From the laboratory into the field: Testing defense mechanisms of bacterial biofilms against protozoan grazing

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Contents

Abstract

Protozoan grazing on bacteria is among the oldest predator-prey interactions in nature. While bacteria developed different defence strategies such as toxicity and microcolony formation to prevent grazing losses, protozoa developed different feeding mechanisms to compass these strategies. One important mode of grazing protection is biofilm formation. Its characteristics such as high bacterial densities and thus possible toxin production, as well as excretion of an extracellular matrix provide bacteria in biofilms with advantages in grazing protection compared to suspended bacteria. However, despite its importance, studies of protozoan grazing on biofilms are rare. This is partly due to the lack of appropriate methods to test mechanisms under complex field conditions. Here, different laboratory as well as field experiments were developed to investigate defence mechanisms of bacterial biofilms against protozoan grazers.

The first part of this thesis demonstrates the impact of the ciliate *Tetrahymena pyriformis* on biofilms of the microcolony forming bacterial strain *Acinetobacter* sp. C6 and toxigenic and non-toxigenic strains of *Vibrio cholerae*, respectively. The grazer had a strong impact on the morphology of *Acinetobacter* sp. biofilms grown under various nutrient conditions. Microcolony formation did not protect the biofilms as such. However, biofilm biovolume of the grazed treatments stayed the same or increased during the course of the experiment indicating possible nutrient recycling. In a comparative study with *T. pyriformis* grazing on a toxigenic wild-type *Vibrio cholerae* strain A1552 and a genetically modified, non-toxigenic *V. cholerae* strain *hapR* it could be demonstrated that biofilms of the toxic *V. cholerae* A1552 supported less ciliates than biofilms of the non-toxic *V. cholerae* hapR. Microcolony abundances and active bacterial cells within the biofilms of *V. cholerae* A1552 increased compared to non-grazed control biofilms arguing for a mutual benefit for grazer and bacteria possibly due to nutrient recycling and chemical cues.

In the second part of this thesis two new tools for environmental biofilm experiments are presented. (i) Diffusion chambers were successfully modified to expose toxigenic and non-toxigenic *V. cholerae* strains into the natural environment. The toxicity of wild-type *V. cholerae* A1552 for the flagellate *Rhynchomonas nasuta*

could be verified. However, in comparison with the natural hapR mutant strain *V. cholerae* N16961, the level of toxicity impact on the flagellate varied dependent on seasonal background. The importance of nutrient concentration on *V. cholerae* toxicity could be demonstrated in subsequent laboratory experiments. This suggested a separate toxicity pathway beside the beforehand known *hapR* pathway. (ii) Two established methods of biofilm and protozoa observation were combined to quantify grazing interactions. The coupling of natural biofilm establishment in flow cells and video microscopic analysis of individual flagellate feeding revealed inter- as well as intra-specific differences and similarities in feeding behaviour and food preferences in three flagellate species. Whereas the three species showed distinct feeding behaviour, individuals of all species were only able to ingest single prey cells. Although microcolonies were contacted no cells were ingested. Thus, microcolony formation did protect bacteria against flagellate grazing.

Taken together these experiments demonstrate the complex interactions of protozoa and bacteria on biofilms. Nutrient recycling, chemical and structural defence strategies of the bacterial community and the physical presence of the grazer have a major impact on biofilms. The presented methods such as the modified diffusion chambers and video microscopy in combination with the flow cell system are powerful tools to unravel the dynamics of predator-prey interactions on biofilms.

Kurzzusammenfassung

Der Fraß von Bakterien durch Protozoen gehört zu den ältesten Räuber-Beute Interaktionen in der Natur. Während Bakterien unterschiedliche Strategien als Schutz gegen Fraß von Protozoen entwickelten, (wie zum Beispiel die Produktion von Toxinen oder die Ausbildung von Mikrokolonien), entwickelten Protozoen unterschiedliche Fraßstrategien um Bakterien trotz Verteidigung konsumieren zu können. Obwohl die Interaktionen zwischen Protozoen und Bakterien im Plankton sehr gut untersucht sind, wurden die Dynamiken in Biofilmen bisher vernachlässigt. Die Ausbildung von bakteriellen Biofilmen, das heißt die Anheftung der Bakterien an ein Substrat, ist ein wichtiger Mechanismus zum Schutz vor Protozoenfraß. Biofilme sind charakterisiert durch eine hohe Dichte an Bakterien und die Produktion von extrazellulärer Matrix. Durch die hohe Dichte an Organismen und die Vielfalt an Verteidigungs- und Fraßstrategien sind die Interaktionen zwischen Bakterien und Protozoen ungleich komplexer als im Plankton. Trotzdem gibt es nur wenige Studien die sich mit diesem Thema beschäftigen. Ein grundlegendes Problem ist ein Mangel an Methoden, welche die Schutzmechanismen unter natürlichen Bedingungen testen könnten. Diese Arbeit stellt sowohl Labor- als auch Feldexperimente vor, die entwickelt wurden, um Verteidigungsmechanismen bakterieller Biofilme gegen den Fraßdruck von Protozoen zu testen.

Der erste Teil der Arbeit zeigte, dass der Ciliat *Tetrahymena pyriformis* einen starken Einfluss auf die Morphologie der Biofilme von *Acinetobacter* sp. hatte. Das Biovolumen der Biofilme blieb während der Experimente kontant, beziehungsweise nahm zu, was auf ein mögliches Nährstoffrecycling durch die Ciliaten hindeutet. Dieses Nährstoffrecycling zeichnet sich dadurch aus, dass Nährstoffe durch Fraß planktischer Bakterien der bakteriellen Biofilmgemeinschaft zugeführt werden. Bei einer Herabsetzung der Nährstoffzufuhr, respektive der Nährstoffqualität, veränderte die Anwesenheit von *T. pyriformis* die Morphologie noch deutlicher. In einer vergleichbaren Studie wurde der gegenseitige Einfluss von *T. pyriformis* und den toxischen *Vibrio cholerae* A1552 (Wildtyp), beziehungsweise den genetisch modifizierten, nicht-toxischen *V. cholerae* hapR (Mutation im Toxin regulierenden

hapR Gen) untersucht. Hier konnte gezeigt werden, dass die Abundanzen des Ciliaten auf Biofilmen des toxischen *V. cholerae* A1552 signifikant geringer waren als auf den Biofilmen des nicht-toxischen *V. cholerae* hapR. Die Anzahl der Mikrokolonien und der aktiven Bakterienzellen im *V. cholerae* A1552-Biofilm stieg im Vergleich zu Biofilmen, welche sich in Anwesenheit des Ciliaten entwickelten, an. Die Bedeutung von indirekten Effekten der Protozoen auf Bakteriengemeinschaften, beispielsweise durch Nährstoffrecycling, wird hierdurch erneut hervorgehoben.

Im zweiten Teil dieser Arbeit wurden zwei neue Methoden zur Untersuchung von naturnahen Biofilmen etabliert. (i) Diffusionskammern wurden erfolgreich modifiziert um toxische und nicht toxische *V. cholerae*-Stämme in ihrer natürlichen Umgebung zu exponieren. Die Toxizität des Wildtyps *V. cholerae* A1552 gegenüber dem Flagellaten *Rhynchomonas nasuta* konnte für das Freiland bestätigt werden. Im Vergleich mit der natürlichen hapR Mutante *V. cholerae* N16961 jedoch variierte das Level der Toxizität je nach Jahreszeit. Darauf aufbauend konnte anschließend im Labor die Rolle der Nährstoffkonzentration auf die Toxizität von *V. cholerae* nachgewiesen werden, was auf einen hapR unabhängigen Pfad der Toxin-Regulierungsmechanismen hindeutet. (ii) Zur Quantifizierung des Fraßverhaltens von Protozoen auf Biofilmen wurden zwei etablierte Methoden, die Fließzelltechnik und die Videomikroskopie, kombiniert. Dabei konnten deutliche Unterschiede im Fraßverhalten von drei oberflächen-assoziierten Flagellatenarten nachgewiesen werden. Obwohl Mikrokolonien kontaktiert wurden, wurden ausschließlich Einzelzellen ingestiert. Dies verdeutlicht die Bedeutung von Mikrokolonien als Fraßschutz gegenüber Flagellaten.

Alle Experimente belegen die komplexen Interaktionen von Protozoen und Bakterien in Biofilmen. Zusammenfassend betrachtend können Protozoen durch Nährstoffrecycling, sowie auch die chemischen und morphologischen Verteidigungsmechanismen der bakteriellen Gemeinschaft einen wesentlichen Einfluss auf die Biofilmgemeinschaften haben. Die hier neu oder weiterentwickelten Methoden, Diffusionskammern sowie Videomikroskopie in Verbindung mit dem Fließzellen-System, können in Zukunft wichtige Werkzeuge bei der Entschlüsselung von Räuber-Beute Dynamiken auf Biofilmen sein.

General Introduction

Biofilms thrive on almost all surfaces in aquatic systems where they harbour high densities of microbial organisms (Carrias and Sime-Ngando 2009). These complex communities play an essential role for ecological processes such as bioremediation and self-purification of river systems (e.g. Battin *et al.* 2003). Nevertheless, they also cause severe damage in the industrial and medical sector (e.g. metal corrosion or persistant infections, Hall-Stoodley *et al.* 2004).

