

Bacterial production and respiration in suspended aggregates — a matter of the incubation method

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ABSTRACT: Bacterial growth rates and carbon production rates were measured by thymidine and leucine incorporation on aggregates formed after incubation, in rolling tanks, of water samples from the River Weser, Germany. Thymidine and leucine incorporation rates per aggregate decreased with increasing pool size when several aggregates were incubated together in a vial under still conditions. The thymidine and leucine incorporation rates per aggregate were on average 5.6- to 5.3-fold lower, respectively, when 5 aggregates were pooled together in vials than those of aggregates which were incubated individually and kept in suspension. The use of oxygen microelectrodes showed that, in contrast to when single aggregates were suspended, the mean diffusion distance for solutes in the surrounding water of the aggregates increased when aggregates were pooled together in vials and incubated under still conditions. Bacterial production and respiration rates were determined by combined radiotracer and microelectrode measurements on 36 individual aggregates, which were suspended during measurements. The average bacterial carbon production and respiration rates were 5.1 ± 6.3 and 8.5 ± 11.6 ng C $\text{agg}^{-1} \text{h}^{-1}$, respectively. The average growth rate constant (μ) was $1.24 \pm 0.32 \text{ d}^{-1}$ and the average growth efficiency was 0.35 ± 0.19 . Our results demonstrate that the exchange of solutes, e.g. oxygen and radiotracers, between the aggregates and the surrounding water is reduced when aggregates are pooled in a vial as opposed to when they are suspended. Previous measurements may, therefore, have underestimated bacterial growth in aggregates due to the incubation method used.

KEY WORDS: Radiotracers · Microelectrodes · Microenvironments · Diffusion · Sinking

INTRODUCTION

A large portion of suspended matter in oceans, rivers and lakes exists as aggregates composed of phytoplankton, bacteria, flagellates, exopolymers, detritus and inorganic matter (Alldredge & Silver 1988, Alldredge et al. 1993, Eisma 1993, Grossart & Simon 1993, Zimmermann & Kausch 1996). The carbon demand for biomass production of the aggregate-associated bacteria has previously been found to be so low that months to years would be required for bacteria to consume all the organic carbon of aggregates sinking out of the

euphotic zone (Karl et al. 1988, Simon et al. 1990, Smith et al. 1992). Hence, other processes such as enzymatic hydrolysis and respiration are considered to be the major microbial processes in aggregate decomposition (Smith et al. 1992, Grossart & Simon 1998, Parparov et al. 1998, Ploug et al. 1999). The turnover time of carbon on marine aggregates due to respiration in darkness has recently been shown to be 1 to 2 wk (Ploug et al. 1999). The high enzymatic hydrolysis rates and respiration rates, but apparently low bacterial production rates, suggest a very low growth yield of attached bacteria, and the question as to why the bacteria appear not to utilize the aggregate carbon and nutrients more efficiently remains open.

Steep gradients in oxygen concentration and pH in marine snow, and even anoxic conditions in large fecal

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pellets, have been demonstrated with microelectrodes (Alldredge & Cohen 1987). It has been hypothesized that diffusion limitation and the chemical microenvironment, e.g. anoxia and low pH, of sinking aggregates may limit growth of the attached bacterial community (Azam et al. 1993). The development of a vertical flow system in which aggregates are stabilized in suspension by an upward flow velocity that opposes the sinking velocity of the aggregates has enabled direct measurements of mass transfer and the oxygen microenvironment in aggregates during sinking (Ploug & Jørgensen 1999). Oxygen concentrations in natural marine snow were seldomly lower than 90% of air saturation during sinking, although the turnover time of the aggregate carbon by respiration was short (Ploug et al. 1999). Sinking of aggregates greatly facilitates diffusion of oxygen from the surrounding water to the aggregates, resulting in a 9-fold higher oxygen concentration inside aggregates compared to that in an aggregate sedimented onto a solid surface. The flow past the sinking aggregates is, therefore, important for its oxygen microenvironment and the exchange of solutes between the aggregate and the surrounding water (Ploug & Jørgensen 1999).

Aggregate-associated bacterial production rates have traditionally been measured by pooling several aggregates in a common sample of a few ml of surrounding water in order to limit the variability among individual aggregates, and, thus, to obtain more representative measurements (Simon et al. 1990, Smith et al. 1992, Grossart & Simon 1993, 1998). Here we examine the effect of the pooling of aggregates on the diffusive exchange of oxygen in the samples during incubation, and its effect on measured bacterial production rates as compared to more natural conditions where the aggregates were maintained suspended. The combination of radiotracer and microsensors techniques allowed us to measure bacterial production rates and respiration rates on the same aggregate and so directly determine the growth efficiency of aggregate-attached bacteria.

