Vol. 52: 283–296, 2008
doi: 10.3354/ame01236

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online September 11, 2008

OPEN ACCESS

Effects of protozoan grazing within river biofilms under semi-natural conditions

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ABSTRACT: Biofilms play an important role in the material flux of many aquatic ecosystems, but little is known about the mechanisms controlling their community structure under natural conditions. In the present study, we focused on the effects of ciliates on the quantity and taxonomic composition of heterotrophic flagellates (HF), and the effects of HF on the quantity and life forms (single cells vs. microcolonies) of bacteria in the early phase of biofilm colonization. For this purpose, we established semi-natural biofilms in flow cells connected to the river Rhine at Cologne, Germany. Using filter cartridges, we size-fractionated the potamoplankton, which is the source of the biofilm community, thus establishing biofilms containing (1) only bacteria (1.2 µm filter), (2) HF and bacteria (8 or 5 µm filter), or (3) ciliates, HF and bacteria (20 µm filter). The presence of ciliates negatively influenced the abundance of biofilm-dwelling HF and selectively altered the taxonomic composition of the HF community. The presence of HF resulted in a significant reduction in the abundance of single bacterial cells, but enhanced the abundance of bacterial microcolonies. Furthermore, the presence of ciliates stimulated the abundance of single-cell bacteria (probably due to an HF-mediated trophic cascade), but had no effect on bacterial microcolonies. Taken together, the results of this study show the importance of protozoan grazing in shaping the species composition and morphology of early river biofilms under semi-natural conditions.

KEY WORDS: River biofilm · Benthic protozoans · Size fractionation · Trophic cascade · Top-down control · Microbial communities

INTRODUCTION

Almost every submersed interface is colonized by complex communities of microbiota (bacteria, fungi, algae and small metazoans) which are together termed biofilms (cf. Wetzel 2001). The organisms which make up the biofilm can reach very high densities compared to the plankton (e.g. Fischer & Pusch 2001, Arndt et al. 2003, Parry 2004). Even though high densities are found for all components within the biofilm and suggest a strong ecosystem-wide impact of biofilm-dwelling organisms, most ecological studies have been on the autotrophic (e.g. Hillebrand et al. 2002) and, more often, on the bacterial (see Hall-Stoodley et al. 2004 for review) parts of this biocoenosis. Such studies have demonstrated that the major proportion of bacterial biomass, activity and function in many natural (e.g. rivers, lakes and oceans) and artificial (e.g. waste water treatment plants) systems is represented by biofilms (e.g. Fischer & Pusch 2001, Battin et al. 2003, Hall-Stoodley et al. 2004). On the one hand, this makes bacterial biofilms an important mediator of the material flux and self-purification processes in both natural and industrial systems. On the other hand, biofilms can cause serious problems in both industrial and medical facilities due to the enhanced stress resistance of biofilm-dwelling bacteria (e.g. Espeland & Wetzel 2001, Mah & O'Toole 2001, Hall-Stoodley et al. 2004). Understanding the mechanisms which control bacterial biofilms is, therefore, a challenge in both environmental and applied microbial ecology.

Among the most important factors controlling natural bacterial communities is grazing by protozoans (Jürgens & Matz 2002, Matz & Kjelleberg 2005). How-

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ever, there is a consensus that biofilm formation can be one mechanism to escape protozoan grazing (Matz et al. 2004, Matz & Kjelleberg 2005). This view is based mainly on studies of planktonic communities which have shown that large bacterial morphs including colonies are frequently formed by bacteria in the presence of flagellated grazers (e.g. Hahn & Höfle 1999, Matz et al. 2002). When settling onto a surface, bacterial cells are also able to undergo a transition from surface-attached single cells via small microcolonies to complex, 3-dimensional, mature biofilms (O`Toole et al. 2000). This complex planktonic-biofilm transition involves the formation of an extracellular polymer matrix (EPS, e.g. Hall-Stoodley et al. 2004). Recent experiments with single-species biofilms have shown that surface-associated microcolony formation is stimulated in the presence of potential protozoan grazers, and that they can resist high grazer densities (Matz et al. 2004, Matz & Kjelleberg 2005, Queck et al. 2006). Nevertheless, this grazing resistance of bacterial microcolonies and biofilms is not *a priori* applicable to all protozoan types. Laboratory studies have demonstrated that certain specialized grazers are able to efficiently utilize mature bacterial biofilms (Huws et al. 2005, Weitere et al. 2005). Whether or not surface-attached microcolonies are resistant to grazing, and if their formation is stimulated by the presence of grazers, might thus be dependent on the dominant grazer types present in the biofilms (Parry et al. 2007). Experiments with natural, complex grazer communities are needed to test the role of protozoan grazers in controlling bacterial biofilms in the field.

Also still unresolved is the trophic structure of the biofilm-dwelling grazer community and whether or not the second order consumers (particularly ciliates) exert a significant grazing pressure on first order consumers (particularly heterotrophic flagellates, HF) within the biofilms. The major origin of food particles (planktonic vs. benthic) for biofilm-dwelling consumers of higher order is still unclear. Theoretical considerations on the basis of dominant feeding types and material flux calculations (Parry 2004, Esser 2006) as well as experimental studies (Weitere et al. 2003) indicate that biofilm-dwelling ciliates utilize significant amounts of planktonic food and thus act as links between planktonic and benthic food webs. In addition, there is little doubt that predation on HF by ciliates also occurs within the benthic food web as shown by the feeding types present (Parry 2004), by grazing studies under controlled laboratory conditions (e.g. Premke & Arndt 2000), as well as by field observations (e.g. Epstein et al. 1992). However, it is as yet uncertain whether this grazing pressure within the biofilm is strong enough to significantly affect the taxonomic structure and/or quantity of HF in natural biofilms, or, as a second step, to generate indirect food web effects such as a trophic cascade (e.g. Jürgens et al. 1994, Zöllner et al. 2003) that ultimately also alters the bacterial biofilm. Summarizing current knowledge on the microbial food web structure within biofilms, it is still unclear whether there are strong trophic links from bacteria to HF and to ciliates within the biofilm food web as it appears in planktonic systems (cf. Azam et al. 1983, Nakano et al. 2001).

In the present study, we tested grazer-mediated effects within the food web of semi-natural biofilms. The biofilms were generated in flow cells fed by natural river water. This water was size-fractionated before passing through flow cells to generate communities with controlled size, and consequently, also controlled trophic structure (cf. Landry 1994). The experiments were designed to test the following hypotheses: (1) Natural bacterial biofilm assemblages form more grazing-resistant morphs such as microcolonies in the presence of HF, whereas single bacterial cells are reduced in abundance due to grazing by HF, and (2) the effects of ciliates on the biofilm structure (HF and bacterial communities) are only of minor importance, as the biofilm-associated ciliates feed primarily on planktonic organisms.

MATERIALS AND METHODS

Overview and study site. The experiments were performed with natural microbial biofilm communities generated by constant flow of field water through a system of flow cells. The biofilm communities thereby established were analysed by both high-resolution light microscopy and confocal laser scanning microscopy (CLSM). The flow cells used here are described by Norf et al. (2007) and were adapted for use as a bypass system. The inflowing field water was experimentally manipulated to selectively exclude specific biofilm components as described below. All experiments were conducted between October 2006 and April 2007 onboard the Ecological Rhine Station of the University of Cologne. This station is a ship that is permanently anchored in the main flow of the Rhine at Cologne-Bayenthal (Rhine km 684.5, which refers to the distance from Lake Constance, the source of the non-alpine Rhine). Rhine water was constantly supplied by pumping it directly from the river to the laboratory.