Although it has been recognized early that bacteria attach to surfaces in the presence of water (Zobell 1943), this mode of life has long been ignored, mostly due to methodological restrictions. However, with the advancement of new visualization techniques, and biotechnological and molecular methods knowledge on biofilms has increased immensely in the last 30 years. Depending on the field of interest different devices and applications for biofilm research were developed such as reactors (Neu and Lawrence 1997), batch- (Watnick 1999) and flow-systems (Wolfaardt *et al.* 1994). The rise of confocal laser scanning microscopy (CLSM) at the end of the 1980s (first described by Cremer and Cremer 1978) was a major breakthrough in biofilm research. The combination of the capillary flow cell system (Wolfaardt *et al.* 1994) with the advancement in fluorescent tagging of bacteria (e.g. green-fluorescent protein) and CLSM allowed non-invasive observation of biofilms over time and led to major achievements in understanding biofilm structure, development, maturation and dispersal (e.g. Sternberg *et al.* 1999, Wimpenny *et al.* 2000, Stoodley *et al.* 2001, Barraud *et al.* 2006).

The majority of studies concerning biofilms were run by research groups in the field of medicine, microbiology and engineering (e.g. de Beer *et al.* 1994, Bradshaw *et al.* 1999). Thus, focus was mainly on the bacterial fraction of biofilms. Nevertheless, more and more studies started to investigate the ecological relevance of biofilms and their persistence in the environment (Huq *et al.* 2008, Lear *et al.* 2009). As mentioned before, biofilms shelter a large fraction of microbial organisms in aquatic systems. Bacteria, but also protists, fungi and small metazoans are an integral part of these communities (Wetzel 2001). It is known from planktonic communities that phagotrophic protozoa exert major grazing pressure on the bacterial community

that is highly selective. But compared to the knowledge on protozoa-bacteria interactions in planktonic environments (e.g. del Giorgio *et al.* 1996, Jürgens and Matz 2002, Pernthaler *et al.* 2009) only little is known about the interplay of protozoa and bacteria on biofilms until today (e.g. Parry 2004).

The focus of the present thesis was on two different protective mechanisms of bacterial biofilms, (i) microcolony formation and (ii) toxicity, against protozoan grazing under laboratory as well as environmental conditions.

The literature review (**chapter 1**) summarizes current knowledge on biofilms, protozoa and the bacterium *Vibrio cholera*. The second part of the thesis presents insights into the effects protozoans can have on bacterial biofilms under different nutrient conditions (**chapter 2**) as well as the mutual impact protozoa and toxic bacterial prey can have (**chapter 3**). The third part reports of the challenge to study protozoa bacteria interactions on biofilms in the natural environment and demonstrates the successful application of two different experimental designs for studying biofilms in the natural environment (**chapter 4** and **5**).

Effects of protozoans on bacterial biofilms – caged in the laboratory

Already mentioned methodological limitations in biofilm research made it difficult to study predator-prey interactions on biofilms. However, the capillary flow cell system allowed observation of three-dimensional biofilm structures under grazing influence. Ecologists started using these techniques and methods developed by medical researchers and microbiologists to investigate basic ecological questions.

One of the earliest interests in biofilm research was biofilm development. Initial attachment of bacteria on surfaces and subsequent formation of microcolonies are essential steps in biofilm formation. Former grazing studies demonstrated that biofilm development is one way of grazing protection and that protozoa even stimulate microcolony formation on biofilms (e.g. Matz and Kjelleberg 2005, Weitere *et al.* 2005). From planktonic communities it is known, that grazing under different nutrient conditions has different impacts on the bacterial community (e.g. Caron *et al.* 1988, Hahn and Höfle 1999). Based on the assumptions that microcolony formation is stimulated by protozoan grazing and protect bacterial biofilms from grazing, these effects were tested in the laboratory under different nutrient conditions. It was tested whether switching to less optimal nutrient supply for

microcolony formation of a bacterial strain might increase biofilm vulnerability towards protozoan grazing and hence, not protect against grazing impact on biofilm structure or biovolume. Biofilms grown under optimal nutrient supply should not experience changes in biofilm morphology or biovolume because they do not benefit from nutrient recycling by protozoans (**chapter 2**).

In addition to microcolony and biofilm formation some bacterial strains (e.g. *Pseudomonas aeruginosa*, Weitere *et al.* 2005) posses the ability of chemical defense. *V. cholerae* wild-type strain A1552 produces an anti-protozoan compound that prevents the flagellate *Rhynchomonas nasuta* from growing and thus prevents grazing losses (Matz *et al.* 2005). This compound is a density dependent, quorum sensing regulated factor that is regulated by the hapR gene (Matz *et al.* 2005). HapR regulates a number of phenotypes in *V. cholerae* including virulence and biofilm formation (Hammer and Bassler 2003). In former experiments the ciliate *T. pyriformis* was able to reduce biofilm biomass of toxic *P. aeruginosa* PAO1 (Weitere *et al.* 2005). Here, we tested whether the anti-protozoan compound of *V. cholerae* A1552 that affected *R. nasuta* had also implications on *T. pyriformis*. Since the anti-protozoan compound released by *V. cholerae* A1552 could be detected in cell free supernatant of *V. cholerae* A1552 biofilms (Matz *et al.* 2005), it was expected that *T. pyriformis* has no impact on the *V. cholerae* biofilms in contrast to *P. aeruginosa PAO1* biofilms but instead gets affected by the anti-protozoan *V. cholerae* A1552 compound (**chapter 3**).

Getting out there – development of methods to investigate protozoa effects on bacterial biofilms in the field

Laboratory experiments helped to understand control mechanisms within microbial communities. However, due to artificial conditions knowledge gained from laboratory experiments cannot be applied to field conditions without thorough investigation. For example, intensely discussed mushroom shaped microcolonies of *P. aeruginosa* biofilms (Klausen *et al.* 2003) might be an artifact of laboratory conditions of flow cell systems and do not necessarily apply to biofilms grown under environmental conditions where several additional structures such as streamers, walls and ripples can be seen (Hall-Stoodley *et al.* 2004). Thus, investigation of biofilms grown under natural conditions is essential.

Chapter 4 reports a new method to expose a defined microbial community into the natural environment. One important question was whether the anti-protozoan toxicity of *V. cholerae* against grazers is a laboratory artifact due to the experimental setting or if the toxicity can also be seen in the field. The challenge to expose a community of a defined bacterial strain and grazer was accomplished by modifying diffusion chambers (McFeters and Stuart 1972) to include a biofilm substratum for consequential CLSM and light microscopic analysis of the biofilm community. Strains of toxic and non-toxic *V. cholerae* as well as *E. coli* strain B were exposed into the marine environment in the presence of the flagellates *R. nasuta* and *Cafeteria roenbergensis*. Since the non-toxigenic *V. cholerae* hapR mutant strain is a genetically modified organism it could not be exposed into the natural environment. Thus, *V. cholerae* strain N16961 that has a natural frameshift mutation in the hapR gene, was exposed for comparison with the wild type *V. cholerae* A1552.

To combine the benefits of environmental experiments with the opportunity of daily monitoring of natural biofilm communities, semi-natural riverine biofilms were grown in flow cells (Norf *et al.* 2007) and flagellate-bacteria interactions quantified by video microscopy (Boenigk and Arndt 2000). This new approach (**chapter 5**) allows monitoring of surface-associated protozoa on the biofilms and quantification of complex predator-prey interactions in microbial biofilm food webs *in situ*. Individuals of three typical biofilm associated bacterivorous flagellates were continuously monitored and video recorded. By analyzing the recorded videos regarding different feeding characteristics inter- as well as intraspecific differences and similarities in feeding behavior and food preferences, respectively, were detected in semi-natural biofilms for the first time.

Chapter 1

Literature Review

Biofilms: an introduction

Biofilms are everywhere around. These microbial communities are present day and night on almost all surfaces that are in aquatic environments. Bacteria attach to surfaces in the presence of water, e.g. the water air interface, a rock in the water or a medical device such as a medial shunt. Depending on the environment surrounding the biofilm these communities also harbour fungi, protists and small metazoans that rely on these consortia in one way or the other (Costerton *et al.* 1995, Carrias and Sime-Ngando 2009).

The structure of biofilms, a result of multiple complex interactions of different organisms, is very heterogeneous with localised areas containing differing nutrient availabilities, pH and oxygen concentrations (Watnick and Kolter 2000). This gives different organisms with diverse requirements the opportunity to live in a community close to each other and take advantage of resources the neighbouring organisms might supply. Due to the large surface area they cover, biofilms play an important role in the self-purification of sediments and water, and the circular flow of nutrients (Hall-Stoodley *et al.* 2004).

Bacteria have three main advantages when living attached to substrate compared to living suspended as single cells: (i) the nutrient availability might be higher for organisms living in mixed species communities. Bacteria that depend on certain metabolites can live in close proximity to bacteria that produce this metabolite and thus gain higher growth rates (e.g. Ylla *et al.* 2009). (ii) The attachment onto surfaces and enclosure in a matrix protects bacteria from threats such as predation, chemical or biological toxins and UV (e.g. Stewart and Costerton 2001), and (iii) the close proximity of cells enables bacteria to interact on a higher level (e.g. quorum sensing, horizontal gene transfer, Carrias and Sime-Ngando 2009).

