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Bacterial colonization of early stages of limnetic diatom microaggregates

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ABSTRACT: Macroscopic organic aggregates in pelagic environments are colonized by bacterial populations that differ from those in the surrounding water. To understand better how this well-adapted bacterial community is established, it is important to examine the initial colonization early in the aggregation process. We studied, therefore, the early formation and bacterial colonization of diatom microaggregates (MA) (<150 µm) during the phytoplankton spring bloom in Lake Constance, Germany. Water samples were incubated in plexiglass cylinders rolled horizontally for 44 to 48 h and subsampled for MA and their bacterial colonization, which was examined by fluorescent *in situ* hybridization with oligonucleotides of various specificity. During the initial 24 h bacterial numbers remained ~70 cells MA^{-1} and finally increased to ~250 MA^{-1}. Detection rates of *Bacteria* by probe EUB338 ranged from 40% to >80% of the DAPI-stainable cells. Initially, α-*Proteobacteria* detected by the probe ALF1b dominated the bacterial community on MA, whereas toward the end of the incubation β-*Proteobacteria* increasingly dominated. Proportions of *Cytophaga/Flavobacteria* detected by the probe CF319a also increased systematically on MA toward the end but constituted lower proportions than β-*Proteobacteria*. In the surrounding water β-*Proteobacteria* dominated during the initial 24 h whereas *Cytophaga/Flavobacteria* consistently dominated in the late phase of the experiments. Applying highly specific probes for narrow clusters of close relatives of the genus *Sphingomonas* (α-*Proteobacterium*), *Duganella zoogloeoides* (formerly *Zoogloea ramigera*) and *Acidovorax facilis* (both β-*Proteobacteria*), we found that these bacteria were present on MA already at the initial sampling or at the latest after 10 h and comprised substantial and sometimes dominant proportions of total α- and β-*Proteobacteria*. These bacteria, also dominant on natural lake snow aggregates in Lake Constance, were never detected among the free-living bacterial community in the surrounding water. Hence, our results indicate that the bacterial community on lake snow aggregates develops largely from seeds on their precursor MA.

KEY WORDS: Aggregates · Diatoms · Bacteria · *In situ* hybridization · Lake Constance

INTRODUCTION

The formation of microscopic (<500 µm; microaggregates [MA]) and macroscopic organic aggregates (>500 µm; marine snow, lake snow) during the course and breakdown of diatom blooms is a well-known phenomenon, eventually leading to their sedimentation (Smetacek 1985, Alldredge & Gotschalk 1989, Kiørboe et al. 1994). Acidic polysaccharides, either attached to algal cells or released as transparent exopolymer particles (TEP), have been identified as a key agent leading to the aggregation event and thus to the formation of a unique microhabitat highly enriched in nutrients, protozoans and bacteria compared with the dilute bulk environment (Shanks & Trent 1979, Caron et al. 1982, Alldredge & Silver 1988, Alldredge et al. 1993, Passow et al. 1994, Logan et al. 1995, Grossart & Simon 1998). Specific populations of bacteria attached to phytoplankton cells or in the phycosphere lead to the formation of different types of exopolysaccharides by diatoms (Grossart 1999). It is,

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however, still unclear whether the bacterial colonization is instrumental in the formation of aggregates. There is good evidence, though, that the aggregate-associated heterotrophic bacterial community rapidly respires and solubilizes at least the labile aggregate components (Smith et al. 1992, Grossart & Simon 1998, Berman et al. 1999, Ploug et al. 1999, Grossart & Ploug 2000).

The composition of the bacterial community associated with macroscopic aggregates (macroaggregates) is highly specific and differs from that of the free-living bacterial community in the surrounding water. Qualitative differences between bacterial communities associated with marine snow and those in the surrounding water have been found in various marine environments (DeLong et al. 1993, Rath et al. 1998, Acinas et al. 1999, Phillips et al. 1999, Simon et al. in press). Highly specific bacterial communities have also been found on lake snow aggregates (Weiss et al. 1996, Schweitzer et al. 2001) and riverine aggregates (Böckelmann et al. 2000). By applying fluorescent *in situ* hybridization with a few highly specific 16S rRNA oligonucleotide probes, targeting narrow clusters of β1-subclass-*Proteobacteria* and of α-*Proteobacteria* closely related to the genus *Sphingomonas*, Schweitzer et al. (2001) identified 16 to 53% of the DAPI-stainable cells on lake snow aggregates in Lake Constance, Germany. In the course of a few days and while the lake snow aggregates sank through the water column, the composition of their bacterial community changed from a slight dominance of α-*Proteobacteria* to a pronounced dominance of β-*Proteobacteria*. This transition was paralleled by that of a consumption of dissolved amino acids from the surrounding water to their release into it. Interestingly, the composition of the lake snow-associated bacterial community differed from that of MA collected during the same period in the lake (Brachvogel et al. 2001). α-*Proteobacteria* were not detected on these MA whereas cells of the *Cytophaga/Flavobacteria* cluster were relatively more abundant than on lake snow aggregates.

