Control of heterotrophic biofilm communities – The importance of grazing and dispersal

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The end of the century
I said my goodbyes
For what it's worth
I always aimed to please
But I nearly died

For what it's worth
Come on lay with me
'Cause I'm on fire
For what it's worth
I tear the sun in three
To light up your eyes

For what it's worth
…

Placebo

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Und nun:

…
Let me take you for a ride
With the devil in the details
…

Placebo

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Zusammenfassung

Bakterien, Algen, Protisten und kleinere Mehrzeller sind integraler Bestandteil von natürlichen Biofilmen und tragen vor allem in flachen Fließgewässern erheblich zum Stoffumsatz und zu weiteren ökologischen Prozessen bei. Wie die Zusammensetzung und Funktion dieser Gemeinschaft reguliert wird, ist jedoch größtenteils ungeklärt und Annahmen darüber stützen sich mehrheitlich auf Ergebnisse aus der Plankton-Forschung oder aus Laborversuchen mit kultivierbaren Arten. Ein Grund hierfür ist die schlechte methodische Zugänglichkeit von Biofilmen, die als substratassoziierte und dreidimensional strukturierte Gemeinschaften nur schwer in vollem Umfang zu untersuchen sind. In der vorliegenden Arbeit wurden von Flusswasser aus dem Rhein (Köln, Deutschland) durchflossene Mikrokosmen (Fließzellen) genutzt und methodisch weiterentwickelt, um die Regulation von Ciliaten-, heterotrophen Flagellaten- und Bakterien-Gemeinschaften in Biofilmen unter natürlichen Hintergrundbedingungen zu untersuchen.

In dem ersten Kapitel wird zunächst eine neue Methode vorgestellt, bei der durch eine Fraktionierung des Rheinwassers mittels Filterkartuschen eine Unterteilung des Planktons in verschiedene Größenklassen erreicht wurde. Diese zeigen eine gute Übereinstimmung mit funktionellen Gilden (Bakterien, heterotrophen Flagellaten (HF) und Ciliaten). Aus diesem größenfraktionierten Plankton wurden dann in Fließzellen Biofilme etabliert, was es ermöglichte, den quantitativen Einfluss von (1) Ciliaten auf HF, (2) Ciliaten auf Bakterien und (3) HF auf Bakterien getrennt voneinander zu untersuchen. Durch die Möglichkeit, die Protozoen mittels Lichtmikroskopie in den Fließzellen *in vivo* zu bestimmen, konnte außerdem der Einfluss von Ciliaten auf die Struktur der HF-Gemeinschaft analysiert werden. Auswirkungen auf den bakteriellen Biofilm wurden mithilfe von konfokaler Laser-Scanning-Mikroskopie (CLSM) in fixierten Biofilmen erfasst. Im Ergebnis konnte gezeigt werden, dass HF die Besiedlung des Substrats durch Bakterien zunächst deutlich fördern. In späteren Stadien wurde eine weitere Stimulation bakterieller Mikrokolonien durch HF nachgewiesen, während einzelne, womöglich noch nicht fest assoziierte Bakterienzellen dezimiert wurden. Insbesondere die Förderung bakterieller Mikrokolonien wurde als Fraß-Schutzmechanismus der Bakterien interpretiert und war so vorher nur aus

Laborstudien mit Modellorganismen bekannt. Die Anwesenheit von Ciliaten hatte einen massiven Einfluss auf die HF und führte zu einer Verschiebung der Gemeinschaftsstruktur hin zu planktivoren, sessilen HF. Als Folge wurden einzelne Bakterienzellen vom Frassdruck entlastet. Damit konnte zum ersten Mal für natürliche Biofilmgemeinschaften eine trophische Kaskade (Ciliaten [+] → HF [–] → Bakterien [+]) nachgewiesen werden.

Im zweiten Teil dieser Arbeit wurde der experimentelle Ansatz um molekularbiologische Methoden (denaturierende Gradientengelelektrophorese (DGGE), Einzelstrang-Konformationspolymorphismus-Analyse (SSCP)) erweitert, um zusätzlich die qualitativen Auswirkungen von Protozoen auf biofilmassoziierte Bakterien erfassen zu können. Dies erforderte eine Anpassung des Versuchsaufbaus um die gleichzeitige Erfassung der Protozoen (mittels Lichtmikroskopie), der bakteriellen Morphologie (CLSM) und der bakteriellen Diversität (DGGE, SSCP) in einer einzigen Fließzelle zu ermöglichen. Die Ergebnisse der in Teil 2 beschriebenen Experimente zeigten, dass die Anwesenheit von HF unabhängig von dem Einfluss auf die Morphologie des bakteriellen Biofilms zu einer veränderten bakteriellen Diversität und einer höheren Artenzahl führte. Dieser Effekt wurde insbesondere in späteren Sukzessionsstadien deutlich und ist in sofern ungewöhnlich, als dass in planktischen Gemeinschaften bisher eine starke Kopplung von morphologischer und taxonomischer Diversität in der bakteriellen Gemeinschaft beobachtet wurde. In einem weiteren Experiment wurde durch nano- und mikrophage Ciliaten, wie bereits in Teil 1 beobachtet, eine Verschiebung der HF-Gemeinschaftsstruktur hin zu sessilen Arten beobachtet. Im Gegensatz zu früheren Versuchen konnte zusätzlich ein direkter Einfluss der Ciliaten auf den bakteriellen Biofilm gezeigt werden. Dabei wurde die bakterielle Diversität und phylogenetische Struktur gegenüber Ciliaten-freien Ansätzen verändert und es wurden deutlich mehr Mikrokolonien bei verminderter bakterieller Artenzahl beobachtet. Die unterschiedlichen saisonalen Hintergrundbedingungen sind als Gründe für die Beobachtung von direkten Effekten anstelle einer trophischen Kaskade anzunehmen. Insbesondere die Artzusammensetzung (mehr bakterivore/ benthivore Ciliaten-Arten im Winter) und das Temperaturregime (veränderte Aktivität von Protozoen und Bakterien) können hier ausschlaggebend sein.

Der dritte Teil dieser Arbeit baute schließlich auf der Theorie auf, dass nicht nur lokale, sondern auch regionale Faktoren wie Immi- und Emigration eine große Rolle in der Regulation von Biofilm-Gemeinschaften spielen. Der Versuchsaufbau wurde erneut dem veränderten theoretischen Gerüst angepasst, um eine Manipulation der HF- oder Ciliaten-Dichten im Plankton zu erlauben und somit den Einfluss verschiedener Immigrationspotentiale auf die HF- oder Ciliaten-Gemeinschaft im Biofilm zu erfassen. Hierbei zeigte sich zunächst ein erstaunlich geringer Einfluss der Planktondichte auf die Regulation ausgereifter HF-Gemeinschaften; im Anschluss an die Manipulation beobachtete (teils erhebliche) Änderungen in der taxonomischen Zusammensetzung, Diversität und Abundanz erfolgten selbst über längere Zeiträume unabhängig vom Immigrationspotential. Für Ciliaten-Gemeinschaften konnten ähnliche Ergebnisse erzielt werden, auch wenn hier eine Art (*Strobilidium caudatum*) gefunden wurde, die deutlich von Immigration abhängig war. Beide Experimente ließen somit auf größtenteils lokal regulierte Protozoen-Gemeinschaften schließen. Immigration spielt jedoch in der Anfangsphase der Besiedlung eine große Rolle; dies wurde in einem abschließenden Experiment überprüft. Hier wurde die anfängliche Besiedlung der Fließzellen durch HF unter verschiedenen Immigrationspotentialen untersucht. In einer Kreuzmanipulation wurde dabei auch der Einfluss der lokalen Nährstoffkonzentration (Fütterung durch suspendierte Bakterien) erfasst. Es zeigte sich eine lineare Zunahme der Besiedlungsgeschwindigkeit und Dichte mit dem Immigrationspotential und eine nachfolgende dichteregulierte Steigerung der Abundanz in der exponentiellen Phase der Besiedlung. Eine Abhängigkeit von der lokalen Ressourcenverfügbarkeit zeigte sich hingegen erst mit zunehmender Sukzession. Immigration spielt somit hauptsächlich bei der Neubesiedlung eines Substrats eine Rolle, während etablierte Gemeinschaften eher intrinsisch reguliert werden.

Zusammenfassend zeigt die vorliegende Arbeit, dass sowohl Ciliaten- und HF-, als auch Bakteriengemeinschaften in natürlichen Biofilmen stark von lokalen Faktoren (Prädation, Konkurrenz, Ressourcenverfügbarkeit) geprägt werden. Diese lokalen Interaktionen können von weiteren Faktoren beeinflusst werden: Immigration wird hierbei besonders wichtig, wenn ein Substrat neu besiedelt wird (vorstellbar z. B. nach einer Störung durch Geschiebetrieb oder Abweiden des Biofilms durch Makroinvertebraten), während die Effekte von Fraß durch Protozoen sowohl vom

Sukzessionstadium der Gemeinschaft als auch von saisonalen Bedingungen beeinflusst werden. Wichtig ist ebenfalls die Ebene (z. B. morphologische oder taxonomische Diversität, Artenanzahl, Artenidentität, Abundanz), auf der die Auswirkung des jeweiligen Faktors betrachtet wird. Die in dieser Arbeit entwickelte Methode kann je nach individueller Fragestellung angepasst werden und birgt daher ein vielfältiges Erweiterungspotential zur weiteren Untersuchung von Steuerungsmechanismen naturnaher Biofilme.

Abstract

Biofilms are essential for the function of many natural ecosystems such as streams and rivers, but what governs their community composition (and thus potential ecosystem services) remains to a large extent elusive. Theories are mainly based on laboratory systems with few cultivable taxa, or on results from studies of planktonic communities. This is in part due to the methodological challenge to study substrate-associated microbial communities in their natural, complex and three-dimensional environment. In this study, the development and expansion of a set-up to test different factors potentially controlling such communities is described. It basically consisted of a river bypass, flow cells, and filter cartridges to allow for the fractionation of the community establishing the biofilm into different size classes and corresponding functional guilds (ciliates, heterotrophic flagellates (HF) and bacteria). First quantitative experiments using this set-up, described in chapter 1, tested the effects of protozoan grazing on the biofilm structure. It was shown that HF initially promote bacterial biofilm formation, but are able to graze on single cells in later successional stages. Ciliates in turn massively influenced the HF community, leading to a switch from benthivorous to planktivorous life forms and a subsequent release of single bacterial cells from grazing pressure (trophic cascade). After extending the set-up and incorporating molecular techniques to also analyse the diversity of the bacterial biofilms (chapter 2), it became obvious that the above described effects can vary depending on background factors like seasonal variations in temperature. Furthermore, it could be shown that both HF and ciliates influence bacterial diversity, and that this effect can be independent of morphological changes in the biofilm. In addition, HF generally increased the number of bacterial phylotypes, while ciliates tended to reduce them. In chapter 3, the set-up was again modified and this time applied to test the influence of different immigration potentials on HF and ciliate communities. In already established HF and ciliate communities, strongly reduced immigration possibilities from the plankton left all but one ciliate taxa uninfluenced. In just establishing HF communities, however, reduced immigration potential resulted in slower colonization of the substrate, but was followed by higher growth rates compensating for the slower colonization. After reaching the equilibrium abundance, the HF community did not longer depend on

immigration but on local resource availability. Together, this study shows that natural biofilm communities are controlled by local factors like grazing, nutrients and competition, but also that regional factors like dispersal and abiotic and seasonal factors might alter the resulting effects depending on the successional stage or community composition. Generally, it was shown that conclusions from laboratory or planktonic studies are not always transferable to natural biofilm communities, thus testing ecological theories under natural background conditions remains crucial to understand the factors shaping them. The presented method is an important and further adaptable tool to achieve this goal.

General Introduction

Algae, protists and small metazoans are an integral part of natural biofilms (Wetzel, 2001), where they occur in much higher densities than in the plankton (Fischer and Pusch, 2001; Arndt et al., 2003; Parry, 2004). Similarly, the major proportion of bacterial biomass, activity and function can be represented in biofilms in both natural (e.g. rivers, lakes and oceans) and artificial environments (e.g. waste water treatment plants; Fischer and Pusch, 2001; Battin et al., 2003; Hall-Stoodley et al., 2004).

Still, the interest in biofilms thus far mainly concentrated on their role as a nuisance in industrial and medical facilities, where their enhanced stress-resistance in relation to planktonic bacteria can cause serious problems (e.g. clogging of pipes, biofouling, spreading of pathogens; reviewed in Hall-Stoodley et al., 2004). In recent years, the study of biofilms in natural environments has gained increasing attention. Studies of bacterial communities have mainly focused on the effects of water quality (attributed to human impact, different streams, or different habitats within a stream) on bacterial community composition (BCC; e.g. Olapade and Leff, 2004; Lear and Lewis, 2009; Lyautey et al., 2010). Other studies considered the influence of physical properties like flow (Besemer et al., 2009) or sediment particle size (Jackson and Weeks, 2008). Studies on the eukaryotic compartments of the biofilm community are less common. Here, the primary producers are the best investigated group with a main focus on their role as an autochthonous carbon source for bacteria (e.g. Augspurger and Kusel, 2010) and as a food source for grazing macrozoobenthos (e.g. Hillebrand et al., 2002). Only few studies consider fungi (mostly in the context of leaf degradation, e.g. Fischer et al., 2009), or protists (e.g. as colonizers of leafs (Franco et al., 1998), or in the context of global warming and resource control (Norf and Weitere, 2010)).

There is a considerable lack of studies investigating the interactions and trophic links between these groups, although the close proximity and high abundances of organisms in natural biofilms results in an interaction potential exceeding that of microbial planktonic communities, where microbial interactions have been shown to be of great importance for e.g. nutrient cycling (Meyer, 1994; Azam, 1998). This lack of studies of benthic microbial consortia is in part due to methodological constrains. However, the application of miniature flow cells (e.g. Stoodley et al., 1999) and

sensitive microscopic methods like Confocal Laser Scanning Microscopy (CLSM; Klausen et al., 2003) made it possible to study attached communities (or, more frequently, populations) in controlled laboratory environments without destroying their three-dimensional structure. Such experiments are restricted to a limited number of (cultivable) species. In recent years, Esser (2006) and Norf et al. (2007) amended the original miniature flow cells to examine multi-species, natural biofilms in a river bypass system. This system was set up onboard the Ecological Rhine Station of the University of Cologne, a boat anchored in the main flow of this large river in Cologne, where Rhine water is permanently provided.

The goal of this thesis was to analyse two understudied factors presumably controlling the species composition and abundance in natural biofilms: trophic interactions within the biofilm community, and the importance of dispersal via connections to the plankton. It was a challenge to achieve this goal under natural background conditions because it was necessary to expand the bypass/flow cell method to be able (1) to separate the focus groups of this study (ciliates, heterotrophic flagellates and bacteria) to perform exclusion experiments assessing trophic interactions and the influence of dispersal, and (2) to integrate the different microscopical and molecular techniques necessary for the analyses of the different trophic levels into the set-up. This study is separated into three chapters illustrating the systematic expansion of the basic set-up and its application to gain novel insights into the mechanisms controlling natural biofilm communities.

Interactions within the biofilm community – The influence of grazing

Grazing is one of the most important factors controlling natural bacterial communities (Jürgens and Matz, 2002; Matz and Kjelleberg, 2005). In comparison to planktonic communities, biofilms possess certain properties that might alter the strength and connectivity of food web pathways, and may thus in turn change the propagating effects of consumers and resources (Fitter and Hillebrand, 2009). Laboratory experiments describe some of these properties: For bacteria, the access to nutrients might be hindered by e.g. boundary layer effects or shear stress leading to aggregation (Rickard et al., 2004). Additionally, the three-dimensional (stratified) architecture leads to nutrient gradients (Bishop, 1997), but nutrient availability could as well be elevated

by the encoating in extrapolymeric substances (EPS) and by recycling effects (Flemming and Wingender, 2010). For biofilm associated protists, feeding on bacteria might be difficult because of grazer defence strategies such as microcolony formation (Weitere et al., 2005), co-aggregation (Rickard et al., 2003), or targeted chemical defence (Matz et al., 2008). On the other hand the high abundance of bacteria and their close proximity to one another lead to a higher contact probability of predators and prey. From the feeding types present it can be concluded that ciliates also exert a significant grazing pressure on HF (Premke and Arndt, 2000; Parry, 2004). However, the magnitude of this inter-protistan grazing pressure in natural biofilms and its effects on the taxonomic structure and abundance of HF is still unknown. As shown by Jürgens et al. (1994) and Zöllner et al. (2003) for planktonic communities, a strong grazing pressure on HF could indirectly also effect bacteria through a trophic cascade.

Chapter 1 concentrates on the establishment of a filtration method to separate different trophic levels in the potamoplankton, and subsequently in the biofilm communities established from this water in flow cells. By successfully applying this new method, both protozoan effects on the morphological structure of biofilm bacteria as well as cascading effects within the biofilm food web were analysed for the first time under natural background conditions.

Interactions within the biofilm community – The influence of grazing, fine-tuned

For bacteria, a well-established model of biofilm formation and succession exists: the settlement of a single cell (with at first loose substrate association) is followed by robust adhesion, aggregation into microcolonies, and growth and maturation into complex, three-dimensional and matrix-embedded biofilms (O'Toole et al., 2000; Hall-Stoodley et al., 2004). In natural communities, the close proximity to other bacterial species additionally results in distinct taxonomical succession patterns in the early period of attachment to an empty substrate (Jackson et al., 2001), but also over seasons (Olapade and Leff, 2004; Lyautey et al., 2005). The different species comprising these distinct succession stages also exhibit different traits, e.g. grazer defence mechanisms (as shown for planktonic communities by Salcher et al., 2005), which may in turn alter the influence of grazers on bacteria. Consequently, and as an addition to the experiments described in chapter 1, the goal of the experiments

described in chapter 2 was to establish a method to concurrently examine the effects of protists on bacterial morphology, abundance and community composition.

The species composition of a complex natural bacterial assemblage, e.g. in a river biofilm, cannot be assessed by microscopic techniques, but the use of molecular fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), Single Stranded Conformation Polymorphism (SSCP), or Terminal Restriction Fragment Length Polymorphism (T-RFLP; reviewed in Kirk et al., 2004) were shown to be effective tools to assess relative changes in bacterial diversity. It was a challenge to integrate such molecular techniques into the already developed flow cell/filtration system onboard the Ecological Rhine Station.

Chapter 2 describes the adaptation of the original set-up to study the effects of external (grazing) vs. internal (succession) control mechanisms on the diversity of riverine bacterial biofilms by DGGE and SSCP. Additionally, the results were related to morphological changes of the bacterial community (analysed by CLSM).

Interactions with the metacommunity – The influence of dispersal

Battin and co-workers (2007) called for the increased employment and rigorous testing of ecological principles and theories by microbial ecologists. One such theory is the metacommunity concept, which states that the species composition of a local community is not only affected by interactions on the local scale, but also by the regional species pool (the metacommunity), to which it is connected via dispersal (immigration and emigration; Leibold et al., 2004). Primarily, four paradigms within this framework are discussed: (1) The patch-dynamics perspective assumes that patches are identical and local species diversity is limited by dispersal and dominated by local extinction and colonization. (2) The species-sorting perspective states that patch quality and dispersal jointly affect the local community composition, but that dispersal is only a way of tracking resource gradients. (3) The mass-effect perspective emphasizes the role of spatial dynamics (immigration and emigration) to counteract local competitive exclusion. (4) The neutral perspective assumes that all species are equal in their competitive ability, movement and fitness, and that species diversity is solely the outcome of random species loss and gain.