But, as mentioned above, biofilms are not only beneficial for man but they can also cause severe damage in industrial and medical settings. Water distribution pipelines and ship hulls are often covered in biofilms that either hinder the flow or corrode the material (Beech and Sunner 2004) and removal of these biofouling layers often lasts only for a short duration. Certain bacteria like *Pseudomonas aeruginosa* or

Streptococcus sp. can cause contamination of indwelling medical devices and serve as a source of chronic infections (Singh *et al.* 2000). A feature of biofilms that causes the severe problems is the production of matrix that enables bacteria to resist stresses such as UV light, chemical agents and antibiotics (Stewart and Costerton 2001).

Since biofilms can cause severe damage to humans, research has mainly focused on medically relevant species (e.g. *Pseudomonas* spp., *Staphylococcus* spp. or *Vibrio cholerae)*. Most research has been done under well-defined laboratory conditions in one or two species experimental set-ups in batch cultures, flow-cell- or rotating reactor systems. However, studies on biofilms from natural environments such as riverbeds and lakes are scarce. The knowledge gained from these studies has opened the doors to more and more specialized questions: how do bacteria attach, which environmental parameters trigger attachment and detachment, do bacteria communicate and if so, how? What enables bacteria in biofilms to resist antibiotics? Is communication possible with higher organisms? And can we apply the knowledge gained from simplified laboratory studies to the natural environment?

To summarize the current knowledge on biofilms, (with focus on freshwater biofilms in the natural environment), protozoa and *Vibrio cholerae*, the following review should give an overview on the aforementioned topics.

Biofilm life cycle

Bacterial biofilms are well studied in the laboratory and thus knowledge about the different stages in the development of biofilms is available: in the initiation phase bacteria attach loosely to the surface followed by a change to irreversible attachment (Fig. 1.1, I). During the second phase bacteria accumulate through growth, cell division and recruitment from the bulk phase. Additionally bacteria start producing extracellular polymeric substances (EPS). This matrix covers the bacterial cells and is responsible for the high resistance of biofilms (Flemming and Wingender 2010). First two-dimensional growth on surfaces occurs followed by growth into the three-dimensional space (Fig. 1.1, II). Microcolonies, the basic living structure unit of biofilms (Carrias and Sime-Ngando 2009) begin forming. After some time, detachment of single bacteria cells can be observed (Fig. 1.1, III).

In mature biofilms equilibrium is reached while new bacteria attach to uncolonised areas while biofilm bacteria detach from the biofilm (Stoodley *et al.* 2002). Grazing or mechanical damage opens space for new bacteria to attach and thus biofilm development is a dynamic, never ending process.

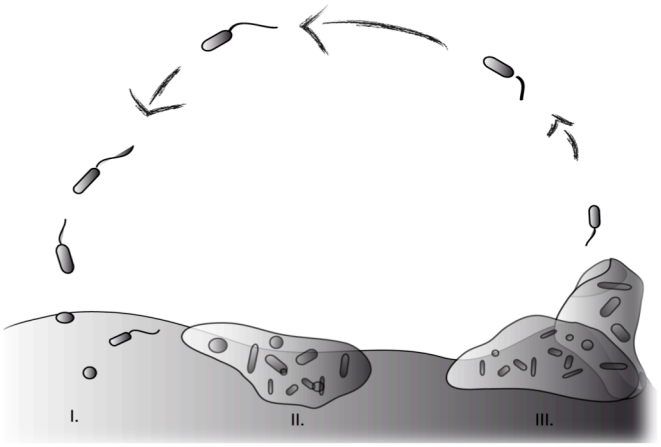

Figure 1.1. Simplified view of the life cycle of a biofilm: (I) loose attachment of bacteria, (II) irreversible attachment and production of EPS, (III) biofilm maturation and dispersal.

Quorum sensing

Bacteria growing in biofilms or consortia have many advantages. One advantage is chemical communication between the cells, known as quorum sensing (QS, for more detailed review see Holden *et al.* 2007). The density dependent release of certain signal molecules enables bacteria to communicate as primitive multicellular organisms (e.g. Waters and Bassler 2005). While a signal of a single cell released into the surrounding environment has no influence on other bacteria, the signal can reach a critical threshold if many bacteria produce the signal and the community can act accordingly. QS plays an important role in biofilm formation, toxin production, and optimisation of population survival by differentiation into diverse morphological forms and other social behaviours of bacteria.

Four criteria have to be considered for a molecule to be a QS signal molecule (after Winzer *et al.* 2002): (i) the production occurs at special stages during growth, under certain physiological conditions or in response to changes in the environment, (ii) the signal accumulates extracellularly and is recognised by a specific receptor, (iii) accumulation generates a concerted response once a critical threshold has been reached, and (iv) the cellular response extends beyond physiological changes

required to metabolise or detoxify the signal. Points 1 to 3 on their own are insufficient to mark a QS signal as they are also met by other molecules.

The first QS signal molecule discovered and the most intensively investigated are the N-acylhomoserine lactones (AHLs). They were first discovered in the marine bacterium *Vibrio fischeri.* This bacterium appears in the planktonic marine environment in numbers of 10^2 cells mL^{-1} where it is non-luminescent. However, *V. fischeri* also appears in the light organ of squids in much higher densities (>10^9 cells mL^{-1}) and are responsible for producing light through bio-luminescence (Boettcher and Ruby 1995, Holden *et al.* 2007). AHLs have now been found to be signal molecules in several Gram-negative bacteria. Many of these are important human pathogens such as *P. aeruginosa*, *Yersinia* spp. and *Vibrio* spp. and are capable of causing infections and diseases in humans, animals and plants. The AHLs produced by these organisms regulate toxin gene expression, genes needed for motility and extracellular enzymes. It seems that the AHLs are a language commonly used by Gram-negative bacteria. In Gram-positive bacteria the signalling molecules are usually peptides rather than AHLs and the systems almost always differ from the Gram-negative QS systems (Waters and Bassler 2005, Obst 2007, Turovskiy *et al.* 2007).

In fact, it is now known that there are several different types of signalling systems in Gram-negative and -positive bacteria. However, only one system is used by both Gram-negative and -positive bacteria: the LuxS/autoinducer-2 system (AI-2). The AI-2 is possibly the basis for a common interspecies language. The *luxS* gene is highly conserved and can be found in more than 60 Gram-positive and -negative bacteria (Holden *et al.* 2007). The AI-2 is of special interest since it is well studied in *Vibrio cholerae*. While QS in bacteria normally induces biofilm formation (e.g. *P. aeruginosa*), it represses biofilm formation by *V. cholerae* (Hammer and Bassler 2003). Cells of *Pseudomonas* start to attach and thus colonise surfaces if a certain density of cells is reached and enough signal molecules are produced, e.g. in lung infections. Cells of *V. cholerae* however detach if a certain threshold is reached and thus leave the biofilm if the density of cells becomes too high. This has been suggested to be an advantage for the cells during the infectious stage of Cholera in humans. *V. cholerae* cells attach to the intestine and once the density of cells is high

enough the cells detach and get released in high numbers into the environment (Zhu *et al.* 2002).

Since different species of bacteria can use the same systems for communication, cross-talk between different species is often possible. In addition, some higher organisms (e.g. plants) have the ability to disturb or destroy the signal, so called *quorum quenching*. Much research effort is being spent on attempts to develop compounds that disturb QS signals in order to manipulate bacterial biofilms, e.g. force bacterial biofilms to detach, as well as other virulence factor expression, and thus develop novel classes of antibiotic agents (Rice *et al.* 2005, McDougald *et al.* 2007).

Protozoa

In general protozoa are microscopic small, single-celled, eukaryotic organisms that range from 2-200 µm on average, yet some species are visible to the naked eye (in the mm range, e.g. Finlay 2001). The term "protozoa" describes distantly related organisms that are grouped together because of their size and ecological function (Sanders 2009). Corliss (1994) defined protozoa as "predominately unicellular, plasmodial, or colonial phagotrophic, colourless protists, wall-less in the trophic state". The general term 'protist' includes the heterotrophic protozoa as well as fungi and autotrophic single celled algae. Representatives can also be mixotrophic or osmotrophic (Sanders 2009).

Free-living protozoa, i.e. protozoa that are not associated with other organisms, constitute a group defined by their key-role in the environment: the ability for phagotrophy, which means they are able to catch and digest food (Berninger *et al.* 1991, Finlay and Esteban 1998). Protozoa can reach large population sizes and are the most abundant group of phagotrophic organisms in the biosphere (Finlay 2001). This makes them the most important grazers for controlling the abundance of bacteria in aquatic environments (Finlay and Esteban 1998, Finlay 2002, Sherr and Sherr 2002, Sleigh 2006).

Protozoa are globally distributed and live everywhere water is available (Laybourn-Parry 1984, Sherr and Sherr 2002). They live in planktonic and benthic communities of freshwater, brackish and marine environments, abyssal plains (Scheckenbach *et al.* 2010) and the arctic (Nitsche *et al.* 2007), and also in soil where they are an important link in bacteria-plant interactions (e.g. Bonkowski and Brandt 2002).

In this review the focus is on the free-living phagotrophic protozoa, whereas unicellular autotrophic and mixotrophic organisms will not be addressed.

Recent Protozoan Taxonomy and Systematics

Before molecular techniques were developed it was very difficult to precisely characterize protists. Since many species posses few morphological differences to distinguish between them, many different species were wrongly considered to belong to one single species. The rise of molecular biology showed that protists are not one phylogenetic group (Fenchel 1991, Hausmann *et al.* 2003) but very diverse indeed. New methods have revealed that solely morphological characterisation is inaccurate for determining taxonomy of protists, similar to what is seen in bacteria.

