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Production of individual marine organic aggregates using Paramagnetic Microspheres: A new tool for examining microbial associations with aggregates

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Abstract

We describe a new method to produce marine aggregates from natural organic material based on the sticking properties of transparent exopolymeric particles. Seawater samples were prescreened and ultrafiltered to concentrate the 30 kDa to 10 µm size fraction. First, we produced small magnetizable aggregates by combining glass microfibers and paramagnetic 1-µm beads with the organic matter present in the concentrated solution. The second step involved clustering the small aggregates into a single macro-aggregate, using a small ring-shaped magnet as an aggregation nucleus. Viral and bacterial densities, determined after dissolution of the newly formed aggregates with methanol, averaged $13.8 \times 10^6 \pm 3.6 \times 10^6$ vir. agg.$^{-1}$ and $4.1 \times 10^6 \pm 1.1 \times 10^6$ bact. agg.$^{-1}$. Bacterial respiration and production measurements of single aggregates averaged 8.47 ± 1.72 nmol O_2 agg.$^{-1}$ h^{-1} and 1.54 ± 0.45 ng C agg.$^{-1}$ h^{-1}, respectively. Particulate organic carbon and nitrogen content of the newly formed macro-aggregates averaged 31.92 ± 2.67 µg C agg.$^{-1}$ and 3.44 ± 0.43 µg N agg.$^{-1}$, respectively. This approach allows the concentration and isolation of the organic matter precursors that compose natural aggregates and provides a simple protocol for recombining those precursors into single newly formed macro-aggregates, which can then be easily manipulated for further investigation. This method is a new tool for investigations into the interactions between microorganisms and marine aggregates and their implications at the ecosystem level, but also into the interactions between aggregates and dissolved organic or inorganic substances.

Large organic aggregates, which are often referred to as marine snow aggregates, can be defined as porous polysaccharide matrices embedding all kinds of living and nonliving particles. They represent the main driving force for vertical fluxes in the sea (Fowler and Knauer 1986; Shanks and Trend 1980) and influence climate by maintaining a constant drawdown of organic carbon to the deep ocean. In addition to being the main carrier of major elements, such as carbon, to the seabed, these particles also play a key role in controlling the fate, transport, and availability of trace metals and organic contaminants in marine systems (Honeyman and Santschi 1992; Santschi et al. 1999).

Large aggregates act as microbial reactors that play a crucial role in the physico-chemical properties of the system at the microscale level. They provide a physical refuge for microorganisms (Alldredge and Cohen 1987; Smith et al. 1992; Blackburn and Fenchel 1999). Indeed, they are highly colonized by an active bacterial community (Simon et al. 2002) that differs in composition from the free living communities in marine (DeLong et al. 1993, 1994; Rath et al. 1998; Acinas et al. 1999; Phillips et al. 1999; Riemann et al. 2000; Knoll et al. 2001; Moeseneder et al. 2001; Grossart et al. 2005, 2006; Kellogg and Deming 2009) and freshwater environments (Weiss et al. 1996; Riemann and Winding 2001; Schweitzer et al. 2001).

The establishment of chemical gradients within aggregates and the complex interactions among attached microorganisms drive the development of specific microbial communities

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(Kiørboe et al. 2004; Long and Azam 2001; Weinbauer et al. 2009). The structure of these microhabitats affects the chemical properties of the system at the microscale level, which in turn, can modify processes relying upon the concentration of dissolved elements such as bacterial activity (Smith et al. 1992; Grossart and Simon 1993; Müller-Niklas et al. 1994; Grossart et al. 1998, 2007). Therefore, these hotspots of prokaryotic activity and of biogeochemical transformation have a structuring effect on bacterial communities by creating ecological niches. In addition, the activity of microorganisms inhabiting aggregates may affect not only microscopic level properties, but also water column processes such as exoenzyme distribution (Ziervogel et al. 2010).

Thus, a macro-aggregate can be considered a full ecosystem characterized by complex interactions between microorganisms and organic and inorganic matters. Understanding the role of such aggregates for the development and activity of microbial communities and for biogeochemical processes is, however, challenged by methodological aspects.

The methods used for studying the development of attached microbial communities on aggregates and related microbial processes are clustered in three general groups. The most direct methods rely on the field sampling of natural marine snow aggregates followed by microscopic observations or incubation in the laboratory (e.g., Alldredge et al. 1986). Despite the advantage of allowing the study of naturally occurring macro-aggregates, this approach has two major drawbacks. First, it requires very careful sampling by scuba diving to avoid disruption. Thus, it does not allow large scale studies involving large numbers of aggregates. Moreover, as they are randomly collected in situ, the composition and age of these aggregates can be very heterogeneous, and so can be the attached microbial dynamics and chemical characteristics. A second cluster of methods that allows studying the dynamics of the attached microbial communities relies on the use of model aggregates. This approach requires the production of artificial aggregates that mimic, as closely as possible, natural ones. For instance, some studies rely on the use of millimeter-sized model agar spheres, the composition and properties of which differ significantly from naturally occurring macro-aggregates, and then follow bacterial colonization of these spheres (e.g., Kiørboe et al. 2002). Whereas the use of such model aggregates allows production of large numbers of aggregate-like particles of known and controlled composition, and investigation of specific mechanisms of colonization, growth, and microbial interactions, they will deviate from natural aggregates due to the differences in organic matter composition. The last cluster brings together the methods that allow the formation of aggregates from natural material. For instance, aggregates can be produced in the laboratory from naturally occurring organic material using roller tanks (e.g., Shanks and Edmondson 1989) or Couette flocculators (e.g., Kiørboe and Hansen 1993, Drapeau et al. 1994). The main limitation of these latter methods is that they only allow the production of a limited number of aggregates, which are difficult to collect and too fragile to be manipulated without risking disruption.

Here, we present a new method that fits into this last cluster. The main advantage of this method is that it allows the production of large numbers of similar, robust, newly formed macro-aggregates from natural material that facilitates the handling of individual macro-aggregate for further investigation.

Materials and procedures

Concentration of marine organic aggregates components

Seawater samples were collected in a bay off Nouméa (Grande Rade, SW lagoon of New Caledonia) in November 2008 using a Teflon air pump at 1 m depth. After sampling, seawater was kept in 25-L polycarbonate carboys, during the transport to the laboratory (within 1 h). In the laboratory, seawater was prefiltered through a 10-µm nylon mesh using a 147 mm diameter filtration unit to remove large particles. The filtrate was then processed with a 30 kDa Biomax filter (Pellicon, Millipore). The ultrafiltrate was kept for rinsing procedures, and the concentrate containing the 30 kDa to 10 µm size fraction was collected into sterile 250-mL tissue culture flasks (concentration factor of 100) used to produce the aggregates. The experimental protocol for the preparation of the newly formed 'natural' aggregates is outlined in Fig. 1.