To understand better the colonization and establishment of specific bacterial communities on macroaggregates, the very early colonization stages and the initial conditions need to be compared with those in the surrounding water. Azam et al. (1993) studied the initial colonization and growth of bacteria on freshly formed diatom MA and found the highest community growth rates during the earliest stages. To understand the colonization process better it is important to know whether the bacteria dominating during later stages on aggregates are already present on very early stages with a low bacterial colonization or whether they appear only later. A related question is whether during these early stages any relation exists between the composition of the bacterial community on aggregates and that in the surrounding water, i.e., how the free-living bacterial community interacts with that on MA and macroaggregates.

We studied the bacterial colonization and composition of the bacterial community on early stages of diatom MA, formed in rolling tanks according to Shanks & Edmondson (1989) during the phytoplankton spring bloom in Lake Constance. By applying rRNA-targeted fluorescent oligonucleotides of various specificity, and including highly specific ones for narrow clusters of α- and β-*Proteobacteria*, we found that the bacteria dominating during later stages on aggregates were already present on the initial stages in substantial amounts. The aggregate-specific bacteria detected by the highly specific probes, however, were never detected in the surrounding water.

MATERIALS AND METHODS

Samples for the experiments were collected on 28 April (Expt 1), and 5 May (Expt 2) and 12 May (Expt 3) 1998 at 3 m depth at the center of Lake Überlingen, the northwestern arm of mesotrophic Lake Constance, Germany. Lake Constance is a warm monomictic prealpine lake with a surface area of 472 km^2, and maximum and mean depths of 253 and 101 m, respectively. The lake has been studied intensively in the recent past (Gaedke 1998, Häse et al. 1998, Simon et al. 1998), also with respect to the significance of lake snow aggregates (Grossart & Simon 1993, 1998, Weiss et al. 1996, Grossart et al. 1997, Schweitzer et al. 2001).

Because the age of natural aggregates cannot be correctly determined, we applied an experimental approach using rolling tanks according to Shanks & Edmondson (1989). To examine the bacterial colonization of truly early stages of aggregates we studied the formation of MA (<150 µm) of the freshly collected samples and their bacterial colonization in these tanks over a period of 44 to 48 h. Within 2 h of collection, the samples were transferred into a series of sterilized 1.2 l plexiglass cylinders and further incubated rolling horizontally at 2 rpm at *in situ* temperature and irradiated with 200 µE m^{-2} s^{-1} in a 12:12 h light:dark cycle. This irradiance is the mean of the growing season at 3 m depth in Lake Constance. During the incubation the tanks were sampled periodically for MA and their bacterial colonization. The surrounding water was sampled as well at each time point. Since each tank was sampled only once, time series were composed of samples from different tanks. Earlier experiments had shown that the formation of aggregates and their bacterial colonization in tanks incubated in parallel were not statistically different (Schweitzer et al. 2001).

For examination of the MA and the associated bacterial community 20 ml was filtered onto 12 µm pore size Nuclepore filters (47 mm diameter, Costar Corp., Cambridge, MA, USA) by gravity. For examination of the free-living bacterial community 2 ml was filtered onto 0.2 µm pore size Nuclepore filters (25 mm diameter) with a gentle vacuum of 150 mbar. Five samples were filtered at each time point such that triplicate samples of ¼ of a filter were available for *in situ* hybridization of each probe (see below). Fixation of the bacteria was done by overlaying the filter with 4% paraformaldehyde-PBS (pH 7.2) for 30 min. After withdrawing the fixative, the filters were rinsed with 2 ml of PBS and Milli-Q water. The samples were stored at 4°C until further processing within a few weeks. Numbers and sizes of MA and total numbers of bacteria on aggregates and in the surrounding water were also determined by epifluorescence microscopy in DAPI-stained samples of the volumes indicated above and filtered onto 12 and 0.2 µm Nuclepore filters, respectively (Porter & Feig 1980). The abundance of MA was determined at 400× magnification on 10 viewfields. Thirty MA were sized according to their longest dimension. For bacteria on aggregates, DAPI-stainable and probe-specific bacteria were counted on 20 randomly selected MA. Because of the variable size of the MA, the coefficient of variation (CV; SD/mean) of the DAPI-cell counts ranged from 20 to 50% and in some cases up to 90% (Table 1, Figs 1 & 2). In samples of the surrounding water 400 to 500 cells were counted on 10 viewfields. In these samples the CV was always <15%.

***In situ* hybridization.** The composition of the bacterial community was determined by *in situ* hybridization with the fluorescence-labeled rRNA targeted oligonucleotides listed in Table 2. The probes LSA225, LSB65 and LSB145 were designed from lake snow clone libraries of Lake Constance in which the clone clusters targeted by these probes were prominent members

Table 1. Total numbers of bacteria (±SD, n = 3) per microaggregate (MA) and in the surrounding water during Expt 1, Expt 2 and Expt 3, carried out on 28 April, and 5 and 12 May 1998