In the last seven years three different taxonomies were published by Cavalier-Smith (2003), Adl *et al.* (2005) and Baldauf *et al.* (2008). All three have in common the view that protists do not fit into one phylogenetic group. The most recent one by Baldauf sorted the eukaryotes into one of eight major-groups (1. Ophistokonts, 2. Amoebozoa, 3. Archeaplastida, 4. Rhizaria, 5. Alveolates, 6. Stramenopiles, 7. Excavates and 8. Discicristates). One example for the broad phylogenetic relationship of protists are the choanomonad with representatives much closer related to the animals than to other flagellates (Caron et al. 2009).

Global Distribution versus Endemism

At the beginning of the 20th century Beijernick (1913) stated that regarding bacteria 'everything is everywhere – the environment selects'. Finlay and Fenchel have taken up this point of view for microbial eukaryotes (1999). The global distribution of protozoa has been widely discussed with two contrary points of view prominently held by Finlay & Fenchel on one side who are in line with Beijernick, and Foissner on the other side who argues for a limited distribution of protozoa. While the three

authors‘ main topic is the diversity of ciliates, the arguments included in the discussion on both sides can be applied to all protozoan species.

While Foissner (1999, 2008) argues for a rich global diversity and a limited geographical distribution of most species, Finlay and Fenchel are convinced that ciliate species are globally dispersed. Even if the local diversity is high, diversity on a global scale might be rather poor (Finlay *et al.* 1999, Fenchel and Finlay 2004, Finlay *et al.* 2004, also Mitchell and Meisterfeld 2005). While the discussion about global distribution versus endemism has been going on for quite some years (e.g. Finlay *et al.* 1996, Fenchel *et al.* 1997, Foissner 1999, Patterson 2001, Fenchel and Finlay 2006, Foissner 2008), it should be considered that the known numbers of ciliate species alone increased from an estimated 3000 species in 1996 to approximately 8000 species (of which approximately 3000 live symbiotic and around 200 are fossil forms) in 2008 (Finlay *et al.* 1996, Lynn 2008). A larger increase in species numbers can be expected in the future due to an increase in taxonomic studies due to advancements in molecular techniques and high-resolution microscopy. Recently the Highlight Report of the ”Census of the Marine Life 2010“ survey was published (CoML 2010). This report presents data collected over ten years of research from scientists from all over the world and shows that the diversity in marine habitats is much higher than previously assumed. Of all newly discovered species 10% belong to the protists. An average of 47 new species were found per year (between 2002-2006), and 90% of the weight of life in the oceans belonged to microorganisms. This data suggest that there might be a much higher diversity in the natural environment than we imagine and that we are just beginning to understand the diversity that exists.

One very important factor that has to be considered when talking about cosmopolitanism versus endemism is that different definitions of a species exist. The classical species concept cannot be applied to protozoa. There are more than 20 different definitions of species (Mayden 1997) and depending on whether the definition is followed rather strictly or loosely you might find more endemic or globally distributed species, respectively (Patterson and Lee 2000). Patterson (2001) applies the concept of weeds and specialists to protozoa. Weed species are those that appear in high numbers in samples but actually represent only a small

portion of the actual diversity of a habitat. Mostly they are consumers of bacteria, have a broad physiological tolerance and high growth rates. *Rhynchomonas nasuta, Bodo* sp., *Cafeteria* sp. and *Planomonas* sp. are typical flagellated weed species while *Tetrahymena* sp.*, Euplotes* sp. and *Vorticella* sp. are typical ciliate weeds. However, the larger proportion of protozoan species are more specialised, diverse and less abundant. Patterson argues that most surveys are most likely "catalogues of weeds" and rather perfunctory and thus result in an underestimation of protozoan species diversity.

Another critical point in protozoan diversity research is the sampling, culturing and surveillance of protozoan species. While, as said before, the majority of protozoa appear in low numbers these species may easily be overlooked, or - because of similar morphologies - considered to belong to another species. Under-sampling or wrong culture conditions might also artificially diminish the number of species in samples. Thus, if talking about global distribution and endemism the definition of species should be taken into account as well as a precise application of methods (Patterson 2001).

Functional Roles and Ecology

As mentioned before, the free-living protozoa are most often characterised by their key function: the ingestion of particulate food particles, so-called phagotrophy (e.g. Fenchel 1987). The morphology and the ecological function of protozoans as grazers are closely linked in all groups of protozoa (Jürgens and Güde 1994) and the huge diversification of the protozoa is related to the evolution of different feeding mechanisms. Also, the small size of the free-living protozoa and their ability to feed on bacteria (partly as sole food source) is the major factor that gives them their importance in the microbial food web (Finlay 2001). The following part will focus on substratum-associated protozoa.

Morphological groups

Free-living heterotrophic protozoa are classically divided into three broad morphological groups: amoeboid, ciliated and flagellated protozoa. These groups are not consistent with any system of classification of protozoa published in recent

years but because the morphology of protozoa is closely related to their function as grazers these groupings still have some utility.

Amoebae

Representatives of the amoeboid morpho-group move and feed with the help of pseudopodia: cellular extensions that form from the cell (Anderson 2001a). Slime molds, heliozoans and rhizopods belong to this assemblage. The rhizopods can be further categorised into the „naked" and the shelled „testate" amoeba. With the naked amoeba there is no clear relationship between the morphology and the ecological function (Finlay 2001). Representatives of this morpho-group are very diverse, appear mostly attached to surfaces and are very abundant in soil. Nevertheless they can also appear in suspension though mostly attached to lake/marine snow (Parry 2004).

Flagellates

Flagellated protozoa are relatively small (2-20 µm on average) but are a fundamentally important group because of their immense abundance in the natural environment. They are ubiquitous on a global scale and appear everywhere from marine and freshwater to terrestrial systems. Because they cover every trophic role from primary producer to carnivore they play a major role in the aquatic food web (Sanders 1991, Sleigh 2000, Anderson 2001b). Due to their ability, especially of heterotrophic nano flagellates (HNF: 2-10µm), to graze on bacteria, they are largely responsible for controlling the bacterial abundance in aquatic environments (Finlay 2001, Sherr *et al*. 2009).

Flagellates possess two, sometimes one or more than two flagella, which they use for locomotion and feeding. In some cases the two flagella are of the same length (e.g. euglenids), and in other, as with heterokonts, one flagellum is longer than the other. Bicosoecids or bodonids are an example for this group. In this case, one trailing flagellum holds the flagellate to a surface while the other is used for locomotion or feeding. Flagellates can exist in solitary or colonial states, attached or free swimming and are typical biofilm dwellers.

The functional role of the flagellates is largely determined by their different morphologies. Choanoflagellates, for example, have a collar around their single

feeding flagellum and can feed on the smallest of bacteria that are trapped in the feeding filter. Others such as *Cafeteria roenbergensis* or *R. nasuta* are raptorial feeders that feed on single bacteria they grab. The different feeding types are explained in more detail later on. Some taxonomic groups, such as choanomonads and bodonids, are exclusively heterotrophic while others contain mixotrophs (euglenids and chrysomonads). The haptomonads and cryptomonads are dominated by autotrophs. Explaining characteristics of the single groups would be beyond the scope of this review but Patterson & Larsen (1991) and Hausmann (2003) give a good overview. Common flagellated groups found on biofilms are euglenids, bodonids, thaumatomastigids, apusomonads and some representatives of protista *incertae sedis*. Some of these can also contribute significantly to the pelagic flagellate community (Arndt *et al.* 2000). Their abundance can range from 10^2 to more than 10^6 flagellates mL^{-1} sediment (Gasol and Vaque 1993). Among the flagellates, the highest biomass is from two groups of euglenozoa: euglenids make up the highest proportion (20 - 80 % of HF biomass), followed by bodonids (5 – 20 %). Both groups are also the most diverse groups of flagellates within biofilms (Patterson *et al.* 1989).

Ciliates

The morphological-functional group of the ciliates is the only one to remain intact as a monophyletic group after the latest phylogenetic studies (Finlay 2001, Baldauf 2008). They belong to the alveolata, along with the dinoflagellates. Ciliates have long been separated from other protists and are the most homogeneous group of protozoa, characterised by three typical attributes: (i) nuclear dimorphism (micro- and macro nucleus), (ii) sexual conjugation and (iii) the possession of cilia at least in some stage of their live cycle. These are derived from kinetosomes with three fibrillar associates that can either cover the whole body or just appear on certain sides of the cell, and are used for locomotion and feeding (Lynn 2008).

The shape and size of ciliates can vary from colonial or stalked ciliates to simple geometric shapes (as the majority of ciliates are) and from lengths of 10 µm to 4500 µm. The body form of the individual ciliate is relatively constant due to the body cortex. In terms of numbers of species ciliates belong to the top five groups of protists with a minimum of 8000 estimated species that are divided into two major

subphyla and eleven classes (Lynn 2008). The life cycle of a ciliate can be divided into three different stages: (1) a vegetative cycle in which growth and cell division occur, (2) a sexual cycle in which conjugation and thus exchange of genetic material occurs and (3) a cryptobiotic cycle in which the cell undergoes encystment (Lynn 2008). All ciliates are heterotrophic and occupy a wide range of ecological niches. They have different feeding modes from filter feeder to interception feeder (described below). Some can temporarily keep the chloroplasts of their algal prey (kleptoplasty) and thus become mixotrophic (Sanders 2009). Typical biofilm ciliates can be found within the peritrichs, cyrtophorids, nassulida, hypotrichs, suctoria and pleurostomadids. They are often attached, or dorso-ventral flattened and posses cilia just on one cell side (Foissner *et al.* 1991).