Formation of magnetizable aggregates

The 30 kDa to 10 µm size fraction concentrated by ultrafiltration contains naturally occurring living and nonliving particulate and colloidal organic material, such as exopolymeric substances, bacteria and viruses. The present method relies on the ability of a specific fraction of exopolymeric substances found in seawater, the transparent exopolymeric particles (TEP) to stick to other particles and form mixed aggregates. The principle of this methodological approach is, first, to form small magnetizable mixed aggregates of TEP, bacteria, viruses, and paramagnetic carboxylated microspheres (PCM) as described in Mari and Dam (2004), and second, to collect and cluster these magnetizable small aggregates into a single macro-aggregate using a magnetic nucleus (Fig. 2).

The superparamagnetic functionalized microspheres (Dynabeads® MyOne™ Carboxylic Acid; Dynal Biotech) of 1 µm diameter used to form magnetizable small aggregates are composed of highly cross-linked polystyrene with evenly distributed magnetic material (26% iron content) and high surface area (10 m^2 g^{-1}). The beads are coated with a hydrophilic layer of glycidyl ether, concealing the iron oxide material inside them, and carboxylic acid groups cover the surface of the beads. These beads are colloidally stable in the absence of a magnetic field and separation from solution is achieved when they are exposed to a magnetic field. The volume of PCM solution (V_i) to add to 250 mL concentrate was calculated to reach a final concentration (C_f) of 2.5×10^5 PCM mL^{-1} in the initial 25 L seawater, as $V_i = C_f V_f / C_i$; where C_i is the initial concen-

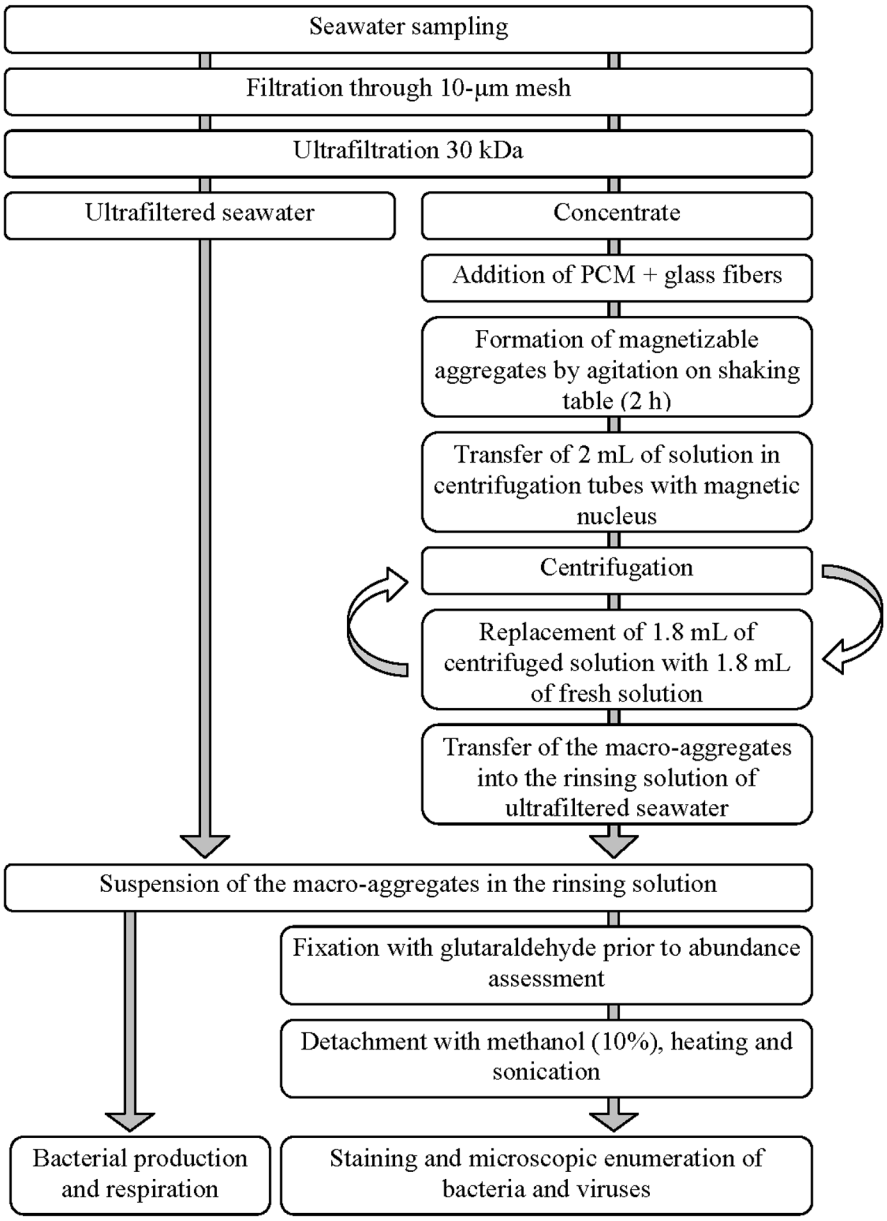

Fig. 1. Experimental setup for the production of 'natural' individual marine organic aggregates using Paramagnetic Carboxylated Microspheres (PCM) of 1-µm diameter.

tration of microspheres and V_f is the volume of seawater (25 L). The initial concentration, C_i, was calculated from: $C_i = 6W10^{12}/\rho\pi d^3$; where W is the mass of PCM per mL solution (10 mg mL^{-1} for 10% solid volume according to manufacturer), ρ is the density of PCM in grams per mL (1.8 g cm^{-3}), and d is the beads diameter in µm. As a result, we added 30 µL PCM solution in the 250 mL concentrate.

To stimulate aggregation, glass fibers were added in the 250 mL concentrate (final concentration = 5 mg L^{-1}). Addition of such fibers has been shown to promote the formation of marine snow aggregates (Passow and Wassmann 1994). A solution containing glass fibers was prepared by adding ripped up precombusted 25 mm GF/F Whatman glass fiber filters into 30 kDa ultrafiltered seawater. Complete dispersion of the fibers into seawater was achieved by sonicating the solution. The 250-mL tissue culture flask containing the 30 kDa-to-10 µm size fraction concentrated by ultrafiltration, the PCM, and the glass fibers was placed on a horizontal culture shaker and was agitated for 2 h at 90 rpm to form mixed aggregates. Visible mixed aggregates were formed (Fig. 2A).