In natural biofilms, the mass-effect perspective seems to be the model best relating to certain properties of HF and ciliates: Fast colonization of substrates from the plankton (Arndt et al., 2003), gliding life forms that can potentially detach and reattach to the substrate at any time (Parry, 2004), as well as more firmly attached species forming swarmer cells (Lee et al., 2000). Additionally, the influence of dispersal might be especially high in riverine systems, where flow is constantly bringing plankton organisms in close proximity to any submerged substrate. A reduced immigration potential should consequently lead to competitive exclusion of certain protozoan taxa and reduced biofilm diversity.

Chapter 3 describes the adaptation of the filtration method illustrated in chapter 1 to test the theory of highly dispersal regulated biofilm communities. HF and ciliates were chosen as model groups for this task. By manipulating plankton densities, the influence of different immigration potentials was tested (1) on established communities of HF and ciliates, and (2) on the succession of HF communities with natural and enhanced resource levels (as a local factor potentially interacting with the regionally spaced one).

Chapter 1 – Effects of protozoan grazing within river biofilms under semi-natural conditions

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.

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). However, 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.

Experimental Procedures

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 constant 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.

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

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.

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

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) 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

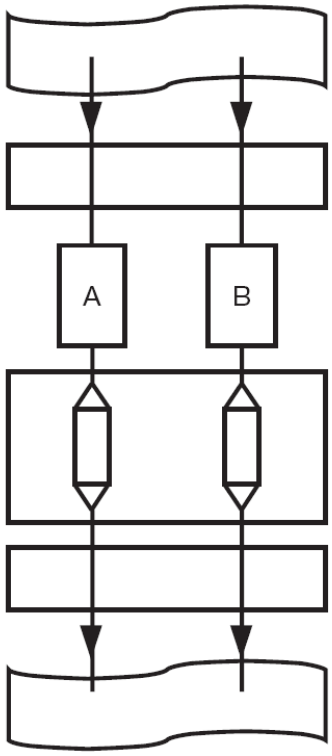

Fig. 1. Experimental set-up. Rhine water was pumped via impeller pumps through a pre-filter (30 to 100 µm mesh size) and filter cartridges (1.2, 5, 8 or 20 µm pore size) into flow cells situated in a water basin held at the actual Rhine temperature. Each treatment was set up in 4 replicates. Water was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

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.

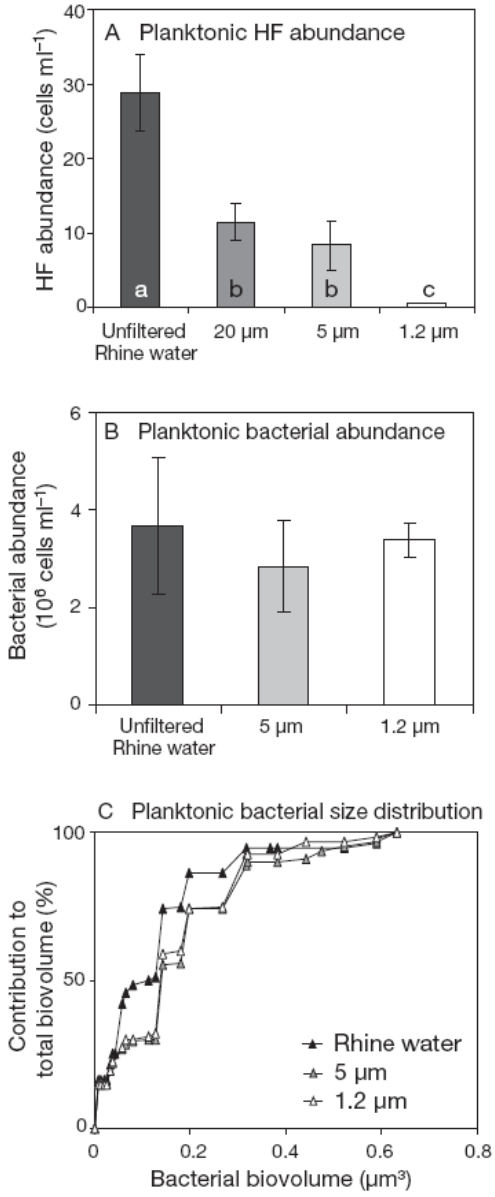

Fig. 2. Tests of filter permeability for suspended heterotrophic flagellates (HF) and bacteria. (A) Test for HF permeability compared with unfiltered Rhine water, as quantified by the liquid aliquot method (LAM). Error bars represent SD. Different letters indicate significant differences in a 1-factorial 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.

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 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

µm² to be single bacterial cells, and all spots >4 µm² to be bacterial colonies (i.e. we explicitly did not use signals of 2.5 to 4 µm² 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 ($\alpha = 0.05$, 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 20010 ± 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 approximately 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 (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.

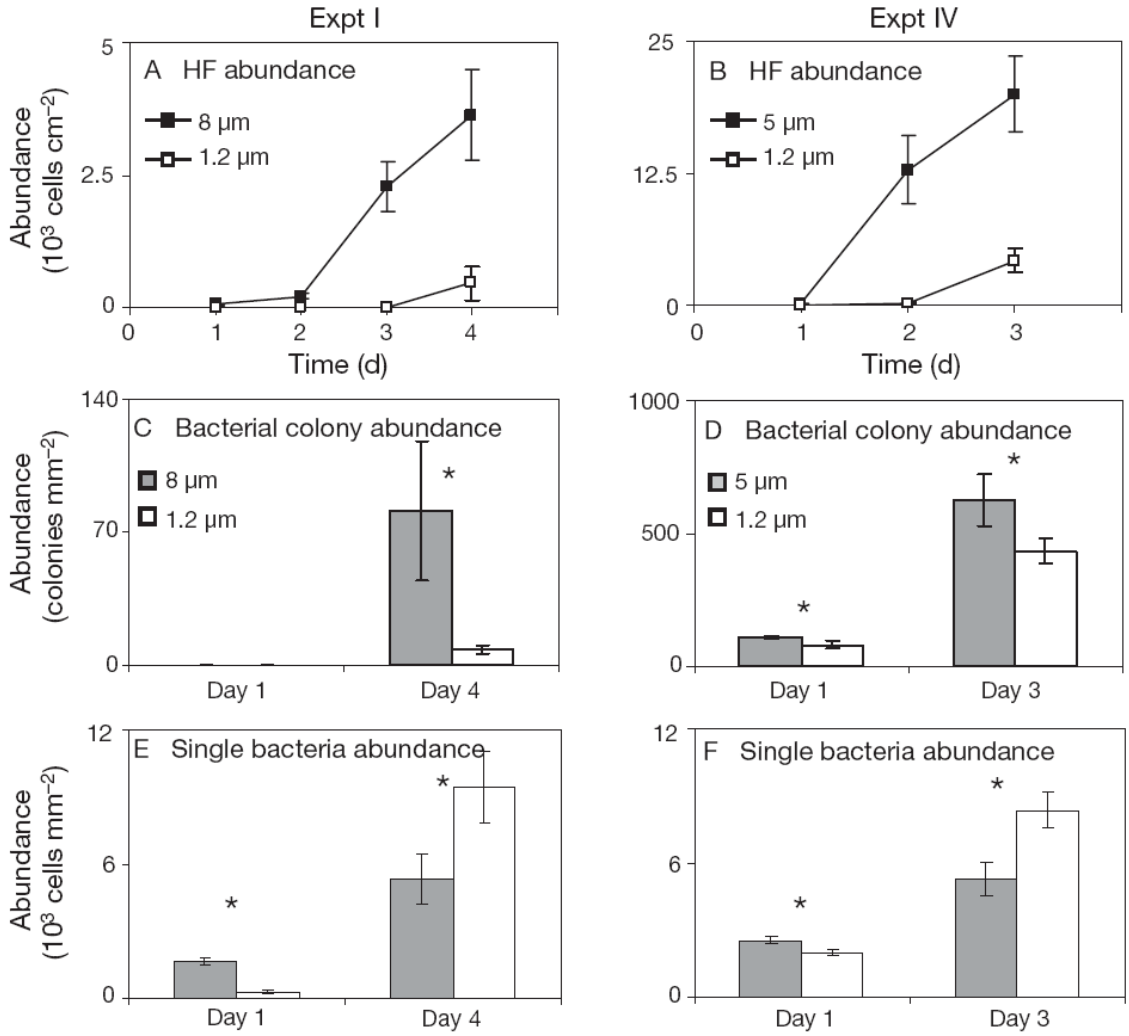

Fig. 3. Results of Expts I and IV on heterotrophic flagellate (HF) grazing effects on biofilm bacteria. (A) HF abundance during Expt I, (B) HF abundance during Expt IV, (C) bacterial colony abundance on Days 1 and 4 of Expt I, (D) bacterial colony abundance on Days 1 and 3 of Expt IV, (E) bacterial single-cell 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.

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.

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 × Treament	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 × Treament	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 × Treament	0.0960	1	53.95	< 0.001
Residuals	0.0213	12		

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).

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.

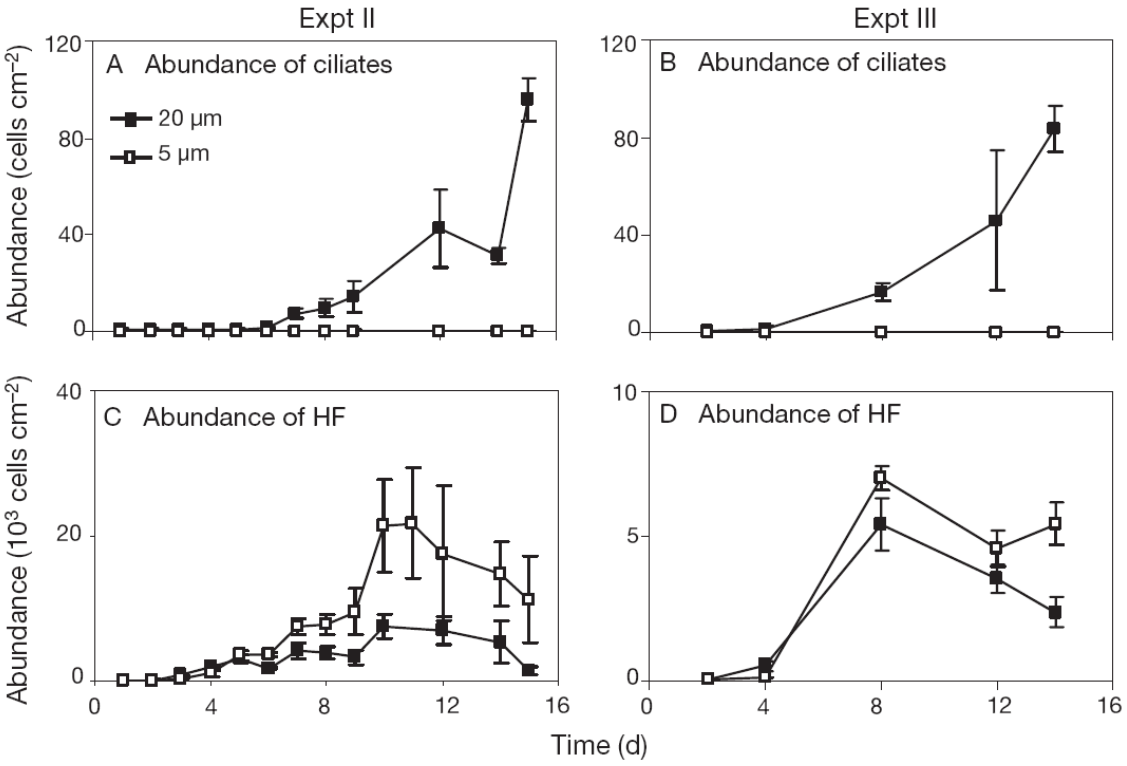

Fig. 4. Development of (A,B) ciliate and (C,D) heterotrophic flagellate (HF) abundance in (A,C) Expt II and (B,D) Expt III in 20 µm and 5 µm filtered treatments. Error bars represent SD. See Table 4 for ANOVAs of HF abundances.

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.

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

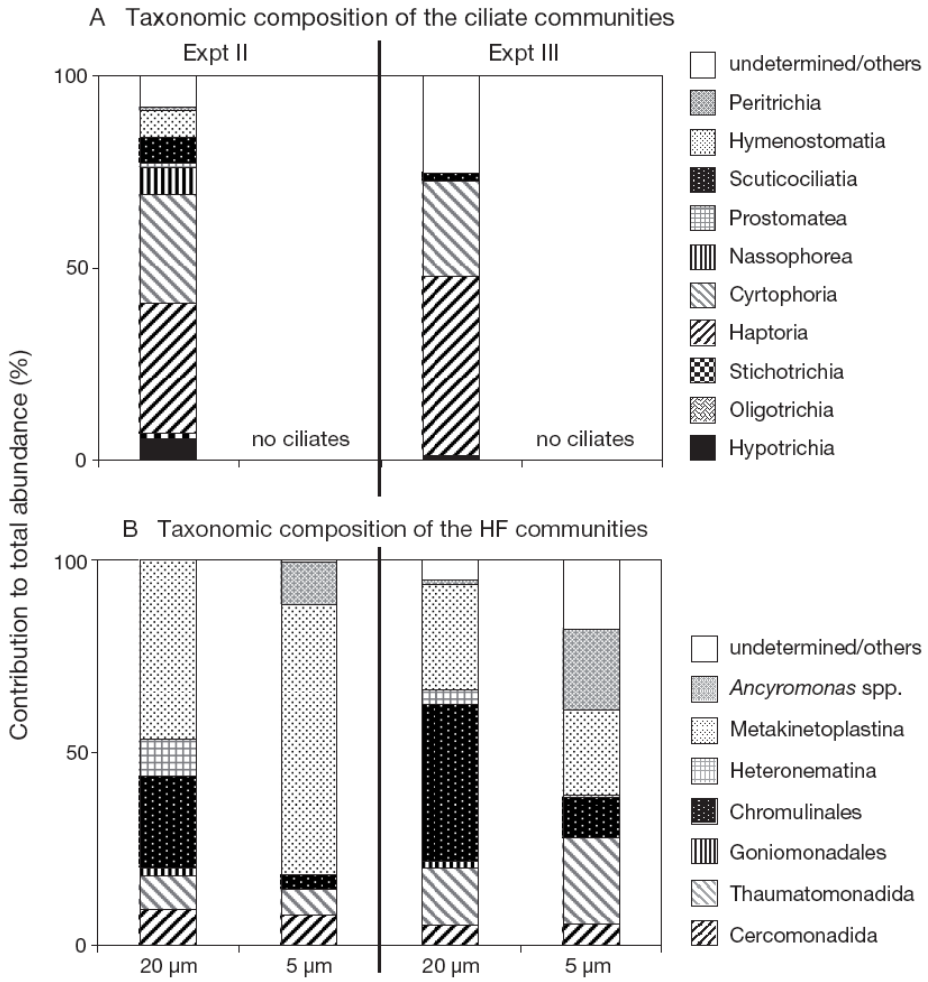

Fig. 5. Taxonomic composition (as relative contribution to total abundance) of (A) ciliate communities, and (B) heterotrophic flagellate (HF) communities in the 20 µm and 5 µm treatments on the final days of Expts II and III (Days 15/14). Oligotrichia abundances were too low to be visible in the figure.

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 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).

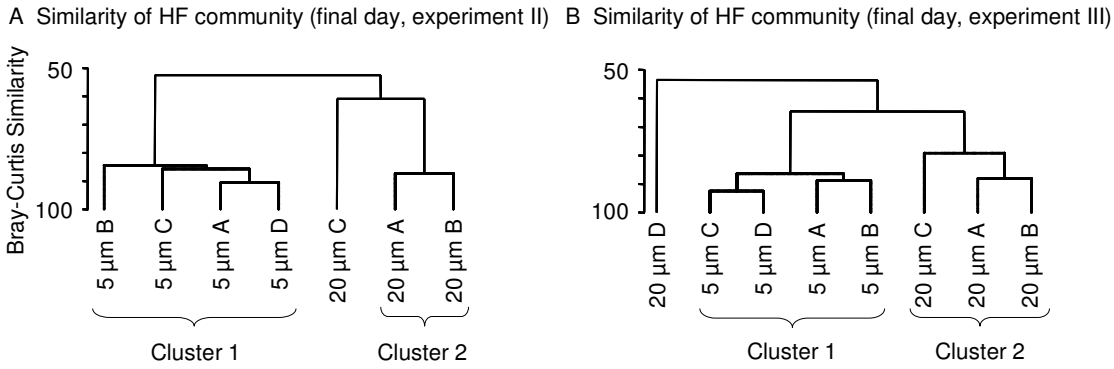

Fig. 6. Bray-Curtis similarity for heterotrophic flagellate (HF) communities on the final day of (A) Expt II, and (B) Expt III. There were 3 instead of 4 replicates for the 20 µm treatment of Expt II because the coverslip of one flow cell cracked during the experiment. Letters A–D indicate the different 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

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).

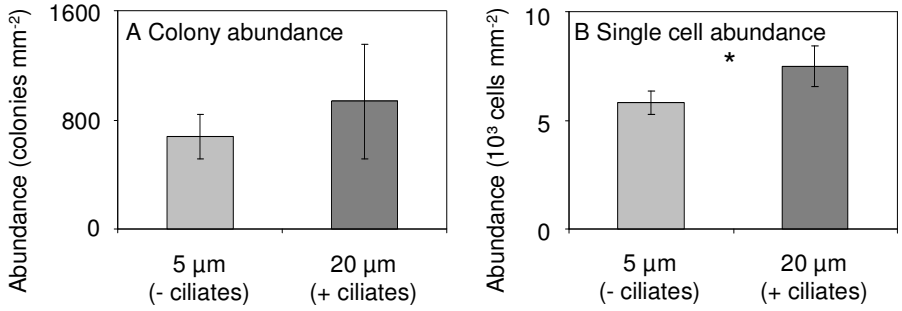

Fig. 7. Effects of ciliates on (A) bacterial colony abundance, and (B) bacterial single-cell abundance for the final day of Expt III (Day 14). Error bars represent SD. (*) indicates significant difference (p < 0.05) in a Student's *t*-test

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 2002a). 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 monospecific 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 monospecific 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 addition 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 ciliates 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).

Conclusion

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.