Experimental set-up. To single out the effects of ciliates and HF on natural biofilms, we used size fractionation of the potamoplankton, i.e. the source community for the biofilm. Filter cartridges (Sartopure Capsules PP2, Sartorius) were employed for this purpose as they provide sufficient filter area when working with a con-

stant flow of particle-rich field water. Due to the large filter area and the low flow rate (2 ml min^{-1}), a sufficient amount of water for the conduct of the experiments was obtained with application of low differential pressures (<50 mbar) between the in- and outflow of the filter cartridges. Four different pore sizes were used (Table 1) according to the specific requirement of the experiment (see next paragraph). Filters of 1.2 µm pore size excluded most protozoans including HF, while they had no measurable effect on the bacterial abundance and size distribution. Most HF passed through filters of 5 µm pore size, while all ciliates were retained by these filters. Filters with 8 µm pore size allowed most HF and very small ciliates to pass through, while the 20 µm filters were also permeable to a large number of different ciliate species besides admitting bacteria and HF. A detailed permeability test of the filters for the different target groups is given in the next subsection.

These filter types were used in different combinations in 4 experiments (Table 2). Expts I and IV (conducted in different seasons, hence with different abundance of planktonic bacteria and HF) dealt with the grazing effects of HF on bacteria. For this purpose, treatments with natural bacterial assemblages but strongly reduced HF abundances (1.2 µm filter) were compared with treatments containing the natural bacterial communities plus natural complex HF communities (8 and 5 µm filters in Expts I and IV, respectively). Furthermore, we tested the grazing effects among biofilm-dwelling protozoans, i.e. grazing of ciliates on HF. Here we compared biofilm communities containing ciliates, HF and bacteria (20 µm filter) with those containing only HF and bacteria (5 µm filter) (Expts II and III, Table 2). Possible HF-mediated cascade effects of ciliates on both single-cell bacteria and bacterial colonies were additionally analysed in Expt III.

Every treatment in all experiments was set up in 4 replicates; however, the number of replicates was doubled when flow cells were needed for fixation and examination under the CLSM (see subsection 'Quantification of biofilm bacteria in the flow cells'). All treatments were generally run with filters of different mesh sizes (i.e. we explicitly did not use unfiltered Rhine water for comparisons) to minimize possible secondary filter effects. To further minimize secondary effects, filter cartridges were changed weekly.

A schematic drawing of the experimental set-up is shown in Fig. 1. After passing a pre-filter of 30 to 100 µm mesh size to prevent clogging of filter cartridges, Rhine water was pumped via an impeller pump (Watson-Marlow) and silicone tubes through the aforementioned filter cartridges. This water was further pumped at a flow rate of 2 ml min^{-1} into the flow cells (Norf et al. 2007) where organisms established biofilms. The flow cells were kept at the actual Rhine temperature (Table 2) in a temperature-controlled water basin. All outgoing and therefore open silicone tubes passed a heat trap (65 to 70°C) to prevent any contamination. The whole system was autoclaved before it was set up.

Permeability test of filter cartridges for suspended bacteria and protozoans. While size-fractionation techniques are frequently used to analyse material flux in planktonic food webs, such techniques have thus far not been used for experimental manipulations of biofilm communities. Methodological investigations were therefore necessary to check for effects of the filters on the quantity of bacteria and protozoans. These investigations were designed to determine whether or not the assumptions underlying the experiments were correct. These assumptions were that (1) the 5 µm filter has little (if any)

Table 1. Filter cartridges used in this study and effects on the community of biofilm-dwelling bacteria and consumers. HF: heterotrophic flagellates

Pore size (µm)	Groups excluded	Resulting community
20	metazoa + large ciliates	ciliates + HF + bacteria
8	metazoa + most ciliates	few small ciliates + HF + bacteria
5	metazoa + ciliates	HF + bacteria
1.2	metazoa + ciliates + HF	bacteria + few HF

Table 2. Summary of the conducted experiments in chronological order. Water temperature is mean daily average Rhine temperature and range over the course of the experiment

Expt	Date	Water temperature (°C)	Filter	Aspects analysed
I	19/10 – 23/10/06	16.0 (15.6 – 16.3)	8/1.2	Top-down effects of HF on bacteria
II	28/01 – 12/02/07	6.6 (5.7 – 7.4)	20/5	Top-down effects of ciliates on HF
III	21/03 – 04/04/07	9.2 (8.0 – 10.7)	20/5	Top-down effects of ciliates on HF and bacteria
IV	23/04 – 26/04/07	17.3 (16.6 – 18.1)	5/1.2	Top-down effects of HF on bacteria

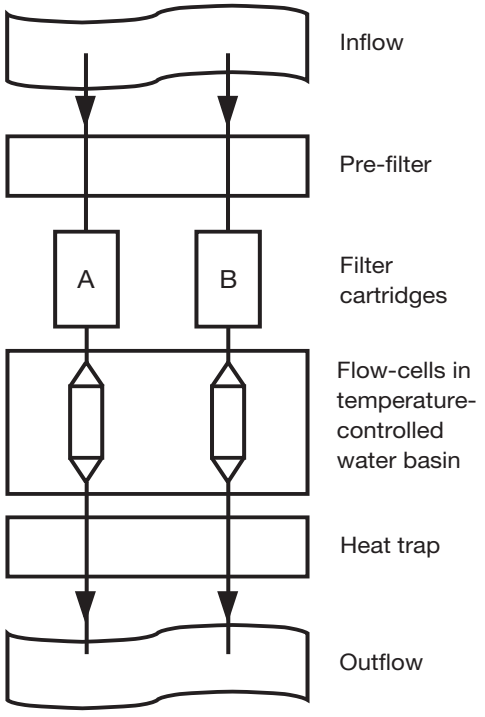

Fig. 1. Experimental set-up. Rhine water was pumped via impeller pumps through a pre-filter (30 to 100 µm mesh size) and filter cartridges (1.2, 5, 8 or 20 µm pore size) into flow cells situated in a water basin held at the actual Rhine temperature. Each treatment was set up in 4 replicates. Water was pumped into the outflow after it was passed through a heat trap (65 to 70°C) to prevent contamination by undesired protozoans

effect on the quantity of the HF in comparison with the 20 µm filter (Expts II and III, Table 2), (2) the 1.2 µm filter efficiently reduces HF abundance at least over the short duration of Expts I and IV, and (3) the 1.2 µm filter has no effect on the quantity and size distribution of bacteria in comparison with the 5 µm filter (Expts I and IV). Regarding filter effects on ciliates, no further methodological investigations were performed because the results of the main experiments clearly showed that (1) all ciliates were excluded by the 5 µm filter, (2) the 8 µm filter was passed by only very few small bacterivorous ciliates, and (3) ciliates passed the 20 µm filter in sufficiently high species and individual numbers to establish complex ciliate communities (see 'Results' section).