Nucleus of aggregation

Specially designed permanent magnets N48 Parylene C coated (Ningbo Lihe Magnetic Industry) were used as aggregation nuclei. These magnets are ring shaped (3 mm external

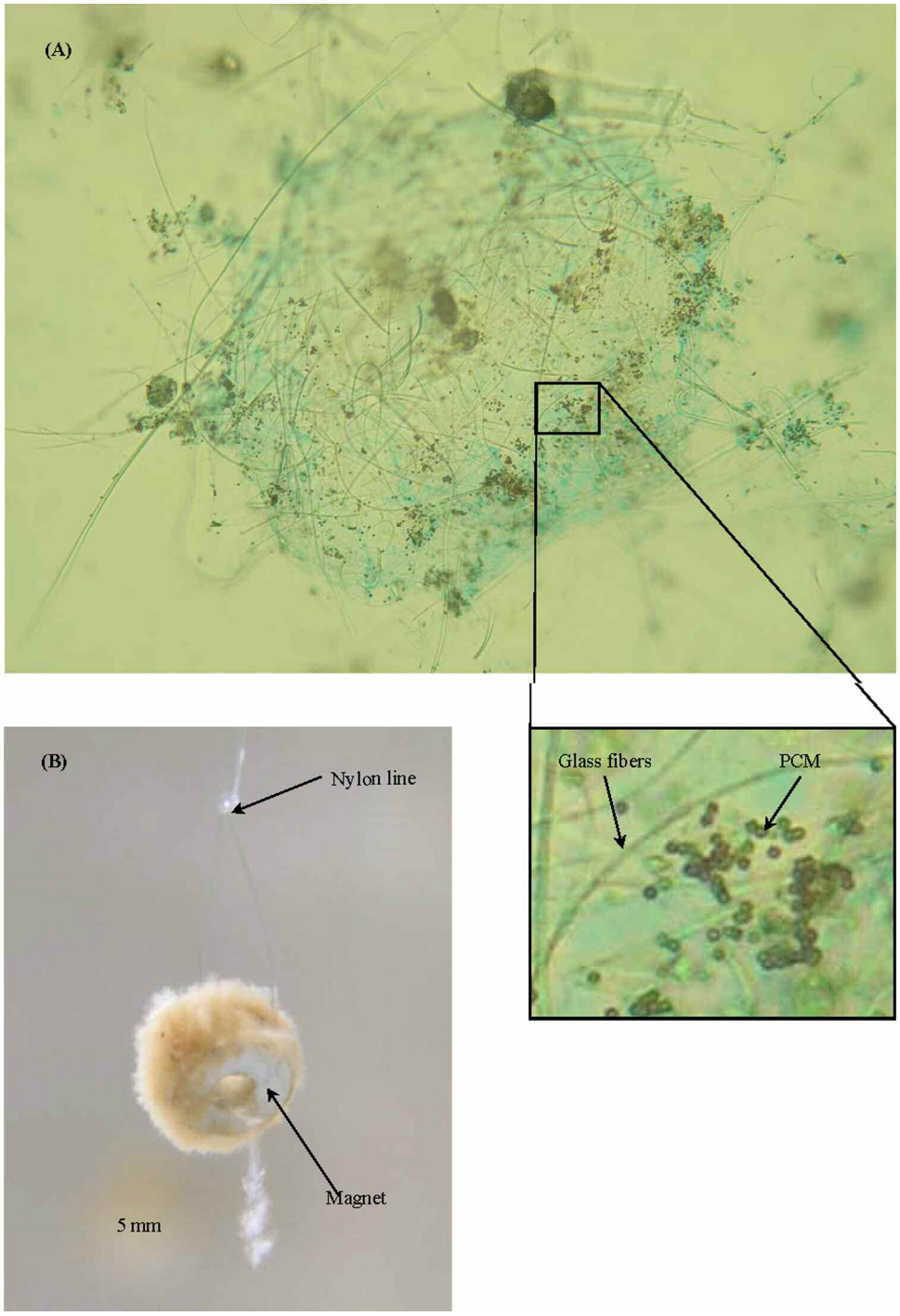

Fig. 2. Magnetizable mixed aggregates of TEP (stained with Alcian blue), microorganisms (not visible), glass fibers, and Paramagnetic Carboxylated Microspheres (PCM; black dots associated with TEP) before magnetic aggregation (A). The picture was taken under a compound light microscope at 250× magnification. Example of a newly formed macro-aggregate suspended in seawater (B). The ring-shaped magnetic nucleus is used to attract the magnetizable mixed aggregates.

diameter, 1 mm thick, and 1 mm internal diameter) and the central hole allows suspension of the final organic aggregates in seawater by means of fine fishing line connected to a small plastic hook (Fig. 2B). The Parylene C coating allows long-term resistance of the magnet to corrosive fluids such as seawater, electrolytes, proteins, enzymes, and lipids. It is an inert, hydrophobic, optically clear biocompatible polymer coating material often used in medical applications.

Formation of the macro-aggregates using the nucleus of aggregation

One magnet was used to form a given aggregate. Each magnet, as mounted on fishing lines as described above, was placed at the bottom of a 2-mL centrifugation tube, and the plastic hook attached to the other extremity of the magnet that was left outside the tube. Two milliliters of solution containing the magnetizable aggregates were sampled from 250-mL tissue culture flask and were transferred into 2-mL centrifugation tubes, enabling the magnetic-driven attachment of the aggregates to the magnet. To enhance the contact rate between the magnetic aggregation nucleus (i.e., the magnet) and the magnetizable aggregates, the tubes were centrifuged at 2000*g* for 10 min in a refrigerated (10°C) microcentrifuge Sigma 1-15K equipped with a swing-out 24-place rotor. After the first centrifugation, 1.8 mL solution was carefully removed from the 2-mL tubes, thereby leaving the nucleus of aggregation coated with magnetizable aggregates at the bottom of the tubes in 0.2 mL solution. To increase the amount of material coating around the nucleus of aggregation, this step was repeated several times by adding 1.8 mL "fresh" solution of magnetizable aggregates into the 2-mL tubes. Two sets of experiments were conducted based on the number of times that this step was repeated.

