pipette with a plastic tube 0.5 cm in diameter and 15 cm long at one end, and added to one tank to obtain from 50–300 part  $l^{-1}$  at the start of the experiments. However, in some experiments the particles could not be analysed all over the 300 h, because their number was too low, or they were extremely fragile to be collected. The size of the LMP-2 was similar to that of LMP-1. Thus, the LMP-2 showed a maximum length of 0.2–8.7 mm, with a mean value of  $1.4 \pm 0.01$  mm. Their volume ranged from 0.1 to 83.0  $\mu l$  part $^{-1}$  with a mean value of  $1.3 \pm 0.23$   $\mu l$  part $^{-1}$  (Table 1).

The comparison of the physical characteristics of the laboratory-made particles with natural macroaggregates reported in other studies is hampered by the fact that only a few studies provide complete information on the size, volume and abundance [38]. Moreover, the sampling devices used for collecting aggregates frequently destroy their fragile structure and therefore the size, volume and abundance of the aggregates could be underestimated. The majority of the available data about marine organic aggregates come from studies where the samples were collected by scuba divers and aggregate abundance was estimated by in situ direct observation. These sampling systems are limited to surface waters, and subjected to error about the size and number of aggregates present in the water column. Systems based on image analysis with underwater video cameras have been developed to measure the size of aggregates in situ [39], but these systems display a problem because the range of particle size is too wide to be correctly detected by a single system.

Maximum length of natural marine aggregates has been reported to range from 0.5 mm (minimum size to consider a particle to be a macroaggregate) to several metres in length, as occasionally detected in the Northern Adriatic Sea during the summer [5]. Disregarding these very large aggregates, the volume of natural aggregates generally ranged from a minimum of 0.1  $\mu l$  to 6 ml (Table 1). On the other hand, the number of aggregates in marine systems varies from 0 to maximum values of 5300 agg  $l^{-1}$ , as observed by Riebesell [24] in the German Bight, North Sea, during a diatom bloom. Due to these high var-

Table 2

Chemical composition (carbohydrates, amino acids and TOC) of the laboratory-made particles analysed in this study and natural macroaggregates

Carbohydrates ( $\mu M$ glucose)	EF carbohydrates	Amino acids ( $\mu M$ glycine)	EF amino acids	TOC ( $\mu M$ C)	EF TOC	Reference
1 217–14 610 (6 716) (66)	120–5 928 (1 414) (66)	1 196–12 390 (6 201) (33)	495–5 752 (1 872) (33)	22–195 (73) (47)	124–905 (384) (47)	This study <sup>a</sup> LMP-1
2 944–8 556 (6 417) (25)	812–3 819 (1 944) (25)	3 027–8 210 (5 124) (23)	823–3 224 (1 448) (23)	26–133 (63) (21)	101–537 (241) (21)	This study <sup>a</sup> LMP-2
1 619	352	254	87	na	na	[28]
1 395	304	na	na	21–318	na	[40]

EF: enrichment factor.

<sup>a</sup>Variation range (mean value) (number of samples).

na: data not available.

iations, the relative importance of the aggregates with regard to the volume in the water column is highly variable in natural marine systems and the volume of aggregates represents 0.001–0.67% of the water column (Table 1). Consequently, LMP-1 and LMP-2 were similar to natural marine aggregates in terms of size and volume. Moreover, the contribution of these particles to the total volume of the microcosms was also similar to the contribution of natural aggregates in marine systems.

The chemical composition, the concentration of TOC, amino acids and carbohydrates of the laboratory-made particles are shown in Table 2. There were no statistically significant differences ( $P > 0.05$ , Student's *t*-test) in these chemical variables between LMP-1 and LMP-2, although LMP-1 were produced from dead and autoclaved diatom cultures. Moreover, the enrichment factors of TOC, amino acid and carbohydrate concentrations were not statistically different ( $P > 0.05$ ) in LMP-1 and LMP-2. Both were enriched in carbohydrates and amino acids by a factor of  $10^3$  as compared to the ambient water, while the enrichment factor for TOC was  $10^2$ .

Microbial abundance was not significantly different between LMP-1 and LMP-2 ( $P > 0.05$ ) (Table 3). Maximum abundances of  $10^9$ – $10^{10}$  bacteria and  $10^6$

flagellates per ml of particle were observed. The enrichment factors of bacterial and flagellate abundances were not statistically different between LMP-1 and LMP-2. Particles were enriched in bacteria and flagellates by a factor of  $10^3$  and  $10^2$  respectively as compared to the ambient water.