The permeability of the different filter cartridges for cultivable HF was tested by comparing HF abundance in Rhine water and the outflow of 20, 5 and 1.2 µm filter cartridges using the liquid aliquot method (LAM; Butler & Rogerson 1995). For each treatment, 3 tissue culture plates with 24 wells were used, resulting in a total of 72 wells per treatment for the 3 replicates. Each well was inoculated with 10 µl (for unfiltered Rhine water and the 20 µm filter), 40 µl (for the 5 µm filter), and 400 µl (for the 1.2 µm filter) of the water sample, and made up to a total volume of 2 ml with autoclaved Rhine water. One sterilized Quinoa grain was added to each well as a source of organic carbon to stimulate microbial growth. After 7 d, the wells were checked for flagellate morphotypes. The abundances of the protozoan morphotypes in the initial inoculum were calculated from the number of wells colonized, and corrected using Poisson distribution (cf. Garstecki & Arndt 2000). Although the abundance of HF was generally lower in filtered than in unfiltered Rhine water, no significant difference was detected when applying 20 and 5 µm filter cartridges (Fig. 2A). Additionally, no effects on the taxonomic composition of the HF community could be detected. The plankton in both treatments and the untreated Rhine water was dominated by Chromulinales and Kinetoplastida. This was also confirmed by the similar taxonomic composition of the biofilm-dwelling HF community in the early colonization (before ciliates became abundant) of the 5 and 20 µm treatments in Expts II and III (data not shown). The 1.2 µm cartridge reduced the HF abundance by 98% in comparison to unfiltered Rhine water and by 93% in comparison to the 5 µm filter (Fig. 2A). This significant reduction is sufficient for short-term experiments with manipulated HF quantities (Expts I and IV, Table 2), but insufficient to maintain low HF abundances on the biofilm in long-term experiments.

The abundance and size distribution of planktonic bacteria in Rhine water, 1.2 µm and 5 µm filtered water were recorded by DAPI counts (Porter & Feig 1980). For each replicate (4 replicates per treatment), a 5 ml sample was taken and diluted with 5 ml of ice-cold glutaraldehyde solution (GA), resulting in a final GA concentration of 1%. After adding 1 ml of DAPI (final concentration: 50 µg ml^{-1}) for 5 min, the organisms within the solution were filtered onto black polycarbonate membrane filters (0.2 µm pore size, Whatman-Nuclepore). The filters were then placed on glass slides, added with 2 drops of non-fluorescent immersion oil and covered with a coverslip. The samples were stored in a dark freezer at –20°C until quantification under an epifluorescence microscope (Zeiss Axioskop). Abundance was recorded until at least 300 bacteria were counted at random spots on the filter. Additionally, bacteria size classes were recorded and converted into volume by assuming simple geometric forms.

Results show equal bacterial abundances in 5 µm and 1.2 µm filtered water and in unfiltered Rhine water (ANOVA $F_{(2,9)} = 0.503$; $p = 0.621$) (Fig. 2B). Furthermore, no difference in the size distribution of bacteria in 5 µm and 1.2 µm filtered water (which were directly compared in the experiments) could be detected (Fig. 2C), although some differences between both

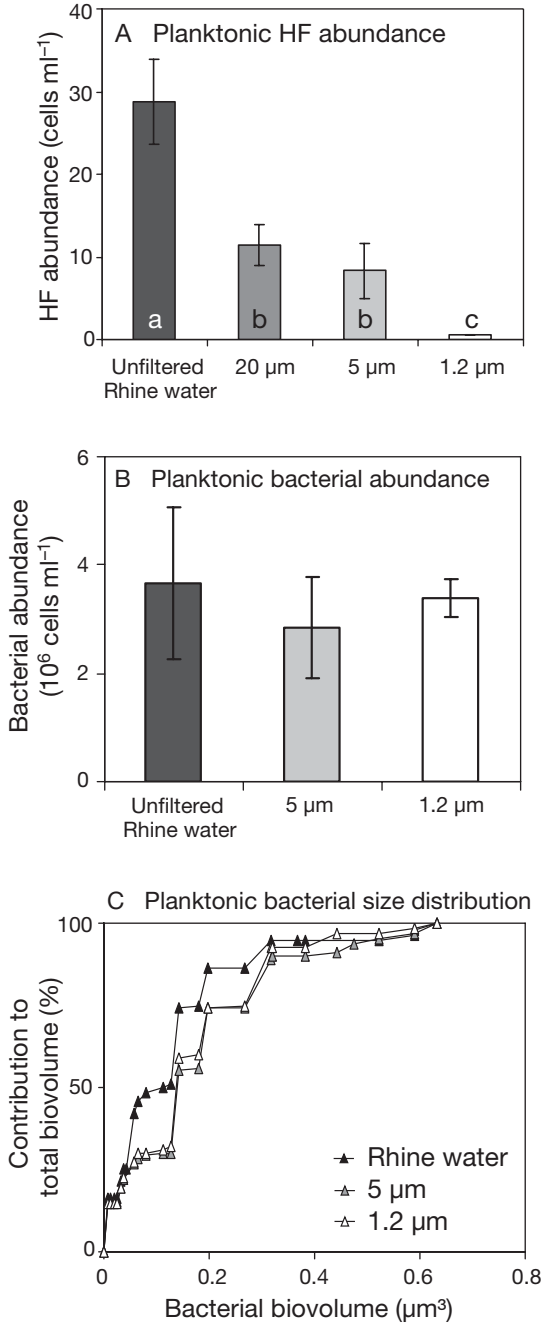

Fig. 2. Tests of filter permeability for suspended heterotrophic flagellates (HF) and bacteria. (A) Test for HF permeability compared with unfiltered Rhine water, as quantified by the liquid aliquot method (LAM). Error bars represent SD. Different letters indicate significant differences in a 1-factorial ANOVA ($F_{(3,8)} = 129.1$; $p < 0.001$) + post-hoc test (Ryan, Einot, Gabriel & Welsch) with log-transformed data. (B) Test for bacteria permeability (DAPI counts). No differences in abundance were detected in a 1-factorial ANOVA ($F_{(2,9)} = 0.503$; $p = 0.621$) with log-transformed data. (C) Cumulative graph of the bacterial size distribution (represented as biovolume) in unfiltered Rhine water, as well as in 5 and 1.2 µm filtered Rhine water. Note similarity between the 1.2 and 5 µm filtered treatments, which were directly compared in the experiments

treatments and the unfiltered Rhine water (which was not considered in the experiments) were noted.

Identification and quantification of biofilm protozoans in the flow cells. Protozoan abundance and taxonomic composition were analysed *in situ* within the flow cells by light microscopy (Zeiss Axioskop, phase contrast). Magnification used was 100 to 200× for ciliate quantification and 200 to 400× for HF quantification. Ciliates and HF were counted repeatedly in defined areas of 0.016, 0.004 or 0.001 cm^2 (depending on the applied magnification) which were randomly distributed on the flow cell. At least 60 specimens were quantified per flow cell except in the early stages of biofilm formation when abundances were extremely low. Protozoans were identified with the help of general keys (e.g. Foissner & Berger 1996) under 400 to 1000× magnification, combined with video recording if necessary for later identification. Taxonomic classification followed the system recently suggested by Adl et al. (2005).

Quantification of biofilm bacteria in the flow cells. The abundances of both single-cell bacteria and bacterial colonies on the biofilm were quantified either *in situ* by light microscopy (Expt I) or in fixed biofilms by CLSM (Expts III and IV).

In Expt I, both single cells and colonies (defined as clusters containing at least 4 closely related single bacterial cells) were counted using 1000 and 400× magnification, respectively, on Days 1 & 4. Randomly distributed spots of defined areas were scanned in each flow cell until at least 60 single cells or colonies were recorded, or until respective areas of 0.0002 and 0.225 cm^2 were scanned. The latter technique was used at the beginning of the experiment when low quantities of single-cell bacteria and colonies were recorded.