In the first set of experiments aimed at determining the optimum number of successive centrifugations (i.e., volume to process) required to form macro-aggregates around the aggregation nucleus, the step described above was repeated four times. These experiments showed that two successive centrifugations were optimum. Consequently, in the second set of experiments, which was aimed at characterizing the attached microbial community composition and activity, and POC and PON concentrations, the centrifugation step was repeated only twice.

Rinsing of the macro-aggregates

Prior to enumeration of aggregate-attached organisms and determination of bacterial production and respiration, the aggregates were rinsed for a minimum of 10 min as follows. Once the final centrifugation was completed, aggregates were transferred into a 15-L polycarbonate rinsing bath filled with ultra-filtered seawater. This transfer was performed without taking the aggregates outside seawater, by using a small ladle adapted to perfectly hold the centrifugation tube in a vertical position. The centrifugation tube was placed into the ladle, the lid of the tube was opened, and the tube was slowly immersed into the rinsing solution. Once into the rinsing solution, each aggregate was suspended by connecting the small plastic hook to a wire stretched horizontally above the solution (Fig. 2B). This procedure minimizes potential disruption of the aggregate and any subsequent material loss. Once the rinsing was completed, aggregates were collected individually using the ladle described above. New tubes were used to collect the rinsed aggregates. The thin fishing line was cut using nonmagnetizable ceramic scissors (magnets may attach to stainless steel scissors, which would damage the aggregates) leaving the individual magnet-associated aggregate at the bottom of each tube, ready for further analyses.

Viral and bacterial abundances within macro-aggregates

The number of bacteria and viruses attached to the newly formed macro-aggregates were determined after detachment, following the procedure described by Lunau et al. (2005). Each aggregate was fixed before detachment by adding glutaraldehyde into the solution (1% final concentration). After fixation, 1.8 mL solution was removed from each tube (the aggregate being left undisturbed in 0.2 mL at the bottom of the tube) and was replaced by 1.8 mL of a solution of ultrafiltered seawater and 10% methanol. The tubes filled with the macro-aggregates and the 10% methanol solution were placed into a heating sonicator and were sonicated at 40 kHz for 5 min at 35°C. This procedure allows solubilizing the polysaccharidic matrix and detaching the particles (i.e., bacteria and viruses). Once the sonication completed, the tubes were disposed inside a magnetic particle concentrator designed to hold 6 microcentrifuge tubes (Dynal MPC-S; Invitrogen Dynal) in order to separate PCM from the organisms left suspended in the solution.

In the first set of measurements, bacterial concentrations were determined after DAPI staining (0.1 µg mL^{-1}; 4′6′-diamidino-2-phenylindole) and filtration onto 0.2 µm black polycarbonate filters (Porter and Feig 1980; King and Parker 1988). In the second set of measurements, determination of viral and bacterial abundances were conducted on duplicate 500 µL samples immediately fixed with 0.02-µm filtered glutaraldehyde (final concentration 1% [vol/vol]) and analyzed the same day. Samples were filtered (<15-kPa vacuum) through 0.02-µm pore size Anodisc membrane filters (Whatman). The membranes of bacterial cells and viruses were stained with SYBR Gold (Molecular Probes Europe) for 15 min in the dark (Chen et al. 2001). Bacteria and viruses were counted under a Leitz Laborlux D epifluorescence microscope.

Carbon and nitrogen content of newly formed aggregates

Four aggregates were collected after rinsing and frozen (–80°C) until analyses. The concentrations of particulate organic carbon (POC) and particulate organic nitrogen (PON) associated with newly formed aggregates were determined following the method of Raimbault et al. (1999). Briefly, aggregates were individually placed in 50 mL Pyrex bottles digestion flasks containing 40 mL deionized water; inorganic carbon was eliminated by acidification (addition of 50 µL 5 N sulfuric acid) and bubbling. Immediately after removal of inorganic carbon, 5 mL oxidizing reagent, prepared with 250 mL deionized Milli-Q water, 30 g disodium tetraborate, and 15 g potassium peroxodisulfate, was dispensed into each sample. After addition of the oxidizing reagent, bottles were capped and autoclaved at 120°C for 30 min. After cooling at room temperature, the digestion mixture was analyzed for nitrate and carbon dioxide. Blanks were prepared to determine the contribution of the magnet and of the microspheres to the POC and PON contents

of the aggregates. As a result, for each aggregate, the magnet contributed to 5.82 ± 2.19 µg C and to 0.30 ± 0.30 µg N, and the microspheres to 0.08 ± 0.02 µg C and to 0.00 ± 0.00 µg N considering that each aggregate contains 0.5 µL of microspheres (assuming that the amount of microspheres added in the initial concentrated solution was divided evenly between each aggregate). The blanks were removed from measurements of POC and PON concentrations.

Bacterial production within macro-aggregates

Four aggregates were collected individually in 2-mL centrifugation tubes ('A') as described above. One mL water was collected from each tube and added to another centrifugation tube ('W') for determination of water column bacterial production (Fig. 3). The remaining water above the aggregates was then carefully removed to leave only 0.2 mL with each aggregate. Twenty microliters ^{3}H-Leu (2.08 µmol L^{-1} at 5.4 Ci $mmol^{-1}$, Perkin-Elmer) was then added to tubes 'A' (189 nmol L^{-1} final concentration). One of the replicate 'A' tubes received 400 µL of the stop solution (methanol 15%, glutaraldehyde 1.5% in 0.2 µm filtered seawater) before ^{3}H-Leu to serve as a zero-time blank. Corresponding 1-mL water samples ('W') received 100 µL of the same ^{3}H-Leu solution and one received 110 µL of 50% TCA before Leucine. After 60 min incubation at 25 ± 0.1°C (close to in situ temperature of 24.7 ± 0.4°C) in the dark, the aggregates received 400 µL of the stop solution. Incorporation in the water samples was stopped by adding 110 µL of 50% TCA. Samples were then stored at 4°C until further centrifugation. The microcentrifuge tubes containing the aggregates were then sonicated at 40 kHz for 5 min at 35°C. Preliminary tests on seawater samples showed that treatment with methanol and sonication did not change the label recovery from TCA precipitate.