MATERIALS AND METHODS

Sampling and aggregate formation. In July 1998 water samples were collected in a Van Dorn bottle from the surface of the River Weser at the station 'Etelsen', ca 20 km south-east of Bremen, Germany. The salinity was <1.0‰. Samples were incubated in 1.4 l plexiglass roller tanks at 2.5 rpm at *in situ* temperature (22°C) in the dark for aggregate formation (Shanks & Edmondson 1989). Aggregates ≥ 0.7 mm in diameter were already formed after 2 h of incubation and were used for production and respiration measurements of the aggregate-associated microbes.

Bacterial production rates. The microcentrifugation method was used (Smith & Azam 1992). Incorporation of [³H]-thymidine (TdR; Fuhrman & Azam 1980) and [¹⁴C]-leucine (Leu; Kirchman et al. 1985, Simon & Azam 1989) into ice-cold trichloroacetic acid precipitate was measured using the dual label approach (Chin-Leo & Kirchman 1988). Triplicate samples and a formalin-killed control were spiked with TdR (75 Ci mmol⁻¹) and Leu (312 mCi mmol⁻¹) (both from Amersham), and incubated at *in situ* temperature for 1 h in the dark. Aggregates of comparable size were incubated in 5 ml of sterile filtered water in 3 different ways: aggregates were pooled in 1 vial, in which they were either (1) separated, or (2) clustered together at the bottom under still conditions; alternatively, (3) single aggregates were kept in suspension by rotating the incubation vial vertically at 2.5 rpm on a roller table. Final concentrations of the radiotracers ranged from 10 to 360 nM and were used to determine uptake kinetics for both tracers. Bacterial cell multiplication was calculated from ³H-TdR incubation assuming an isotope dilution factor of 4 (Simon 1990) and a theoretical conversion factor of 0.5×10^{18} cells mol⁻¹ TdR. Carbon biomass production by attached bacteria was calculated from ¹⁴C-Leu incorporation, assuming an intracellular isotope dilution factor of 2 and a carbon:protein ratio of 0.86 of the bacterial biomass (Simon & Azam 1989). Bacterial production was calculated as in Grossart & Simon (1998), assuming 53 fg protein cell⁻¹ on aggregates.

Bacterial enumeration. Bacteria on aggregates were counted after DAPI staining by epifluorescence microscopy (Porter & Feig 1980). To remove attached bacteria, single aggregates were treated by ultrasound in 2 mM Na-pyrophosphate (Velji & Albright 1986) prior to filtration onto 0.2 µm Nuclepore membranes.

Microelectrode measurements. Oxygen gradients were directly measured at steady state in pooled samples of aggregates in vials similar to those used for bacterial production measurements, and in individual sinking aggregates which were kept in suspension by an upward-directed flow opposing their sinking velocities (Ploug & Jørgensen 1999). Ten liters of surface water were filtered through microfibre glass filters (GF/C) and filled into the flow system. All measurements were done at *in situ* temperature (22°C), using a slender Clark-type microelectrode (Revsbech 1989) with a 4 µm wide sensing tip, a 90% response time of 0.5 s, and a stirring sensitivity of <0.3%. The microelectrode was calibrated in air-saturated and in N₂-flushed water. The electrode signal was measured by a picoammeter connected to a strip chart recorder. The position of the aggregate surface was determined by slowly advancing the microelectrode towards the aggregate until it visibly touched the upper surface, as

observed under a dissecting microscope. The oxygen gradients were measured in the diffusive boundary layer (DBL) at 50 μm step intervals at steady state in darkness. The dimensions of the aggregates were measured under the dissecting microscope while they were in the flow system. The same aggregates were used for bacterial production measurements immediately after the microelectrode measurements.

Analysis of oxygen gradients. The measured oxygen gradients in the DBL during sinking were used to determine the oxygen fluxes and thereby the respiration rates in the aggregates (Ploug et al. 1997). A respiratory quotient of 1.2 mol O_2 :1 mol CO_2 was assumed. The diffusion coefficient for oxygen in water at salinities <1‰ and at 22°C is $2.24 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Broecker & Peng 1974). The diffusion calculations were made for an ellipsoid as described in Ploug et al. (1999).