CLSM analyses were performed on Day 14 in Expt III, and on Days 1 & 3 in Expt IV. Four flow cells of each treatment were fixed with formaldehyde (2% final concentration) and stored in the dark in a refrigerator at 4°C. They were stained with 4 ml of propidiumiodide (100 µg ml^{-1}) immediately before biofilm analyses under the CLSM (Zeiss Axiovert 100M). We used a helium-neon laser as an excitation source (543 nm), and xy-scans of the biofilm directly adhering to the coverslip to analyse bacterial abundance. Each flow cell was scanned for both bacterial colonies and single bacterial cells at 6 randomly distributed spots under a magnification of 400× (total scanned area = 0.318 cm^2 flow cell^{-1}). We used the software LSM 510 Meta (Version 3.2 SP2, Zeiss 2003) to produce images which were further analysed using the shareware ImageJ (Version 1.37v; http://rsb.info.nih.gov/ij/). A major adjustment done before this analysis was the setting of a minimum threshold of 2 × 2 pixels

(= 0.2 µm^2 at 400× magnification) to eliminate random noise. After transferring the processed data from ImageJ into Microsoft Excel, we had to set a size threshold to discriminate between single bacterial cells and bacterial colonies. In Expt I, single bacterial cells of up to 4 µm^2 and bacterial colonies (cluster of at least 4 cells) as small as 2.5 µm^2 were recorded by light microscopy. In contrast to the direct count in Expt I, it was difficult to distinguish between colony and single cells in the automatic image analysis in Expt IV. Therefore, to ensure a clear separation of single cells and colonies in Expt IV, we excluded the range with overlapping sizes in the CLSM data and assumed all spots ≤2.5 µm^2 to be single bacterial cells, and all spots >4 µm^2 to be bacterial colonies (i.e. we explicitly did not use signals of 2.5 to 4 µm^2 in further calculations for Expt IV).

Data/statistical analysis. Statistical analyses were performed using SPSS 15.0 for Microsoft Windows. For multiple comparisons, 1-, 2-factorial, and rmANOVAs (the latter for repeated measurements over time) were employed. Data were generally log-transformed to achieve homogenous variances. Abundance of nanoflagellates in the plankton as observed by the LAM method (Fig. 2A) was compared using a 1-factorial ANOVA with subsequent post-hoc test (Ryan, Einot, Gabriel & Welsch) for unplanned multiple comparison. Bacterial abundance obtained by DAPI counts (Fig. 2B) was also compared using a 1-factorial ANOVA. Since there was no significant group effect, a post-hoc test was not applied. For comparisons of single-cell bacteria or bacterial colony abundances in Expts I and IV (see Fig. 3C–F), 1-factorial ANOVAs were applied. If the ANOVA indicated significant effects, LSD test for planned pairwise comparison was used; differences between treatments on the same day were considered *a priori*. To show possible effects of time, treatment (grazer density) and their interaction on bacteria (Expts I and IV) (see Fig. 3D–F), 2-factorial ANOVAs were conducted with bacterial abundance (single cell or colony) as the dependent variable, and time and treatment as independent variables. Ciliate grazing effects on HF in Expts II and III were repeatedly recorded over a time period of 2 wk (Table 2, see Fig. 4). Here, we used rmANOVAs with HF abundance as the dependent variable, time as within-subject factor and treatment as between-subject factor. Due to the low HF abundances in the earlier succession stages (and consequently much lower variances compared to later succession stages; see Fig. 4C,D), the abundance data collected from Day 7 until the final day of the experiments was used for the calculation of the rmANOVA. Data for Expt II was log-transformed prior to the analysis; no such transformation was necessary for Expt III, in which the Student's *t*-test was employed for pairwise comparison of bacterial data (see Fig. 7).

To compare the HF communities in Expts II and III, the Bray-Curtis similarity was calculated using the software Primer (Version 6.1.5, PRIMER-E 2006). All data was log (x + 1)-transformed prior to the analysis to avoid overemphasizing the effects of dominant groups. Results were then displayed as a dendrogram based on the group average and significantly distinct clusters were indicated (see Fig. 6). Significance was calculated using the SIMPROF test (α = 0.05, Primer 6.1.5 software).

RESULTS

Top-down effects of HF on bacteria

In Expts I and IV, we tested the effects of HF on the natural Rhine bacteria assemblage by comparing the early stage of biofilm formation in 1.2 µm treatments with 8 µm and 5 µm treatments, respectively. High HF abundances were reached rapidly, with 3631 ± 852 cells cm^{-2} on Day 4 in the 8 µm treatment in Expt I (Fig. 3A) and 20 010 ± 3602 cells cm^{-2} on Day 3 in the 5 µm treatment in Expt IV (Fig. 3B). The 1.2 µm treatment had much lower HF abundances during the first few days; nevertheless, the experiments were terminated on Day 4 (Expt I) and Day 3 (Expt IV) after the HF showed a significant increase in the 1.2 µm treatment (462 ± 319 and 4209 ± 1147 cells cm^{-2} in Expts I and IV, respectively) (Fig. 3A,B).

The parameters tested for the bacteria were microcolony and single-cell abundances. Consistent patterns were detected for both parameters in both experiments despite the variation in absolute abundances. Microcolony abundance generally increased over time in all treatments in both experiments (Fig. 3C,D), although no microcolonies were detected on Day 1 in either treatment of Expt I. Bacterial colonies had been growing continuously in both treatments until the experiments were stopped on the 4th day, and their abundance was significantly enhanced in the 8 µm treatment (*t*-test: $p < 0.01$). In Expt IV, colonies were found on both test days (Days 1 and 3), and significantly more microcolonies were found in the treatment with enhanced grazer presence (5 µm filter). The average microcolony size did not differ between the treatments in either experiment and was ~10 µm^2 (data not shown).

The abundance of surface-attached single bacterial cells generally increased with time in both experiments and in all treatments, but showed a more differentiated reaction to HF abundance (Fig. 3E,F). On Day 1 of biofilm formation, significantly more single cells were found in treatments with more HF present