The extraction procedure for aggregates was designed to separate the magnet from the TCA precipitate. Indeed, the magnet would likely scrap the pellet in the bottom of microcentrifuge tube during rinsing procedures. The 'A' tubes were centrifuged 30 s at 4000*g* to remove any drops remaining on the tube walls. After immobilizing the magnet midway up the

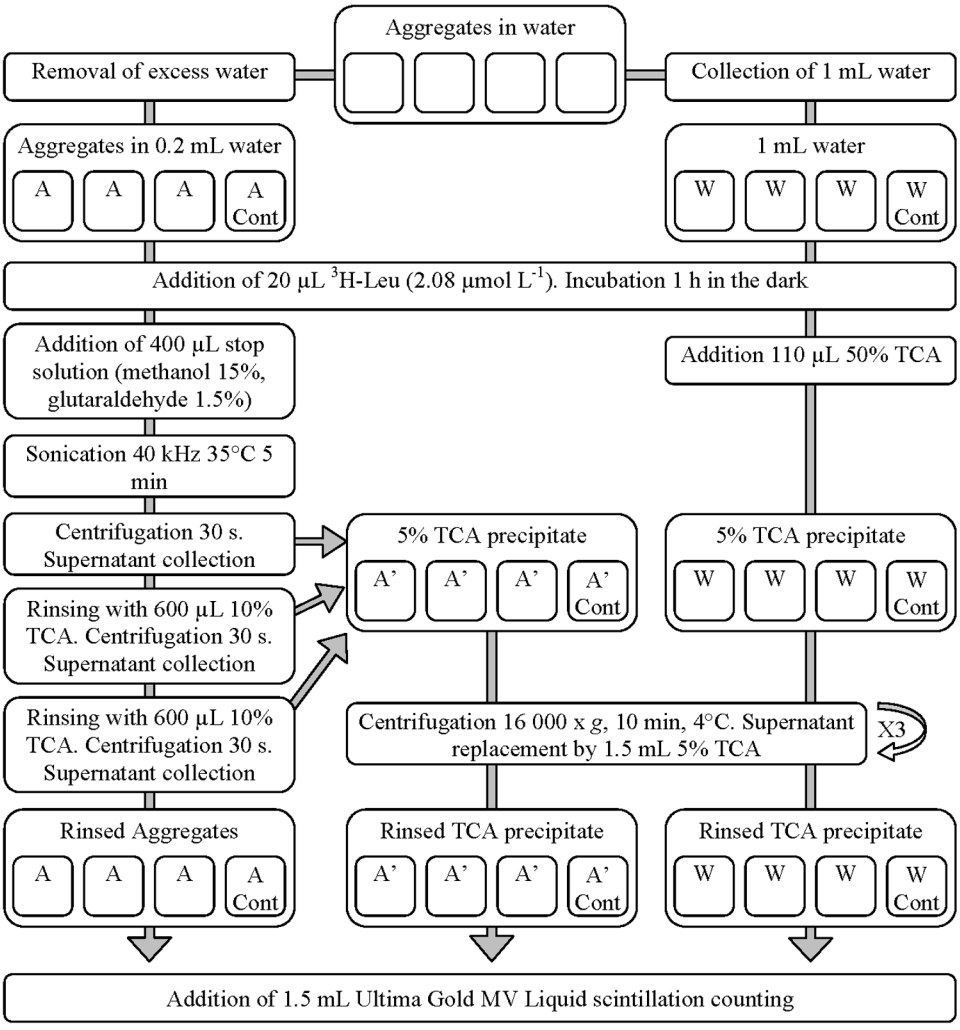

Fig. 3. Experimental setup for the determination of bacterial production of newly produced marine organic aggregates.

tube using the magnetic particle concentrator described above, the solution (~620 µL) was pipetted into another microcentrifuge tube ('B'). The magnets in the 'A' tubes received 600 µL of 10% TCA, were mixed, centrifuged 30 s at 4000*g*, and the rinsing was added to tube 'B'. This procedure was repeated once with 600 µL of 5% TCA. After 15 min precipitation at 4°C, samples 'B' were processed as water samples ('W'). Samples were centrifuged at 16000*g* for 10 min at 4°C, the supernatant was discarded, and the pellets were resuspended in 1.5 mL 5% TCA. This procedure was repeated 3 times. The pellets of tubes 'B' and 'W' and the magnets of tubes 'A' received 1.5 mL of Ultima Gold MV, and radioactivity was determined after 24 h to avoid chemiluminescence using a TriCarb 1600 TR scintillation counter. Quench correction was made using external standards. Radioactivity recovered on the aggregates (R) was computed as R = A + B – (W × 0.195/1), where A, B, and W is radioactivity counted in tubes A, B, and W, respectively (mean of triplicates – control), and 0.195/1 to normalize activity in tubes B to the same volume than aggregates (200 µL – 5 µL, volume of the magnet).

Bacterial respiration within macro-aggregates

Bacterial respiration was measured using a microelectrode of oxygen according to the protocol described by Briand et al. (2004) using micro-respiration chamber (MicroResp, Unisense) (Marshalonis and Pinckney 2007). Micro-respiration chambers of 1000 µL were constructed from glass and fitted with glass stoppers with a capillary hole that allows the microelectrode to penetrate the glass stopper and protrude into the respiration compartment. The capillary also acted as a vent during closure of the chamber so that pressurization of the chamber, and hence of the aggregates, was avoided. The small pore size of the chambers means that gas and liquid exchange with the surroundings is assumed as minimal. Each chamber was gently filled with a single aggregate with the water sampled around the aggregate. Chambers were placed on the top of small magnetic stirrers to allow mixing of the water. Since aggregates were formed using magnetic nuclei, water mixing was achieved by stirring the magnetic nucleus coated with the aggregate. Chambers were incubated under dark conditions in a temperature-controlled bath to maintain a constant temperature of 25 ± 0.1°C (mimicking the in situ temperature of 24.7 ± 0.4°C) during the measurement. Oxygen was continuously monitored using an oxygen microelectrode (Unisense) with a guard cathode that prevents any oxygen consumption by the electrode itself (Revsbech 1989). Microelectrodes were calibrated using a two-point procedure with 0% (0.1 M NaOH/ascorbate) and 100% (bubbling with air) saturation dissolved O_2 concentrations as endpoints. Oxygen concentration in air-saturated water was calculated from the solubility equation according to Garcia and Gordon (1992). Oxygen consumption of the water surrounding the aggregates was measured with the same procedure, and oxygen consumption of a single aggregate was deduced from the difference between both respiration (water plus aggregate and water without aggregate, respectively). Measurements were performed in triplicates.