Chapter 2 – Influence of succession and protozoan grazers on the phylogenetic structure of riverine bacterial biofilms

Abstract

Recently, protozoans have been recognized to profoundly alter the morphology of bacterial biofilms with a stimulation of biofilm formation in early succession stages versus a mixture of negative and positive grazing effects in later stages. Here, the effect of protozoans on the phylogenetic structure of bacterial biofilms was tested under natural background conditions by applying size fractionation in a river bypass system. With the help of fingerprinting methods (Single Stranded Conformation Polymorphism (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE)), we could show that flagellates significantly affect the phylogenetic structure of both early and late biofilms, and that they generally positively affect species richness. Such effects were recorded in two experiments with different seasonal background conditions (May and January), whereas the morphological structure of the biofilm was only affected in the May experiment. It was also demonstrated that ciliates dramatically change the structure of the HF community, leading to a switch from benthivorous to planktivorous life forms. Concurrently, a massive increase in the abundance of bacterial microcolonies, a change in the morphological structure of the bacterial community and a trend towards decreased bacterial species richness in late succession stages were found when ciliates were present. Our data highlights the importance of community shifts in bacterial biofilms under protozoan grazing, which has been overlooked in previous quantitative studies.

Introduction

Bacterial biofilms play a prominent role for the function of many natural ecosystems such as streams and rivers (Watnick, 2000; Findlay, 2010), as well as several industrial facilities including sewage plants (Hao, 2010). Correspondingly, there is a great interest in understanding mechanisms which control bacterial biofilms. Among them, protozoan grazing has been recognized as an important factor (Parry, 2004). Protozoan grazing of bacteria represents the most ancient predator-prey interaction and both groups thus share a long history of co-evolution (Pernthaler, 2005; Matz and Kjelleberg, 2005). Only in recent years attention on grazing interactions within biofilms is increasing, and first quantitative studies in controlled laboratory environments showed that in early stages of succession, protozoans can stimulate the attachment of bacteria and the formation of microcolonies (Matz et al., 2004), but also that certain protozoan species are well adapted to grazing of and within bacterial biofilms in later stages (Huws et al., 2005; Weitere et al., 2005). It was proposed that by altering the morphology of the bacterial biofilm, protozoa indirectly also influence other biofilm properties, e.g. nutrient uptake and dispersal probability (Sabater et al., 2002; Böhme et al., 2009).

Several authors have shown for planktonic or suspended laboratory systems that a change in the morphology of bacteria due to grazing by protists is coupled with pronounced changes in diversity (Hahn and Höfle, 2001, Salcher et al. 2005), and a link between taxonomic diversity and potential functional diversity (ability to utilize different carbon sources) was demonstrated by Leflaive et al. (2008). It is likely, that protozoan grazing also shapes the phylogenetic structure of bacterial biofilms, thus driving functional changes. Effects of predation on diversity depend on both predator and prey properties as well as on abiotic factors such as nutrients (Chase et al., 2002), additionally Wey et al. (2009) showed that a later successional stage leads to a higher influence of community processes (species interactions) and nutrients for semi-natural biofilms. It is a challenge to extend studies of predator-prey interactions in biofilms to complex natural environments to be able to test these hypotheses under natural background conditions. However, as yet there was a lack of appropriate approaches to

experimentally test the effects of grazing on the phylogenetic structure of complex biofilm communities under field conditions.

In an earlier study we developed a system to examine near-natural biofilms in a river bypass, and showed that HF initially promote biofilm formation and in later successional stages reduce the abundance of single bacterial cells, while microcolonies are further promoted (Wey et al., 2008). If ciliates are also present, they alter the HF community composition (from benthivorous to planktivorous HF taxa) and relieve the bacterial single cells from predation pressure, presumably by a trophic cascade. In the present study, we expanded this approach by incorporating the possibility to harvest the bacterial biofilm, thus allowing the protozoan community analysis (live counts) as well as the analysis of both the phylogenetic and morphological structure of the biofilm in parallel. This system was used to test the hypotheses that protozoans (either flagellates or ciliates) alter the phylogenetic structure of bacterial biofilms in late stages of the succession (controlled by grazing and other community interactions) but not in early stages of biofilm formation (controlled by random physical attachment of the bacteria).

Experimental Procedures

Study site

All experiments described here were conducted on the Ecological Rhine Station of the University of Cologne, which is a ship permanently anchored in the River Rhine in Cologne, Germany (50°54'25''N, 6°58'43''E). The ship is equipped with a pump permanently pumping fresh Rhine water into the onboard laboratory. The water is pre-filtered through a 300 µm mesh to keep out coarse debris and large animals and is then directly used for experiments.

Experimental set-up

In a river bypass system we filtered the potamoplankton (the source of the biofilm community) according to size classes (see below), and thus established experimentally manipulated, diverse biofilms under natural background conditions. The details of the

experimental set-up as well as methodological pre-investigations are described in Wey et al. (2008).

In brief, the Rhine water was filtered through filter cartridges (Sartopure Capsules PP2, Sartorius) to obtain water containing bacteria, heterotrophic flagellates and ciliates (20 µm pore width), bacteria and HF (5 µm pore width), or bacteria and a severely reduced number of HF (1.2 µm pore width). These organisms established biofilms in flow cells (after Norf et al., 2007) connected to the filter cartridges under a constant flow of 2 ml min^{-1} and exposed to the Rhine temperature conditions at the time of the experiment. The flow cells had to be modified in order to allow (1) live counts of protozoans, (2) confocal laser scanning microscopy for the analyses of the biofilm morphology and (3) DNA extraction for subsequent molecular fingerprinting analysis of the bacterial community. Protozoa were counted in vivo by light microscopy (see "Abundance and taxonomic composition of protists" below); for the bacterial community analyses, two sterile cover slips of 15×15 mm were placed inside of each flow cell (Fig. 1, see "Morphology of bacterial biofilms (CLSM analyses)" and "DNA extraction" below for details of analysis procedures).

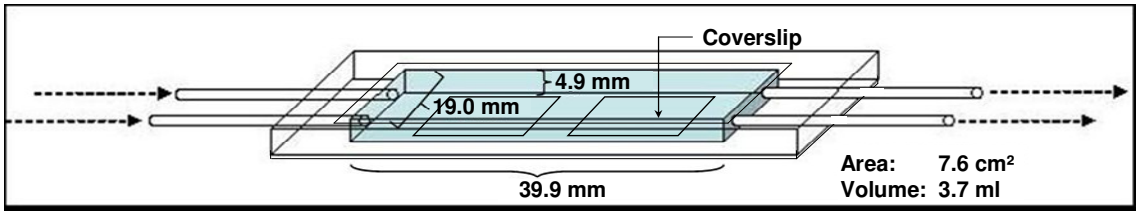

Fig. 1: Schematic drawing of a flow cell including the two coverslips inserted for molecular and morphological analyses of the bacterial biofilm.

We performed two types of experiments, one comparing bacterial biofilms in the presence of high numbers and strongly reduced numbers of HF (Experiments I and II, Table 1), and one comparing bacterial and HF biofilms in the presence and absence of ciliates (Experiment III, Table 1). The exact time periods, the water temperature and the applied filter cartridges are given in Table 1.

Every treatment was set up in four parallels. On days were we analysed the bacterial community, it was necessary to open the flow cells and discard them afterwards (see "Morphology of bacterial biofilms (CLSM analyses)" below), thus we had to replicate the four parallels of every treatment for each day the bacterial community was investigated (that is, two replicates each for experiments I and II, and 4 replicates for experiment III).

Table 1: Summary of experiments and background conditions. Water temperature lists the mean daily average Rhine temperature and range over the course of the experiment.

Experiment	Date	Water temperature (°C)	Filter treatments*	Aspect analysed
I	08/05 – 11/05/10	13.8 (13.7 – 13.9)	5/1.2	Effects of HF on bacteria
II	18/01 – 25/01/10	4.5 (4.0 – 4.9)	5/1.2	Effects of HF on bacteria
III	18/01 – 16/02/10	4.1 (3.4 – 4.9)	20/5	Effects of ciliates on HF and bacteria

* Filter treatments result in communities according to (Wey et al., 2008): 1.2 µm: bacteria (plus small numbers of HF), 5 µm: bacteria and HF, 20 µm: bacteria, HF and ciliates.

Abundance and taxonomic composition of protists

Protists were counted and classified by live counting with a Zeiss Axioskop equipped with phase contrast. Exact procedures are described in Wey et al. (2008). Protozoa were always counted in the set of flow cells destined for the next sampling of bacterial biofilms. We taxonomically classified the protists after the recent system of Adl et al. (2005).

Morphology of bacterial biofilms (CLSM analyses)

We generally investigated bacterial biofilms at the beginning of the biofilm colonization (day 1 or 2), and on the final day of the experiment (days 4, 7, or 29). In experiment I, we additionally harvested bacterial biofilms just two hours after the start of the experiment (day "0") to account for early successional effects; and in experiment III on days 7 and 14 because of major changes in protist taxonomy and/or abundance on those days. On the respective days, flow cells were opened with a sterile razor blade and the two cover slips inside were removed for further investigation of the bacterial biofilm. One of the cover slips was frozen at -20°C immediately after sampling for later DNA extraction (see "DNA extraction" below), the other one was immediately fixed in 2% formaldehyde and stored in a dark refrigerator at 4°C for later analysis under the Confocal Laser Scanning Microscope (CLSM, Zeiss Axiovert 100M). For this purpose, the cover slip was stained with propidiumiodide (100 µg ml^{-1}) and placed on an object slide. The further procedure under the CLSM and the analysis of the pictures with ImageJ (Version 1.37v; http://rsb.info.nih.gov/ij/) is described in Wey et al. (2008). One major setting based on this study was that all spots

≤2.5 µm^2 were counted as single bacterial cells and all spots >4 µm^2 were counted as bacterial colonies.

DNA extraction

To extract DNA from the biofilms we first added glass beads to the microcentrifuge tubes containing the coverslips, then froze them in liquid nitrogen and crushed the samples in a FastPrep Homogenizer (MP Biomedicals) at 4 m s-1 for 20 seconds. Further steps followed a modified protocol from Murray and Thompson (1980) and involved incubation in 500 µl cetyltrimethylammonium bromide (CTAB) extraction buffer at 65°C for one hour, washing with chloroform-isoamyl alcohol (24:1) and precipitation of DNA with 1 volume of isopropanol. After washing with 70 % ethanol the pellet was dried and dissolved in 50 µl ultra-pure water over night.

On day 1 of experiment II we additionally took four plankton samples; one sample each from the overflow of the 1.2, 5, and 20 µm filter cartridges, and one sample of the 300 µm pre-filtered water. 250 ml of water from each sample was filtered on a 0.2 µm filter (Whatman glass microfiber filter, GF/F grade) by a vacuum pump, and the filter was immediately stored at -20°C and further treated as described for the biofilm samples (see above).

Diversity of bacterial biofilms (PCR-DGGE analyses)

We amplified DNA for the DGGE with the bacteria specific primers 341F (5'CCTACGGGAGGCAGCAG3') and 907R (5'CCGTCAATTCMTTTGAGTTT3', Muyzer et al., 1995). The forward primer carried a 40 bp GC clamp (5'CGCCCGCCGCGCCCCGCGCCCG GCCCGCCGCCCCCGCCCC3') at its 5' end. PCR mixtures (50 µl) contained 1 µM of each primer, 1.25 U Taq DNA polymerase (5 Prime advance, 5 Prime), 200 µM of each desoxynucleoside triphosphate (5 Prime), 0.6 mg ml^{-1} bovine serum albumine (Fermentas) and 1 µl of each sample. PCR started with denaturing for 5 minutes at 95°C, 35 cycles of 1 minute each at 95°C, 52°C (annealing) and 72°C (elongation), and a final step of 5 minutes at 72°C.

DGGE itself was performed using the IngenyPhorU system as described by Labrenz et al. (2007). After trying wider ranges of denaturant, we settled for a range of 35-65 % (100 % of denaturant consisted of 7 M urea and 40 % deionised formamide), because it

showed the highest resolution of bands while still depicting the whole community. Gels were stained with SYBRGold (1:5,000 in TAE buffer) for 1 hour and were visualised by UV radiation. Images were taken using a digital gel documentation system (GeneSnap, Syngene).

Diversity of bacterial biofilms (PCR-SSCP analyses)

To amplify DNA for the SSCP, we used the bacteria specific primers Com1F (5'CAGCAGCCGCGGTAATAC3') and Com2R-Ph (5'CCGTCAATTCCTTTGAGTTT3', with a 5`-terminal phosphate group) after Schwieger and Tebbe (1998) in 50 µl reactions (385 nM of each primer, 200 µM of each desoxynucleoside triphosphate (5 Prime), 1.25 U Taq DNA polymerase (5 Prime advance, 5 Prime), 0.8 mM $MgCl_2$ (5 Prime), 0.6 mg ml^{-1} bovine serum albumine (Fermentas), 1 µl of each sample). PCR followed the protocol by Labrenz et al. (2007). We were not able to amplify all samples of experiment I, in detail, all samples of day 0 (except one 1.2 µm sample) and three samples of the 1.2 µm treatment of day 1 could not be amplified.

We generated single stranded DNA (ssDNA) by digesting the PCR products with λ exonuclease (49 µl PCR product, 17 U λ exonuclease, 1× λ exonuclease buffer, final volume: 56.3 µl) and incubating the mixture at 37°C and 300 rpm for 2 hours. The samples were then purified with the NucleoSpin Extract II kit (MachereyNagel) according to the manufacturer's instructions. After checking the purity of the product on a 1.2 % agarose gel, the remaining 10 µl were constricted to 4 µl with a vacuum concentrator at room temperature. After adding 4 µl of SSCP loading buffer to each sample, samples were incubated for 3 minutes at 95°C and then immediately cooled on ice for 10 minutes. The SSCP was performed at 400 V and 20°C for 16 hours. Gels were silver stained and dried at room temperature according to Bassam et al. (1991). Images were taken using a high resolution flatbed scanner.

Analysis of fingerprints for bacterial diversity in biofilms

The 8 bit TIFF files of both fingerprint techniques were imported into the GelCompar II software (Version 5.1, Bionumerics) and analyzed according to van Verseveld and Röling (2004). We used the Pearson's product moment correlation coefficient, which does not depend on band assignment but on the whole lane intensity to produce a

similarity matrix, which was subsequently pictured as a dendrogram applying unweighted pair group method with arithmetic mean (UPGMA) clustering. To test the goodness of fit between the UPGMA dendrogram and the original similarity matrix the cophenetic correlation coefficient (implemented in the GelCompar II package) was calculated and is shown at the nodes of each dendrogram. Values above 80 are generally considered to account for a robust cluster. We additionally let GelCompar II assign bands to check if the number of bands differed between treatments or over time.

Statistical analyses

Statistical analyses were performed with Prism 5 for Windows (GraphPad Software, Inc., Version 5.00). Data were generally log transformed to achieve homogenous variances. To show possible effects of time, treatment (grazer density) and their interactions on the abundance of HF (Expt. III only), the area covered by bacteria, the abundance of bacterial microcolonies, the abundance of bacterial single cells and the number of bands in fingerprint gels, we performed two-factorial ANOVAs with the respective abundance, covered area or number of bands as the dependent variable, and time and treatment as independent variables. The Student´s t-test was employed for pairwise comparison of the number of bands in the SSCP gel of experiment I (Fig. 5d).

Results

Development of protists

In experiments I and II, HF abundances in the 5 µm treatments steadily rose, though colonization was slower and final abundances lower in experiment II (1,213 ± 260 HF cm^{-2} compared to 6,350 ± 962 HF cm^{-2} in exp. I; Figs. 2a, 3a). Although HF were also found in the 1.2 µm treatments, abundances were severely reduced by the filter compared to the 5 µm treatments. In these, typical biofilm grazers (gliding HF) were present at all times and constituted at least 51 % of the community, with at times up to 78 % (Figs. 2b, 3b). The grazer community predominantly consisted of Metakinetoplastina (mainly *Rhynchomonas nasuta* and *Neobodo designis*),

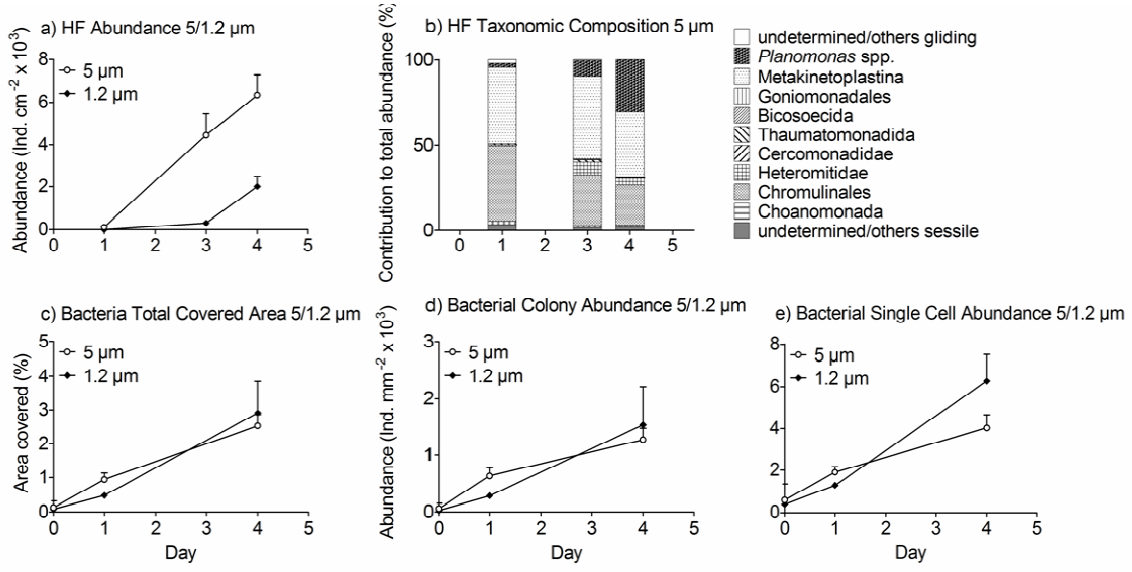

Fig. 2: Biofilm structure in experiment I: (a) HF abundance, (b) taxonomic composition of the HF community in the 5 µm treatment, (c) total area covered by bacteria, (d) abundance of bacterial microcolonies, (e) abundance of bacterial single cells. Error bars represent standard deviation (SD). Refer to table 2 statistical analyses.

Table 2: Experiment I: Results of two-factorial ANOVAs testing for effects of treatment (grazer density), time (days) and interactions thereof. All data were log-transformed before running the ANOVAs to achieve homogenous variances. Given are the sum of squares (SS), degrees of freedom (df), *F*- and *p*-values; n.s. = not significant.