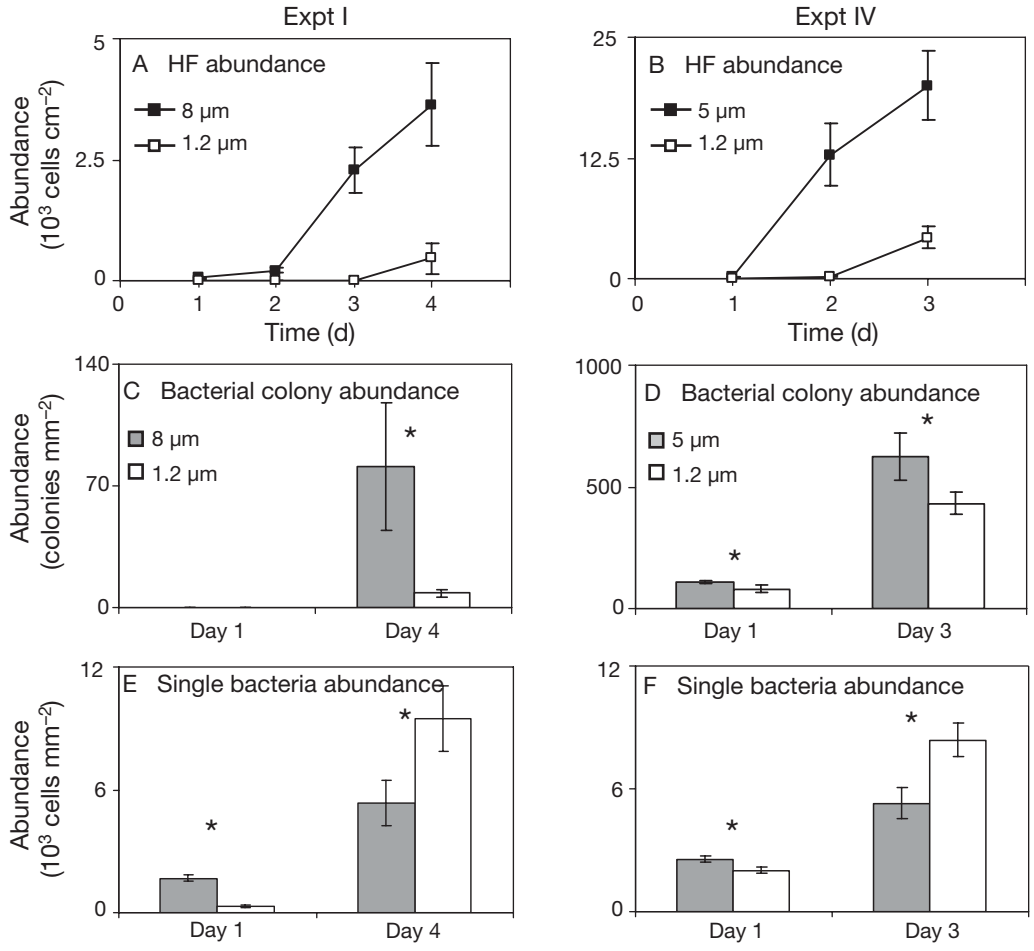

Fig. 3. Results of Expts I and IV on heterotrophic flagellate (HF) grazing effects on biofilm bacteria. (A) HF abundance during Expt I, (B) HF abundance during Expt IV, (C) bacterial colony abundance on Days 1 and 4 of Expt I, (D) bacterial colony abundance on Days 1 and 3 of Expt IV, (E) bacterial single-cell abundance on Days 1 and 4 of Expt I, and (F) bacterial single-cell abundance on Days 1 and 3 of Expt IV. Error bars represent SD. (*) indicates significant difference ($p < 0.05$) in a 1-factorial ANOVA with subsequent LSD test for planned pairwise comparisons. Table 3 shows results of 2-factorial ANOVAs testing for influences of Time (Days 1 and 3, respectively), Treatment (HF density) and interactions of these factors. In Fig. 3C, (*) indicates significant difference ($p < 0.01$) in a Student´s *t*-test

(8 and 5 µm treatments). In contrast, significantly more cells were detected in grazer-reduced treatments (1.2 µm) on the final days of both experiments.

As revealed by the 2-factorial ANOVAs and subsequent pairwise comparisons, these grazer-induced effects on both single cells in Expts I and IV, and on colonies in Expt IV were significant (Table 3). Furthermore, the ANOVAs revealed a significant interaction between treatment (HF density) and time for single cells in both experiments, giving further evidence of the differential grazer-induced effects on Day 1 (increased abundance at high grazer densities) and Day 3 (decreased abundance at high grazer densities). In contrast, no treatment × time interaction was found for colony abundance in Expt IV, showing consistently positive grazer effect on colony abundance.

Top-down effects of ciliates on HF

In Expts II and III, we tested the effects of ciliates on HF by comparing the 20 µm with the 5 µm treatments. The regular ciliate counts confirmed that no ciliates were present over the 2 wk duration of the experiments in the 5 µm treatments (Fig. 4A,B). In the 20 µm treatments, ciliate abundance increased continuously over the course of the experiments, with a final ciliate abundance of 96 ± 9 cells cm^{-2} on Day 15 in Expt II (Fig. 4A) and 84 ± 9 cells cm^{-2} on Day 14 in Expt III (Fig. 4B). At that time, the dominating ciliate groups in both experiments were the predatory haptorids (mainly *Litonotus* sp. and *Acineria* sp.) and the generally smaller, omnivorous cyrtophorids (mainly *Pseudochilodonopsis* sp., *Chilodonella uncinata* and *Thigmogaster* sp.) (Fig. 5A).

Table 3. Grazer (heterotrophic flagellates, HF) impact on biofilm bacteria (Expts I and IV). Results of 2-factorial ANOVAs testing for the influence of Time (Days 1 and 3/4), Treatment (HF density) and Time × Treatment on bacterial microcolony and single cell abundance (log-transformed). Colony abundance in Expt I (Fig. 3C) was compared using a *t*-test since no colonies were present on Day 1

	SS	df	*F*	p
Colony abundance, Expt IV (Fig. 3D)				
Time	2.1861	1	546.36	< 0.001
Treatment	0.0858	1	21.44	< 0.001
Time × Treatment	0.0003	1	0.09	= 0.764
Residuals	0.0480	12		
Single cell abundance, Expt I (Fig. 3E)				
Time	1.9537	1	170.85	< 0.001
Treatment	0.0953	1	8.34	= 0.013
Time × Treatment	0.4596	1	40.19	< 0.001
Residuals	0.1372	12		
Single cell abundance, Expt IV (Fig. 3F)				
Time	0.8620	1	484.23	< 0.001
Treatment	0.0091	1	5.16	= 0.042
Time × Treatment	0.0960	1	53.95	< 0.001
Residuals	0.0213	12		

In both experiments, the HF abundances showed a similar and almost parallel development in the 2 treatments, but were in general significantly less abundant when ciliates were present (Fig. 4C,D; see Table 4 for rmANOVA). The gap between the HF abundances of the 2 treatments increased in both experiments with increasing ciliate abundance during the succession, a finding which is supported by the significant interaction between time and treatment (Table 4).

During the final days of the experiments (Days 15/14), the sessile Chromulinales (chrysomonads, e.g. *Spumella* sp.), the Goniomonadales (exclusively *Goniomonas truncata*) and the Heteronematina (euglenids, e.g. *Petalomonas* sp.) increased their relative contribution to HF abundance in the presence of ciliates in both experiments (Fig. 5B). In contrast, *Ancyromonas* sp. (incertae sedis) completely disappeared in Expt II and almost completely disappeared in Expt III in the presence of ciliates. Other HF groups did not seem to be profoundly influenced by grazer presence in terms of their relative contribution to HF abundance. Together, the differences in the HF community structure between the treatments were supported by the cluster analysis based on Bray-Curtis similarities (Fig. 6) for the final days of both experiments (Days