Assessment

Efficiency of the concentration procedure on the abundance of attached bacteria

During the first set of measurements, the effect of successive centrifugations on the abundance of attached bacteria was assessed. Bacterial abundance in the original field sample was $2.2 \times 10^6 \pm 0.3 \times 10^6$ mL^{-1}. The abundance of bacteria after ultrafiltration reached $131.8 \times 10^6 \pm 11.6 \times 10^6$ mL^{-1}, which corresponds to a concentration factor of ~60. The specific bacterial density of the aggregates produced after a single centrifugation of 2 mL concentrated solution containing the magnetizable aggregates was $3.0 \times 10^6 \pm 0.5 \times 10^6$ agg.$^{-1}$, which corresponds to the number of bacteria in 1.4 mL natural seawater. Considering the bacterial abundance in the concentrate (i.e., 131.8×10^6 mL^{-1}), a single centrifugation allowed attachment onto the aggregation nucleus of only 2.3% of the bacterial population. The bacterial concentration in the supernatant, i.e., after centrifugation, decreased by about 15%, which is higher than the fraction of bacteria retained by the aggregate, suggesting that not all the bacteria at the bottom of the tubes were attached to the aggregation nucleus. Additional centrifugations increased the specific bacterial density of the aggregates, with a bacterial abundance of $26.1 \times 10^6 \pm 2.2 \times 10^6$ agg.$^{-1}$ after 4 centrifugations (equivalent to the number of bacteria in ~12 mL of natural seawater).

After the 10 min rinsing period in the bath containing ultrafiltered seawater, each aggregate was individually collected in a 2 mL tube. Before analyses, 1.8 mL water was removed from the tube, leaving the aggregate ready for analyses. Water in the tube is the ultrafiltered seawater used for rinsing and so contains free bacteria resulting from the rinsing of the aggregate. Therefore, to assess the contribution of the free bacteria coming from the rinsing solution to the specific bacterial density of the aggregate, the concentration of bacteria in this solution was determined (0.5×10^6 mL^{-1}). Because only 0.2 mL of this solution was left at the bottom of the tube before determination of the specific bacterial density, the contribution of free bacteria to the estimation of the specific bacterial density of the aggregates was very low (<0.37%) and thus was ignored.

Interestingly, the relationship between the number of aggregate-attached bacteria and the number of centrifugations was not linear, and followed an exponential curve (Fig. 4). Such a relationship suggests that successive centrifugation not only allows the attachment of new bacteria to the aggregate, but also renders the aggregate more compact, potentially reducing losses during handling of the aggregate.

Bacterial and viral abundance within newly formed macro-aggregates

During the second set of measurements, the relative abundance of attached bacteria and viruses was assessed. Viral and

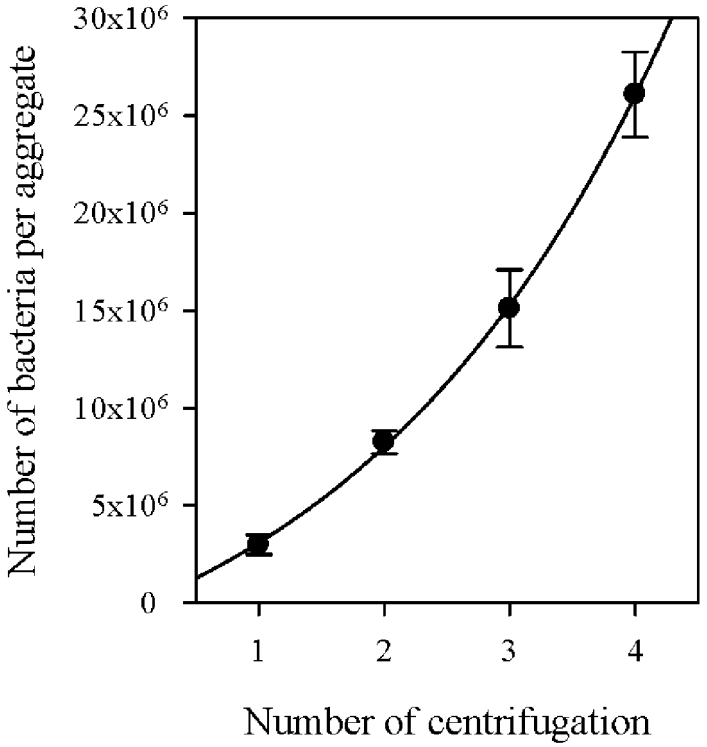

Fig. 4. Effect of successive centrifugation on the bacterial density in newly formed aggregates.

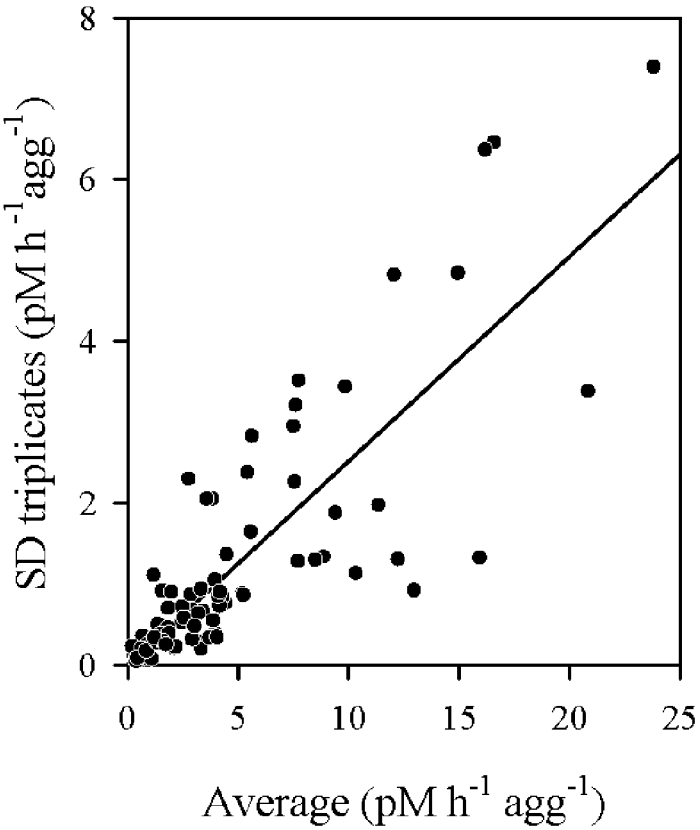

Fig. 5. Standard deviation of triplicate determinations of bacterial production in aggregates versus mean values.