		SS	df	*F*	*p*	Significance
Fig. 2c	(Bacteria Total Covered Area)					
	Time	1.1320	2	171.2	< 0.001	***
	Treatment	0.0063	1	1.893	= 0.186	n.s.
	Time×Treatment	0.0248	2	3.744	0.044	*
	Residuals	0.0595	18			
Fig. 2d	(Bacterial Colony Abundance)					
	Time	14.420	2	52.83	< 0.001	***
	Treatment	0.0286	1	0.210	= 0.652	n.s.
	Time×Treatment	0.2362	2	0.866	= 0.438	n.s.
	Residuals	2.4560	18			
Fig. 2e	(Bacterial Single Cell Abundance)					
	Time	5.2340	2	62.40	< 0.001	***
	Treatment	0.0019	1	0.045	= 0.835	n.s.
	Time×Treatment	0.1326	2	1.581	= 0.233	n.s.
	Residuals	0.7549	18			
Fig. 5c	(Species richness in DGGE gel)					
	Time	0.2525	2	10.51	= 0.001	**
	Treatment	0.0000	1	0.000	= 0.980	n.s.
	Time×Treatment	0.0886	2	3.689	= 0.048	*
	Residuals	0.1921	16			

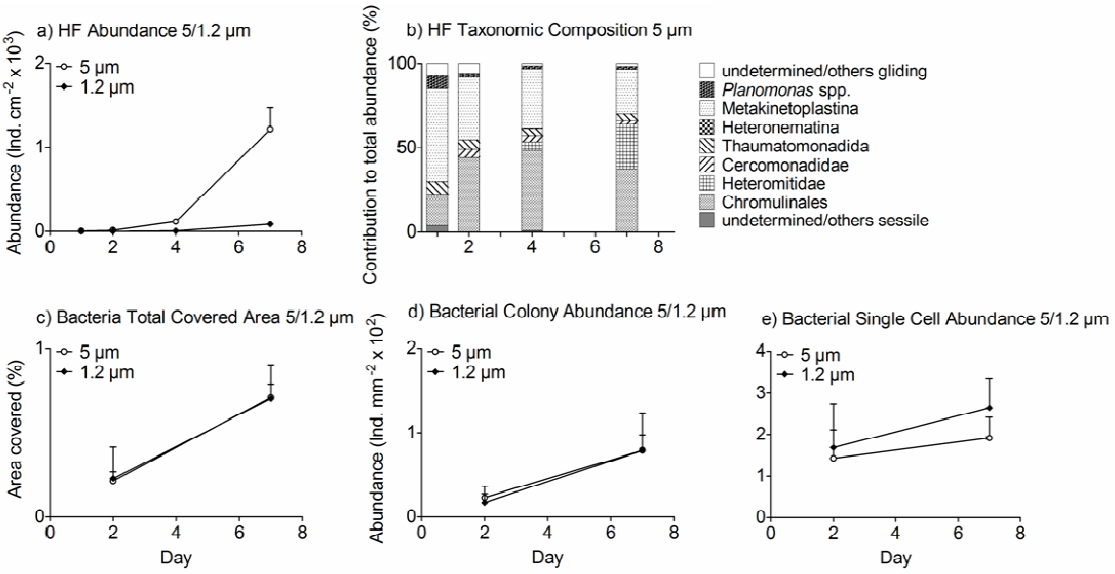

Fig. 3: Biofilm structure in experiment II: (a) HF abundance, (b) taxonomic composition of the HF community in the 5 µm treatment, (c) total area covered by bacteria, (d) abundance of bacterial microcolonies, (e) abundance of bacterial single cells. Error bars represent SD. Refer to table 3 for statistical analyses.

Table 3: Experiment II: Results of two-factorial ANOVAs testing for effects of treatment (grazer density), time (days) and interactions thereof. All data were log-transformed before running the ANOVAs to achieve homogenous variances. Given are the sum of squares (SS), degrees of freedom (df), *F*- and *p*-values; n.s. = not significant.

		SS	df	*F*	*p*	Significance
Fig. 3c	(Bacteria Total Covered Area)					
	Time	0.0785	1	46.77	< 0.001	***
	Treatment	0.0000	1	0.000	= 0.981	n.s.
	Time×Treatment	0.0000	1	0.022	= 0.885	n.s.
	Residuals	0.0185	11			
Fig. 3d	(Bacterial Colony Abundance)					
	Time	1.4350	1	24.51	< 0.001	***
	Treatment	0.0217	1	0.371	= 0.555	n.s.
	Time×Treatment	0.0020	1	0.035	=0.855	n.s.
	Residuals	0.6440	11			
Fig. 3e	(Bacterial Single Cell Abundance)					
	Time	0.1397	1	4.314	= 0.062	n.s.
	Treatment	0.0348	1	1.075	= 0.322	n.s.
	Time×Treatment	0.0054	1	0.168	= 0.690	n.s.
	Residuals	0.3563	11			
Fig. 6c	(Species richness in DGGE gel)					
	Time	0.0260	1	3.105	= 0.100	n.s.
	Treatment	0.0546	1	6.523	= 0.025	*
	Time×Treatment	0.0010	1	0.120	= 0.735	n.s.
	Residuals	0.1005	12			
Fig. 6d	(Species richness in SSCP gel)					
	Time	0.0710	1	13.37	= 0.003	**
	Treatment	0.0630	1	11.86	= 0.005	**
	Time×Treatment	0.0004	1	0.069	= 0.798	n.s.
	Residuals	0.0637	12			

Planomonas (formerly *Ancyromonas*) spp. and Heteromitidae (exclusively *Bodomorpha* spp.).

In experiment III, ciliate abundances reached a first peak after 14 days and rose to 99 ± 17 individuals cm^{-2} on the final day of the experiment (Fig. 4a). Gliding, bacterivorous and omnivorous ciliates (scuticociliates like *Cyclidium glaucoma* and *Cinetochilum margaritaceum*, but also prostomatids) dominated the community with the exception of day 14, when the predatorial haptorids constituted 56 % of the total abundance (Fig. 4b). The composition of HF in the 5 µm treatment was dominated by gliding HF throughout the experiment (again mainly Heteromitidae and Metakinetoplastina; Figs. 4c, 4e), while in the 20 µm treatment sessile HF (mainly Choanomonada like *Codosiga*

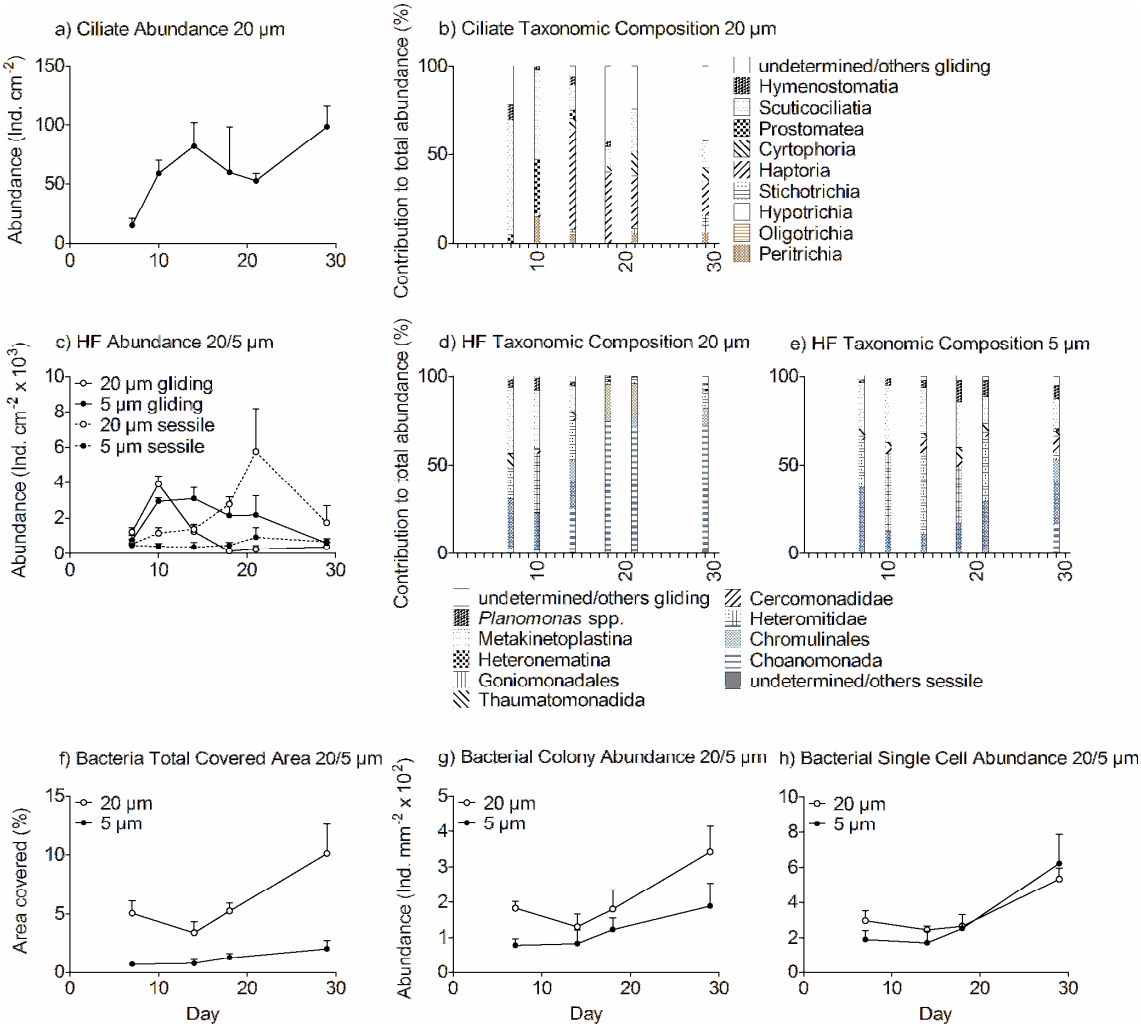

Fig. 4: Biofilm structure in experiment III: (a) ciliate abundance, (b) taxonomic composition of the ciliate community in the 20 µm treatment, (c) HF abundance, (d) taxonomic composition of the HF community in the 20 µm treatment, (e) taxonomic composition of the HF community in the 5 µm treatment, (f) total area covered by bacteria, (g) abundance of bacterial microcolonies, (h) abundance of bacterial single cells. Error bars represent SD. Refer to table 4 for statistical analyses.

Table 4: Experiment III: Results of two-factorial ANOVAs testing for effects of treatment (presence of ciliates), time (days) and interactions thereof. All data were log-transformed before running the ANOVAs to achieve homogenous variances. Given are the sum of squares (SS), degrees of freedom (df), *F*- and *p*-values; n.s. = not significant.

		SS	df	*F*	*p*	Significance
Fig. 4c	(HF Abundance)					
	Time	1.654	5	9.067	< 0.001	***
	Treatment	5.342	3	48.82	< 0.001	***
	Time × Treatment	8.679	15	15.86	< 0.001	***
	Residuals	2.407	66			
Fig. 4f	(Bacterial Total Covered Area)					
	Time	0.390	3	21.33	< 0.001	***
	Treatment	1.660	1	272.3	< 0.001	***
	Time × Treatment	0.036	3	1.973	= 0.149	n.s.
	Residuals	0.128	21			
Fig. 4g	(Bacterial Colony Abundance)					
	Time	0.621	3	10.72	< 0.001	***
	Treatment	0.479	1	25.16	< 0.001	***
	Time × Treatment	0.042	3	0.736	= 0.542	n.s.
	Residuals	0.400	21			
Fig. 4h	(Bacterial Single Cell Abundance)					
	Time	0.859	3	21.66	< 0.001	***
	Treatment	0.057	1	4.343	= 0.050	*
	Time × Treatment	0.081	3	2.044	= 0.139	n.s.
	Residuals	0.277	21			
Fig. 7c	(Species richness in DGGE gel)					
	Time	0.1673	3	7.519	= 0.002	**
	Treatment	0.0132	1	1.783	= 0.200	n.s.
	Time × Treatment	0.0368	3	1.654	= 0.217	n.s.
	Residuals	0.1187	16			
Fig. 7d	(Species richness in SSCP gel)					
	Time	0.0007	1	0.624	= 0.452	n.s.
	Treatment	0.0033	1	3.207	= 0.111	n.s.
	Time × Treatment	0.0307	1	29.48	< 0.001	***
	Residuals	0.0083	8			

sp. and *Monosiga* sp., but also Chromulinales like *Anthophysa vegetans* and *Spumella* sp.) dominated after day 14 (Figs. 4c, 4d). After day 18, sessile HF were so abundant in the 20 µm treatment that the combined abundance of gliding and sessile HF was higher than in the 5 µm treatment for the remaining experiment (Fig. 4c), and statistical analysis confirmed a highly significant impact of time and treatment on the HF abundance (Table 4).

Effects on the morphology of bacterial biofilms

There were no significant treatment effects on the area covered by bacteria in experiments I and II (Figs. 2c, 3c; Tables 2, 3), but in experiment III area coverage was significantly enhanced by the presence of ciliates throughout the experiment (Fig. 4f; Table 4). Bacterial colonies were more abundant when ciliates were present (Fig. 4g), while single cells were slightly promoted up to day 14 and slightly reduced at the last day of the experiment (Fig. 4h). In experiments with only HF as grazers (experiments I and II) we could not detect a significant influence of grazing on the abundance of microcolonies (Figs. 2d, 3d) or single cells (Figs. 2e, 3e), although a trend to less single cells was observed on the last days in both experiments, and a stimulation of biofilm formation on day one could be observed in experiment I.

Effects on the phylogenetic structure of bacterial biofilms

We consistently found an influence of succession on the bacterial diversity in all experiments. In experiment I this was especially pronounced for the diversity of 5 µm treatments, which differed considerably after 0, 1 and 4 days in the DGGE gel (Fig. 5a). Additionally, species richness (Fig. 5c) increased significantly over time in both treatments (Table 2), though this increase was caused by different species in the two treatments (see red bars in the DGGE gel on day 4, Fig. 5a). We did not have enough parallels to statistically test the effect of time in the SSCP gel (Fig. 5b), but a trend to more bands (a higher species richness) in later succession stages was found (Fig. 5d). The effect of succession was smaller in the winter experiments (II and III), nevertheless DGGE and SSCP fingerprints generally showed clusters according to days well below a 60 % cut-off value (Figs. 6a, 6b and 7a, 7b). We again found a trend for the species richness to increase with time (Figs. 6c, 6d and 7c, 7d), albeit the effect was only significant for the SSCP of experiment II (Table 3) and the DGGE of experiment III (Table 4).

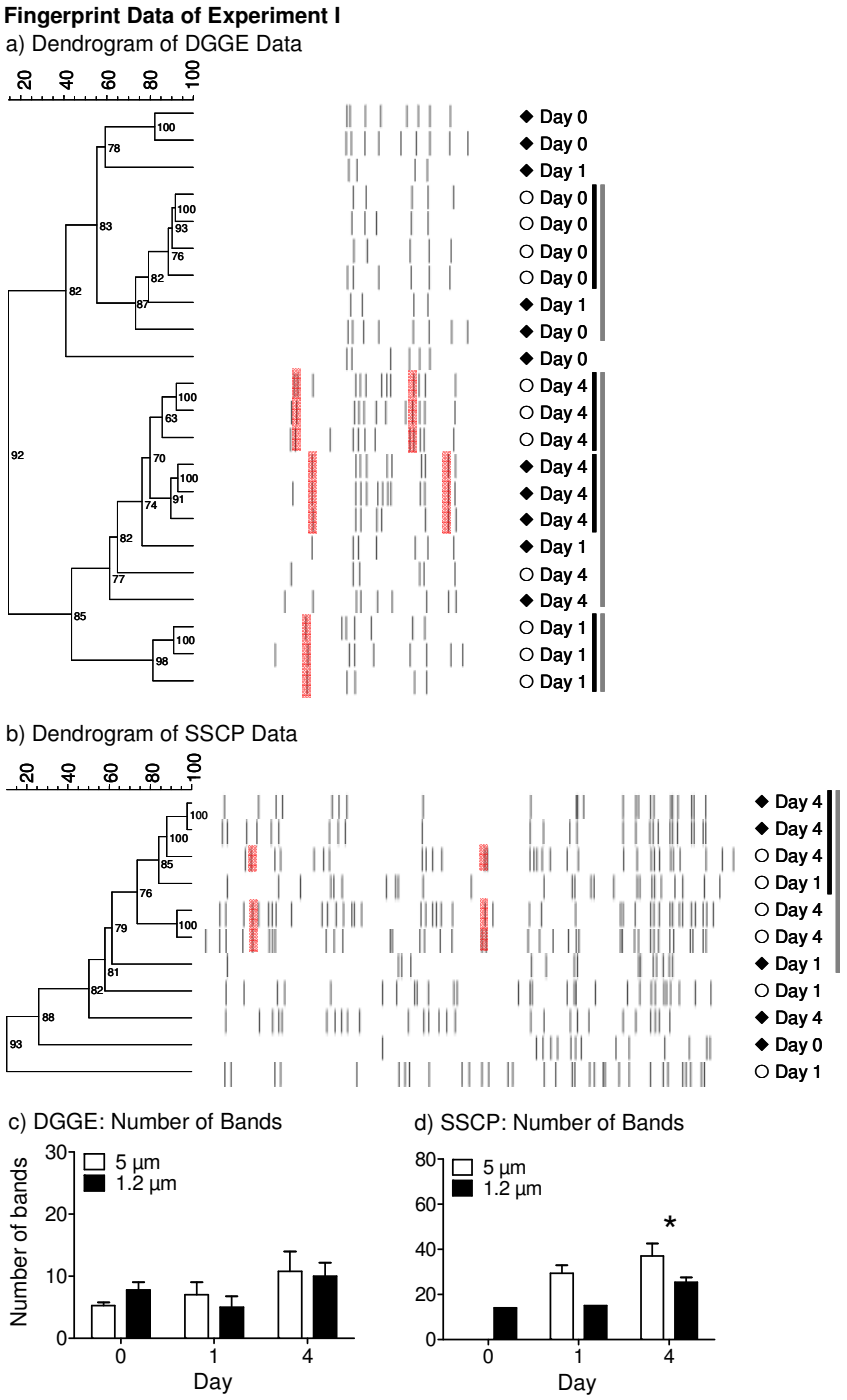

Fig. 5: Experiment I: UPGMA dendrograms of DGGE and SSCP gels with banding patterns, and numbers of bands in these gels: (a) dendrogram for DGGE gel, (b) dendrogram for SSCP gel. Symbols: ○ = 5 µm treatment, ◆ = 1.2 µm treatment. The Pearson's correlation coefficient is shown in % (scale bars), numbers at dendrogram branches are cophenetic correlation coefficients. Red bars indicate treatment specific bands (within the same day). Samples clustering together and consisting of at least 3 samples are indicated by bars at the right hand side of each dendrogram: black bars for a cut-off level of ≥ 80%, and grey bars for a cut-off level of ≥ 60 %. (c) Species richness (number of bands) for DGGE in panel a, (d) species richness (number of bands) for SSCP in panel b. Error bars represent SD. Refer to table 2 for statistical analyses. (*) in panel d indicates a significant difference in a Student's t-test (p = 0.05).
Remark: We were not able to amplify DNA from all samples for SSCP, thus only single data points (1.2 µm treatments, day 0 and 1) or no data at all (5 µm treatment, day 0) are shown for some treatments.