RESULTS

The aggregates contained very few phytoplankton cells, but also high amounts of detritus and some resuspended mineral grains. Bacterial numbers per aggregate ranged from 1.0×10^6 to 1.5×10^6 . The average diameter of single aggregates was $1.14 \pm 0.36 \text{ mm}$, and their average volume was $4/3\pi \times (0.57 \text{ mm})^3 = 0.79 \text{ mm}^3$. The longest axis was always less than 2 times larger than the shortest axis. TdR and Leu incorporation rates per aggregate were measured in samples of different numbers with densely clustered aggregates and with aggregates which were well-separated within the vials (Fig. 1). The bacterial production rates were systematically higher on aggregates which were well-separated than on clustered aggregates. Incorporation rates of both TdR and Leu tracers per aggregate were highest when only 2 aggregates were pooled in a vial, and decreased with increasing number of aggregates within a vial. Oxygen concentrations within the samples also decreased with increasing number of aggregates per vial (Fig. 2). Oxygen concentration was saturated in the upper few mm of the vials, but a gradient of oxygen developed towards the bottom of the 5 cm long vial on which the aggregates were sedimented (data not shown). The steepest oxygen gradients occurred at the interface of the aggregates and the surrounding water at distances $\leq 1.5 \text{ mm}$ above the surface of the sample. Mass transfer of oxygen into the aggregates occurred mainly by molecular diffusion in this boundary layer, since diffusion of oxygen from below was prohibited by the solid surface of the vial. Anoxia was only detected when 40 of these relatively small aggregates were pooled together in a volume of ca 31 mm^3 , leading to increased diffusion distances within the 2 mm deep sample with concurrent oxygen

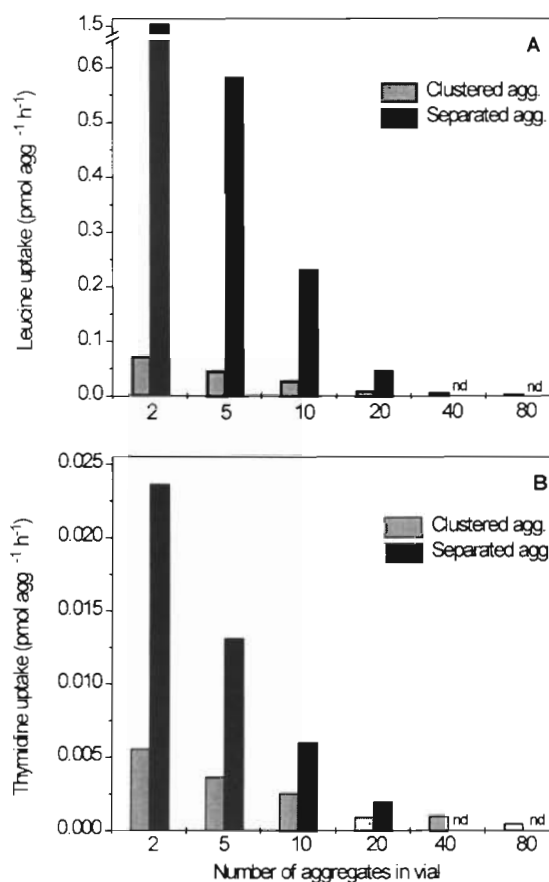


Fig. 1. (A) Leucine and (B) thymidine incorporation rates per aggregate measured with different pool sizes of the samples when the aggregates were separated and clustered in the vials. Bars: mean value of 3 series of measurements for pool sizes up to 20 agg sample⁻¹. nd: not determined for separated aggregates

uptake. In contrast, single aggregates were always $\geq 85\%$ saturated with oxygen due to a much smaller diffusion distance from the surrounding water into single aggregates, which had a high exposed surface area to volume ratio. The respiration rates in sedimented aggregate samples could not be calculated from the oxygen gradients because the exposed surface area was difficult to quantify.

Incorporation kinetics for TdR and Leu were studied in detail with pools of 5 aggregates densely clustered and with 5 aggregates separated within the vials (Fig. 3). We used triplicate samples of aggregates for each measuring point, i.e. 130 to 175 aggregates for each series of measurements. The measured data were fitted to Monod kinetics by a non-linear regression Levenberg-Marquardt algorithm (Origin 3.0, MicroCal Software, Inc.). Maximum incorporation rate, V_{max} , increased 1.9- and 2.3-fold for Leu and TdR, respectively, when aggregates were separated within the sample, compared to clustered aggregates. The maximum in-

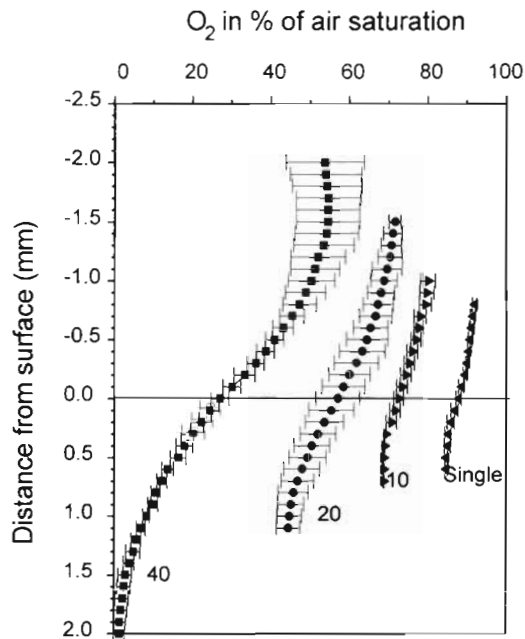


Fig. 2. Oxygen distributions in samples of different pool sizes. Numbers on the curves: numbers of aggregates being clustered together in the sample. Each profile represents the mean value of 3 series of measurements with the SD of the mean

corporation rate for TdR was 46-fold lower than that of Leu when aggregates were separated within the samples. The apparent half saturation constant, K_M , did not change significantly under different incubation conditions. K_M was 25 nM for Leu and 14 to 16 nM for TdR. Uptake rates thus increased at all substrate concentrations when the aggregates were separated, compared to when they were clustered within the vials.