bacterial densities, determined on 24 aggregates after dissolution of the aggregates, averaged $13.8 \times 10^6 \pm 3.6 \times 10^6$ vir. agg.$^{-1}$ and $4.1 \times 10^6 \pm 1.1 \times 10^6$ bact. agg.$^{-1}$. The virus to bacterium ratio on newly formed aggregates (VBR_{Agg}) averaged (±SD) 3.5 ± 0.7, which is less than a third of the VBR measured in the field at the sampling station (11.1 ± 2.2). This suggests that the concentration/aggregation procedure is more efficient for concentrating bacteria than viruses. Such a differential attachment could occur if viruses are less affected by centrifugation than bacteria, and thus, remain in suspension. Alternatively, the method used to solubilize the aggregates and release the attached viruses and bacteria may prove less efficient for disaggregating viruses than bacteria. The efficiency of the methanol-detachment method (Lunau et al. 2005) for counting viruses has not assessed, and thus, at present, it is still unclear whether the observed diminution of the VBR is due to a methodological artefact, or to a preferential attachment of bacteria during the centrifugation step. Also, it is known from other studies that the VBR on organic aggregates can be lower than in ambient water (e.g., Weinbauer et al. 2009). The observed bacterial abundance within the newly formed macro-aggregates is within the range of that observed for naturally occurring organic aggregates (i.e., from 2.5×10^5 to 5.4×10^8 bact. agg.$^{-1}$; Simon et al. 2002).

Bacterial production within newly formed macro-aggregates

With the procedure employed to extract bacteria from macroaggregates, the distribution of bacterial production (BP) in the three operationally defined fractions is on average (± SE, $n = 84$) 24 ± 3% in water, 28 ± 2% extracted from the aggregate and 48 ± 2% remaining attached to the magnets. Whereas the contribution of BP due to water surrounding the aggregates is moderate, the label (^{3}H) remaining attached to the magnets after methanol and ultrasound treatment is still high. Tests showed that the first rinsing of the magnet removed average 9% more label, and the second on average 3% more label. Hence, additional rinsing would not increase the amount of labeled TCA precipitate extracted from the magnets. Occasionally, BP was determined on aggregates using a higher concentration of ^{3}H-Leu (i.e., 378 nmol Leu L^{-1}). BP was not significantly greater than with 189 nmol Leu L^{-1}, suggesting that the chosen concentration was optimum for the determination of bacterial production within the formed aggregates. Variability (SD) between triplicate determinations of BP on aggregates was significantly related to activity (Fig. 5). The coefficient of variation was on average 26% of the mean ($n = 84$) and initial value of Leucine incorporation was 1.00 ± 0.29 pmol Leu agg.$^{-1}$ h^{-1} (average ±SD). Using a conservative theoretical conversion factor of 1.55 kg C mol^{-1} Leu (Simon and Azam 1989), this is equivalent to a bacterial production of 1.54 ± 0.45 ng C agg.$^{-1}$ h^{-1}, which is within the range of that measured for naturally occurring marine aggregates (i.e., from 0.01 to 188 ng C agg.$^{-1}$ h^{-1}; Simon et al. 2002).

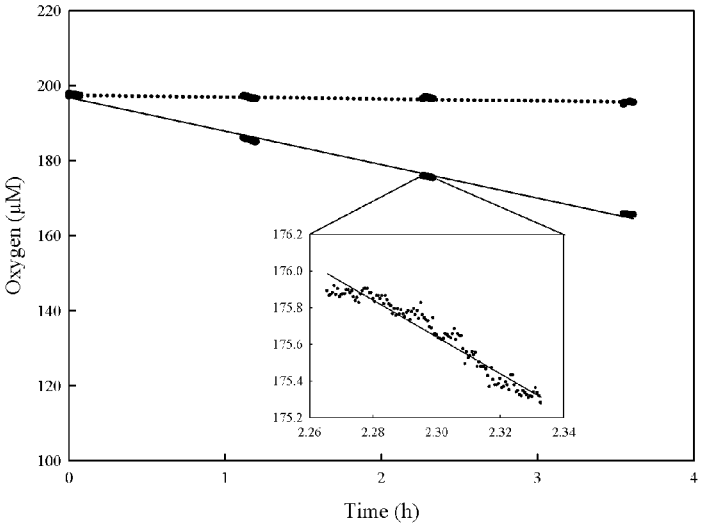

Fig. 6. Oxygen time course in the microrespiration chamber filled with a single newly formed aggregate and water (solid line; y = 196.8 – 8.93 x; *r* = 0.99) and filled only with water (dashed line; y = 197.5 – 0.51 x; *r* = 0.92). The insert shows enlarged view of raw data measurements.

Bacterial respiration within newly formed macro-aggregates

Figure 6 presents the oxygen time course in the microrespiration chamber filled with a single aggregate and the surrounding water. Within a few hours of dark incubation (<4 h), a sharp linear decrease of oxygen concentration was observed. The slope of the fitting curve corresponds to the amount of oxygen consumed by the aggregate itself and the surrounding water, i.e., 8.93 ± 0.03 µmol O_2 L^{-1} h^{-1}. When the water was incubated in the same conditions without the aggregate, the oxygen decrease was still linear but weaker with a slope of 0.51 ± 0.01 µmol O_2 L^{-1} h^{-1}. The calculated respiration value for the aggregate was 8.42 ± 0.03 µmol O_2 L^{-1} h^{-1}, which corresponds to a respiration of 8.42 ± 0.03 nmol O_2 $agg.^{-1}$ h^{-1}. Respiration rates measured in three different aggregates formed with the same procedure and incubated under the same conditions averaged 8.47 ± 1.72 nmol O_2 $agg.^{-1}$ h^{-1} (20.3% of the mean), which is within the range of that measured for naturally occurring marine aggregates (i.e., from 0.3 to 167 ng C $agg.^{-1}$ h^{-1}; Simon et al. 2002). Bacterial growth efficiency calculated from bacterial production and respiration [BGE = BP/(BP + BR)], using a respiratory coefficient of 1, averaged 1.5 ± 0.5%.