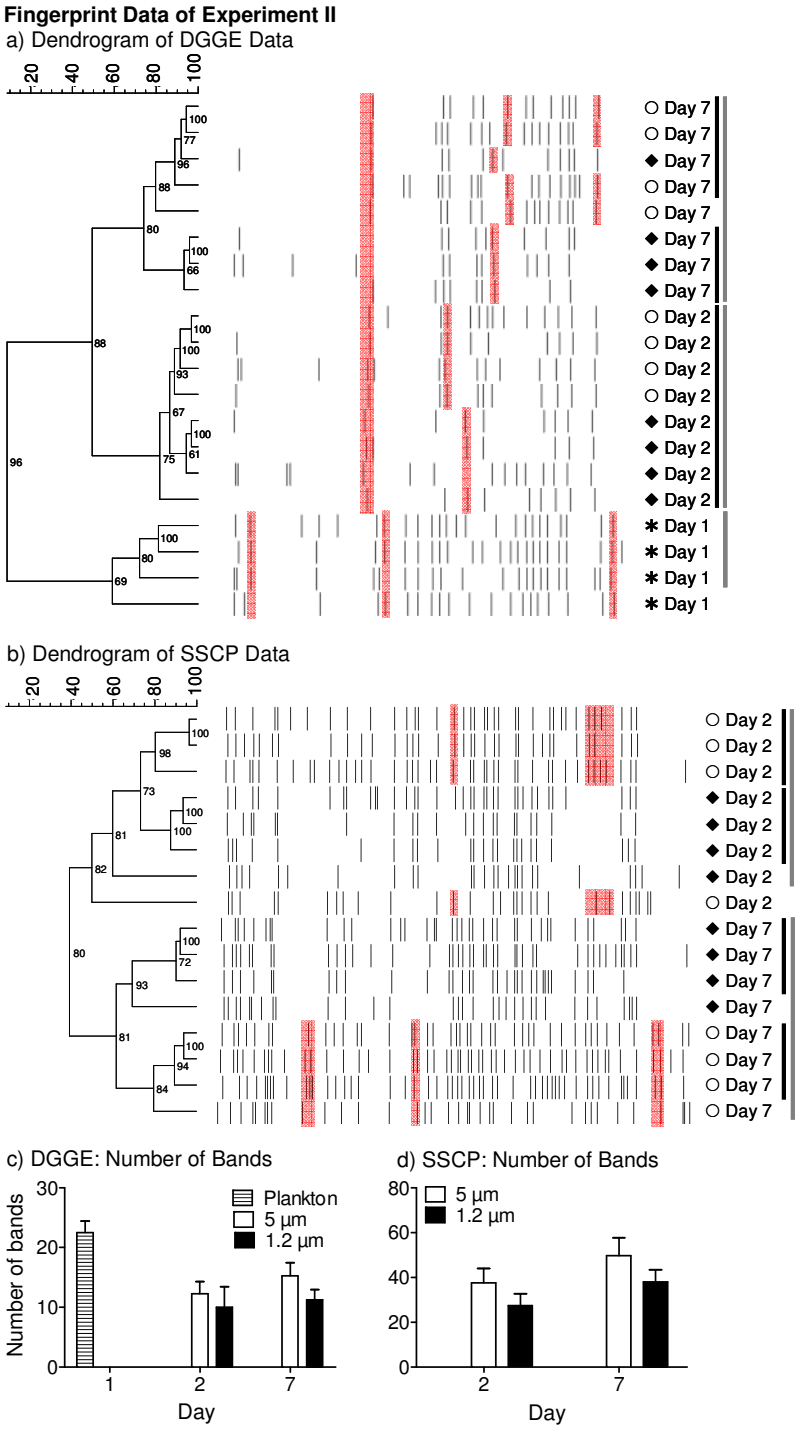

Fig. 6: Experiment II: UPGMA dendrograms of DGGE and SSCP gels with banding patterns, and numbers of bands in these gels: (a) dendrogram for DGGE gel, (b) dendrogram for SSCP gel. Symbols: ○= 5 µm treatment, ◆ = 1.2 µm treatment, ✱ = plankton samples. The Pearson's correlation coefficient is shown in % (scale bars), numbers at dendrogram branches are cophenetic correlation coefficients. Red bars indicate treatment specific bands (within the same day), or bands only showing in plankton or biofilm samples. Samples clustering together and consisting of at least 3 samples are indicated by bars at the right hand side of each dendrogram: black bars for a cut-off level of ≥ 80 %, and grey bars for a cut-off level of ≥ 60 %. (c) Species richness (number of bands) for DGGE in panel a, (d) species richness (number of bands) for SSCP in panel b. Error bars represent SD. Refer to table 2 for statistical analyses.

In addition to successional effects, we found pronounced treatment specific effects in almost all gels and experiments. The most distinct one was found in experiment II between plankton and biofilm samples (Fig. 6a). There were considerably more bands in lanes with plankton samples (Fig. 6c), and apart from planktonic phylotypes that were not found in the biofilm, the opposite was also true with biofilm phylotypes not detectable in plankton samples (red bars, Fig. 6a).

Regarding the presence of grazers, the SSCP gel of experiment I again was an exception to the rule with no meaningful clusters appearing, nevertheless the species richness was enhanced by the presence of elevated numbers of HF on the last day of the experiment (Fig. 5d). The presence of grazers also significantly enhanced the number of bacterial phylotypes in the second HF experiment, as shown in the respective SSCP gel (Fig. 6d, Table 3), and additionally treatment specific diversity clusters were found for both tested days (Fig. 6b). The enhanced species richness could be attributed to different phylotypes on the respective days (red bars in Fig. 6b). Results of the DGGE gels of the HF experiments (I and II) differed from those obtained by SSCP insofar as clusters according to treatment occurred at later successional stages and were not visible at the beginning of the respective experiment (Figs. 5a, 6a). Nevertheless, we already observed treatment-specific phylotypes at the first examined days in both experiments (red bars in Figs. 5a, 6a).

In the ciliate experiment (III), bacterial diversity also differed in concurrence with different grazer communities, here again the SSCP gel showed a better resolution than the DGGE gel (Figs. 7a, 7b). Both gels showed that bacterial species richness was slightly lower during the experiment when no ciliates were present, but that it was enhanced at the end of the experiment in this treatment (Figs. 7c, 7d; Table 4). The SSCP gel showed an especially high loss of bands in the 20 µm treatment on day 29, and one of these lost bands was present in all other treatments displayed on the gel (red bars in Fig. 7b).

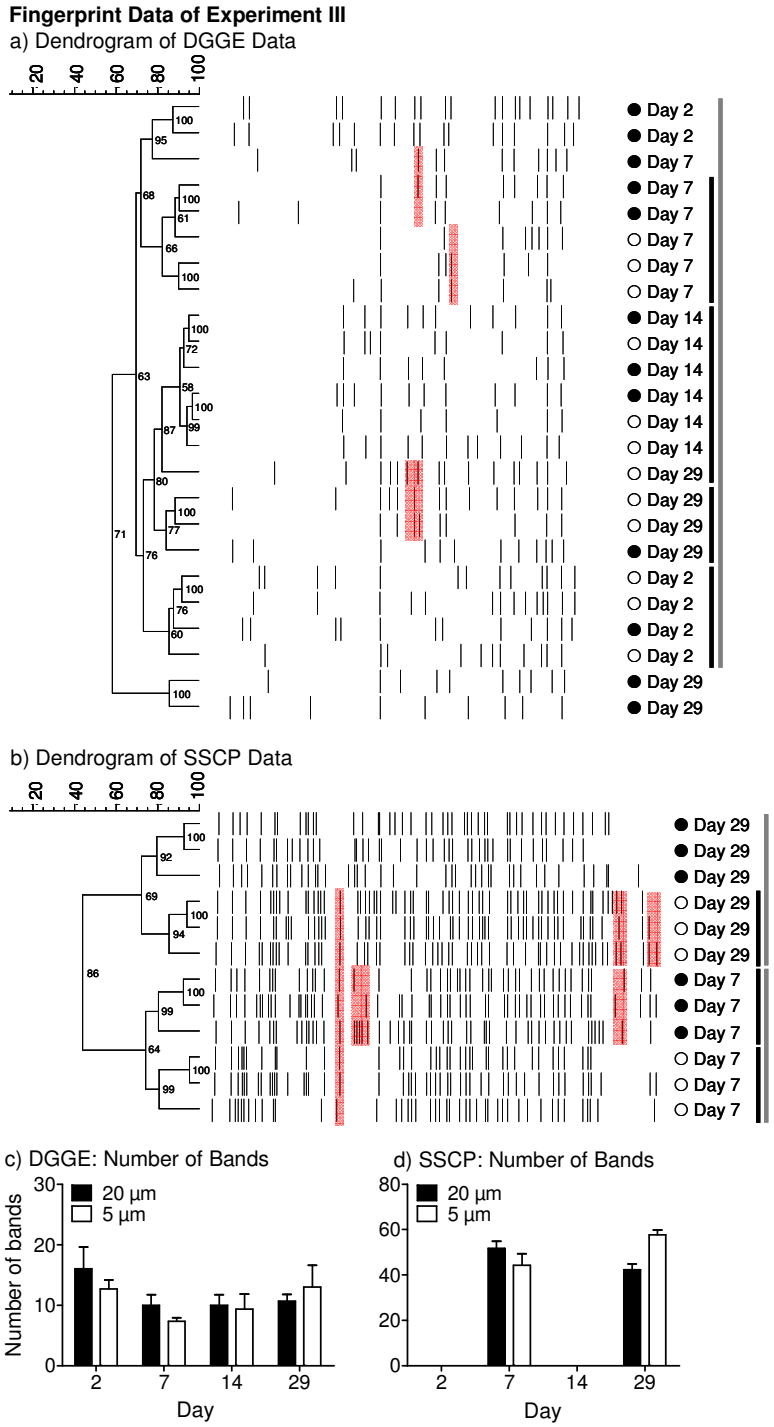

Fig. 7: Experiment III: UPGMA dendrograms of DGGE and SSCP gels with banding patterns, and numbers of bands in these gels: (a) dendrogram for DGGE gel, (b) dendrogram for SSCP gel. Symbols: ● = 20 µm treatment, ○ = 5 µm treatment. The Pearson's correlation coefficient is shown in % (scale bars), numbers at dendrogram branches are cophenetic correlation coefficients. Red bars indicate treatment specific bands (within the same day), and in one case a band showing in all treatments/days except for the 20 µm treatment on day 29. Samples clustering together and consisting of at least 3 samples are indicated by bars at the right hand side of each dendrogram: black bars for a cut-off level of ≥ 80 %, and grey bars for a cut-off level of ≥ 60 %. (c) Species richness (number of bands) for DGGE in panel a, (d) species richness (number of bands) for SSCP in panel b. Error bars represent SD. Refer to table 2 for statistical analyses.

Discussion

General remarks

Drawbacks of PCR based approaches of highly diverse natural samples have been discussed before and include e.g. the unequal amplification of taxa by a given primer and the formation of artefactual PCR products (Kanagawa, 2003). To test the robustness of our results, we therefore used two different fingerprinting methods, SSCP and DGGE, were the fragments amplified by PCR comprise different variable regions and are of different length, thus probably targeting different taxa of our original samples. Both methods have their own strengths and weaknesses (Nocker et al., 2007) and were shown to complement each other before (Smalla et al., 2007). Generally, the cluster analyses of bacterial diversity in this study lead to comparable results from both SSCP and DGGE gels within experiments (Figs. 5-7) and thus confirmed the observed patterns of bacterial species diversity. Although we found a higher number of bands in SSCP than in DGGE gels in all experiments (see Figs. 5-7), taxa richness in general was within the range of other studies of bacterial diversity in streams (Lyautey et al., 2005; Hullar et al., 2006; Beier et al., 2008).

HF alter the phylogenetic structure of bacterial biofilms independent of the effects on bacterial morphology

The differences in season and water temperature (Table 1) were directly transcribed into different abundances of both HF and bacteria in experiments I and II, resulting in very low abundances of both groups in the winter experiment. Seasonal changes in the abundance of both prokaryotic (Lyautey et al., 2005; Anderson-Glenna et al., 2008) and eukaryotic (Aguilera et al., 2007; Reiss and Schmid-Araya, 2008) biofilm inhabitants with lowest abundances during low temperatures were also found in various other rivers.

Although the taxonomic composition differed between the two experiments, typical surface-feeding HF like *N. designis* and *R. nasuta* (Caron, 1987) were always abundant. As a reaction to this significant grazing pressure, we expected the promotion of biofilm and microcolony establishment in early stages of biofilm formation, and a reduction of bacterial single cells in later stages as demonstrated earlier in quantitative studies (Weitere et al., 2005; Wey et al., 2008). Such a trend was demonstrated in experiment

I of the present study, even though the effects were not significant. However, no grazer effects were found in experiment II in winter. Low HF abundances in combination with reduced biological activity at low temperatures could explain these missing effects. Another explanation for this finding would be a natural seasonal variation in bacterial species (described for example by Brümmer et al., 2000; Olapade and Leff, 2004; Hullar et al., 2006) and thus a different response to grazing (Hahn and Höfle, 2001).

Even though quantitative effects of HF on the bacterial biofilms were lacking, a clear response of the bacterial community composition towards the presence of grazers was shown. The changes in bacterial diversity due to the higher abundance of HF were accompanied by a trend to increase the bacterial richness (sometimes in conjunction with time), with possible consequences for the functional diversity of the bacterial community (Loreau, 2001; Leflaive et al., 2008). In planktonic systems, morphological and taxonomical changes are usually coupled (Hahn and Höfle, 2001; Salcher et al., 2005), thus this is an unusual finding and shows that effects on the community composition can occur even though no effect on the biofilm morphology is detectable.

In experiment II, we also compared the diversity of the initial planktonic bacterial community to the biofilm community and found that they are considerably different. This confirms findings by Besemer et al. (2007) and Beier et al. (2008) of large dissimilarities in bacterial community composition between stream water and benthic habitats (sediments, biofilms) in flowing water systems. It is noteworthy that although species richness was reduced in the biofilm compared to the plankton, certain phylotypes were only detectable in the attached bacterial community. This suggests that some bacteria are either especially adapted to life in biofilm habitats or that they are bad competitors if suspended, thus being to low in abundance to be detected by DGGE. Moreover, we also found differences in the initial bacterial biofilm community depending on the presence of HF. This demonstrates that the presence of grazers can modify the bacterial community initially colonizing an empty substrate, thus creating a different basis for subsequential biotic interactions and abiotic processes.

Ciliates at the same time alter the phylogenetic and morphological structure of bacterial biofilms, and the taxonomic composition of HF

We showed massive effects of ciliates on the HF community composition and abundance. Particularly benthivorous (gliding) HF were strongly reduced in the presence of ciliates, whereas planktivorous (sessile) HF were stimulated; this is in accordance with our previous study (Wey et al., 2008). It is likely, that two different mechanisms lead to this shift in the HF community: Firstly, competition by benthivorous, bacterivorous ciliates (e.g. scuticociliates like *Cinetochilum margaritaceum* and *Cyclidium glaucoma*, but also prostomatids (Foissner and Berger, 1996), both of which were abundant at all times) and secondly, predation by haptorids (like *Acineria uncinata* (Foissner and Berger, 1996), these were especially numerous at times of reduced abundances of gliding HF). The most abundant sessile HF in this study, *Codosiga* sp., *Monosiga* sp., *Anthophysa vegetans*, *Spumella* sp. and *Paraphysomonas* sp., are all attached to the substrate by plasma threads, thus keeping the actual cell body out of the plane preferentially searched for prey by haptorids (M. Erken, pers. com.).

The presence of high numbers of bacterivorous ciliates presumably also lead to a considerably larger area covered by bacteria and a higher number of microcolonies. We propose that this is the consequence of a grazer defence strategy, hereby confirming results of laboratory experiments with single ciliate species (Lawrence and Snyder, 1998; Böhme et al., 2009) for near-natural biofilms. The direct link of bacteria to ciliates in the present study stands in contrast to our previous study (Wey et al., 2008), where we found a top-down cascade from ciliates to HF to bacteria, resulting in a relieve of bacterial single cells from HF grazing pressure, but no change in microcolony abundance. Both studies were conducted under different background conditions, e.g. temperature, season, and grazer community composition, thus emphasizing the importance of theses factors for protist-bacteria interactions in biofilms. In planktonic communities, differential effects of protozoa on bacterial community composition and/or abundance under varying background conditions were for example described by Gasol et al. (2002), and there is evidence for both direct effects of HF and ciliates on bacteria (Sherr and Sherr, 2002), as well as for trophic cascades under certain circumstances (Sanders et al. 1992).

The direct influence of ciliates on the bacterial biofilm also led to changes in bacterial diversity, and certain phylotypes were reduced in the presence of ciliates. Although size-selective grazing is the most studied factor in characterizing the link between protists and bacteria in the plankton, selection according to phylotype was also described before, for example by Corno and Jürgens (2008), who also found that these taxonomical changes were accompanied by changes in substrate-utilization profiles. Again, this suggests that changes in diversity as observed in the present study can consequentially affect the functioning of the community.

The authors of several quantitative studies of marine and limnic benthic systems concluded that the carbon flow between bacteria and ciliates might be limited in these habitats (Kemp, 1988; Marxsen, 2006; Königs and Cleven, 2007). The present study suggests that the accumulation of bacterial biomass and not its reduction, as well as changes in the diversity of the bacterial community might (at least under certain background conditions) be the principal influence of ciliates on bacterial biofilms; with nevertheless potentially far reaching consequences for carbon fluxes in benthic food webs.

Conclusion

We showed that both HF and ciliates have the power to alter the bacterial morphological structure and diversity in natural biofilms. Furthermore, changes in diversity are not necessarily coupled to changes in the morphology of the bacterial biofilm, and effects in this study were slightly more pronounced in later successional stages. It is thus necessary to consider both the morphology and phylogenetic structure of the biofilm, as well as the stage of community succession in order to fully understand the effects of protozoan grazers in natural benthic habitats. Our combined approach to study diverse natural communities with a combination of live counting, fixation, image processing and molecular methods offers a tool to study such complex effects and has a great potential for further studies regarding factors controlling the food web structure within biofilms.

Chapter 3 – Role of dispersal in shaping communities of ciliates and heterotrophic flagellates within riverine biofilms

Abstract

We tested the role of immigration from the plankton on the structure of local surface-associated communities of ciliates and heterotrophic flagellates (HF) in flow cells fed with fresh riverine water (Rhine, Germany). By applying size-fractionation, we reduced the immigration potential of HF from the plankton in mature, pre-grown biofilms by 93%, and observed no significant effects on the abundance and taxonomic composition of HF. Compared to a treatment with natural plankton density, a 100% reduction in the number of planktonic cells flowing over mature biofilms resulted in a reduced total abundance of ciliates, whereas only slight effects on the relative composition of present morphospecies were detectable. When starting with sterile flow cells, we found an initial linear increase in HF abundance proportional to their planktonic abundance; additionally supplemented planktonic bacteria showed that this initial colonization rate was resource independent. The initial phase was followed by an exponential increase, which was also independent from the resource level and strongest when initial colonization was low. In contrast to the early succession stages, the final abundances reached were independent of the plankton abundance but strongly dependent upon the local resource level. Immigration is an important factor controlling the initial substrate colonization and early growth, and ciliate and HF communities in biofilms become increasingly independent of immigration with maturation, being then controlled by local factors such as resources.