Maximum incorporation rates for TdR in clustered and separated aggregates (Fig. 3) were nearly similar to the incorporation rates shown in Fig. 1 when 5 aggregates were pooled. The measured uptake rates of Leu were more variable. Maximum incorporation rate for Leu in the 5 separated aggregates shown in Fig. 3 was similar to the incorporation rate shown in Fig. 1. In clustered aggregates, however, V_{max} was 6-fold higher in Fig. 3 compared to the uptake rate shown in Fig. 1. Uptake rates at saturating tracer concentrations were almost constant, with small standard deviations in samples where the aggregates were clustered together. In contrast, the uptake rates were relatively more scattered in samples when the aggregates were separated (Fig. 3). These differences may have been caused by differences between individual aggregates. Variable

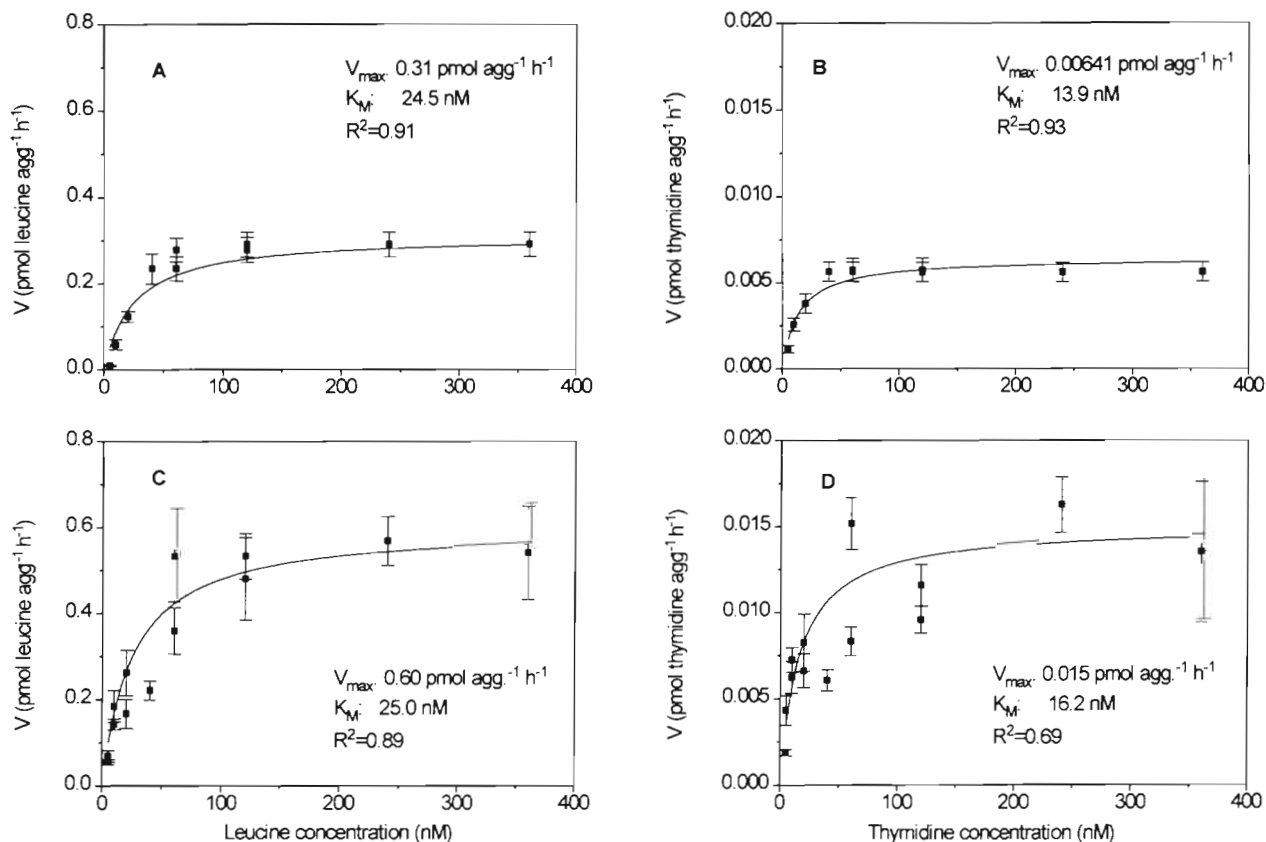


Fig. 3. Leucine and thymidine incorporation rates per aggregate measured with 5 aggregates in each sample, when the aggregates were (A, B) clustered and (C, D) separated in the vials. Each data point represents the mean value with the SD of the mean ($n = 3$)

distances between aggregates could also create different microenvironments in terms of substrate concentrations and hence alter the general growth conditions in the bacterial community.