Time (h)	Microaggregates (cells MA^{-1})	Surrounding water (10^6 cells ml^{-1})
Expt 1		
3	71 ± 32	1.1 ± 0.1
15	69 ± 32	1.3 ± 0.2
24	68 ± 29	1.1 ± 0.2
41	101 ± 53	1.5 ± 0.1
48	254 ± 131	1.8 ± 0.2
Expt 2		
2	64 ± 23	1.1 ± 0.7
14	66 ± 24	1.5 ± 0.1
24	74 ± 24	1.6 ± 0.2
30	117 ± 105	2.0 ± 0.3
45	267 ± 193	2.9 ± 0.4
Expt 3		
2	68 ± 40	1.3 ± 0.3
13	60 ± 29	1.9 ± 0.1
24	87 ± 37	3.2 ± 0.4
37	84 ± 30	2.5 ± 0.5
45	265 ± 181	3.1 ± 0.7

(Huber 1997). The target molecule of probes BET42a and GAM42a is the 23S rRNA and that of all other probes the 16S rRNA. The probes were linked to the fluorochrome 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (FLUOS) or Cy3 (derivate of succinimide ester Cy3 of a cyanine). For the analysis of the aggregate-associated bacterial community, the probes were linked to FLUOS, except the CF319a probe, which was always linked to Cy3. Free-living bacteria were analyzed exclusively with Cy3-labeled probes because they have a higher intensity than FLUOS. Because of the smaller size of the free-living bacteria (<0.08 µm^3) than that of the aggregate-associated cells (0.1 to

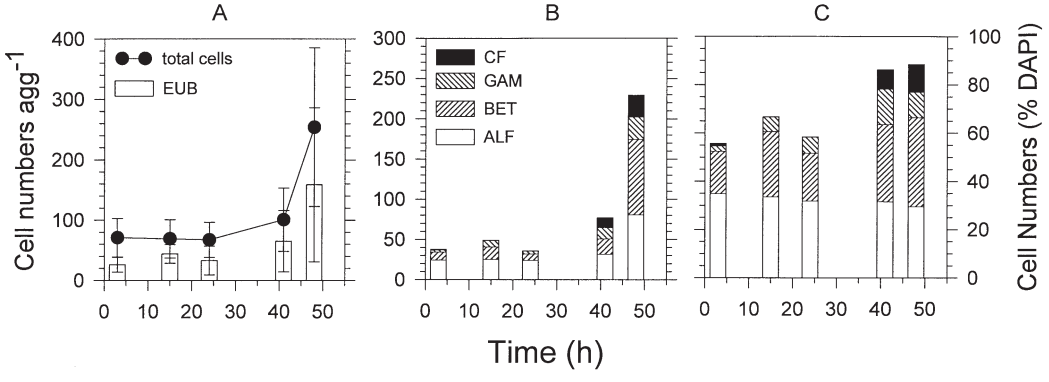

Fig. 1. Bacterial colonization of microaggregates in Expt 1 from 28 April 1998. Total numbers of cells and of *Bacteria* detected by (A) probe EUB338; (B) numbers; and (C) percentages of DAPI-stainable cells of α- (ALF) , β- (BET) and γ-*Proteobacteria* (GAM), and of the *Cytophaga/Flavobacteria* cluster (CF)

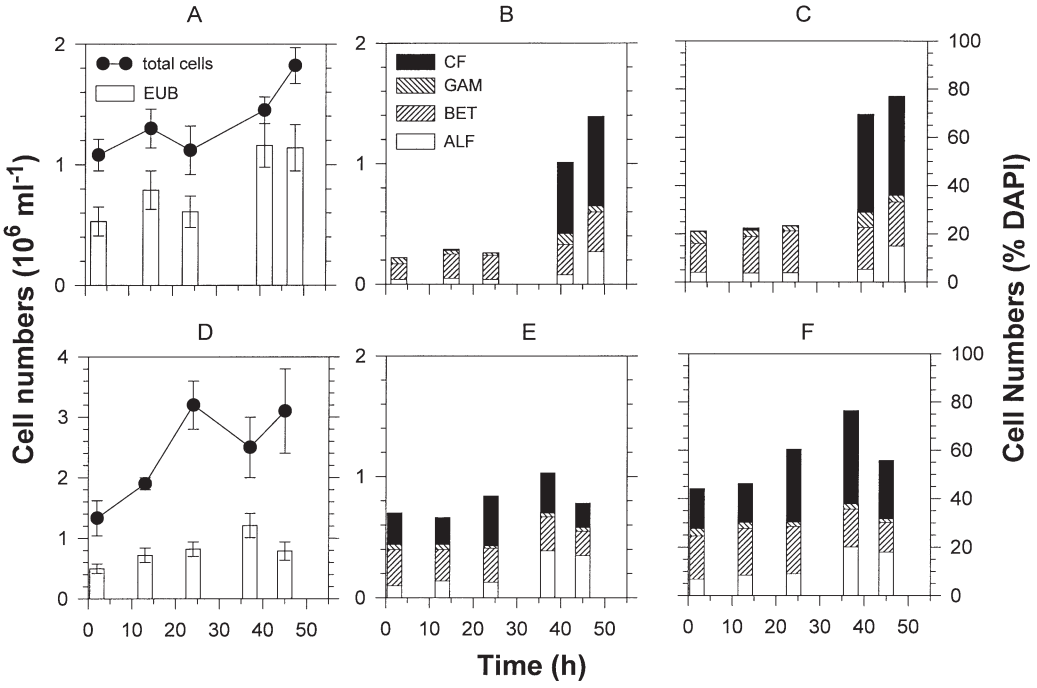

Fig. 2. Numbers of bacteria in the surrounding water in (A–C) Expt 1 (28 April) and (D–F) Expt 3 (12 May). Numbers of total cells and of *Bacteria* detected by (A,D) probe EUB338; (B,E) numbers; and (C,F) percentages of DAPI-stainable cells of α-, β- and γ-*Proteobacteria*, and of the *Cytophaga/Flavobacteria* cluster. See Fig. 1 for abbreviations