Feeding types

In addition to groupings due to morphological features, there are also categories based on the feeding type displayed by protozoa:

Fenchel (1987) distinguished the phagotrophs according to their feeding mode and morphological features into diffusion feeders, filter feeders and raptorial or interception feeders. Franco (1998) characterised ciliates according to their ecological function in dependence to Fenchel (1987) with modifications in the mechanism of food particle collection. Boenigk and Arndt (2002) thought this way of categorising to imprecise, and divided the protozoa according to their degree of contact with substrate, e.g. whether they are free swimming, loosely attached or attached. Parry (2004) differentiated them according to the contact with the substrate but also included the degree of contact of the prey (suspended and free swimming, loosely attached or attached?). As a result the author grouped protozoa into transient, sessile, browsing and amoeboid types, and either preferably feeding on attached or suspended prey. While all of these groupings have their advantages no single grouping covers all without discriminating certain groups or species. In the following the single types will be explained according to Fenchel's categories including Boenigk's and Parry's types where appropriate:

Diffusion feeder

Very motile prey in sufficiently high numbers is necessary for this feeding type to be effective (Fenchel 1991). The protozoan waits for the prey to make contact and then traps it with sticky tentacles, e.g. suctorians (Fenchel 1987). Diffusion feeders can be attached to surfaces as some suctorians are, or free swimming as the heliozoans.

Filter feeder

This feeding mode depends on the production of water currents by the protozoan that transports water through a collar of cilia or tentacles and traps bacteria in these filters. The capture rate is proportional to the food concentration, the size of the filter and the flow velocity produced by the filter. Filter feeders are relatively big compared to their prey. They feed predominately on suspended prey and make up approximately 2/3 of the planktonic community and just 1/5 of the biofilm community (Boenigk and Arndt 2002). Attachment to surfaces guarantees the highest capture rate, even if attachment is just transient as with ciliates such as *Euplotes* and *Cyclidium* (Fenchel 1986, 1987). To avoid repeated filtering of already processed water attached flagellates and browsing ciliates move regularly. Ciliates like *Vorticella* sp. contract their stalk to presumably mix the water phase around the protozoan (Fenchel 1986). Ciliates like *Vorticella* sp. and *Stentor* sp. and the flagellate *Codosiga* sp. are well known attached biofilm protozoa that feed on suspended prey (e.g. Eisenmann *et al.* 2001).

In flagellates such as choanomonads the flagellum produces a current that drives water away from the cell body and bacteria as well as other food particles get trapped on the outside of the collar. Pseudopods then draw these bacteria to the cell body and enclose the prey in a food vacuole. All acceptably sized particles are ingested. The choanomonads are specialised feeders on the smallest of prey. The chrysomonads and helioflagellates use a similar mechanism. Here, the water current gets directed against the cell and the bacteria are collected on the inside of the tentacle collar (Fenchel 1987).

The biggest diversity of filter feeding apparati occurs within the ciliates: a membranell zone on the left side of the mouth, the adoral zone of membranells ("AZM"), which generates a water current that filters the water according to two

principles: upstream and downstream filtration. Upstream filter feeders produce the water current with adoral polykinetids that function as a filter at the same time (prey size ≥ 2µm). The downstream filter feeders produce the water current with the adoral polykinetids but filter the particles out of the water with the paroral or undulating membrane ("UM", prey size < 2µm; Fenchel 1987, Franco *et al.* 1998). So called "hoover feeders" (e.g. cyrtophorida, nassulida) possess a basket of microtubular rods which is used to filter prey such as diatoms or bacteria (Franco *et al.* 1998).

Raptorial or direct interception feeder

Raptorial feeding can be found in many small flagellates and amoebae and some ciliate groups. It is the most widespread mechanism of the three feeding types (Sanders 1991). A raptorial feeder is usually not much bigger than its prey. Single prey cells (bacteria or smaller flagellates and ciliates) are handled individually. These feeders show discrimination in handling and ingestion of food particles. Here, the differentiation between the three classifications of feeding types after Fenchel, Parry and Boenigk & Arndt is not easy to discriminate. There are raptorial feeders like *Neobodo* sp. that are gliding on the surface of the biofilm and feed on single loosely and more strongly attached bacteria. *Spumella* can attach to surfaces and feed from the planktonic phase on single bacteria or can swim freely in the planktonic phase. Raptorial ciliates can be attached or loosely attached and feed on suspended or loosely attached prey.

The most specialised groups of flagellates are chrysomonads, bicosoecids and all kinetoplastids. Possible prey particles get drawn towards the cell surface along a flow line and are then intercepted on the cell surface (Fenchel 1987). Feeding efficiency increases with particle size (Fenchel 1991). The kinetoplastids have a characteristic structure which is a cytopharynx made of microtubular rods (e.g. *R. nasuta*). Some predatory ciliates such as pleurostomatids and prostomatids have extrusomes or "toxicysts" that are used to immobilise prey cells before ingesting. The „browser feeders" scrap prey organisms from surfaces of substrates (e.g. colpodida, Franco et al. 1998).

Feeding process & behaviour

It should be noted that the feeding behaviour of phagotrophic protozoa depends on both biotic and abiotic environmental factors (e.g. older cells exhibit higher feeding rates compared to freshly divided cells Boenigk and Arndt 2000). Although the behaviour of protozoa can be influenced by these factors the feeding process can generally be divided into 2 different phases: 'search time' spent looking for prey (or waiting for prey for attached protists), and 'handling time'. This can further be subdivided into: (i) contact, (ii) processing, (iii) ingestion and (iv) refractory phase (Boenigk and Arndt 2000). These phases can be very different for different species. Even with flagellates that seem to occupy the same niche differences in the search phase and handling phase as well as during processing or ingestion phases can be seen. For example, *Neobodo designis* and *Planomonas* sp. both feed on single attached bacteria. *N. designis* has a longer search time and a very short handling time while *Planomonas* sp. has a longer handling time and a shorter search time (see chapter 5). The same applies not only to flagellates but also to ciliates and possibly to amoeba as well. In case of high prey density the maximum ingestion rate is limited by the time used to handle the particle. But if the prey density is low a high capture efficiency is advantageous while the handling time is unimportant (Boenigk and Arndt 2000).

Protozoan adaptations for life on biofilms

Although life in a biofilm community might promise many advantages (e.g. higher density of organisms, higher nutrient availability, protection), there are always challenges for all members of the community. Advantages for bacteria existing in the biofilm mode of life were previously highlighted, however, protozoa also have certain traits that allow them to fully exploit the advantages of a substrate-defined life style.

As mentioned above, life in a biofilm can be rather stressful compared to life in suspension. Different groups of protozoa differ in their ability to attach to the biofilm. While some are loosely associated with the biofilm community (such as browsing ciliates e.g. cyrtophorida) or glide on the biofilm (e.g. the before mentioned kinetoplastida), others attach firmly to the surface. Amoebae for example attach very strongly to surfaces because of the large cell-surface-contact area (Arndt

et al. 2000). One important factor that must be considered, especially in flowing environments, is shear stress. While smaller flagellates that are attached directly to the substratum (e.g. *Spumella* sp., *C. roenbergensis*) experience low shear stress, larger ciliates like *Vorticella* sp. and *Stentor* sp. can experience high shear. Nonetheless, the attachment to the surface and the resistance of the shear forces can be of great advantage especially for suspension feeding protozoa. These protozoa have a much higher grazing success if attached to surfaces than if suspended (Fenchel 1986, 1987). This is a very important factor in the bentho-pelagic coupling and one of the important ways to enhance nutrient availability in biofilm communities (Fig. 1.2). But not all protozoa are attached to the substratum. Browsing ciliates and gliding flagellates for example are typical biofilm inhabitants. These protozoa feed on attached and loosely attached prey organisms and are often dorso-ventraly flattened, a common adaptation of surface crawling protozoa (Fenchel 1987).