In a second series of experiments, single aggregates were incubated separately and maintained in suspension during measurements of bacterial production and respiration, to allow the measurements to be done under more natural conditions. Sinking velocities of the aggregates were $>100 \text{ m d}^{-1}$ or 1.15 mm s^{-1} . Oxygen gradients were measured in the aggregates prior to the bacterial production measurements. An example of the oxygen distribution around and within a sinking aggregate is shown in Fig. 4. The shortest axis of the aggregates was 0.6 mm and the longest axis was 1.1 mm. Oxygen concentration was $>97\%$ of that of the surrounding water in the central part of the aggregate because the flow past the aggregates during sinking enhances mass transfer of oxygen from the surrounding water to the entire surface of aggregates. Oxygen gradients were steepest at the aggregate-water interface. This indicates that respiration mainly occurred close to the aggregate surface. The DBL thickness was 0.125 mm upstream and 0.310 mm downstream. The diffusion distance from the surrounding water to the aggregates was thus greatly reduced during sinking as compared to sedimented aggregates (Fig. 2).

The TdR incorporation rate for 36 aggregates ranged from 0 to $0.20 \text{ pmol agg}^{-1} \text{ h}^{-1}$, with a mean value of $0.036 \pm 0.052 \text{ pmol agg}^{-1} \text{ h}^{-1}$ (Table 1). The Leu incorporation rate ranged from 0.06 to $7.77 \text{ pmol agg}^{-1} \text{ h}^{-1}$,

Table 1. Bacterial activities in sinking aggregates. The data show the mean values with the SD of the mean ($n = 36$). The data range is shown in parentheses

Thymidine incorporation rate ($\text{pmol agg}^{-1} \text{ h}^{-1}$)	0.036 ± 0.052 (0–0.20)
Leucine incorporation rate ($\text{pmol agg}^{-1} \text{ h}^{-1}$)	1.60 ± 2.02 (0.06–7.77)
Growth rate constant, μ (d^{-1})	1.24 ± 0.32 (0–6.5)
Bacterial production rate ($\text{ng C agg}^{-1} \text{ h}^{-1}$)	5.1 ± 6.3 (0.19–24)
Respiration rate ($\text{ng C agg}^{-1} \text{ h}^{-1}$)	8.5 ± 11.6 (1.0–51)
Growth yield	0.35 ± 0.19 (0.04–0.69)

with a mean value of $1.60 \pm 2.02 \text{ pmol agg}^{-1} \text{ h}^{-1}$. Measurements on single aggregates showed a high variability compared to those on pooled aggregates. However, the average incorporation rates of TdR and Leu in suspended aggregates were 5.6- and 5.3-fold higher than those of the 5 clustered aggregates shown in Fig. 3, and up to 20-fold higher than those of the clustered aggregates shown in Fig. 1. The average TdR incorporation rate of sinking aggregates was 2.4-fold higher, and that of Leu 2.6-fold higher than when 5 aggregates were separated within the vials, as shown in Fig. 3.

The respiration rates, as calculated from the oxygen gradients measured at $50 \mu\text{m}$ step intervals in the DBL, also varied among the 36 aggregates, ranging from 1 to $51 \text{ ng C agg}^{-1} \text{ h}^{-1}$. The average respiration rate for single aggregates was $8.5 \pm 11.6 \text{ ng C agg}^{-1} \text{ h}^{-1}$. Bacterial growth rates and net bacterial carbon production (BPP) were calculated from the TdR and Leu incorporation rates, respectively. The bacterial growth rate constant (μ) was $1.24 \pm 0.32 \text{ d}^{-1}$ and ranged from 0 to 6.5 d^{-1} . BPP ranged from 0.19 to $24 \text{ ng C agg}^{-1} \text{ h}^{-1}$, with a mean value of $5.1 \pm 6.3 \text{ ng C agg}^{-1} \text{ h}^{-1}$. Since bacterial production rates in single aggregates were measured

immediately after the respiration measurements on the same aggregates, we were able to directly determine the growth efficiency of the bacterial community, assuming that dark respiration was exclusively due to bacteria. The growth efficiency determined as $\text{BPP}/(\text{BPP} + \text{dark respiration})$ varied from 0.04 to 0.69 in single aggregates, with a mean value of 0.35 ± 0.19 .