0.2 µm^3), this differential labeling maximized the fraction of free-living bacteria that was detected but did not affect that of the aggregate-associated cells. *in situ* hybridization was performed at 46°C for 90 min in preheated hybridization chambers. The procedures of Manz et al. (1992, 1996) were applied but modified as follows. Each filter was cut into quarters, of which 2 were put on 1 glass slide for *in situ* hybridization. To each filter quarter, 20 µl of the hybridization buffer was applied. It contained 0.9 M NaCl and formamide at a concentration of 20% (EUB338, ALF1b) or 35% (other probes), 20 mM Tris-HCl (pH 7.4), 0.01% SDS and the probe in a concentration of 2.5 ng µl^{-1} each. Probes BET42a and GAM42a were used with a competitor oligonucleotide (Manz et al. 1992, Snaidr et al. 1997). To stop hybridization, the filter on the glass slides were rinsed and incubated at 46°C for 15 min in washing buffer, containing 180 mM (20%) or 40 mM (35%) formamide, NaCl and 0.01% SDS. After rinsing with Milli-Q water, the slides were dried and stained with DAPI (0.01%). Finally, they were embedded in Cityfluor (Chemical Laboratory, University of Kent, Kent, UK) on glass slides and covered with cover slides. The hybridized samples were visualized by a Nikon epifluorescence microscope equipped with the filter sets UV-2A for DAPI (Nikon), B-2A for FLUOS (Nikon, Tokyo, Japan) and XF32 NM198 for Cy3 (Omega Optical Inc., Brattleboro, VT, USA) at a magnification of 1250×. As

Table 2. Identification, target groups and sequences according to Brosius et al. (1981) of the oligonucleotide probes used. For the probes specific for α- (LSA) and β-subclass *Proteobacteria* (LSB), the closest related and identified bacterial species is given

Probe	Target group	Sequence (5′→3′)	Source
EUB33	*Bacteria*	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)
ALF1b	α-*Proteobacteria*	CGT TCG (C/T)TC TGA GCC AG	Manz et al. (1992)
BET42a	β-*Proteobacteria*	GCC TTC CCA CTT CGT TT	Manz et al. (1992)
GAM42a	γ-*Proteobacteria*	GCC TTC CCA CAT CGT TT	Manz et al. (1992)
CF319a	*Cytophaga/Flavobacteria*	TGG TCC GTG TCT CAG TAC	Manz et al. (1996)
LSA225	*Sphingomonas* sp. and relatives	TCC TAC GCG GGC TCG TCC	Schweitzer et al. (2001)
LSB65	*Duganella zoogloeoides* and relatives	GTT GCC CCG CGC TGC CGT	Schweitzer et al. (2001)
LSB145	*Acidovorax facilis* and relatives	CTT TCG CTC CGT TAT CCC	Schweitzer et al. (2001)

negative controls for the highly specific probes we used *Agrobacterium rubi* (LSA225), *Ectothiorhodospira halophila* (LSB65) and *Hydrogenophaga palleroni* (LSB145). Cells were counted as described above. The CV of the triplicate analyses of the various probes ranged between 0.06–0.34 (EUB338), 0.12–0.49 (ALF1b), 0.12–0.49 (BET42a), 0.10–2.20 (GAM 42a), and 0.11–1.50 (CF319a). The CV exceeded 0.6 when very low cell numbers were recorded.

RESULTS

Samples were collected at the height and breakdown of the phytoplankton spring bloom as indicated by chlorophyll *a* concentrations at 3 m of 8.3, 12.6 and 2.3 µg l^{-1} on 28 April, and 5 and 12 May, respectively (B. Beese unpubl. data). The microscopic examination of the DAPI-stained samples withdrawn from the rolling tanks showed that the phytoplankton was dominated by diatoms such as *Asterionella formosa*, *Fragilaria crotonensis* and *Stephanodiscus* spp. MA were always composites of diatoms and organic material gluing the algal cells together. During the initial hours of the experiments, together with the diatoms, MA of a size of 50 to 80 µm were present at abundances of <50 ml^{-1}. After 24 to 30 h the mean size of the MA increased to >100 µm and the abundance to 60 to 80 ml^{-1} even though MA of the initial smaller size class were still present. When the experiments were stopped after 44 to 48 h, a few aggregates of >3 mm had formed in each tank.