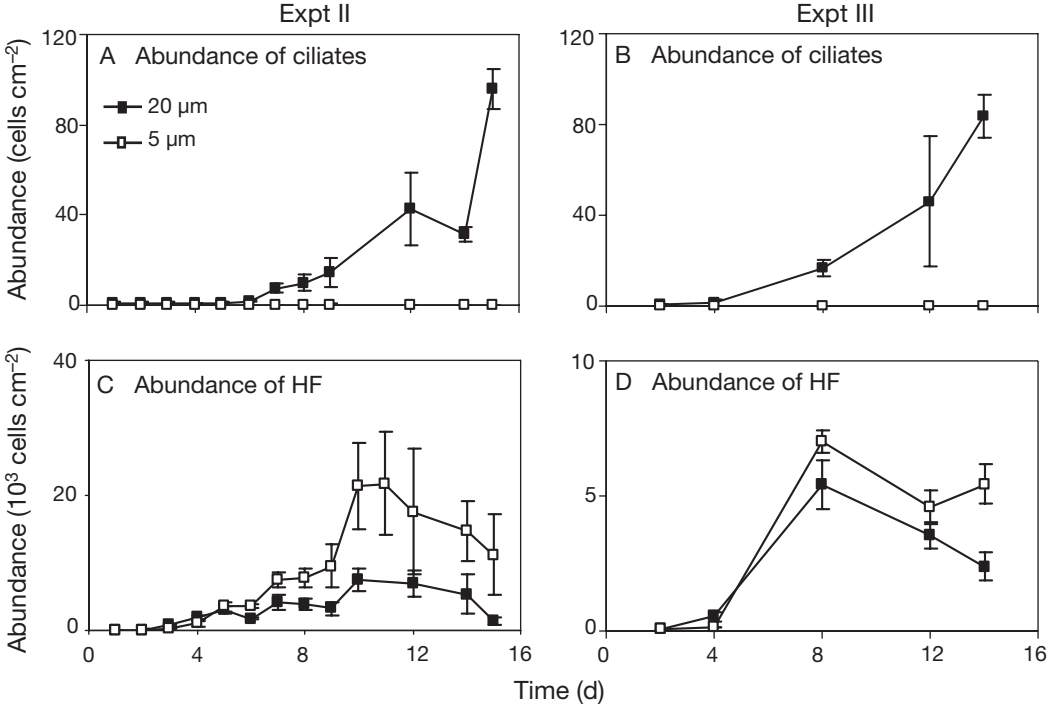

Fig. 4. Development of (A,B) ciliate and (C,D) heterotrophic flagellate (HF) abundance in (A,C) Expt II and (B,D) Expt III in 20 µm and 5 µm filtered treatments. Error bars represent SD. See Table 4 for ANOVAs of HF abundances

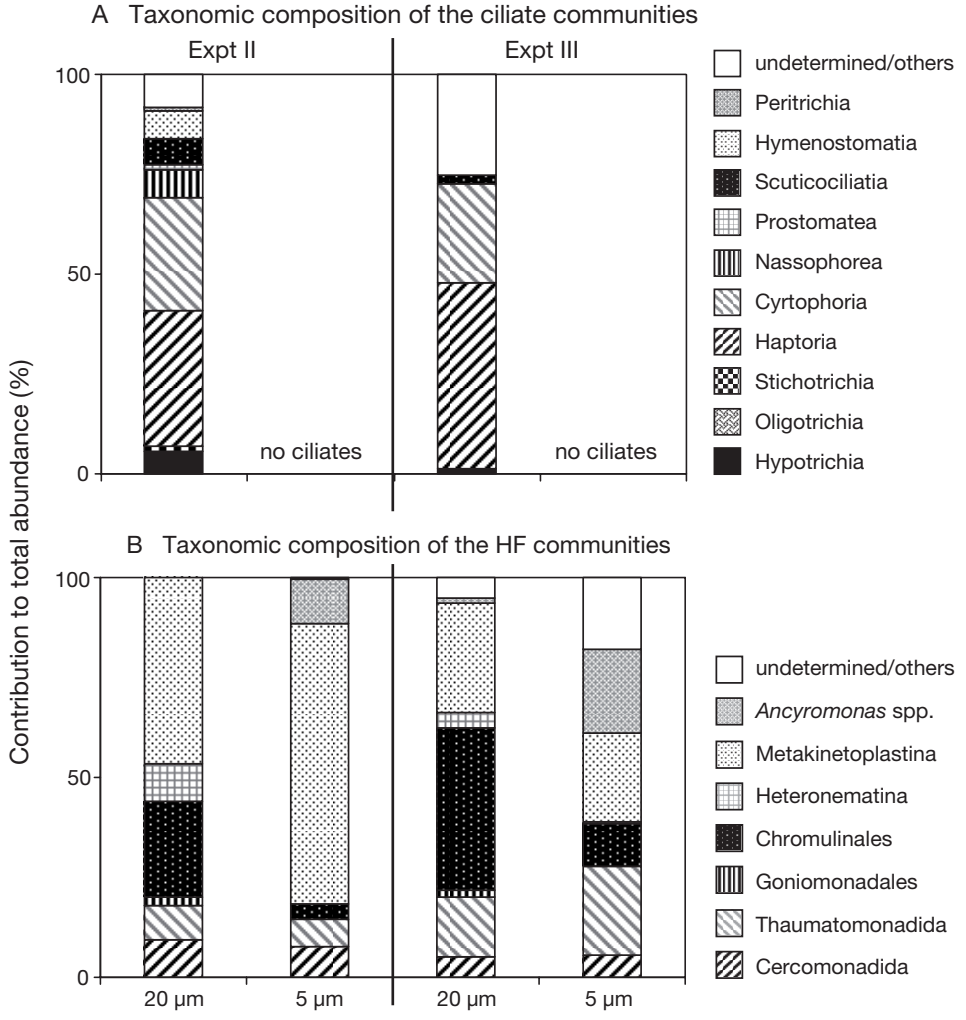

Fig. 5. Taxonomic composition (as relative contribution to total abundance) of (A) ciliate communities, and (B) heterotrophic flagellate (HF) communities in the 20 µm and 5 µm treatments on the final days of Expts II and III (Days 15/14). Oligotrichia abundances were too low to be visible in the figure

15/14): The 5 µm treatment replicates formed 1 significant cluster in both experiments and all but one 20 µm treatment replicate also formed 1 significant cluster. Cluster analysis based on the Jaccard index, which takes only presence/absence data into account, revealed the same significant clusters (data not shown).

Table 4. Results of rmANOVAs testing for the influence of Time, Treatment (ciliates present/absent) and the interaction of Time × Treatment on heterotrophic flagellate (HF) abundance. p-values are shown

Experiment	Between subjects	Within subjects	
	Treatment	Time	Time×Treatment
II (Fig. 4C)	= 0.002	<0.001	= 0.001
III (Fig. 4D)	<0.001	<0.001	= 0.041

Effects of ciliates on bacteria

Besides the effects of the presence or absence of ciliates on the HF community, we also tested HF-mediated cascade effects of ciliates on both bacterial single cells and bacterial colonies in Expt III. As shown above, the presence of ciliates strongly reduced the total HF abundance on the final day (Day 14) of the experiment (Fig. 4D), and clearly altered the HF community structure (Figs. 5B & 6B). However, this reduced HF abundance (at high ciliate abundances) had no effect (*t*-test: $p > 0.05$) on the abundance of bacterial colonies on Day 14 (Fig. 7A). In contrast, there was a significant treatment effect (*t*-test: $p < 0.05$) on the abundance of single bacterial cells, which were reduced in the 5 µm treatment at high HF abundances and in the absence of ciliates (Fig. 7B).