DISCUSSION

Bacterial production rates in marine and lake snow measured in previous studies show high variability (Table 2). Compared to most of the earlier studies, sizes of aggregates in the present study were relatively small, whereas bacterial numbers were similar to those of previous measure-

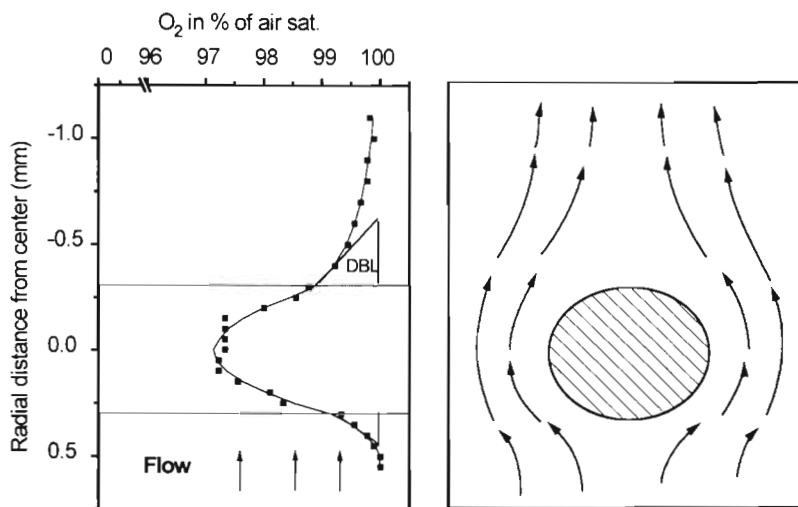


Fig. 4. Oxygen concentrations measured around and within sinking aggregates during darkness. Aggregate was suspended by an upward flow velocity of 140 m d^{-1} , which opposed its sinking velocity

Table 2. Bacterial production rates measured on aggregates in this and previous studies. nd: data not determined or shown

Study site	Aggregate size (mm)	No. of bacteria ($\times 10^6$)	Thymidine uptake (pmol $\text{agg}^{-1} \text{h}^{-1}$)	Thymidine uptake ($\times 10^{-21} \text{ cell}^{-1} \text{h}^{-1}$)	Leucine uptake (pmol $\text{agg}^{-1} \text{h}^{-1}$)	Bacterial net production (ng C $\text{agg}^{-1} \text{h}^{-1}$)	Source
Subtropical Atlantic	>3	3–30	0.025–1.3	nd	nd	0.5–78.1 ^a	Allredge & Youngbluth (1985)
Southern California Bight	>3	1.2–1.7	0.004–0.047	2.5–34.3	nd	0.67–8.04 ^a	Allredge et al. (1986)
North Atlantic	>3	1.8–278	0.004–0.099	1.12–2.27	nd	0.32–13.8 ^a	Allredge et al. (1986)
Southern California Bight	>2	1.2–14.9	nd	0.9–20.8	nd	0.06–2.3 ^b	Allredge & Gotschalk (1990)
Southern California Bight	>2	0.41–1.71	nd	nd	0.042–0.45	0.13–1.25 ^b	Simon et al. (1990)
Southern California Bight	>2	<0.79	nd	nd	0.030–0.045	0.01–0.14 ^b	Smith et al. (1992)
NE Atlantic	1–6	0.2–0.41	nd	nd	0.0002–0.0025	0.0006–0.0077 ^c	Turley & Mackie (1994)
Lake Constance	>3	1.0–20	<0.001–0.039	<0.1–39	0.032–0.583	0.1–1.8 ^b	Grossart & Simon (1993)
Lake Constance	3–20	5–80	<0.0001–0.033	<0.1–33	0.032–0.486	0.1–1.5 ^b	Grossart & Simon (1998)
Weser, Germany	0.5–2.2	1.0–1.5	0.–0.20	0–131	0.06–7.77	0.19–24 ^b	This study

^aCalculated from thymidine uptake rates^bCalculated from leucine uptake rates^cCalculated according to Grossart & Simon (1993)

ments on larger marine and limnetic aggregates. The BPP and the respiration on aggregates similar to those of the present study have been shown to increase with increasing particle size (Grossart & Plough in press). The bacterial numbers as well as the respiration rates on the aggregates of the present study were similar to those measured for similar sizes of marine snow (Plough et al. 1999). The incorporation rates of TdR and Leu measured in the present study also showed a very large variability, but the average values fell in the upper range of previous measurements in marine and lake aggregates (Table 1). The average doubling time measured in the present study was 0.56 d. The doubling time of bacteria attached to aggregates has previously been shown to range between 0.5 and 10 d. Bacterial production rates on aggregates are highly dependent on the origin of the aggregates, e.g. phytoplankton, larvacean or fecal pellets, and miscellaneous aggregates (Allredge & Gotschalk 1990, Simon et al. 1990, Grossart & Simon 1998). Bacterial production rates have been measured by TdR incorporation rates in single, disrupted aggregates (Allredge & Gotschalk 1990). Those rates fell in the lower range of bacterial net production per aggregate, but the cell-specific rates were similar to those estimated by TdR incorporation rates in intact and pooled aggregates of other studies (Table 2). Bacterial production rates estimated by Leu incorporation rates, however, have always been low, and in all of those studies 4 to 10 aggregates were pooled during incubation. In the present study, both the TdR and Leu incorporation rates per aggregate increased when intact aggregates were incubated individually and suspended, compared to when the aggregates were pooled during incubation.