Introduction

Microbial biofilms can contribute strongly to processes of shallow aquatic ecosystems, particularly of running waters (Mulholland et al. 1994; Fischer and Pusch 2001; Battin et al. 2003). Because of the prominent role of these microbial communities, factors controlling biofilm structure and composition are of great interest. A well-established model of development exists for bacterial populations within the biofilm (for review *see* Hall-Stoodley et al. 2004): The settlement and loose association of planktonic cells to substrate is followed by robust adhesion, which is in turn followed by the aggregation of cells into microcolonies and subsequent growth and maturation to complex, three-dimensionally structured and matrix-embedded biofilms. Dispersal is achieved either by physically enforced sloughing or by programmed release of planktonic cells. While these processes are well understood on the population level, the factors controlling the community composition of complex natural biofilms have not been thoroughly investigated. So far, studies have concentrated mostly on the effects of nutrients, predation and flow (Gong et al. 2005; Haglund and Hillebrand 2005; Besemer et al. 2007).

The application of ecological theory on community control to biofilms could lead to a better understanding of the regulation of complex, surface-attached communities (Warren 1996; Mouquet et al. 2003). Particularly the effect of dispersal on the local community structure has received increasing attention in recent years (Mouquet and Loreau 2003; Leibold et al. 2004). Battin and co-workers recently presented a promising framework by considering ecological theory from landscape ecology in biofilm research (Battin et al. 2007). This concept also points out the open nature of biofilms and the possibility of dispersal between local biofilm patches (Sloan et al. 2006). Such an introduction of Hubbell's neutral theory (Hubbell 2001) and other metacommunity concepts (for review *see* Leibold et al. 2004) into biofilm research acknowledges that dispersal could play an important role in controlling local biofilm community structures. However, most of these theoretical considerations are as yet untested.

Protozoans represent an abundant and, in their function as grazers, important component of natural biofilms (Arndt et al. 2003; Parry 2004). Depending on their feeding mode, biofilm-dwelling protozoans can either graze on bacterial biofilms

(Huws et al. 2005; Weitere et al. 2005) or consume planktonic resources, thus acting as a link between planktonic and benthic food webs (Weitere et al. 2003; Kathol et al. 2009). Protozoans, particularly ciliates and heterotrophic flagellates (HF), are often not as obligatorily associated with the substrate as matrix-embedded bacterial biofilms are (Lee et al. 2000; Parry 2004); many forms glide or crawl freely on the substrate and can potentially move from the local biofilm patch into the plankton at any time. Other forms are attached to the substrate either more firmly via stalks or loricae or more loosely via plasma threads or flagella. Such groups can, however, also migrate into the plankton either by direct detachment or by the formation of swarmer cells. Thus the life forms of biofilm-dwelling ciliates and HF suggest a high migration potential. It has further been shown that new substrates exposed to aquatic environments can be rapidly colonized by protozoan groups from the plankton (Arndt et al. 2003; Wey et al. 2008). This rapid colonization soon leads to high densities of HF and ciliates in biofilms, and the initial assemblage of individual protozoans develops into a diversely interacting community, as e.g., indicated by the early occurrence of predacious taxa. The biofilm communities have similarities with their planktonic counterparts, further supporting the conclusion of a high dispersal potential of biofilm-dwelling ciliates and HF (Arndt et al. 2003). Planktonic aggregates, which offer a suspended biofilm habitat and which can be densely colonized by protozoans having a surface-associated life mode (Kiørboe et al. 2004), offer a further link between biofilm-associated and planktonic protozoan communities.

Taken together, the current knowledge on biofilm-dwelling ciliate and HF communities suggests a high exchange rate with the plankton and thus a largely dispersal-regulated community structure. However, this hypothesis is as yet untested, even though it is possibly very important in the regulation of local biofilm communities. In the present study we therefore tested the role of regional (immigration potential from the plankton) vs. local (growth and mortality in response to the local conditions) effects on different stages of the biofilm development under semi-natural conditions. For this purpose we manipulated the densities of natural planktonic ciliate and HF communities with the help of size fractionation and tested their potential to influence the local biofilm community structure in flow cells.

Experimental Procedures

Overview and study site

The experiments were conducted between January and November 2007 on board the Ecological Rhine Station of the University of Cologne, which is a ship permanently anchored in the Rhine at Cologne-Marienburg (Rhine km 684.5). Here, fresh Rhine water is constantly supplied by pumping it directly from the river into the laboratory. The natural microbial community in the plankton of this field water was the source community for the biofilm colonization described here.

To achieve different densities of suspended organisms and thus different immigration potentials, we experimentally manipulated the Rhine water by size fractionation (Wey et al. 2008) before conducting it further to flow cells (Norf et al. 2007) in which the contained organisms established biofilms. We tested the effects of reduced immigration potential on mature biofilm communities in two experiments, using pre-grown biofilms and a reduction or exclusion of planktonic HF (experiment I) or ciliates (experiment II). In a third experiment (experiment III) we considered the full development of biofilm-dwelling HF communities from sterile substrate to mature communities at steady state abundance under manipulated plankton abundances and resource densities.

General experimental set-up and tools

For the size fractionation of the potamoplankton we employed filter cartridges (Sartopure Capsules PP2, Sartorius) of pore sizes 1.2, 5, and 20 μm in different combinations, depending on the specific question. Schematic drawings of the two general experimental set-ups applied here, including the filter cartridges used, are shown in Fig. 1, and detailed descriptions of the set-ups for the different experiments are given in the next two sections. Generally, after passing a pre-filter of 100 - 300 μm mesh size, Rhine water was pumped via an impeller pump (Watson-Marlow GmbH) and silicone tubes (2 mm inner diameter, Deutsch and Neumann) through the aforementioned filter cartridges, and then into flow cells in which the suspended organisms established biofilms. The flow cells were identical to those used by Norf et al. (2007), having a total inner volume of 3.8 mL and a total inner surface area of 20.9 cm^2 (cover-slip area for biofilm observation: 7.2 cm^2). We applied a flow rate of 2.5 mL

min^{-1} (150 mL h^{-1}), resulting in an average flow velocity of 4.5 × 10^{-4} m s^{-1} under laminar flow conditions (Reynold's number [*Re*] = 3.5, calculated according to Stoodley et al. [1998]). The residence time of the water in the flow cells was 1.5 min. Every treatment in every experiment was set up in four replicates and the flow cells were kept constantly at the actual Rhine temperature in a temperature-controlled water bath. All outgoing and therefore open silicone tubes passed a heat-trap (maintained

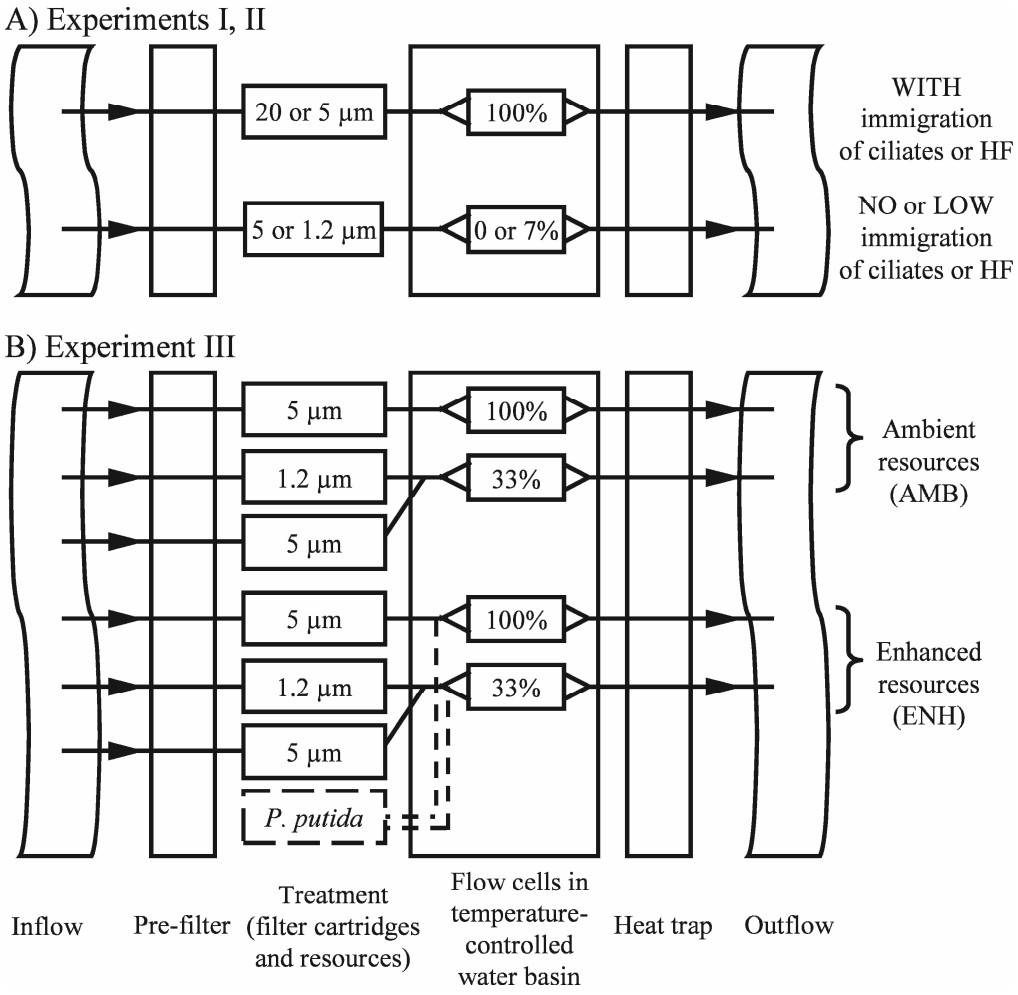

Fig. 1. Schematic drawing of the experimental setup. After passing a prefilter of 100 – 300-µm mesh size, the Rhine water was pumped through filter cartridges of different mesh sizes into the flow cells where the biofilms were established. The percentage written in the flow cells gives the percentage of organisms (ciliates or HF) passing the particular filter in relation to the plankton abundance passing the larger of the two filters. The discharged water finally passed a heat trap to prevent contamination. (A) Setup for experiments I and II. Biofilm communities with HF (experiment I) or ciliates (experiment II) were pre-grown from 5-µm or 20-µm filtered water, respectively. Then, further immigration was stopped (or significantly reduced) after 1 week in experiment I and 2 weeks in experiment II by connecting the flow cells to 1.2-µm or 5-µm filter cartridges, respectively. (B) Setup for experiment III. Biofilm communities with HF were established receiving 100% or 33% of the planktonic HF passing the 5-µm filter. The two immigration potentials were crossed with resource manipulations by considering ambient resources (no further resources added, AMB), and enhancement of resources with planktonic bacteria (addition of *P. putida*, ENH).

between 65°C and 70°C) to prevent any contamination by undesired protozoans. The whole system was sterilised by autoclaving before it was set up. In order to account for possible secondary filter effects, all treatments within a given experiment were generally run with filters of different pore size; we did not consider unfiltered Rhine water as a reference. Filter cartridges were changed weekly to further minimise secondary effects.

Mature biofilm studies (experiments I and II)

A schematic drawing of the set-up for mature biofilm studies is shown in Fig. 1A. Experiment I was run from 28 January until 12 February 2007, during which period the mean of the daily average of the Rhine temperature was 6.5°C (range: 5.7 - 7.4°C). The HF abundance in the plankton during this period was 147 ± 61 cells mL^{-1} (routine live count on 6 February 2007, B. Gräfe, pers. comm.). Biofilms for experiment I were pre-cultivated for eight days in eight flow cells connected to 5 μm cartridges (which excludes all metazoans and ciliates, Wey et al. 2008), allowing the establishment of a biofilm community containing HF and bacteria. On day eight, four randomly chosen flow cells were connected to 1.2 μm filter cartridges (which reduce the planktonic HF abundance by 93% [Wey et al. 2008]; this treatment was thus termed LOW immigration treatment), while the remaining four flow cells were connected to fresh 5 μm filter cartridges to allow further HF immigration (WITH immigration treatment). The filter cartridges used have no effect on the planktonic bacterial abundance or size distribution (Wey et al. 2008), thus ensuring comparable resource availability for HF in both treatments.

Experiment II was run from 21 March until 18 April 2007, during which period the mean of the daily average of the Rhine temperature was 11.1°C (range: 8.0 – 16.1°C). The average HF abundance in the plankton during this period was 407 ± 46 cells mL^{-1} (mean of routine live counts on 22 March and 10 April 2007, B. Gräfe, pers. comm.). To study the effects of immigration on mature ciliate communities, we pre-cultivated biofilms in flow cells connected to 20 μm cartridges (exclusion of metazoans and large ciliates, Wey et al. 2008) for 14 days, after which time a complex community containing ciliates, HF, and bacteria had been established. Then, one set of flow cells was changed to 5 μm filter cartridges to completely exclude further ciliate immigration

(NO immigration treatment, Wey et al. 2008), and the other set of flow cells was supplied with a fresh 20 μm filter cartridge (WITH immigration treatment). There was no difference in effect on the quantity of the pico- and nanoplankton between the 5 μm and the 20 μm filter cartridges, thus ensuring comparable resource availability for ciliates in both treatments.

Early biofilm studies (experiment III)

Experiment III was run from 23 October until 1 November 2007, during which period the mean of the daily average of the Rhine temperature was 11.9°C (range: 11.5 – 12.1°C). The average HF abundance in the plankton during this period was 147 ± 62 cells mL^{-1} (mean of routine live counts on 18 October and 8 November 2007, B. Gräfe, pers. comm.). The effect of different plankton densities, and thus immigration potentials, on pioneer biofilm colonization by HF was analyzed by comparing biofilms derived from 5 μm filtered water (exclusion of metazoans and ciliates) to a treatment in which the planktonic HF density had been reduced to 33% (in comparison to the 5 μm treatment) by a combination of 5 μm and 1.2 μm cartridges (Fig. 1B). Since 7% of the HF contained in 5 μm filtered water pass through the 1.2 μm filter (Wey et al. 2008), the 33% treatment was achieved by combining the filtrate of the 5 μm cartridges (flow rate: 0.7 mL min^{-1}) with that of the 1.2 μm cartridges (flow rate: 1.8 mL min^{-1}). The resulting flow rate was the same as in the 5 μm (100%) treatment (2.5 mL min^{-1}).

In addition to this regionally scaled factor we tested the effect of a local factor, i.e., the resource availability, by crossing the manipulation of the immigration potential with resource manipulations (ambient resource conditions and resource elevation by supplementation of planktonic bacteria). Resource addition was achieved by adding a suspension of *Pseudomonas putida* MM1 bacteria (Duetz et al. 1994) into the flow cells shortly before the inflow of Rhine water. This treatment has been shown to efficiently stimulate different feeding types (suspension feeding and surface feeding) within biofilm-dwelling protozoan communities (Norf et al. In press).

For the preparation of the bacterial suspension we followed the protocol used by Norf et al. (In press): We cultured cryo-preserved *P. putida* in 50% M9 culture medium (Hahm et al. 1994) containing 0.04 g L^{-1} glucose at room temperature (20°C). The

cultures were harvested after two days by centrifugation at 3400 g. The pellet was resuspended in 50 mL Pratt medium (Pratt and Salomon 1980). After refrigeration (4ºC) for two days we again harvested the cultures by centrifugation as described above. The cell density was determined with a Helber counting chamber (W. Schreck) and a suspension containing 1×10^8 cells mL^{-1} (final abundance) was prepared in Pratt medium. The bacterial suspension was attached to the flow cells with sterile miniature silicone tubes at a flow rate of 0.05 mL min^{-1}. The dilution factor with original Rhine River water was 1:50, yielding a final supplement of 2×10^6 cells mL^{-1} *P. putida* to the Rhine River water within the flow cells. The effect of the supplement on the overall flow rate is thus very small and the immigration potential (as determined by the discharge of protozoans) is not affected by the supplement. The concentration of supplemented bacteria was chosen in order to significantly enhance the abundance of the naturally occurring planktonic bacteria in the River Rhine, which was $1.1\times10^6 \pm 1.1\times10^5$ cells mL^{-1} on the day before the experiment was started (22 Oct 2007, M. Kathol, pers. comm.). The bacterial suspension was kept at 6°C during the experiments and was renewed every two days.

In summary, this cross-experiment (experiment III) was comprised of four treatments: 1) 100% immigration potential under ambient resource level (hereafter 100% AMB), 2) 100% immigration potential with enhanced resources (100% ENH), 3) 33% immigration potential with ambient resource level (33% AMB), and 4) 33% immigration potential with enhanced resources (33% ENH). All treatments were set up in four replicates.

Identification and quantification of protozoans

The abundance and taxonomic composition of ciliates and HF in the flow cells were analysed in situ by light microscopy (Zeiss Axiostar, phase contrast) every 1-2 days. We occasionally counted at greater intervals (of up to four days) in experiment II (because the ciliates grew much more slowly than the HF), and at shorter intervals in the first phase of experiment III, during which time we determined HF abundances approximately every twelve hours to be able to calculate the colonization rate more accurately. A 100 – 200× magnification was used for ciliate enumeration and 200 – 400× magnification for HF enumeration. Ciliates and HF were repeatedly counted in randomly distributed defined areas of 0.016, 0.004, or 0.001 cm^2 (depending on the

applied magnification). At least 60 specimens were normally quantified per flow cell; exceptions were only made in cases of extremely low abundances in early stages of biofilm formation. Protozoans were identified with the help of general keys (Foissner and Berger 1996; Lee et al. 2000) using a 400 – 1000× magnification, combined with video recording if necessary for later identification. The taxonomic classification was followed by the recent system suggested by Adl et al. (2005).

Data analysis

The statistical analyses were performed with the software SPSS 15.0 for Microsoft Windows. The immigration effects on HF and ciliates in experiments I and II were repeatedly recorded over a time period of one to two weeks after the pre-cultivation. Here we used repeated measurement ANOVAs (rmANOVAs) with the HF abundance, respectively ciliate abundance as dependent variable, time as within-subject factor and immigration potential as between-subject factor. Only the days after the filter change were taken into account (days 9-15 for experiment I on HF, days 15-28 for experiment II on ciliates). The data were log transformed prior to the analysis to achieve homogenous variances.

To analyse the similarity of HF or ciliate communities, the Bray-Curtis and Jaccard similarity was calculated with the software Primer (version 6.1.5, PRIMER-E, 2006). All data were based on morphotype (mostly on species or genera level) abundance counts which were log (X + 1) transformed. The results were then displayed as dendrograms based on the group average. Significantly similar clusters as calculated by the SIMPROF test (α = 0.05, Primer 6.1.5 software) were indicated. Additionally, we used SPSS to compare the number of morphotypes (in the different treatments on the final day of a given experiment) with each other. A Student's *t*-test was used when comparing two treatments (expts. I and II), and a one-factorial ANOVA when comparing four treatments (expt. III). Since no significant differences were found by the ANOVA in experiment III, we applied no post-hoc test.

For experiment III we calculated the initial colonization rate (cells cm^{-2} d^{-1}) and the rate of increase (d^{-1}) of the HF with the help of regression models separately for each independent replicate. The colonization rate was calculated with the help of linear regressions based on abundance data collected during the first two days. The rate of

increase was calculated on the basis of abundance data taken from the exponential phase (after four to six days). The rate is given by the slope of the linear regression of the dependency of (ln-transformed) abundance on time. The data extracted from the regression analysis as well as the final abundance reached at equilibrium were then taken to perform two-factorial ANOVAs on the effects of immigration potential (33% and 100% plankton density) and resource level (ambient and enhanced) on HF colonization rate, rate of increase or final abundance. The final abundance was log-transformed prior to the analysis to achieve homogenous variances. If the ANOVA indicated significant effects, the Ryan-Einot-Gabriel-Welsch (REGW) test was used for pairwise comparison.