Total numbers of bacteria on the MA subsampled 2 to 3 h after the start of the experiments ranged from 64 to 80 cells MA^{-1} and remained constant within 24 h (Table 1, Fig. 1A). Thereafter the numbers of MA-associated bacteria increased to 254 to 267 cells MA^{-1} at the end of the experiments. Bacterial numbers in the surrounding water initially were ~1 × 10^6 ml^{-1} and increased to 1.8×10^6 ml^{-1} in Expt 1, and to 3×10^6 ml^{-1} in Expts 2 and 3 (Table 1, Fig. 2A,D). Because the results of the bacterial colonization of the MA in the 3 experiments were rather similar, we present the detailed results only of Expt 1.

Probe EUB338 detected at least 40% of the DAPI-stainable cells on MA. Toward the end of the experi-

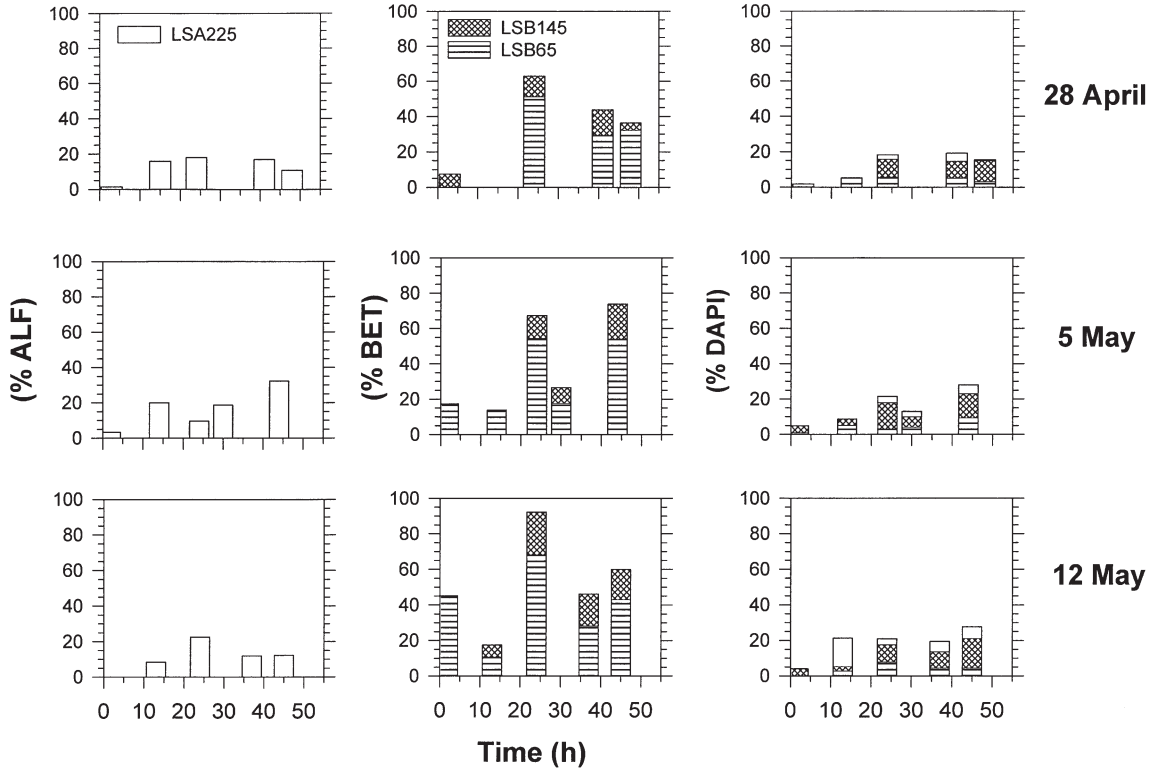

Fig. 3. Colonization of microaggregates by specific bacterial populations during Expt 1 (28 April), Expt 2 (5 May) and Expt 3 (12 May). Left panels: percentages of α-*Proteobacteria* detected by the probe LSA225 (*Sphingomonas* and relatives); central panels: percentages of β-*Proteobacteria* detected by the probes LSB145 (*Duganella zoogoeoides* and relatives) and LSB65 (*Acidovorax facilis* and relatives); right panels: cumulative percentages of DAPI-stainable bacteria detected by the probes LSA225, LSB145 and LSB65

ments the detection rate increased to >70%. The detection rate of the free-living bacteria by probe EUB338 was also 40 to 80% of the DAPI-stainable cells but only 20 to 40% in Expt 3 (Figs 2D & 3A). α-*Proteobacteria* comprised 25 to 45% of the DAPI-stainable cells on MA in all 3 experiments with only minor fluctuations during a single experiment (Fig. 1C). Proportions during the initial 24 h were not significantly different from those during the late period after 30 to 48 h (*t*-test, $p < 0.01$; Table 3). The proportions of β-*Proteobacteria* on MA during the first 24 h were lower than those of α-*Proteobacteria* but significantly increased toward the end of the experiments. In the late phase of Expt 1 and Expt 2 they were significantly higher than those of α-*Proteobacteria* (*t*-test, $p < 0.01$) and in Expt 3 were not significantly different from those of α-*Proteobacteria* (Table 3). Proportions of γ-*Proteobacteria* on MA always comprised substantially lower proportions than α- and β-*Proteobacteria* but also consistently increased during the course of the experiments (Fig. 1B,C, Table 3). Cells of the *Cytophaga/Flavobacteria* cluster in Expt 1 and Expt 2 occurred only occasionally during the initial 24 h but accounted for 9.7 and 15.8% of the DAPI-stainable cells, respectively, in the late phase (Fig. 1B,C, Table 3). In Expt 3, cells of this phylogenetic cluster were present already at the initial sampling at 2 h and comprised proportions of 12% of the DAPI cell counts during the entire experiment (Table 3). All cells of the *Proteobacteria* subclasses and *Cytophaga/Flavobacteria* together accounted for ~100% of the counts by probe EUB338.