Therefore, on the basis of the analysed variables and after the comparison between LMP-1 and LMP-2, we may conclude that there are no significant differences between the two kinds of laboratory-made particles in terms of chemical composition and microbial abundances in spite of the fact that LMP-1 were produced from diatom cultures in artificial seawater, while LMP-2 were produced from unaltered natural seawater.

However, when comparing the chemical composition and microbial abundances of the laboratory-made particles produced in this study with those of natural aggregates reported in the literature we found some differences. Despite the fact that the values found in the literature are highly variable, the bacterial and flagellate abundance as well as the chemical composition of our particles and the enrichment factors are higher than those of natural aggregates (Tables 2 and 3). The minimum values of TOC, amino acid and carbohydrate concentrations of the

Table 3  
Bacterial and flagellate abundances in the laboratory-made particles analysed in this study and in natural macroaggregates

Bacterial abundance ( $10^6$ bact ml <sup>-1</sup> )	EF bacterial abundance	Flagellate abundance ( $10^4$ flag ml <sup>-1</sup> )	EF flagellate abundance	Reference
150–9 000 (3 499) (75)	50–6 803 (1 381) (75)	5–160 (60) (24)	10–957 (155) (24)	This study <sup>a</sup> LMP-1
711–5 360 (1 658) (16)	141–3 081 (1 030) (16)	10–130 (57) (11)	80–811 (267) (11)	This study <sup>a</sup> LMP-2
1–33	na	0.02–0.6	na	[41]
748	1 247	0.37	123	[29] <sup>b</sup>
12	67	na	na	[29] <sup>c</sup>
1–464	9–560	na	na	[42]
151–352	83–298	14.9–34.1	107–987	[31]
1–1 390	2–5	na	na	[43]
1–110	2–133	na	na	[40]
1–3	2–4	na	na	[44]
190	100	na	na	[45]
30–2 540	100–5 700	130–3 300	1 300–33 000	[46]
na	3 200	1–100	3 200	[5]

EF: enrichment factor.

<sup>a</sup>Variation range (mean value) (number of samples).

<sup>b</sup>Data obtained during an upwelling event.

<sup>c</sup>Data obtained after an upwelling event.

na: data not available.

laboratory-made particles are comparable with the maximum values observed in natural aggregates.

There are only a few published papers where the chemical composition of natural aggregates have been analysed, and concentrations are generally given per aggregate and not per ml of aggregate. Depending on the origin of the aggregates, the chemical composition and abundance of microorganisms in aggregates may vary considerably. As also pointed out by Alldredge and Gotschalk [34] and Karner and Herndl [44], aggregates of phytoplankton origin are, compared to other types of aggregates, specially enriched in both nutrients and microbial communities. In addition, Prézelin and Alldredge [29] analysed aggregates formed by appendicularian houses of *Oikopleura dioica* colonised by diatoms during an upwelling event, and found bacterial abundance and enrichment factors of bacterial and flagellate abundance higher than those generally reported for natural aggregates, and similar to those found in this work. Therefore, the laboratory-made particles formed in this study might resemble natural macroaggregates formed during an upwelling event in terms of bacterial and flagellate abundances. Moreover, the concentration of TOC, amino acids and carbohydrates, as well as the microbial abundances determined in aggregates collected by scuba divers with syringes may be underestimated due to the inclusion of unknown amounts of ambient water [28,44,47]. In this study the samples of laboratory-made particles were collected without ambient water and consequently this difference in the sampling method can explain why the concentration of TOC, carbohydrates and amino acids as well as microbial abundances are higher in the laboratory-made particles than in the natural aggregates.

In summary, both kinds of laboratory-made particles appear to be suitable models to analyse microbial processes in marine macroaggregates, since their chemical and microbiological characteristics are close to those of natural aggregates. Since studying natural aggregates is difficult due to their seasonal occurrence, and considering the problems with sampling, these laboratory-made particles can be useful when the availability of natural aggregates is not sufficient to carry out extensive analysis of microbial processes. Both LMP-1 and LMP-2 present advantages and disadvantages, and their utility will depend

on the objective of the study. Following the first experimental approach (LMP-1) the experimental conditions to create particles are controlled and therefore the experiments can be reproduced maintaining the same number and quality of particles and varying microbial communities. In the second experimental approach (LMP-2) particles are produced from the material present in the natural seawater and therefore they vary qualitatively and quantitatively from one sampling date to another. The advantage of the second approach is that LMP-2 are produced from autochthonous material and therefore they are a better model of natural aggregates than the particles produced from diatom cultures.

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