Results

Effects of immigration on mature HF communities

In experiment I we tested the effect of the immigration potential (100% vs. 7%) on HF communities in pre-cultivated (one-week-old) biofilms. Effects of the treatment were found neither on the abundance (Fig. 2A, Table 1) nor on the taxonomic composition of the HF (Fig. 2C, D).

The development of the abundance showed similar dynamics in both treatments. The HF communities at the time of filter change (after eight days of pre-growth) were dominated by Metakinetoplastina (56% of the total abundance, particularly *Neobodo designis* and one undetermined species), *Ancyromonas* spp. (25%) and Thaumatomonadida (18%). These three groups still contributed significantly to the community composition at the end of the experiment under both immigration potentials. The abundances of two further groups, Cercomonadida (genus *Cercomonas*) and Chromulinales (genus *Spumella*), increased slightly in both treatments.

Calculation of the Bray-Curtis similarity revealed a significant difference between the taxonomic composition of the eight-day-old starting community and all communities at the end of the experiment (day 15), but we found no significantly different treatment-specific clusters between the open and closed communities (SIMPROF-test, $\alpha = 0.05$). We also calculated the similarities using the Jaccard index, which takes only absence and presence data into account, but still could not find any significant

treatment-specific clusters. Additionally, the number of morphotypes (10.3 ± 1.5 and 10.5 ± 1.0 in the closed and open communities, respectively) did not differ significantly between the two treatments on the final day of the experiment (Student's *t*-test, $p > 0.05$).

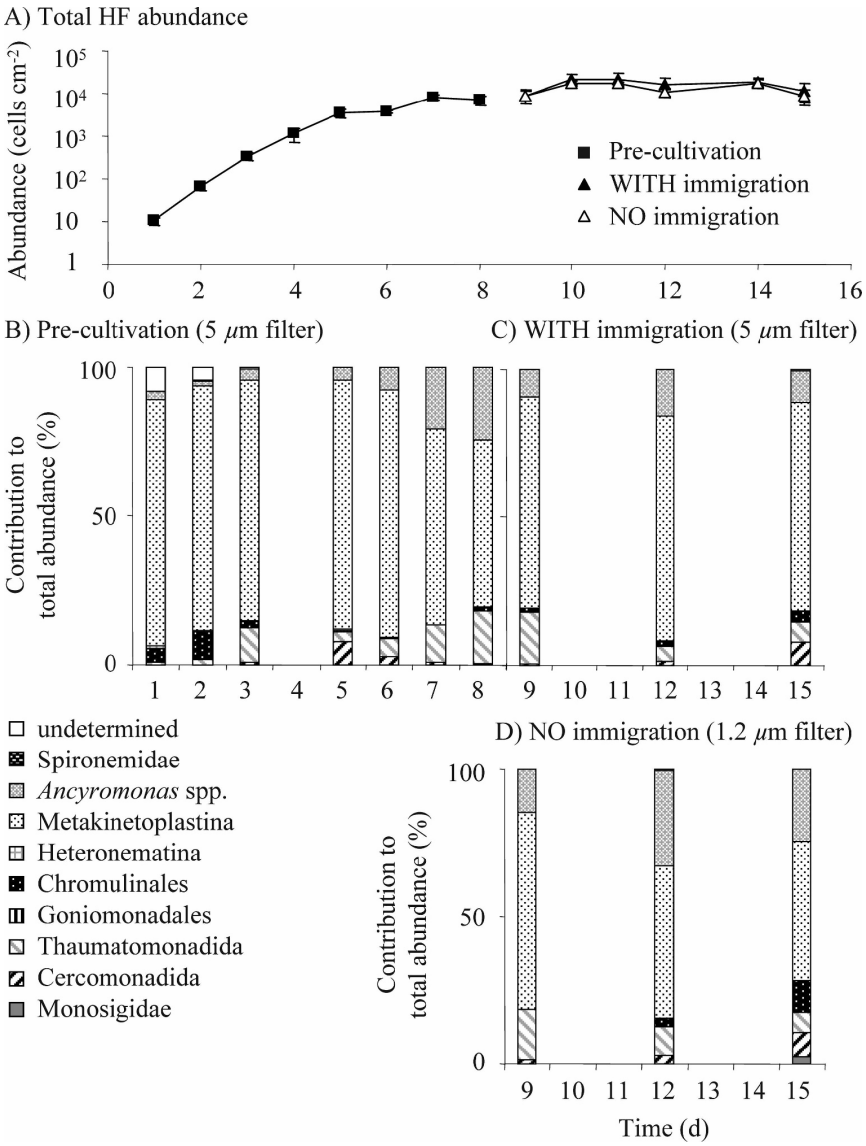

Fig. 2. Effects of strongly reduced immigration potential on HF communities in mature biofilms (experiment I). After a pre-cultivation period of 8 d with 5-µm filter cartridges, we strongly reduced further immigration into one charge of flow cells by applying 1.2-µm filter cartridges. (A) Development of HF abundance (log-scaled). See Table 1 for results of the rmANOVA. (B) Development of the taxonomic composition of the HF community before the filter change (precultivation). (C) Development of the taxonomic composition in the 100% immigration potential treatment (5-µm filter, as indicated by ''WITH immigration'') after the filter change. (D) Development of the taxonomic composition in flow cells with strongly reduced immigration potential (1.2-µm filter, as indicated by ''LOW immigration'') after the filter change.

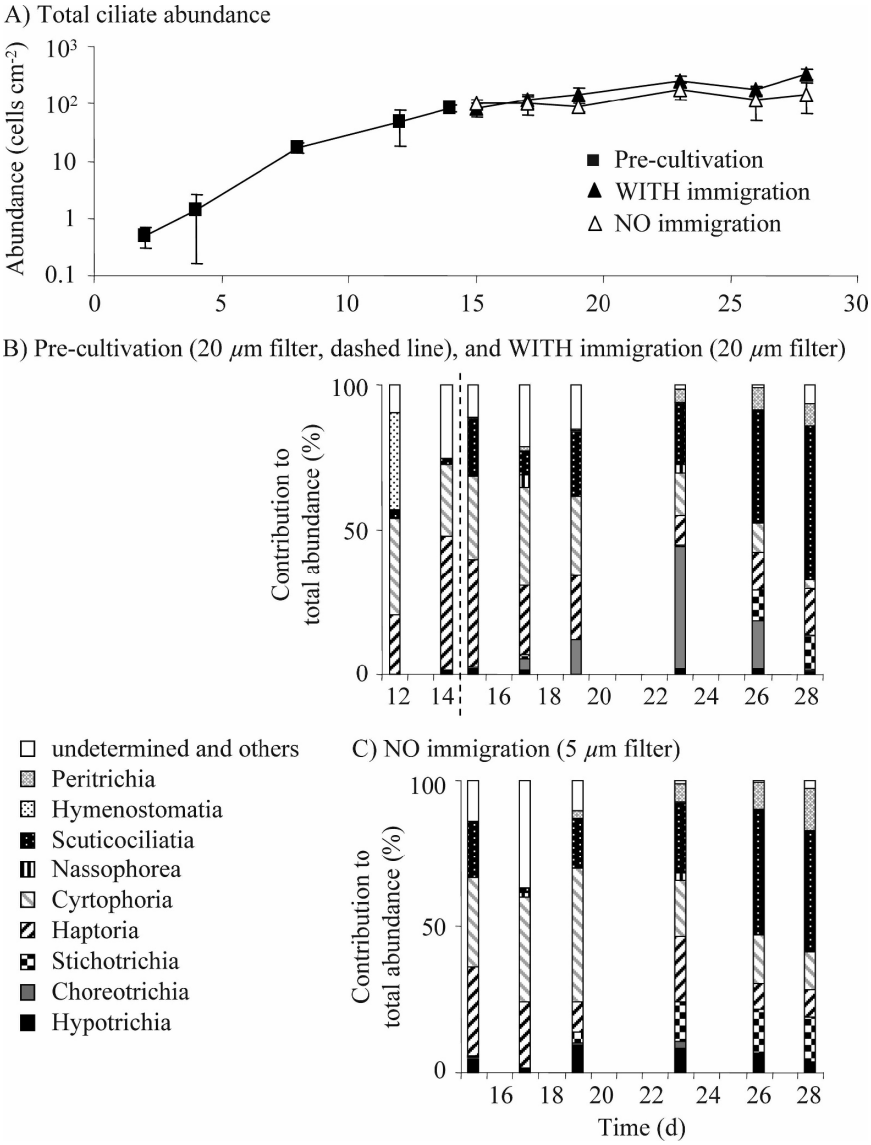

Fig. 3. Effects of the immigration potential on ciliate communities in mature biofilms (experiment II). After pre-cultivating the biofilms for 14 d with 20-µm filter cartridges, we completely stopped further immigration into one charge of flow cells by applying 5-µm filter cartridges. (A) Development of ciliate abundance (log-scaled). See Table 1 for results of the rmANOVA. (B) Development of the taxonomic composition of the ciliate community before the filter change (dashed line) and in flow cells with 100% further immigration (20-µm filter, as indicated by ''WITH immigration'') after the filter change. (C) Development of the taxonomic composition in flow cells with no further immigration (5-µm filter, as indicated by ''NO immigration'') after the filter change.

Table 1. Results of rmANOVAs testing for the influence of time (within-subject factor), immigration potential (between-subject factor), and the interaction of time and immigration potential on HF and ciliate abundance in the mature biofilm experiments (experiments I and II). The rmANOVAs were run for the period from after the filter change until the final day of the respective experiment (days 9–15 for experiment I, days 15–28 for experiment II). All data were log transformed before the analysis. Given are the sum of squares (SS), degrees of freedom (df), mean sum of squares (MSS), the *F*-ratios, and the *p*-values.

	SS	df	MSS	*F*	*p*
Experiment I (Fig. 2A)					
Time	0.485	5	0.097	14.800	< 0.001
Immigration	0.019	1	0.019	0.293	= 0.612
Time × Immigration	0.032	5	0.006	0.972	= 0.454
Residuals	0.164	25	0.007		
Experiment II (Fig. 3A)					
Time	0.929	5	0.186	6.949	< 0.001
Immigration	0.336	1	0.336	6.124	= 0.048
Time × Immigration	0.329	5	0.066	2.461	= 0.055
Residuals	0.802	30	0.027		

Effects of immigration on mature ciliate communities

In experiment II we tested the effects of the immigration potential (100% vs. 0%) on a ciliate community in a two-week-old biofilm. The abundance in both treatments developed similarly. Nevertheless, abundances in the closed community (NO immigration treatment) slowly decreased over time in comparison to the open community (WITH immigration treatment, Fig. 3A). The rmANOVA confirmed a weakly significant (p=0.048) effect of the treatment on the ciliate abundance (Table 1).

Regarding the taxonomic composition, there was a clearly higher contribution of choreotrichs (exclusively *Strobilidium caudatum*) in the open compared to the closed community between days 19 and 26 (Fig. 3B, C); nevertheless, at the end of the experiment (day 28) the two communities were similar, with a clear dominance of the scuticociliates (mainly *Cinetochilum margaritaceum*), peritrichs (mainly *Vorticella* sp.), haptorids (*Litonotus alpestris*, *Litonotus* sp., and *Acineria uncinata* or *incurvata*) and stichotrichs. The latter group was mainly composed of *Holosticha pullaster* in the closed community, and displayed a more diverse assemblage (particularly *Oxytricha setigera*, *Holosticha pullaster*, and one undetermined species) in the open community. These compositions, which developed independently from the immigration potential, differed considerably from the community compositions at the start of the

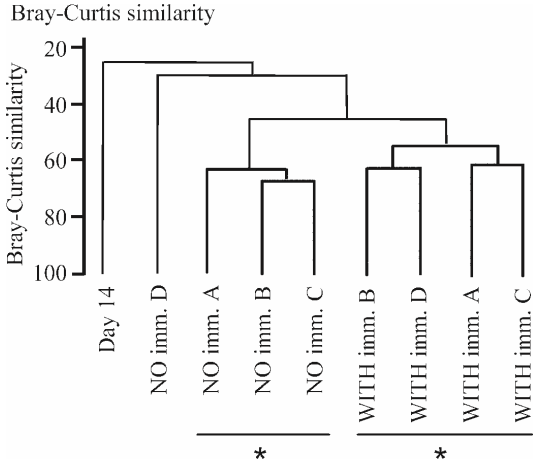

Fig. 4. Similarities of ciliate communities on the final day of experiment II, calculated with the Bray–Curtis index. Capital letters indicate different replicates. The mean of all replicates on day 14 (before the filter change) is also given as reference. Significant clusters according to the SIMPROF-test (α = 0.05) are indicated by an asterisk.

experimental manipulation (day 14) with its dominance of the haptorids (mainly *Acineria uncinata* or *incurvata* and *Litonotus alpestris*) and cyrtophorids (comprised of at least seven undetermined species), whereas the contribution of scuticociliates (mainly *Cyclidium glaucoma*) was low at that time.

The cluster analysis based on the Bray-Curtis similarity revealed a significant separation of the replicates of closed (apart from parallel D) and open communities on the final day of the experiment, and a separation of these clusters from the 14-day-old starting community (SIMPROF-test, α = 0.05, Fig. 4). In contrast, the analysis based on the Jaccard-index (which only takes presence and absence data into account) revealed no significant treatment-specific cluster, only a clear separation of the 28-day-old communities from the 14-day-old community (SIMPROF-test, α = 0.05, data not shown). The number of morphotypes (8.5 ± 2.9 and 14.5 ± 2.1 in closed and open communities, respectively) differed significantly between the two treatments on the final day of the experiment (Student's *t*-test, $p < 0.05$).

Effects of immigration and resources on the development of biofilm-dwelling HF communities

In experiment III we tested the interactive effects of the immigration potential with resources (as local factor) on the colonization of biofilms by HF. The general colonization pattern could be separated into three phases (Fig. 5), i.e., into phases of 1) an initial linear increase in abundance, 2) an exponential increase, and 3) an equilibrium.

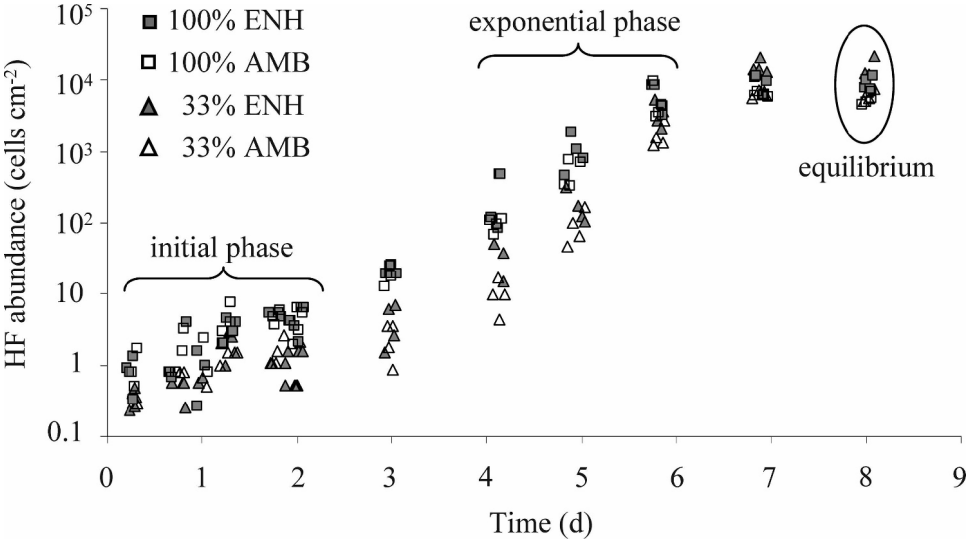

Fig. 5. Development of HF abundance with time depending on the immigration potential and resource addition (log-scaled, experiment III). Phases that were further analyzed in Fig. 6, i.e., the initial colonization, the phase of exponential increase, and the final abundance at equilibrium, are indicated.

The colonization rate during the initial phase depended significantly on the immigration potential, whereas it was independent from the resource level (Table 2, Fig. 6A). The colonization rates under the high (100%) and low (33%) immigration potentials were 2.32 ± 0.31 cells cm^{-2} d^{-1}, respectively 0.80 ± 0.35 cells cm^{-2} d^{-1} for the AMB treatments, and 2.34 ± 0.21 cells cm^{-2} d^{-1}, respectively 0.71 ± 0.16 cells cm^{-2} d^{-1} for the ENH treatments. The ratios (high vs. low immigration potential) of the colonization rates thus resembled the ratios of the plankton abundance of 3:1.

The rate of increase during the exponential phase was still independent from the resource level, but depended significantly on the immigration potential (Table 2, Fig. 6B). In contrast to the initial colonization rate, however, the highest increase rates were found in the treatments with low immigration potential. When reaching final abundance at equilibrium, resource quantity turned out to be the strongest predictor of total HF abundance, having highly significant effects (Table 2, Fig. 6C) and leading to abundances of 9318 to 13,977 HF cm^{-2} in ENH treatments vs. 5504 to 6357 HF cm^{-2} in AMB treatments. There also was a weaker effect of the immigration potential on the final abundance, with a trend to lower abundances at high immigration potentials.