The composition of the free-living bacterial community in the surrounding water differed greatly from that on MA. During the initial 24 h it was dominated largely by β-*Proteobacteria* comprising 15 to 25% of the DAPI cell counts. These proportions were always significantly higher than those of α-*Proteobacteria* (Table 3), which comprised <10% and often <5% (Fig. 3B,C,E,F). In contrast to the aggregate-associated β-*Proteobacteria*, proportions of this phylogenetic cluster in the surrounding water remained constant (Expt 1) or decreased in the late phase (Expt 2 and Expt 3). An inverse trend was also true for α-*Proteobacteria*, whose proportions increased in 2 of the 3 experiments during the late phase. γ-*Proteobacteria* always comprised only minor proportions. Even though cells of the *Cytophaga/Flavobacteria* cluster occurred in the surrounding water at the same time as on MA, their proportions in the former were significantly higher and accounted for at least 16% but after 24 h for 24 to 41% of the DAPI-stainable cells (Fig. 2B,C,E,F). During the late phase they were the single most abundant bacterial group. In Expt 1 and Expt 2 the free-living bacteria detected by the group-specific probes accounted for 50% of the EUB338 counts during the initial 24 h but for 100% thereafter. In Expt 3 they accounted for 100% of the EUB338 counts during the entire course.

Bacteria related closely to *Sphingomonas* sp. detected by probe LSA225 were present already at the initial sampling in Expt 1 and Expt 2 and, after 12 h and including Expt 3, comprised proportions of 10 to 32% of total α-*Proteobacteria* (Fig. 3, left panels). *Duganella zoogloeoides* (formerly *Zoogloea ramigera* ATCC 25935) and relatives and *Acidovorax facilis* and relatives, detected by probes LSB65 and LSB145, were also detected already at the initial sampling (Fig. 2, center panels). Probe LSB65 in most cases detected as much as 27 to 70% of total β-*Proteobacteria* and always higher proportions than probe LSB145. Probes LSB65+LSB145 together in some cases detected 60 to 100% of total β-*Proteobacteria*. All 3 probes together detected between 2 and 28% of the DAPI-stainable bacteria (Fig. 3, right panels). None of the 3 highly specific probes detected any bacteria among the free-living bacterial community in the surrounding water.

Table 3. Proportions of α- (ALF), β- (BET) and γ-*Proteobacteria* (GAM) and of *Cytophaga/Flavobacteria* (CF) on microaggregates (MA) and in the surrounding water (bulk) during the initial and late phase of the Expt 1 (28 April), Expt 2 (5 May) and Expt 3 (12 May). Mean values ± SE for the respective periods are given

Time (h)	ALF (%DAPI)	BET (%DAPI)	GAM (%DAPI)	CF (%DAPI)
Expt 1				
MA				
3–24	33.5 ± 3.0	21.5 ± 2.5	5.0 ± 0.7	0.3 ± 0.7
41–48	30.6 ± 5.3	34.5 ± 0.2	12.6 ± 1.6	9.7 ± 1.2
bulk				
3–24	3.9 ± 0.2	14.9 ± 0.8	3.1 ± 0.3	0.4 ± 0.4
41–48	10.2 ± 1.4	17.9 ± 0.6	4.5 ± 0.3	40.7 ± 2.1
Expt 2				
MA				
2–24	27.5 ± 1.6	25.5 ± 2.6	0.6 ± 0.8	0
30–44	25.5 ± 1.4	29.5 ± 1.0	5.6 ± 1.5	15.8 ± 0.5
Bulk				
2–24	4.0 ± 0.4	20.7 ± 0.9	0.6 ± 0.2	0
30–44	3.5 ± 0.5	14.8 ± 1.2	1.3 ± 0.1	35.9 ± 1.9
Expt 3				
MA				
2–24	38.7 ± 2.7	15.8 ± 2,3	9.0 ± 1.4	11.5 ± 1.5
37–45	38.0 ± 1.8	35.3 ± 1.0	12.7 ± 1.2	12.0 ± 0.3
Bulk				
2–24	8.2 ± 0.1	18.9 ± 4.2	2.4 ± 0.1	20.8 ± 1.2
37–45	19.1 ± 0.8	13.9 ± 0.5	1.5 ± 0.2	31.3 ± 1.8