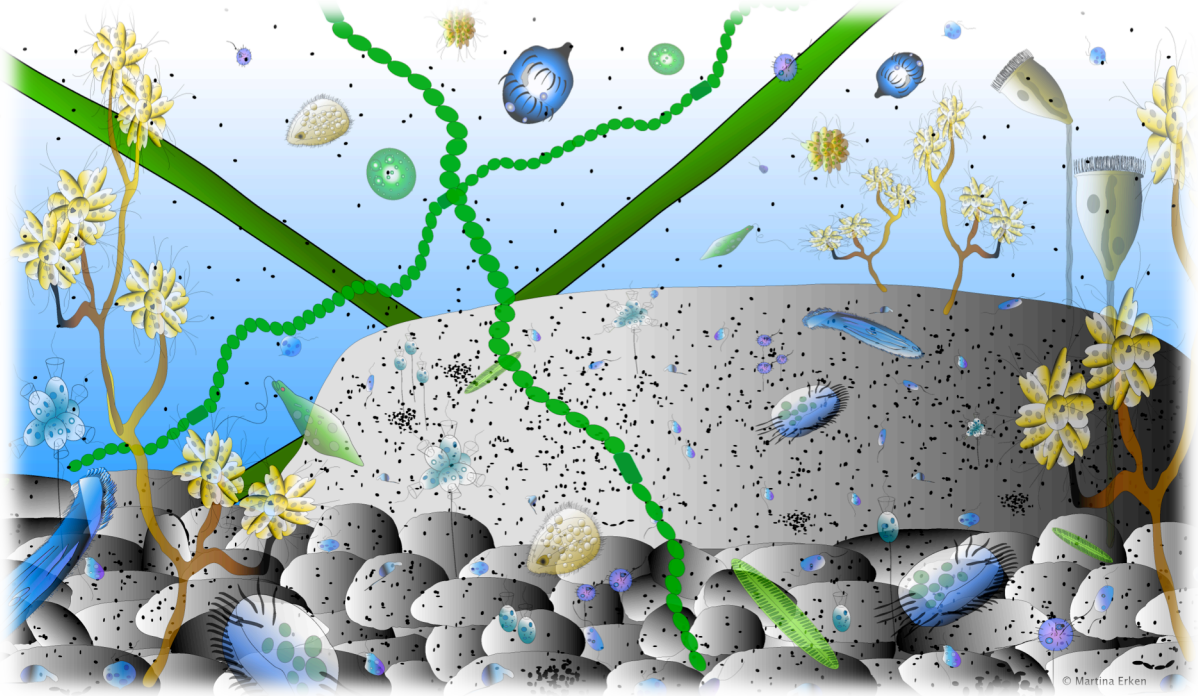

Figure 1.2. Illustration of a natural riverine biofilm community with protists and bacteria.

Biofilm succession

In the last couple of years research has begun focusing on the succession and taxonomic composition especially of riverine biofilms. Since the biofilm develops and matures in a rather conservative pattern, the same morphological groups can be found during the same respective time phases of succession, at least in comparable environments. The colonisation of a biofilm is in accordance with the island colonisation model after MacArthur-Wilson (1967). The colonisation is highest at the beginning of the process after which immigration and extinction are at equilibrium and the biofilm community changes only in taxonomic composition. Early in the development of the biofilm, single bacteria attach to the surface. Heterotrophic nanoflagellates follow, especially kinetoplastids like *Neobodo* sp. and *R. nasuta.* Small chrysomonads such as *Spumella* sp. can also be found. These are "initial" or "pioneer" species (Franco *et al.* 1998) that appear after only a couple of hours (Wey *et al.* 2008). These species find it a perfect environment for predation due to the attachment of single bacteria, which they feed on. In the first week "initial" and "early coloniser" ciliate species appear on the biofilm. Mostly, these are bacterivorous filter feeders like *Cyclidium* sp. or *Cinetochilum* sp. with only some raptorial feeders (Norf *et al.* 2009b). In more mature biofilms the HNF disappear and are replaced by attached filter feeding flagellates like choanoflagellates and bicosoecids. "Hoover feeding" ciliates like *Glaucoma* sp. and predatory ciliates like *Acineria* sp. appear on the mature biofilm. *Glaucoma* sp. can feed on attached filamentous bacteria and thus can prey on the bacterial microcolonies that developed during biofilm maturation (Norf *et al.* 2009b). Hypotrich ciliates like *Euplotes* sp. or *Aspidisca* sp. are browsing ciliates that can feed on more mature bacterial biofilms and also appear after the early biofilm state. Although the disappearance of single bacteria on more mature biofilms can be one reason for the disappearance of vagile flagellates such as the kinetoplastids, the appearance of nanophagous ciliates like pleurostomatids may be another factor. In mature biofilms, where immigration and emigration are in a steady state filter feeding sessile ciliates like *Vorticella* sp. or *Stentor* sp. appear on the biofilms (Franco *et al.* 1998, Norf *et al.* 2009b). Predatory ciliates like *Litonotus* sp. that feed on other ciliates also appear on mature biofilms where a diverse protozoan community has already developed.

Bacteria -Protozoa Interactions

Protozoan Grazing

The interactions of phagotrophic protozoa and bacteria are very complex and considered to be one of the oldest predator-prey interactions in nature (Jürgens and Güde 1994). Predation by protozoa, especially by HNFs (< 5 µm), is one of the main mortality factors for bacteria in aquatic environments (e.g. Hahn and Höfle 2001, Jürgens and Matz 2002). The pressure exerted by the grazers is highly selective and gave rise to the development of a multitude of defence mechanisms (morphological features or chemical defence) in bacteria. Some authors even suggest that the toxicity of pathogenic bacteria is primarily a defence against grazing and the toxicity for higher organisms is secondary (Matz and Kjelleberg 2005).

While there is a large amount of information on protozoa-bacteria, protozoa-protozoa or metazoa-protozoa interactions in planktonic communities, hardly anything is known about the interactions in surface-attached communities. While in recent years more groups have began to work with environmental biofilm communities there is still much to learn. While some information gained from planktonic studies can be applied to biofilm communities, not all information is relevant. The major differences between plankton and biofilms are that (i) the abundances are much higher in biofilms than in the suspended phase (up to 3-4 orders of magnitude, e.g. Caron *et al.* 1986, Artolozaga *et al.* 1997), (ii) the taxonomic composition of bacteria as well as that of the protozoa differs immensely and (iii) the proximity of predator and prey is much closer on biofilms than in plankton (Sherr and Sherr 2002). The impact of grazing is based on a complex interplay of several parameters. Grazing has a major impact on the taxonomic composition, morphology, physiological state and nutrient availability of the bacterial community (Hahn and Höfle 2001). All of these topics will be mentioned separately to give each the credit it deserves. The knowledge on planktonic communities will be discussed with additional information about biofilm communities where available.

Taxonomic Composition…

…of the bacterial prey community

It is known from planktonic communities that grazing by protozoa has a major impact on bacterial communities. The phenotypic heterogeneity of bacterial communities is largely due to grazing (Jürgens and Matz 2002). If given the opportunity to choose between active and inactive prey cells, HNF were four times more likely to graze on active cells (Gasol *et al.* 1995). This might be one explanation for the fact that metabolically active cells are found in fewer numbers than inactive cells in planktonic assemblages. This reduction of active bacteria might also be a reason for a community species shift as shown before in plankton samples when the community was threatened with grazers (Jürgens *et al.* 1999, Van Hannen *et al.* 1999). Recently, investigations have been conducted on whether the shift in taxonomic composition can also be seen in biofilm communities (e.g. Wey 2010, Lear *et al.* 2008). The exclusion of certain trophic levels in semi-natural biofilm communities might influence the taxonomic composition of biofilm communities, although it seems that the succession is more important than the influence of grazers (Wey, 2010).

…of the protozoan community

In exclusion experiments with natural biofilm communities Wey *et al.* (2008) could show that the exclusion of certain size fractions can have a major direct impact on the protozoan community composition and an indirect impact on the structure of the bacterial community. The exclusion of ciliates in 8 µm filtered natural water resulted in a high abundance of HNF and bacterial microcolonies and a low abundance of single bacteria. If the ciliates were present on the biofilm (water filtered through 20 µm filters) the abundance of HNFs did not reach such high levels as when the ciliates were excluded. The bacterial community showed a significantly higher abundance of single bacteria if the ciliates were present on the biofilm. The ciliates preyed upon the flagellates and thus relieved grazing pressure on the bacterial biofilm. It appears that predatory ciliates exhibit a non-random grazing pressure. From direct video observations it was revealed that individuals of *Acineria* sp. feed preferentially on vagile flagellates like *Neobodo* sp. and *Planomonas* sp.

whereas individuals of *Holosticha* sp. seem to preferentially graze on sessile flagellates like *Spumella* sp.. The presence of these ciliates could alter the protozoan community towards a more sessile or more vagile HNF community (own observations, data not published).

Morphological Adaptations of the bacterial prey

From environmental and laboratory experiments it is well documented that the presence of protozoan grazers induces a size shift in freshwater, brackish and coastal planktonic bacterial communities. Larger, smaller as well as bimodal size shifts have been observed. Pernthaler *et al.* (1996) suggested a size fractioning of grazing resistant (cells > 2.4 µm), grazing suppressed (1.6 - 2.4 µm) and grazing vulnerable (0.4 - 1.6 µm) bacteria. While there is an upper size limit where prey cannot be ingested there is no lower size limit (Jürgens and DeMott 1995). Hahn and Höfle (2001) differentiated between grazing-resistant and grazing-protected bacteria, the former being too large to be ingested, the others being subject to a lower grazing efficiency and thus less grazing pressure. This division should be taken as a guideline but is certainly not fixed. While small cells (< 0.4 µm) are indeed "weakly affected" (Pernthaler *et al.* 1996) by certain grazers like kinetoplastids, these cells are highly endangered by grazing by choanoflagellates. Depending on the taxonomic composition of the surrounding organisms the prey fractions can be affected very differently. Filaments resistant to protozoan grazing appear in certain seasons and are highly vulnerable to grazing of cladoceran metazoans (Pernthaler *et al.* 2009).

While in freshwater environments filament formation and thus enlargement of bacterial aggregates is a common grazing protection this phenomenon cannot be seen in open ocean environments. In contrast, protozoa graze preferably on larger cells, which are in most cases the actively growing part of the bacterial assemblage (Gasol *et al.* 1995, del Giorgio *et al.* 1996). Some bacterial strains show filament formation due to nutrients made available by grazing (nutrient recycling) and thus are indirectly pushed by protozoa to form filaments (Hahn *et al.* 1999).