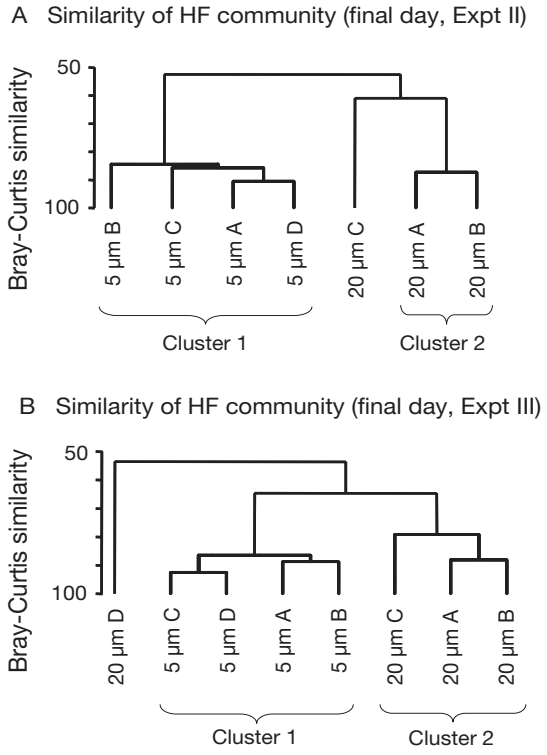

Fig. 6. Bray-Curtis similarity for heterotrophic flagellate (HF) communities on the final day of (A) Expt II, and (B) Expt III. There were 3 instead of 4 replicates for the 20 µm treatment of Expt II because the coverslip of one flow cell cracked during the experiment. Letters A–D indicate the different replicates for each treatment (20 µm or 5 µm filtered). The Bray-Curtis similarity was calculated from log (x + 1)-transformed abundance data of HF morphotypes. Significant ($p < 0.05$) clusters based on the SIMPROF-test are indicated

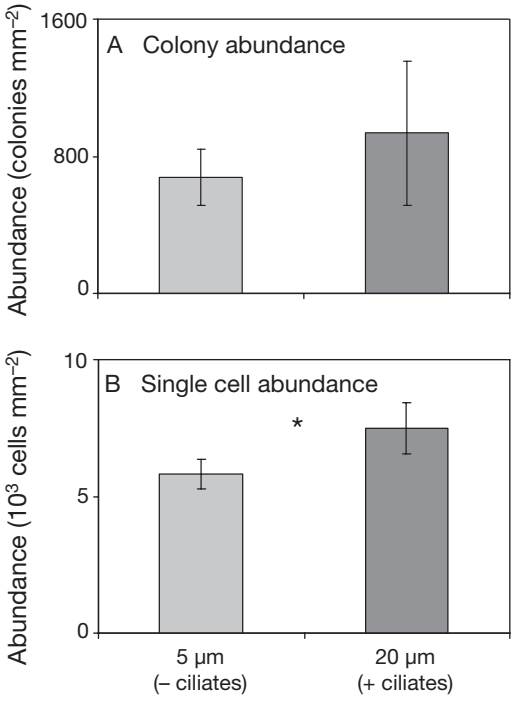

Fig. 7. Effects of ciliates on (A) bacterial colony abundance, and (B) bacterial single-cell abundance for the final day of Expt III (Day 14). Error bars represent SD. (*) indicates significant difference ($p < 0.05$) in a Student's t-test

DISCUSSION

Methodological remarks

This study is among the first few experimental studies to test grazer effects on bacterial biofilms under natural conditions. Before performing the experiments, we searched for alternative methods to test grazer effects within the biofilms. The application of eukaryotic inhibitors which were used for grazing experiments in the 1980s was rejected because of inhibitor effects on bacteria (e.g. Tremaine & Mills 1987). Furthermore, some protozoan taxa survive the application of inhibitors (e.g. Sanders & Porter 1986), which was also confirmed in trials with cycloheximide and colchicine as inhibitors (data not shown).

The present size fractionation method was thus seen as the best available method to test grazing effects on natural biofilms. Weaknesses include possible effects on the plankton community due to filtration. Such effects were reduced by the parallel application of filter cartridges (i.e. the 5 and 8 µm treatments were compared with the 1.2 µm treatments, and the 5 µm treatments with the 20 µm treatments). The methodological investigations revealed no effects either on bacterial size distribution or on HF abundance and taxonomic composition between treatments considered within 1 experiment. Furthermore, we did not detect any pore size effects (5 vs. 1.2 µm and 5 vs. 20 µm) on the abundance of suspended bacterial colonies in our DAPI counts (data not shown), which was expected because suspended planktonic bacteria in the highly turbulent Rhine usually occur as suspended single cells (Weitere & Arndt 2002b). Nevertheless, we cannot fully exclude slight filter effects on the colonies in intermediate size ranges below the detection level of our DAPI counts, as these could have passed through the larger of the 2 filter cartridges in any given experiment.

Top-down effects of HF on bacteria

In 2 independent experiments (Expts I and IV) conducted in different seasons (with different abundance of planktonic bacteria and HF) and different filter combinations (1.2 vs. 8 µm, and 1.2 vs. 5 µm), results consistently show that the presence of HF in semi-natural

biofilms results in (1) an enhanced number of bacterial microcolonies, (2) an enhanced number of bacterial single cells in the early stages of biofilm formation (Day 1), and (3) a reduction in the number of single bacterial cells in later stages of biofilm formation (Days 3 to 4), while bacterial microcolonies still grow.

The differences in the absolute HF and bacterial abundance between the experiments are probably due to seasonal differences in plankton abundance in October (Expt I) and at the end of April (Expt IV), since temperatures were the same in both experiments (Table 2). Abundances of planktonic HF are generally higher in April than in October (Weitere & Arndt 2002a). In contrast, the abundances of planktonic bacteria are much more stable throughout the year, and differences between October and April are usually small (Weitere & Arndt 2002b). Such differences in the abundance patterns of planktonic groups (which could colonize the biofilms) probably explain the different abundances of surface-attached HF and the similar abundances of the biofilm bacteria in Expts I and IV. However, there were large differences in the abundances of bacterial colonies between both experiments. It seems likely that the higher abundances of HF in Expt IV stimulated the formation of more bacterial colonies (see next paragraph). Nevertheless, such conclusions remain to be validated as the bacterial colonies were quantified using different methods in Expts I and IV.

The observed stimulation of surface-attached bacterial microcolonies can be interpreted as a grazer-defence strategy. This finding in the natural biofilm setting is in accordance with earlier studies in which the formation of grazing-resistant bacterial microcolonies in the presence of HF in the plankton was reported (Hahn & Höfle 1999, Šimek et al. 2001, Matz et al. 2002). Subsequent laboratory studies on single-species bacterial biofilms have further shown that the formation of surface-attached microcolonies and consequently of bacterial biofilms was stimulated by the presence of HF (Matz et al. 2004, Weitere et al. 2005, Queck et al. 2006).

The initial enhancement of the abundance of surface-attached single cells further shows that the pioneer stage of biofilm formation, i.e. the attachment of planktonic cells to the surface, is also stimulated by the presence of grazers. Thus, not only microcolony formation but also the total number of bacteria contributing to biofilm formation are promoted by the presence of HF. The cellular basis of mechanisms leading to the grazer-induced enhancement of surface colonization and microcolony formation are, to our knowledge, still unknown. However, our data show the significance of grazers in the formation of biofilms in complex natural communities.

In older biofilms, the number of microcolonies was still enhanced by high grazer densities in both Expts I and IV, while the abundance of single bacterial cells was negatively affected by high HF abundances. This suggests that surface association alone does not fully protect bacteria from HF predation (see also Zubkov & Sleigh 1999, Boenigk & Arndt 2000a for HF grazing on surface-attached bacteria). Rather, the surface attachment of single planktonic cells is a necessary step towards the formation of matrix-embedded microcolonies which are better able to protect bacteria from grazing (cf. Matz & Kjelleberg 2005). On the other hand, the reduction of single bacterial cells might also be due to the stimulated formation of colonies and thus the incorporation of single cells into colonies. Both mechanisms (grazing and colony stimulation) probably contribute to the observed reduction of single bacterial cells in later biofilm stages.

The contrasting effects on single bacterial cells at the early (Day 1) and later (Day 3) biofilm stages also suggest that grazing pressure on surface-attached bacteria increases with biofilm age. Our taxonomic monitoring (data not shown) revealed that dominant early colonizers were typical suspension feeders such as the chrysomonad *Spumella* sp. (Boenigk & Arndt 2000b). Surface-associated gliding HF, such as the surface-feeding *Rhynchomonas nasuta* (Boenigk & Arndt 2000a), became increasingly abundant after the first day of colonization. We interpret this as an adaptation of grazer feeding mode to the succession of the bacterial prey. Weitere et al. (2005) also demonstrated different effects of early versus late biofilm colonizing grazers in single-species biofilms.