DISCUSSION

Our experiments with lake water incubated in rolling tanks showed that MA of a size of 50 to 80 µm increased to >100 µm and formed macroaggregates within 40 h. This time period of macroaggregate formation is very similar to that reported previously for the same lake (Weiss et al. 1996, Grossart et al. 1997, Schweitzer et al. 2001). Many studies in various environments and including Lake Constance have shown that TEP, either as single MA or associated with diatom cells, are of key importance in gluing together these phytoplankton algae and forming macroaggregates (Alldredge et al. 1993, Kiørboe et al. 1994, Passow & Alldredge 1994, Passow et al. 1994, Logan et al. 1995, Grossart et al. 1997). Therefore, even though TEP was not examined specifically, we assume that it primarily mediated the aggregation in our experiments. Small MA were still present after 40 h, presumably because the phytoplankton was still growing in the rolling tanks incubated in a light-dark cycle and continuously produced new MA. We did not examine the formation of TEP from dissolved precursor material but did examine the formation of MA of diatom origin, which were always identified by the diatom structures. The MA were colonized by a bacterial community dominated by narrow clusters of α- and β-*Proteobacteria* and very similar to those on natural and naturally derived lake snow aggregates several days old in Lake Constance (Schweitzer et al. 2001). These authors examined the temporal succession of the bacterial community on lake snow aggregates 2 to 4 d old and found a systematic decrease in the proportion of α-*Proteobacteria* and an increase in that of β-*Proteobacteria* such that the latter usually exceeded the proportion of the former on lake snow aggregates 3 to 4 d old. Hence, this succession is a consistent follow-up of the one we found and indicates that the MA we studied were indeed precursors of lake snow aggregates. Natural MA <60 µm in Lake Constance, stained by DAPI, were shown not to be precursors of lake snow aggregates but the older decomposition stages of lake snow aggregates and of other detrital particulate organic matter (Brachvogel et al. 2001). These authors showed that the abundance of natural DAPI-stained MA did not covary with that of phytoplankton biomass and TEP, and that their associated bacterial community was dominated by β-*Proteobacteria* and cells of the *Cytophaga/Flavobacteria* cluster, whereas α-*Proteobacteria* were never detected. The MA we studied presumably have a very short half-life under natural conditions and rapidly form or are scavenged by larger aggregates because of their high sticking coefficient. Our experimental observations and this conclusion are consistent with observations made by Azam et al. (1993) during the aggregation of marine diatoms.

In our experiments the bacterial numbers MA^{-1} ranged between 61 and 80 for the initial 24 h when the size of the MA was still <80 µm but increased thereafter with doubling times of 5 to 12 h until 44 to 48 h. This increase was presumably due to cell multiplication as well as gluing together of smaller MA. Similar observations with even higher growth rates are reported by Azam et al. (1993). Our bacterial numbers MA^{-1} are well in the range of other reports including TEP, protein containing MA, and DAPI yellow particles and other types of MA (Passow et al. 1994, Mostajir et al. 1995, Long & Azam 1996, Brachvogel et al. 2001).

Bacteria of close relatives of *Sphingomonas*, *Duganella zoogloeoides* and *Acidovorax facilis* detected by probes LSA225, LBS 65 and LSB 145, respectively, were present on MA already at the initial sampling or after 10 h of incubation (LSA225) and continuously constituted substantial and sometimes dominating proportions of total α- and β-*Proteobacteria*. In the 3 experiments detection rates of *Bacteria* and of total group-specific cells initially were 40%, but in most cases 55 to 75%, of the DAPI-stainable cells and increased to >80%, indicating that the majority of the bacterial community on MA was detected by the probes applied. The initial detection rates are at the upper end of those of natural MA in Lake Constance and of reports of free-living bacterial communities in lacustrine ecosystems (Glöckner et al. 1999, Pernthaler et al. 1999, Zwisler 2000, Brachvogel et al. 2001). In particular, bacteria detected by probe LSB65 and closely related to *D. zoogloeoides* (formerly *Zoogloea ramigera*) dominated total β-*Proteobacteria* to a great extent. These observations suggest that these bacteria are already present on senescent diatoms and are actively involved in the aggregation process. *D. zoogloeoides* is known to produce mucopolysaccharides, occurs on activated sludge flocs in sewage treatment plants and has also been found on mucilage of filamentous cyanobacteria (Caldwell & Caldwell 1978, Ikeda et al. 1982, Dugan et al. 1992). α- and β-*Proteobacteria* also dominated on natural lake snow aggregates in Lake Constance collected at 25 m depth and on lake snow aggregates 2 to 3 d old and formed in rolling tanks (Schweitzer et al. 2001). On natural lake snow aggregates, however, the proportions of β-*Proteobacteria* and of bacteria detected by the probe LSB145 and closely related to *A. facilis* and relatives were higher than on MA, whereas that of bacteria detected by the probe LSB65 was lower. On the other hand, the proportion of bacteria detected by the probe LSA225 and closely related to *Sphingomonas* sp. and relatives was higher on lake snow aggregates than on MA, even though the proportion of total α-*Proteo-*

bacteria was lower. As outlined in more detail by Schweitzer et al. (2001), aerobic chemoorganoheterotrophic bacteria occurring on activated sludge flocs are the closest known relatives of those identified on aggregates. These bacteria exhibit high potentials of hydrolytic enzyme activities and metabolize labile organic matter such as amino acids and carbohydrates. Schweitzer et al. (2001) further showed that the bacterial community on lake snow aggregates consumed labile organic matter, mainly amino acids, from the surrounding water as long as it was dominated by α-*Proteobacteria*, until an aggregate age of 2 to 3 d. Thereafter, when β-*Proteobacteria* dominated more and more, dissolved amino acids were released into the surrounding water. Hence, we assume that during the early formation of aggregates we examined and when α-*Proteobacteria* dominated or shared equal proportions with β-*Proteobacteria* labile organic matter was consumed from the surrounding water, presumably leading at least partly to the formation of mucus-like sticky organic material and the aggregation of senescent diatom cells.