The steady-state oxygen gradients showed that the diffusion distance of solutes from the surrounding water to the aggregates is increased when aggregates are sedimented compared to when they are suspended in a flow. The aggregates were too small and the respiration rates too low to produce anoxia in single aggregates. Anoxic conditions were only detected in a ca 31 mm³ large aggregate sample of 2 mm depth (Fig. 2). The half-saturation constant, K_M , for oxygen respiration is on the order of 1 μM in heterotrophic microorganisms (Focht & Verstrate 1977). The observed increase in bacterial production rates due to flow in the present study was, therefore, not due to diffusion limitation by oxygen and, hence, energy limitation of the bacterial activities for the relatively small pool size of 5 aggregates.

The DBL thickness of sinking aggregates has been shown to be an important limiting factor for metabolic rates under diffusion limitation (Plough et al. 1997). Diffusion limitation of biological processes only occurs when the potential biological uptake rates exceed the

maximum diffusive supply to the microorganisms. At steady state, the substrates are taken up by the microorganisms as fast as they are continuously supplied from the surrounding water through diffusion and advection. The uptake rates increase linearly with the concentration of the substrate in the bulk water phase under diffusion limitation. Hence, the uptake systems are not saturated as long as the biological processes are diffusion limited by a substrate from the surrounding water. In the present study, the saturation of incorporation rates of both tracers apparently started to occur at tracer concentrations >60 nM. At tracer concentrations of 60 to 360 nM the incorporation rates were independent of the bulk water concentration, which indicates that the bacterial uptake could be saturated by adding Leu and TdR at these concentrations (Fig. 3). The uptake rates, however, increased when aggregates were separated or incubated in a flow, compared to densely clustered aggregates. The concentration levels of substrates in the microenvironment of the aggregates may, therefore, be dependent on the exposed surface area and flow.

Cell-specific growth rates of bacteria on aggregates have been shown to decrease, whereas hydrolytic activities increase, with the age of the aggregates, reflecting the quality and bacterial availability of the particulate organic matter (Smith et al. 1995, Unanue et al. 1997). Ecto-enzymatic hydrolysis, e.g. by aminopeptidase, has been shown to play an important role in the solubilization of marine and limnetic aggregates, with a concurrent efflux of hydrolysates to the surrounding waters (Smith et al. 1992, Grossart & Simon 1998). Bacterial production as measured by Leu incorporation rates was very low in both studies (Table 2), and it was concluded that bacterial production and hydrolysis were loosely coupled on these aggregates. The measurements of hydrolysis were also done with pooled aggregate samples incubated under still conditions. An efflux of hydrolysate to the surrounding water implies that the concentration of the hydrolysate inside the aggregates is higher than that of the surrounding water, i.e. opposite to the oxygen concentration as shown in the present study and in earlier studies (Alldredge & Cohen 1987, Ploug & Jørgensen 1999, Ploug et al. 1999). The concentration levels of the hydrolysis products may be close to saturation levels at the uptake sites, whereby hydrolysis and bacterial production appear to be loosely coupled during incubations of pooled and settled aggregates due to relatively large diffusion distances. The isotope will thus be diluted within the sample if Leu is among the hydrolysis products being produced within the aggregate, leading to lower incorporation rates of the added tracer from the surrounding water if the reduced specific activity is not taken into account.

The impact of flow on mass transfer in porous aggregates has been analyzed theoretically and shown to enhance fluxes of solutes, especially those with low diffusion coefficients (Logan & Hunt 1987). The diffusion coefficient of Leu is $0.73 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 25°C (Longworth 1952 cf. Hayduk & Laudie 1974), and thus 3-fold lower than that of oxygen. The diffusion and equilibrium time of any solute between the aggregate and the surrounding water is strongly dependent on the diffusion distance as well as on the dimensions of the diffusion field. In a 1-dimensional diffusion field the mean diffusion time, t , of any solute is described by (Berg 1983):

$$t = \frac{s^2}{2D} \quad (1)$$

where s is the diffusion distance and D is the diffusion coefficient of the chemical solute.