Laboratory as well as environmental experiments have shown that microcolony formation of bacteria on surfaces in the presence of grazers such as *Neobodo* sp. and

Tetrahymena sp. can protected the biofilm from being grazed (see chapter 2, 3 and 5). Grazing of some protozoa even enhanced the biofilm biomass. Microcolony formation might function as grazing protection like filament formation in the planktonic environment (e.g. Matz *et al.* 2004a, Wey *et al.* 2008). However, it is important to consider the feeding mode of the predator when talking about grazing protection. For flagellates like *N. designis* and *R. nasuta* it might be true that microcolony formation protects from grazing, amoeba like *Acanthamoeba* sp. or ciliates like *Tetrahymena* sp. can consume bacteria grown in microcolonies (Weitere *et al.* 2005).

Non-morphological bacterial defences

Morphological adaptations (microcolony formation and filament growth) defend bacteria from protozoan grazing, but non-morphological features also function to avoid predation such as motility, growth in spatial refuges, chemical resistance to digestion, exoploymer production (e.g. coating) and toxin production (Jürgens and Matz 2002). Matz and Kjelleberg give a good overview regarding these factors (2005).

All these factors apply to bacteria in planktonic as well as in biofilm communities. However, some might be more important in the plankton (e.g. motility) while others are more important in attached communities. Although a higher motility means a higher contact and possibly higher grazing rates, these can be compensated for by higher escape probabilities, e.g. “run & reverse“ swimming pattern (Mitchell *et al.* 1995, Matz *et al.* 2002). Productions of extracellular polymeric substances (EPS) are advantageous for single bacteria in suspension as well as for attached bacterial communities. Extracellular polymeric secretion by the prey organisms can affect the grazing rates of predators (Liu and Buskey 2000). Bacterial biofilm communities can produce large amounts of EPS. A biofilm of the alginate overproducing bacterial strain, *P. aeruginosa* PDO300, was resistant to grazing of the early biofilm colonizers *N. designis* and *R. nasuta* while the ciliate *T. pyriformis* and the amoeba *A. castellanii* were able to reduce the biofilm biomass significantly (Matz *et al.* 2004a, Weitere *et al.* 2005). Toxin production occurs in bacteria such as *P. aeruginosa* and *V. cholerae* (Matz *et al.* 2005, Weitere *et al.* 2005, Matz *et al.* 2008). The density dependent toxin production of *V. cholerae* cannot be seen in the suspended phase but only in high-

density biofilms. Therefore, biofilm communities have an advantage of defence that suspended cells do not possess. Some bacteria produce violacein, a toxin that can kill protozoa and thus defend the bacterial community (Singh 1942, Matz *et al.* 2004b). Just as predators have the ability to chemically sense prey (Fenchel and Blackburn 1999), prey organisms can sense the predator (Wicklow 1997). Some bacterial strains can even resist the digestion inside food vacuoles (Boenigk *et al.* 2001).

Microbial loop & nutrient recycling

Since Azam *et al.* (1983) the "microbial loop" is widely accepted not only in marine but also in freshwater and terrestrial environments. While it was formerly believed that dissolved organic matter (DOM) and dissolved organic carbon (DOC) are a "sink" of nutrients that can not be reintroduced into the food chain, it is now known that protozoa and bacteria are a link from DOC to higher trophic levels. Bacteria feed on the organic matter (e.g. dissolved matter from faeces of metazoans or carcases of fish), protozoa graze on the bacteria and are themselves then grazed upon by metazoans. Depending on the environment, grazing can have different implications for the microbial community.

In nutrient-rich environments zooplankton communities exhibit a high pressure on phagotrophic protists and thus release pressure on bacteria. The bacterial community shows a reduction in diversity due to a competition for nutrients (Corno *et al.* 2008). In nutrient poor environments the impact of the phagotrophic grazers is bigger and induces shifts in the taxonomic composition of the bacterial community (Jürgens *et al.* 1999, Hahn and Höfle 2001). Corno *et al.* (2008) found a strong correlation between predation pressure and relative bacteria diversity. Increases in bacterial production lead to an increase in the relative importance of competition as opposed to predation (Bohannan and Lenski 2000).

As previously mentioned, information gained from the study of planktonic systems cannot be directly applied to the study of biofilms. The differences already mentioned have a strong impact on the nutrient recycling: (i) higher abundances of bacteria and predators indicate that nutrient demand is much higher in biofilms than in suspended communities. Since biofilms can be very thick a nutrient gradient can be assumed (Bishop 1997) with the lowest nutrient availability at the substrate

interface. This has direct implications for (ii) the taxonomic composition. Since the nutrient availability of a biofilm differs between base and top, different species can thrive in different layers of the biofilms. Anoxic bacteria might thrive at the base were the oxygen concentration is too low for aerobic bacteria. (iii) The closer proximity has different advantages and disadvantages for predator and prey. Grazing of bacteria, be it single bacteria by HNF or large "chunks" of biofilm by hoover feeding ciliates, releases and reintroduce nutrients into the close environment of the grazer. Sloughing and sloppy feeding can damage non-grazed cells and release nutrients for other cells. The mechanical damage the grazers cause in the biofilm can create channels that supply the lower middle levels of the biofilm with higher nutrient concentrations.

Laboratory experiments with fluorescently labelled bacteria showed that protozoa can reach high grazing rates on attached bacteria (see Parry 2004 for review). However, due to nutrient recycling bacterial biofilms could benefit from grazing. Most studies dealing with nutrient availability on biofilms were done in laboratory studies or focused on autotrophic biofilms (Hillebrand and Kahlert 2001, Hillebrand *et al.* 2002), while very little is known about grazing on natural biofilms. Norf *et al.* (2009a, 2009b) studied the influence of substrate enrichment on the ciliate community of natural biofilms. They could show that resource enrichment could significantly affect the colonisation speed of ciliates on biofilms, influencing both the abundance and the taxonomic composition.

Vibrio cholerae

The disease Cholera has a major impact on human populations all over the world, especially on the Indian subcontinent. To understand the disease and its epidemiology, it is important to understand the causative agent and its ecology. Since it has had such a major influence on human populations from the beginning of time until the present day, a lot of research has been done on the disease and the genetics, the epidemiology and the ecology of this bacterium. However, surprisingly little is known about the interactions of *V. cholerae* with phagotrophic protozoa considering the major impact grazers can have on the bacterial community. The following chapter summarizes the most important characteristics of the organism. Since the bacterium has such a medically important influence, a large volume of literature has been devoted to aforementioned topics of *V. cholerae* and its disease. This chapter can only provide a brief overview. For more details see the mentioned references.

Discovery

Recordings of diseases similar to Cholera can be traced back as far as 2500 years ago, but the modern era of Cholera epidemics started with the first pandemic in 1817 in India (Colwell 2002, Thompson 2004, Prouty and Klose 2006). In 1854, a young Italian medicine student, Filippo Pacini, discovered the first Vibrio species as the causative agent of Cholera during the late second and third pandemic in Florence. He published the article 'Microscopical observations and pathological deductions on cholera' that failed to be noticed by the scientific community (Howard-Jones 1984, Faruque *et al.* 1998). At the same time, the Cholera pandemic was also spreading in England. In 1849, the British physician John Snow was able to track down the source of a Cholera breakout in central London to a single contaminated well and by shutting down the well the epidemic was stopped (Colwell 2002, Thompson 2004). John Snow's investigations are broadly acknowledged as the first epidemiological investigation in the history of medicine (Colwell 2006).

It was not until the fifth pandemic in 1884 that Robert Koch described the bacterium causing Cholera. He and his team went to Egypt and India and were able to isolate and grow pure cultures of *V. cholerae* from the stools of Cholera patients. In his publication 'An Address on Cholera and its bacillus' Robert Koch described the bacterium as 'comma bacilli, on account of their peculiar shape' (Koch 1884).

Epidemology

There have been seven distinct pandemics since the first recorded one in 1817 (Faruque *et al.* 1998) and all but the 7th pandemic arose from the Indian subcontinent (Kaper *et al.* 1995). The disease is endemic to southern Asia, especially the Bangladesh delta from where most pandemics spread. The disease shows a seasonal pattern with peaks in spring and fall especially when water temperature rises. The 7th pandemic – which is still on-going – started in 1961 and has the most extensive spread temporally and geographically. This is the first pandemic to be caused by *V. cholerae* serogroup O1 biotype El Tor (five and six were caused by the classical biotype), and it arose from Sulawesi, Indonesia, not the Indian subcontinent. The O1 El Tor strain has an enhanced ability to survive in niches that *classical V. cholerae* do not have which is one of the reasons that the 7th pandemic is so widely spread. In addition, the El Tor strains cause many more mild infections of diarrheal diseases per case of Cholera gravis and thus there are many more asymptomatic excretors who spread the disease (Kaper *et al.* 1995).

In 1992 epidemic Cholera was reported in Madras and has since spread. This time a beforehand-unknown serogroup O139 was the cause of the epidemic. The emergence of a new serogroup is referred to by some as the start as the current and 8th pandemic (Kaper *et al.* 1995). It is interesting that the causative agent throughout the pandemics changed (Prouty and Klose 2006). However, it is important to know that O139 has not replaced O1 El Tor strains - they are still cause of epidemics (Sack 2004, WHO 2010).