Bacteria of the *Cytophaga/Flavobacteria* cluster were also present on the MA we studied but constituted much lower proportions than α- and β-*Proteobacteria*. During the first 2 experiments they occurred only after 40 and 30 h, respectively. In the last experiment carried out at the decline of the phytoplankton spring bloom, however, they were already present at the initial time point. Bacteria of the *Cytophaga/Flavobacteria* cluster are known to degrade a variety of complex polymers including recalcitrant carbohydrates (Reichenbach 1992). Hence, we assume that the occurrence of bacteria of the *Cytophaga/Flavobacteria* cluster reflects the presence or production on MA of low but increasing amounts of more refractory organic matter during the course of the bloom. On natural MA in Lake Constance bacteria of the *Cytophaga/Flavobacteria* cluster constituted 8 to 20% of the DAPI-stainable cells, equivalent to 23 to 37% of *Bacteria* detected by the probe EUB338 (Brachvogel et al. 2001). This proportion was equal to or only slightly lower than that of β-*Proteobacteria*, suggesting that natural MA in Lake Constance were much more refractory than the MA and precursors of macroaggregates we studied. On lake snow aggregates in Lake Constance, however, bacteria of the *Cytophaga/Flavobacteria* cluster were relatively less abundant than on natural MA and increased in proportion only at 50 m and below. Natural biofilm communities in the Elbe River, Germany, and its estuary are also colonized by bacteria of this cluster to high proportions (Brümmer et al. 2000, Simon et al. in press). Böckelmann et al. (2000), however, found high proportions of this cluster (i.e. 30 to 40%) of DAPI-stainable bacteria only in spring on riverine aggregates in the Elbe River, which in general were also dominated by β-*Proteobacteria* and specifically by populations closely related to *Aquabacterium commune*, which is not closely related to the bacteria we detected on MA.

In the free-living bacterial community initial detection rates of *Bacteria* in the 3 experiments ranged from 38 to 50% of the DAPI-stainable cells and that of total group-specific bacteria from 22 to 44% with increasing proportions during the course of the bloom and covering 100% of *Bacteria*. At the end of the experiments, proportions of 50 to 75% were detected. These detection rates are well in the range of those found in free-living bacterial communities in other lacustrine ecosystems including Lake Constance (Glöckner et al. 1999, Pernthaler et al. 1999, Zwisler 2000). In contrast to the bacterial community on MA, that in the surrounding water was not initially dominated by α-*Proteobacteria* but by β-*Proteobacteria*. In the last experiment, the initial proportion of bacteria of the *Cytophaga/Flavobacteria* cluster was only slightly lower than that of β-*Proteobacteria*. During the course of the experiments mainly *Cytophaga/Flavobacteria* were growing such that at the end of all 3 experiments they clearly dominated the bacterial community and constituted greater proportions than on MA. Hence, and in accordance with the above mentioned assumption, the composition of the bacterial community in the surrounding water suggests that during the experiments and the spring bloom the supply and turnover of labile dissolved organic matter decreased at the expense of polymeric and more refractory constituents. Interestingly, the free-living bacterial community in Lake Constance usually is dominated by β-*Proteobacteria*, and *Cytophaga/Flavobacteria* constitute only about 50% of the former (Zwisler 2000). These findings indicate that substrate supply to the free-living bacteria in our experiments was somehow different from the long-term *in situ* conditions and may reflect short-term dynamics that become undetectable after a few days and when lower, i.e. weekly, sampling frequencies are applied.

None of the probes highly specific for narrow clusters of MA-associated α- and β-*Proteobacteria* detected any single free-living bacterium. This observation suggests that there was no measurable release and propagation of these aggregate-associated bacteria into the surrounding water. Obviously they were very closely associated with or even firmly attached to the MA such that after division newly formed cells did not reach the surrounding water. Hence, in the case of these bacteria, aggregates did not serve as a means of their propagation into the surrounding water as previously postulated (Jacobsen & Azam 1984). We hypothesize, however, that during later stages, when

macroaggregates progressively decompose and disintegrate, aggregate-specific bacteria are released and colonize newly produced senescent diatoms as seeds and may propagate again. Accordingly, they would propagate only a little in the substrate-dilute bulk water.

In conclusion, we have shown that the bacterial community on diatom macroaggregates is already seeded and largely determined on their precursor MA which obviously already provide a suitable microenvironment. During the course of roughly 2 d the composition undergoes slight changes with increasing proportions of β-*Proteobacteria* and bacteria of the *Cytophaga/Flavobacteria* cluster. The significantly different composition of the MA-associated bacterial community from that in the surrounding water, which exhibits much higher proportions of *Cytophaga/Flavobacteria*, indicates pronounced differences in the turnover of organic matter between these 2 habitats.

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