In a 3-dimensional diffusion field, the mean diffusion time over a similar diffusion distance is 3 times shorter, as described by (Berg 1983):

$$t = \frac{s^2}{6D} \quad (2)$$

Most aggregate studies have been carried out for aggregates larger than 3 mm. The diffusion field of the solutes between the aggregates and the surrounding water will be 1-dimensional if a pooled aggregate sample occupies the whole bottom of a vial, with increased diffusion distances if the aggregates sit on top of each other. The mean diffusion and equilibrium time for Leu to exchange between the surrounding water and through a pooled aggregate sample with several 3 mm large aggregates occupying the lower 6 mm of the vial would be 6.8 h ($s = 0.6 \text{ cm}$, cf. Eq. 1), if the boundary layer was ignored. In contrast, the exchange will be much faster if single aggregates sink through the surrounding water. In a 3-dimensional diffusion field, the mean diffusion time for Leu to or from the center of a 3 mm large aggregate is only 9 min ($s = r_0 = 0.15 \text{ cm}$, cf. Eq. 2), if the boundary layer is ignored. The minimum equilibrium time in our aggregates would be 11.4 ($s = 0.1 \text{ cm}$; 1 to 10 pooled aggregates) to 45 min ($s = 0.2 \text{ cm}$; 40 aggregates) in a 1-dimensional diffusion field, whereas it would be as low as 1.4 min ($s = r_0 = 0.06 \text{ cm}$) for single aggregates in a 3-dimensional diffusion field, if the boundary layer were ignored and an effective diffusion coefficient inside the aggregates similar to that in water were assumed.

The DBL thickness and a lower diffusion coefficient in the aggregates imply longer diffusion and equilibrium times. We did not measure the diffusion coefficient inside aggregates, but the diffusion distance for oxygen was directly shown to be larger for sedimented aggregates than for suspended aggregates. The time to reach steady state for oxygen gradients in a 2 to

3 mm large, sedimented aggregate with a DBL thickness of 1 mm has been experimentally shown to be ca 20 min (Ploug & Jørgensen 1999). The diffusion and equilibrium time would accordingly be 60 min for Leu, as the diffusion coefficient for O₂ is 3 times larger than that of Leu. The apparently lower bacterial production rates as measured by ¹⁴C-Leu in samples with many aggregates may thus partly be an artifact of the isotope dilution due to Leu being produced by ecto-enzymatic hydrolysis inside the aggregates, and the non-equilibrium of the isotopes due to their diffusion time relative to the incubation time of 1 h, even in the relatively small aggregates of the present study.

The growth efficiency of the bacterial community in single suspended aggregates was calculated assuming that dark respiration was exclusively due to bacteria. The aggregates of the present study contained very few phytoplankton, and the aggregates were never net autotrophic communities in light (data not shown). Respiration due to protozoa is difficult to measure separately in microbial communities. At steady state, protozoan grazing equals the growth rate of the bacteria. With an average bacterial doubling time of 0.5 d, the bacterial number is doubled within 12 h. The carbon content of aggregate-associated bacteria is on average 60 fg C cell⁻¹ (Simon et al. 1990). At steady state, the protozoan respiration would be (0.5 × 60 ng C agg⁻¹)/12 h = 2.5 ng C agg⁻¹ h⁻¹, assuming an average bacterial carbon content of 60 fg C cell⁻¹, 10⁶ bacteria agg⁻¹, and a 50% growth efficiency of the protozoa. The average dark respiration of the aggregates was 8.5 ng C agg⁻¹ h⁻¹, and protozoan respiration would thus comprise 30% of dark respiration at steady state, whereby the bacterial growth efficiencies may be underestimated in the present study.

Estimates of bacterial growth efficiencies for natural free-living pelagic bacteria range from <0.05 to 0.60, as recently reviewed by del Giorgio & Cole (1998). Our estimates of the growth efficiency in aggregates were in the same range or higher than that determined for natural free-living pelagic bacteria (Table 1). Further study with time-series measurements of aggregates similar to those of the present study has shown that the average growth efficiency can be as high as up to 0.50 ± 0.13 during the 3 d after aggregate formation, and it decreases with decreased substrate availability concurrent with changes in the bacterial community composition on the aggregates (Grossart & Ploug in press).

The incubation of single aggregates in rotating vials results in increased exchange of solutes between the aggregates and the surrounding water and, thus, decreased equilibrium time for the tracers, which allows for short incubation times. In addition, suspension of aggregates creates more realistic conditions of mass

transfer and, thus, chemical microenvironments of all substrates for the aggregate-attached microorganisms as experienced in the field. The high bacterial production rates and high growth yields measured in the present study show that bacterial communities attached to aggregates can indeed be highly active, and that bacterial hydrolysis and hydrolysate uptake may be more closely coupled in sinking aggregates than previous studies on sedimented and pooled aggregates have suggested. Whereas bacterial carbon production will transform the aggregate carbon into bacterial biomass, respiration results in a loss of the aggregate carbon as CO₂ to the surrounding water when the aggregates are net heterotrophic communities. Carbon transformation due to growth and respiration by aggregate-associated microbial communities can, therefore, be significantly higher than previously assumed in estimates of carbon fluxes in aquatic environments.

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