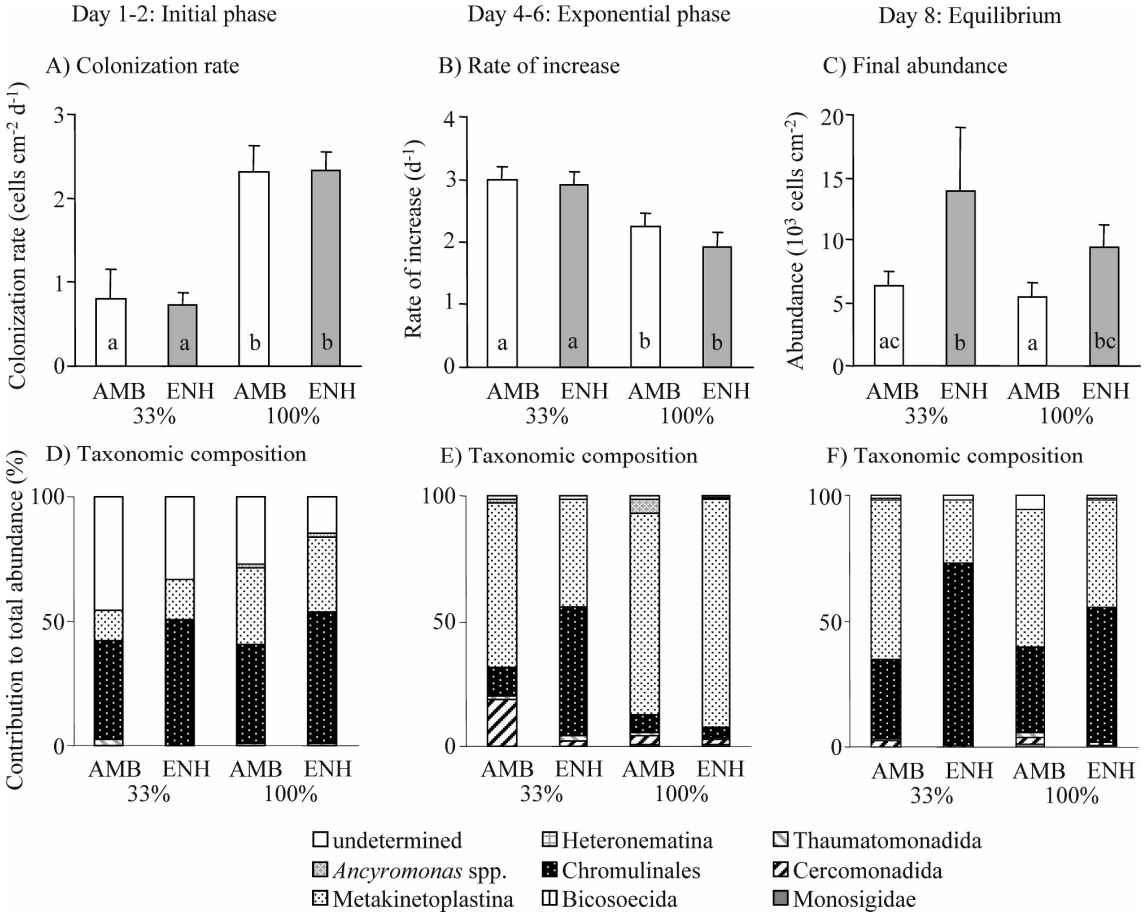

Fig. 6. Effects of immigration potential and resource availability (supplement of planktonic bacteria) on the development of the HF community during the three different phases of biofilm formation in experiment III as indicated in Fig. 5. Different letters (a–c) in the bars in panels A–C indicate significant differences in REGW-test (see Table 2 for ANOVA results). (A) Initial colonization rate during the phase of linear abundance increase in the first 2 d. (B) Rate of increase during the phase of exponential increase (days 4–6). (C) Final abundance after reaching the equilibrium abundance on day 8. (D–F) Taxonomic composition during the three phases.

Regarding the taxonomic composition, the initial phase of the experiment (plotted for all samples taken within the first two days) was dominated by the Chromulinales (genus *Spumella*) which contributed 40% of the total abundance to both AMB treatments, and more than 50% of the total abundance to the ENH treatments (51% at 33% immigration potential, 53% at 100% immigration potential, Fig. 6D). The second most abundant group in this phase were the Metakinetoplastina with 13% to 31% contribution in the different treatments, followed by much smaller numbers of *Ancyromonas* spp. and Thaumatomonadida, as well as a relatively large share of undetermined HF genera.

In the exponential phase the Chromulinales dominated only in the 33% ENH treatment (52% of the total abundance), while in all other treatments Metakinetoplastina dominated, with especially high contributions in the 100% immigration potential

treatments (up to 91% in the 100% ENH treatment, Fig. 6E, plotted for all samples taken within days 4 to 6). Apart from the Cercomonadida in the 33% AMB treatment, other HF groups contributed only in minor proportions to the taxonomic compositions of all treatments.

Table 2. Effects of immigration potential and resource level on the colonization of the substrate by HF (experiment III, Fig. 6A–C): Results of two-factorial ANOVAs testing for the effects of plankton abundance (100% or 33%) and resource level (ambient or enhanced) on initial colonization rate, rate of increase, and final abundance (log- transformed). Given are the sum of squares (SS), degrees of freedom (df), mean sum of squares (MSS), the *F*-ratios, and the *p*-values.

	SS	df	MSS	*F*	*p*
Initial colonization rate (Fig. 6A)					
Immigration potential	9.853	1	9.853	144.996	< 0.001
Resource	0.004	1	0.004	0.066	= 0.802
Residuals	0.883	13	0.068		
Rate of increase during exponential phase (Fig. 6B)					
Immigration potential	2.976	1	2.976	59.155	< 0.001
Resource	0.165	1	0.165	3.287	= 0.093
Residuals	0.654	13	0.050		
Final abundance (log-transformed, Fig. 6C)					
Immigration potential	0.052	1	0.052	5.110	= 0.042
Resource	0.309	1	0.309	30.512	< 0.001
Residuals	0.132	13	0.010		

After reaching the equilibrium abundance, the Chromulinales again dominated the treatments with resource addition, but with stronger differences between AMB and ENH treatments than during the initial phase (i.e., 32% and 34% in the low (33%) and high (100%) AMB treatments respectively, compared to 72% and 54% in the low and high ENH treatments, Fig. 6F). Although the Chromulinales were mainly composed of a sessile *Spumella* sp., the likewise sessile, colonial *Anthophysa vegetans* also reached high abundances, especially in the enhanced treatments. These two dominating Chromulinales-taxa were accompanied by sessile *Paraphysomonas* spp. The AMB treatments were dominated by Metakinetoplastina (63% at 33% immigration potential, 54% at 100% immigration potential, Fig. 6F), which mainly consisted of *Neobodo designis* and *Rhynchomonas nasuta*, together with *Neobodo curvifilis* and one undetermined *Bodo*-like species. These different taxonomic compositions of the communities at equilibrium abundance did not result in a significant, treatment-specific separation of groups in the cluster analysis based on the Bray-Curtis similarity

and the SIMPROF-test (data not shown). Also, no differences in the number of morphotypes (which was in the range of 7.0 to 8.8 in all treatments) were found by a one-factorial ANOVA ($F_{3, 12} = 2.3$, $p = 0.13$).

Discussion

Limited effects of immigration on mature communities

The limited dependence of the local dynamics of the examined mature ciliate and HF communities on immigration is one remarkable finding of experiments I and II. Abundances as well as taxonomic compositions showed similar dynamics under the different immigration potentials. Moreover, the relative community composition in the ciliate experiment (in which a 100% exclusion was possible due to the 5 μm filter) changed strongly during the course of the immigration manipulations, but with similar developments in both open and closed communities. This shows that the development of the mature communities is driven by other factors than by immigration, at least in the experimental setting described here.

However, the lack of immigration led to a small but significant decrease in the abundance and number of ciliate morphotypes in experiment II, whereas no such effects were found on the HF (experiment I). The results for the ciliates are in accordance with a microcosm study by Cadotte and Fukami (2005). In the present study, ciliate densities (and thus the total number of individuals within the flow cells) were about two orders of magnitude lower than found for the HF experiment (Figs. 2A, 3A; Arndt et al. 2003). Furthermore, ciliates usually display lower growth rates than HF (Hansen et al. 1997). These lower growth rates (and thus a lower potential to compensate losses due to emigration) and a smaller population size (resulting in a higher extinction risk; Boorman and Levitt 1973; Leibold et al. 2004) could thus have prevented the establishment of a ciliate community totally independent of immigration. Furthermore, it should be considered that a residual immigration was possible in the HF communities (reduction of plankton densities by 93%) but not in the ciliate communities (reduction of plankton densities by 100%).

At the population level, distinct negative effects of the lack of immigration were found for the choreotrich ciliate species *Strobilidium caudatum*. The attachment of *Strobilidium caudatum* to the substratum (via fine stalks) is highly reversible and

dynamic in that the cells frequently detach and re-attach themselves (Grim and Halcrow 1979). Thus it appears likely that, when import is interrupted, this species disappears from the local patch. However, *Strobilidium caudatum* was the only example found here which clearly benefited from immigration.

As outlined in the introduction, there were several reasons to expect dispersal to be much more important for ciliate and HF communities in mature biofilms. Although we found some small immigration effects which are probably due to a mix of different factors, we have to reject the hypothesis that immigration from the water column is a major factor in controlling mature, largely undisturbed biofilm-dwelling communities. However, we did not consider the full range of plankton abundances which can occur in field communities because the natural densities were already reduced in the controls due to filter effects. Furthermore, the natural plankton densities can occasionally be distinctly higher than during our investigations (Weitere and Arndt 2003). Therefore we cannot fully exclude that plankton abundances higher than those considered here can have immigration effects on biofilm communities.

Effects of immigration and resources depend on stage of biofilm development

The sigmoid development of the HF abundances in experiment III is typical for the initial colonization of a habitat (Pearl 1927), and can be divided into three phases. In the present experiment, both the initial colonization of the substrate and the exponential phase were strongly affected by the immigration potential, but in accordance with experiments I and II we found no effect of different plankton densities on the mature HF community.

In the first phase of the colonization, the ratio of the colonization rates in 100% vs. 33% immigration treatments was 2.9 (AMB) and 3.3 (ENH), suggesting a linear relationship between plankton abundance and rate of colonization as found earlier for the flagellate *Spumella* sp. (Kjørboe et al. 2004). However, our results demonstrate a rapid compensation of the limited immigration potential in the second, exponential phase. Furthermore, the rates of increase were not affected by enhanced resources in both phases, leading to the conclusion that the communities were not resource limited in this intermediate succession stage at relatively low abundances. Therefore, our results suggest either a density-dependent immigration mechanism or density-

dependent growth rates at this succession stage. Density-dependent immigration mechanisms for protozoan communities have been demonstrated by Hauzy et al. (2007) for ciliates, and Kjørboe et al. (2004) for the colonization of aggregates by flagellates. Nevertheless, the extremely high rates of increase during the exponential phase in comparison to the initial phase (which was basically immigration controlled) suggest that density-dependent growth rather than density-dependent immigration plays an important role here.

After reaching the abundance plateau, treatments with enhanced resources (ENH) generally showed higher abundances than those without resource addition (AMB), indicating that the HF community was now resource limited. However, in comparison to the high immigration potential (100%), the low immigration potential (33%) had no negative effect on the final abundance. Together with the results of experiments I and II (*see* Discussion above), this further supports the conclusion of an independence of the observed protozoan biofilm communities from immigration after the initial colonization of the substrate has been completed, and the local steady state abundance is reached (*see* Kjørboe et al. 2004 for a similar finding for the flagellate *Neobodo designis*).

Regarding the taxonomic composition, the development of the community composition in experiment III followed the general pattern of a domination of a few species in early and late succession stages and a higher diversity in mid-stages (Mouquet et al. 2003; Fukami 2004). Some members of the Metakinetoplastina such as *Neobodo designis* (which occurred frequently in the experiments) are known as typical 'pelagic-benthic species' (Arndt et al. 2000), which frequently colonize aggregates in the water column and thus have potentially high dispersal rates; Metakinetoplastina indeed seemed to be supported by a high immigration potential in the initial phase and subsequent growth in the exponential phase. Sessile Chromulinales on plasma threads (*Spumella* sp.) contributed more to communities with enhanced resources (especially in the initial phase and after reaching the equilibrium), suggesting a successful exploitation of the added planktonic bacteria (Christensen-Dalsgaard and Fenchel 2003). Furthermore, elevated rates of increase in the 33% treatments in the exponential phase could be due to the generally higher

proportion of other (apart from Metakinetoplastina) groups, allowing for a higher productivity by a more diverse resource exploitation (Venail et al. 2008).

Contribution to metacommunity framework

The metacommunity concept (Leibold et al. 2004) offers a theoretical framework for studying the effects of dispersal on local and regional scales and has recently been introduced into biofilm research (Battin et al. 2007). With our present approach we considered local biofilm patches and manipulated the immigration potential from the plankton, over which local biofilm patches are linked by dispersal. One perspective, i.e., the species-sorting perspective (Leibold et al. 2004), emphasizes that while the species number entering a local patch is dispersal controlled, the relative composition in that patch is mainly controlled by the local conditions. In such communities, dispersal only plays a role if the local conditions change. The results of all present experiments support this perspective for ciliate and HF communities in biofilms, as differences in the final community composition and abundance were mainly controlled by the resource availability (i.e., patch quality), not by the immigration potential.

In contrast to our initial hypotheses, only a few species-specific dispersal effects occurred in the three experiments (at least after the initial colonization was completed). These findings are in agreement with Hubbell's neutral theory (Hubbell 2001), which is based on the assumption that all species in a community are similar in their competitive ability, movement and fitness. Therefore, only disturbances (which could occur in our experiments due to predatorial protozoans, but probably at a low intensity due to the exclusion of the grazing macrofauna) and random relocations (which were possible, but may only have stretched over short distances, i.e., within a particular flow cell) would have the power to alter the species frequencies of the communities. Such processes then lead to open spaces which can be colonized by immigrating species (Shurin 2001). Field communities often experience high disturbance rates, e.g., through sediment rafting and river bed discharge (Gibbins et al. 2007) or due to grazing (Hillebrand 2008), and depending on the re-colonization source (i.e., the surrounding biofilm or the water column), founder effects could then lead to different communities. Since the present study clearly showed immigration effects on

the early biofilm development, disturbance should be emphasized in studies on the dispersal effect on biofilms as a next step.

Conclusion

Together, the results of the three experiments demonstrate a limited effect of immigration on the mature biofilm-dwelling ciliate and HF communities, and highlight the importance of local factors, particularly resource availability (*see* also Mouquet et al. 2003; Cadotte et al. 2006). Significant immigration effects, which occurred in the early stage of succession in experiment III were compensated for during the exponential phase, probably by density-dependent growth. The results thus suggest that immigration by planktonic HF and ciliates into biofilms is of quantitative importance only when open spaces exist, as achieved for example by disturbance.

Concluding Remarks & Perspective

Despite the ecological significance of biofilms, little is known about the factors regulating community structure and diversity. This thesis presents the successful application of a new method to test ecological concepts and local interactions in distinct compartments (bacteria, HF, ciliates) of semi-natural biofilms. The results obtained contribute to the general understanding of biofilm community control on different trophic levels and spatio-temporal scales.

Interactions within the biofilm community – The influence of grazing

A new method was developed and employed to investigate top-down effects of ciliates on HF and bacteria, and of HF on bacteria in the context of natural communities. To test these effects, the density of HF and ciliates was manipulated by filtration of the plankton, the source for the biofilm community. Higher densities of HF distinctly influenced the morphology and abundance of the bacterial cells, leading to enhanced biofilm growth in early phases of biofilm formation (more single cells, more microcolonies), as well as in later stages (more microcolonies). Nevertheless, HF reduced single cells in these later stages; presumably because they are not firmly enough attached to the substrate and could still be grazed upon. The presence of ciliates significantly reduced the abundance of gliding HF, leading to a change in HF taxonomic structure towards sessile, planktivorous forms. This resulted in a trophic cascade with reduced grazing pressure on single bacterial cells. Thus, these experiments showed for the first time for semi-natural, complex biofilms that (1) top-down effects of grazers shape biofilm communities (shown for both bacteria and HF), and (2) bacterial microcolonies are stimulated in the presence of HF, which probably is a grazer defence strategy.

That the newly developed method provides room for further development was shown in the second chapter, where molecular fingerprinting methods were introduced to assess the bacterial component on a finer scale of resolution. Again, the potamoplankton was size-fractionated to achieve biofilms with different trophic structures (density/presence of HF and ciliates), but bacterial biofilms were, in addition to the morphological structure, also examined concerning their diversity. Here, the

influence of protozoa on the structure of the bacterial biofilm, e.g. the occurrence of microcolonies and single cells, varied between seasons and confirmed the findings of chapter 1 in one case, while no influence on bacterial morphology was found in a second experiment. Nevertheless, SSCP and DGGE profiles revealed that HF altered the bacterial diversity and increased the number of bacterial phylotypes in both experiments, thus interestingly demonstrating independence of phylogenetic and morphological changes.

In accordance to chapter 1, ciliates altered the HF community structure, although this time the effect of bacterivorous ciliates exceeded that of predatorial ones and lead to direct effects on the bacterial biofilm (enhanced microcolony abundance and a trend to less bacterial phylotypes) rather than a trophic cascade. Furthermore, the presence of grazers altered the diversity at most times during biofilm succession, although the successional stage proved to be a strong predictor of bacterial diversity in all experiments. Chapter 2 thus shows that under certain circumstances (seasonal influences) microcolony formation is not provoked by enhanced grazer abundance and that protozoa alter the phylogenetic structure of bacteria in biofilms regardless of the occurrence of morphological effects or the influences of season and succession.

Further investigations of top-down effects should incorporate other seasons, as well as additional parameters such as temperature and nutrient levels to further assess the effects of abiotic factors on the community composition of both protozoa and bacteria. In conjunction with the taxonomic diversity, the assessment of the functional diversity of the bacterial component (e.g. by community level physiological profile systems like “Biolog” plates) are also of interest and promise to take research on biofilms another step forward towards the understanding of nutrient cycling at the basis of stream food webs.

Interactions with the metacommunity – The influence of dispersal

Chapter 3 shows that the set-up described here can also be used to combine the analysis of regional effects with that of local interactions within the biofilm community. Different plankton densities of HF and ciliates were used to assess the influence of immigration on different successional stages and under different resource conditions. The view of biofilms as largely immigration controlled communities was

challenged, when surprisingly little effects of the planktonic community on mature HF and ciliate biofilm communities were found. Further experiments revealed that the succession stage plays an important role in mediating immigration influences: In early stages of colonization, the immigration potential indeed was the main predictor of biofilm community composition and abundance, but after the initial settlement the biofilm community became increasingly controlled by local factors such as nutrient availability and presumably also species interactions.

The metacommunity concept includes a very distinct set of models to describe the influence of dispersal on local communities and provides a promising framework for future biofilm studies. Open questions that could be tested by modifying the set-up described in this thesis comprise, but are not limited to: (1) the scale of community patches (how are different local patch (community) sizes influenced by dispersal?), (2) the importance of different sources of immigrating individuals (plankton vs. neighbouring biofilm patches), and (3) the influence of disturbance (which brings communities back to early, immigration-controlled succession stages).

Conclusion

This thesis shows for the first time that ciliate, HF and bacterial communities in natural biofilms are strongly controlled by local factors (predation, competition, availability of resources). Nevertheless, these local interactions can be altered by other factors: The effects of immigration and predation both depend on the successional stage of the biofilm, and additionally seasonal influences can modify the answer of biofilm communities to internal grazing pressure. Also important is the level on which the effect of a certain factor is analysed (e.g. morphological or taxonomical diversity, number of species, species identity, abundance). The method developed in this study is highly adaptable to test different ecological theories on distinct time scales and resolutions under natural background conditions. For that reason it has a large potential for further exciting experiments on the factors controlling biofilm communities.

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Publikationen

[1]Wey,J.K., Scherwass,A., Norf,H., Arndt,H., and Weitere,M. (2008) Effects of protozoan grazing within river biofilms under semi-natural conditions. *Aquatic Microbial Ecology* **52:** 283-296.

[2]Wey,J.K., Norf,H., Arndt,H., and Weitere,M. (2009) Role of dispersal in shaping communities of ciliates and heterotrophic flagellates within riverine biofilms. *Limnology and Oceanography* **54:** 1615-1626.

[3]Wey,J.K., Jürgens,K., Weitere,M. (in Vorber.) Protozoan grazers alter the phylogenetic structure of riverine bacterial biofilms.

[1] inhaltlich Kapitel 1 dieser Dissertation

[2] inhaltlich Kapitel 3 dieser Dissertation

[3] inhaltlich Kapitel 2 dieser Dissertation