This study has shown that, similar to the situation in the plankton, HF are able to significantly decrease bacterial abundance and stimulate microcolony formation in semi-natural biofilms. Nevertheless, the semi-natural grazer community, which consisted of various species with different food preferences, was not able to reduce the biofilm bacteria within microcolonies. This agrees with the results of other field studies, such as that of Epstein et al. (1992) for a sandy tidal flat, Wieltschnig et al. (2003) for the sediment of an oxbow lake, and Esser (2006) for Rhine biofilms: all 3 studies concluded that HF were not numerous enough to control the bacterial population. Thus, due to their ability to form grazing-resistant morphotypes, bacteria in biofilms are able to establish large biomasses despite the presence of flagellated grazers.

Top-down effects of ciliates on HF

The presence of ciliates in the biofilm clearly reduced the abundance of HF and altered their community structure. Thus, the results show that ciliates significantly interact with biofilm-dwelling HF in addi-

tion to their previously demonstrated effect on plankton through grazing (Weitere et al. 2003).

One of the innovations of the present study is the use of complex, semi-natural biofilm communities for experimental manipulations. The protozoans were in fact composed of various taxonomic groups including many species and functional groups. The ciliate community contained picophagous (which feed on bacteria, e.g. scuticociliates and hymenostomates) as well as nano- and microphagous (which feed on other protists, e.g. haptorids, cyrtophorids) groups; thus, the ciliates could potentially affect the HF as predators and/or as competitors for food. There are several indications, however, that predation plays an important role in explaining the demonstrated ciliate-induced effects on the biofilm-dwelling HF. It is known from studies on planktonic and laboratory-grown communities that some ciliates prey preferably on HF (e.g. Cleven 1996, Jürgens et al. 1996, Premke & Arndt 2000).

On the final days of Expts II and III, the ciliate community was clearly dominated by the predatorial haptorids (Foissner et al. 1995) and the omnivorous cyrtophorids (Foissner et al. 1991), which both at least partly feed on HF. Video-supported observations of ciliates in the same flow cell system as that used in the present study revealed several HF-consuming ciliate groups within the semi-natural biofilms (A. Walterscheid unpubl. data). Among them were selective grazers such as *Thigmogaster* sp. (a cyrtophorid also common in our experiments), which preyed on gliding HF and particularly on *Ancyromonas* sp. Such selective grazing probably explains the disappearance of *Ancyromonas* sp. under the presence of ciliates as was observed here. Selective grazing of ciliates on HF has also been reported in other studies on natural and experimental mixtures of prey organisms (Epstein et al. 1992, Pfister & Arndt 1998). It is important to note that *Ancyromonas* sp. was among the small HF and was present in the early biofilm formation of both treatments (5 and 20 µm) in similar densities. The effect on *Ancyromonas* sp. in the later stages of biofilm formation under the presence of ciliates is thus not a filter effect.

In our experiments, Chromulinales (a grouping which roughly corresponds to the former group 'chrysomonads'), Heteronematina (euglenids) and Goniomonadales were generally promoted by the presence of ciliates. Chromulinales (mostly comprised of the colony-forming species *Anthophysa vegetans*) are probably not as easily reached as they are firmly substrate-associated prey due to their plasma threads, and are therefore avoided by the ciliates. Heteronematina and Goniomonadales could have been avoided due to their relatively large size or could have benefited from the reduction of competitors. However, results on the Heteronematina are tentative, as large specimens could have been excluded by the filtration procedure.

The total abundance of ciliated grazers in our study was relatively low compared to findings of other studies performed under similar conditions (same flow cells fed with unfiltered Rhine water; Norf et al. 2007). The low abundances are mostly due to the under-representation of sessile ciliates such as *Strobilidium caudatum* and peritrichs, while the vagile ciliates occurred in comparable abundances. Sessile ciliates (as well as other larger cilates) were most likely excluded by the 20 µm filtration, as they occurred in significant numbers in a different but concurrent experiment with untreated Rhine water in the same laboratory (H. Norf pers. obs.). These sessile ciliates are usually abundant in biofilms (e.g. Gong et al. 2005), but their impact on biofilm-dwelling HF is probably low as they are believed to feed primarily on plankton (Foissner et al. 1991, 1992, Parry 2004). However, it is possible that we have underestimated the ciliate-induced effects on HF in natural biofilm communities because of the relatively low ciliate abundances observed here.

Effects of ciliates on bacteria

Our data also demonstrated that the presence of ciliates can positively affect single-cell bacteria in the biofilm. This phenomenon could be due to a HF-mediated trophic cascade. In the presence of ciliates, HF abundances were reduced, subsequently enhancing the abundance of bacterial single cells. Trophic cascades (including HF-mediated cascades) have often been reported from planktonic systems (e.g. Jürgens et al. 1994, Zöllner et al. 2003). Evidence for the existence of HF-mediated trophic cascades in benthic environments is scarce (but see Epstein et al. 1992). This is probably partly due to an overlap in feeding preferences among organism groups within the benthos, such as the grazing of bacteria by both biofilm-dwelling HF and ciliates (see last subsection). Thus, it was unclear in our experiments whether ciliates further reduced biofilm bacteria or enhanced bacterial abundance due to their grazing on HF. Even though bacterivorous, benthic-feeding ciliates were present (see last subsection), the positive net effect of ciliates on the biofilm-dwelling, single-cell bacteria suggests a stronger cascade effect than a direct predation effect of the ciliates. However, this conclusion was drawn from a single experiment and should be further studied.

In contrast to that of single bacterial cells, the number of bacterial microcolonies which were enhanced in the presence of HF in Expts I and IV (see subsection

'Top-down effects of HF on bacteria'), did not differ between the treatments of Expt III. This is not surprising because both ciliated and flagellated grazers have been shown to induce microcolony and biofilm formation in laboratory studies (Weitere et al. 2005).

CONCLUSIONS

The results of the present study show some of the complex mechanisms behind the dynamics of the early formation of natural biofilms. Methodological difficulties facing scientists when dealing with biofilms may partly explain why these issues (particularly protozoan-mediated top-down effects) have thus far been barely studied in natural biofilms. Here, we established a new tool: the combination of filter cartridges, direct observations and CLSM imaging of bacterial biofilms in flow cells to show that biofilm-dwelling HF can act as a significant trophic link between surface-attached bacteria and ciliates. Both trophic interactions (bacteria with HF as well as HF with ciliates) were shown to be highly selective in terms of the effects on single bacterial cells versus microcolonies, and on specific HF groups. However, as demonstrated earlier for mono-grazer laboratory experiments, the complex semi-natural HF communities stimulated the formation of surface-attached bacterial microcolonies rather than reduced them. The HF-mediated carbon transfer within biofilms thus seems to be limited to certain bacterial morphotypes.

Acknowledgements. This study was funded by a grant from the German Research Foundation (DFG) to M.W. and H.A. We thank M. Kathol for help with the DAPI counts, B. Gräfe for assistance with the CLSM and for helpful comments on the processing of the CLSM data, and F. Bartlett for critical comments on the manuscript.

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Editorial responsibility: Staffan Kjelleberg, Sydney, New South Wales, Australia

Submitted: February 12, 2008; Accepted: July 9, 2008
Proofs received from author(s): September 7, 2008