Particulate organic carbon and nitrogen content of newly formed macro-aggregates

The POC and PON content of the newly formed macro-aggregates averaged 31.92 ± 2.67 µg C $agg.^{-1}$ and 3.44 ± 0.43 µg N $agg.^{-1}$, respectively. The mean C:N molar ratio was 11.0 ± 2.1, which is significantly higher than that of Redfield, but within the range observed for organic aggregates (i.e., from 5.1 to 20.1; Simon et al. 2002). This supports the idea of a high content of polysaccharidic material, such as TEP, within aggregates. Because these newly formed aggregates are, in fact, biofilms coated at the surface of a magnet, it is not possible to determine their volume. However, an estimate of their volume can be obtained from their POC and PON content. According to Ploug and Grossart (2000), the POC and PON content (µg $agg.^{-1}$) of diatom aggregates increase with increasing aggregate volume (V; mm^3) as POC = $3.25V^{0.66}$, and as PON = $0.42V^{0.67}$, respectively. Therefore, considering the measured POC and PON content, the volume of the organic aggregates formed around the magnet should be between 12.2 and 14.9 mm^3. Assuming a spherical shape for those newly formed aggregates, they should be comparable to natural aggregates of 1 to 1.2 mm diameter.

The respiration rate of a diatom aggregate (R; µg C $agg.^{-1}$ d^{-1}) can be derived from its POC content (µg C $agg.^{-1}$) according to the relationship R = $0.083(POC)^{0.98}$ (Ploug and Grossart 2000). Considering that the average POC content of the magnetically formed aggregates is ~32 µg C $agg.^{-1}$, and assuming that these aggregates present similar characteristics to diatom aggregates, the respiration rate should be ~2500 ng C $agg.^{-1}$ d^{-1}, similar to the rate measured from the oxygen consumption assuming a respiratory coefficient of 1. Interestingly, derived and measured rates are within the same range as the respiration rates reported for marine diatom and detrital aggregates, i.e., between 7 and 4000 ng C $agg.^{-1}$ d^{-1} (Ploug et al. 1999; Ploug and Grossart 2000; Ploug 2001).

Discussion

Macro-aggregate production

During our study, we produced aggregates from seawater collected in situ after prefiltration through a 10-µm nylon mesh to remove large particles and organisms. Moreover, the present method can easily be followed using other size fractions as the initial building blocks for the final aggregates. For instance, one could consider using the bulk organic matter to form larger aggregates that more closely mimic naturally occurring ones. One could also consider producing aggregates from the sub-micrometer size fraction to address in more detail bacteria-virus interactions within aggregates or the bacteria-aggregate interactions. Thus, the proposed method can be easily expanded to test several hypotheses.

During the development of the present method, our goal was to produce a large number of similar aggregates. To achieve this, we included a centrifugation step aimed at increasing reproducibility between aggregates. The centrifugation step also increases the stability of the aggregates by rendering them more compact. This intermediate step can be avoided if the goal is to produce a single giant aggregate to be used for a specific purpose (e.g., grazing experiments with ciliates or copepods). In an attempt to produce such a giant aggregate, we directly suspended a nucleus of aggregation (i.e., a magnet) inside a 250-mL culture flask filled with a concentrated solution of magnetizable aggregates. Interestingly, we could see by the naked eye the macro-aggregate gradually growing with the small magnetizable aggregates originally located at about 2 cm from the magnet moving toward this

nucleus. Because such a giant aggregate would likely be more fragile, its use would require avoiding additional manipulation after formation. This would also mean that the rinsing step would need to be adapted, for example, by gently replacing the remaining solution after aggregate formation by flushing it out with clean incubation seawater. This latter procedure would effectively eliminate the transplant step.

Applications

This study presents a new protocol for the formation of large marine aggregates from natural organic matter, and shows a nonexhaustive panel of measurements that could easily be performed on the newly formed macro-aggregates. The main improvement of the proposed method in comparison with other model aggregates also produced from natural material (e.g., Ploug and Grossart 2000) is the easy manipulation of aggregates that are more robust to disruption. The macro-aggregates produced following the proposed method could be used to study the colonization by bacteria or metazoans and the release of microorganisms (e.g., bacteria and viruses) from the aggregate to the surrounding environment during ageing experiments. The fact that the proposed protocol allows immobilization of the newly formed macro-aggregates in a nonturbulent system allows close and precise sampling near the aggregate, and thus, precise determination of the variations of microorganisms' concentration or community composition in the vicinity of the aggregate. In short, we argue that the procedure described here will prove a useful tool for investigations into the interactions between microorganisms and marine aggregates and their implications at the ecosystem level, but also into the interactions between aggregates and dissolved organic or inorganic substances.

Comments and recommendations

Potential improvements

One of the main limitations comes from the percent recovery of label still attached to the magnet (48% on average) after measuring bacterial production on such aggregates. This high percentage, while not affecting results of BP measurements, suggests that the detachment procedure used to count bacteria should be improved. Repeated extractions with methanol and ultrasound or other physico-chemical or enzymatic treatments (Bockelmann et al. 2003; Amalfitano and Fazi 2008; Kallmeyer et al. 2008) could be tested to improve recovery. Another potential improvement could be to use smaller (i.e., 0.2 mL) microtubes to measure BP in aggregates. Indeed, the use of 2 mL microtubes requires removing a large proportion of the overlying water to minimize the use of ^{3}H-leucine. Using 0.2 mL samples would suppress this step and hence the risk of removing a part of the activity due to loosely attached bacteria.

An additional drawback of this method is linked to the inclusion of microspheres and glass fibers in the aggregate matrix. Such additions aimed at increasing the stability of the aggregates may potentially generate aggregates with physical characteristics (e.g., porosity, surface area) somewhat diverging from that of naturally occurring aggregates, and may, in turn, affect various chemical and biological variables (e.g., bacterial colonization, growth, exchange of solutes, binding of ions). The presence of the magnetic nucleus obviously modifies the density and porosity of the aggregates, which, in turn, may modify the water flow within aggregates. This could be assessed using micro-flow sensors. The newly formed aggregates should probably be viewed as biofilms formed from natural organic material coated at the surface of solid central core. A way to produce aggregates more closely comparable with natural aggregates would be to significantly reduce the size of the central magnet and to decrease the concentrations of microspheres and glass fibers in the aggregate matrix. One could also simply choose to avoid the use of glass fibers. Whereas these precautions may allow producing aggregates more tightly mimicking natural aggregates, it would most likely reduce the stability of the produced aggregates.

Finally, each parameter studied requires a separate aggregate. Indeed, determining the abundance, the composition or the activity of attached microbial communities implies sacrificing one aggregate per analysis. The variability between aggregates cannot be improved because this limitation is intrinsic to the method; it is, however, small because the coefficient of variation for triplicate determinations averaged 26% for BP and 20% for bacterial respiration.

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