One of the severe dangers with this disease is that it spreads rapidly and infects many people in a short period of time. It strikes almost always in locations that have poor sanitation and low water quality. Since it is a faecal-oral transmitted disease, water and food are the most important vehicles of transmission (Faruque and Nair

2006). Cholera patients excrete a high number of bacteria, which leads to a higher risk of contaminated water. Since October 2010, a new Cholera outbreak threatens Haiti, where, due to the devastating earthquake in January, the infrastructure and sanitation system collapsed. On 24 November 2010, the World Health Organisation reported that 60240 cholera cases (including 1415 deaths) were recorded by the Haitian Ministry of Public Health and Population as of 20, November 2010 (WHO 2010). The causative strain was verified by the "Centre for Disease Control and Prevention" as the *V. cholerae* O1 strain, serotype Ogawa (CDC 2010).

Biology

The genus *Vibrio* (family Vibrionaceae, also includes the genera *Photobacterium*, *Aeromonas* and *Pleisiosomonas*) belongs to the Gammaproteobacteria. They are Gram-negative, rod-shaped bacteria, with a curved or straight axis, 0.5 x 3 μm long and appear single-celled, or sometimes united in S-shapes or spirals. They are mesophillic, chemoorganotrophic, and their metabolism is facultative fermentative. Some species have polar flagella with which they are motile (Said 1996). They can be found in a wide range of aquatic habitats, including estuarine, sea- and freshwater (Thompson 2004).

There are 65 species within the genus *Vibrio* of which more than 12 are known to cause disease in humans, including *Vibrio parahaemolyticus*, *Vibrio fluvialis* and *Vibrio mimicus*. Of these pathogenic species, *V. cholerae* is the biggest threat to human health, however, there are differences in the pathogenic potential of strains of the species. *V. cholerae* strains are classified by serogroup, which is based on the structure of the somatic O-antigen. Of the more than 200 serogroups of *V. cholerae* just two serogroups, O1 and O139 have been associated with epidemic and pandemic Cholera disease (Kaper *et al.* 1995, Said 1996, Faruque and Nair 2006). Nevertheless, some of them have been shown to cause moderate to severe forms of gastroenteritis. These are often referred to as non-O1/non-O139 *V. cholerae* strains. It is important therefore to assess if isolates possess the genes encoding the cholera toxin (CT) and the O1 or O139 antigens in order to assess the pathogenic potential of these (Kaper *et al.* 1995).

All somatic (O) antigens are thermostable and cannot be destroyed by treatment with Ethanol and n-HCl (Sakazaki 1992). The O1 serogroup is further differentiated into three serotypes: Inaba, Ogawa and a rarely reported Hikojima. These serotypes are further categorised into two biotypes (classical and El Tor) based on a number of immunological and biochemical properties. This differentiation is important epidemiologically as the O1 El Tor strains have replace the classical strains, although the molecular basis for the differentiation is not well understood (Sakazaki 1992, Kaper *et al.* 1995). The biotype El Tor was first isolated in 1905, but until the 7th pandemic had been associated only with sporadic diarrhoea.

Virulence

The genetic pathway of virulence factor production is quite complex and diverse. The following should just give a short overview:

Cholera toxin (CT) is an ADP-ribosylating toxin that is responsible for the main symptoms of the disease. Composed of two subunits (A and B), it leads to increased cyclic adenosine monophosphate (cAMP) levels in human epithelial cells and ultimately to an increased chloride-ion secretion (Prouty and Klose 2006). The osmotic imbalance causes diarrhoea with large amounts of water flowing into the lumen of the intestine (Prouty and Klose 2006). Two factors are essential for the virulence of *V. cholerae*: a lysogenic phage, CTX φ, and the toxin regulating pilus (TCP, Prouty and Klose 2006). The phage encodes the genes *ctx*A and *ctx*B, both of which encode CT (Waldor and Mekalanos 1996). Non-toxic strains of *V. cholerae* can be converted to toxic strains by infection with the *ctx*AB carrying phage CTX φ (Boyd et al. 2000). The TCP is under the same genetic control as the CT (Faruque *et al.* 2004a, Faruque *et al.* 2004b). This pilus is expressed during the colonisation of the human intestine (Taylor *et al.* 1987) whose genes are carried on the Vibrio Pathogenicity Island (VPI, Sack 2004). The pilus has two important roles: the colonisation of the host (the pilus is mandatory for that (Taylor *et al.* 1987, Prouty and Klose 2006)) and to serve as a receptor for the CTX phage (Waldor and Mekalanos 1996). The way TCP works is not fully understood, but may induce microcolony formation by direct pilus-pilus contact (Kirn *et al.* 2000). It has been suggested that environmental strains have the ability to become toxic through acquisition of the virulence genes. Two steps are required for the strain to become

toxic: (i) infection with the CTX φ phage and (ii) the acquisition of VPI which is possible through horizontal gene transfer (Faruque *et al.* 1998, Sack 2004).

Clinical disease

The clinical disease is characterised by rapid loss of watery stools (often referred to as rice water stools due to its appearance) that cause rapid dehydration and demineralisation. Death can occur as soon as 12 h after the first symptoms if a fast and suitable treatment is not initiated (Cash *et al.* 1974, Bennish 1994). The disease is transmitted through contaminated food or drinking water.

The inoculum required to establish Cholera is quite large compared to other diseases. In volunteer studies it was shown that the infectious dose was 10^{11} cells mL^{-1} unless sodium bicarbonate was given to neutralize gastric acid. *V. cholerae* O1 is largely acid labile and the acidy environment of the stomach kills most bacteria. However, if the pH of the stomach is increased, more bacteria survive and the infectious dose is much lower (Faruque *et al.* 2004b). Pre-enrichment of the bacteria in contaminated food or water can cause the first case of Cholera. It has been suggested that the bacteria can be further enriched in the human host, leading to subsequent higher numbers of *V. cholerae* in the environment, which in turn might lead to epidemic outbreaks (Sleigh, 2006).

Ecology

As mentioned above, *V. cholerae* is part of the natural bacterial flora in aquatic environments (Colwell and Spira 1992). Although all strains can be isolated from water samples, non-O1/non-O139 strains are more common than O1 and O139 strains (Minami *et al.* 1991, Faruque *et al.* 1998). Environmental factors such as nutrient supply and temperature play an important role in the persistence, evolution and transmission of this bacterium (Vezzulli *et al.* 2010). Thus, the occurrence of cholera outbreaks is strongly linked to biotic and abiotic factors. In times of human infection it colonizes the human intestine and between epidemics it can be found in its original aquatic habitat (Sack 2004). The seasonal pattern of cholera can be correlated with seasonal patterns of zooplankton blooms and favourable climate (Halpern *et al.* 2008).

All *Vibrio* species show a strong association with marine plankton, with a higher number of bacteria on zooplankton than phytoplankton (Simidu 1977, Islam *et al.* 1994). *V. cholerae* attaches to plants, phytoplankton (e.g. *Anabena*), crustaceans, and insects (chironomonids, Vezzulli *et al.* 2010) and has a strong association with copepods (Huq *et al.* 1983, Heidelberg *et al.* 2002, Lizarrága-Partida *et al.* 2009). Zooplankton is considered to be the largest reservoir for *V. cholerae* in the natural environment (Colwell *et al.* 1996). The attachment seems to be specific for certain areas on the copepods: the highest bacterial densities found on the oral region and egg sack of copepods (Huq *et al.* 1983). Colwell et al. (1996) showed that *V. cholerae* cells survive longer if attached to zooplankton than non-attached cells. Bacterial numbers can reach high enough densities on copepods that ingestion of a few in unsanitary water is enough to cause infection (up to 104 cells copepod^{-1}, Huq and Colwell 1995). In environmental studies in Bangladesh Colwell *et al.* (2003) were able to lower the number of Cholera cases by nearly 50% by simply filtering water through a sari cloth. Through filtering the water, phyto- and zooplankton was removed and with this the abundance of *V. cholerae* in the water decreased. However there are other reservoirs. For example, *V. cholerae* has been shown to be transported for several miles by attachment to waterfowl birds (Bennish 1994, Faruque and Nair 2006, Halpern *et al.* 2008).

V. cholerae and protozoa

Although protozoa heavily influence the bacterial community in aquatic environments only a few studies have investigated the influence protozoa have on *V. cholerae* and vice versa.

While most studies suggest that most *V. cholerae* cells attach to zooplankton (Heidelberg *et al*. 2002) the majority of *V. cholerae* cells in mesocosm experiments run by Worden *et al.* (2006) were suspended and heavily grazed upon by heterotrophic nanoflagellates. Another study investigated ciliate grazing on suspended *V. cholerae* cells. Here, the grazers were not able to reduce the numbers of *V. cholerae* but instead drove the bacterial cells into a viable but non-culturable state (Macek *et al.* 1997). More importantly, *V. cholerae* have been shown to grow and survive intracellularly in the amoeba, *Acanthamoeba castellanii* in the environment, which has implications for the persistence of this organism in the

environment. The bacterial cells can possibly escape into the cytoplasm of the amoeba and thus avoid digestion (Abd *et al.* 2005). Amoeba are considered to be trojan horses and might play a role in the transmission of pathogenic bacteria (Barker and Brown 1994).

Although *V. cholerae* attaches to surfaces and protozoa have a major impact on bacterial biofilms, until now only one study has been published that focused on the interactions of *V. cholerae* and heterotrophic flagellates on biofilms. Matz *et al*. (2005) studied the interactions of the two heterotrophic flagellates *C. roenbergensis* and *R. nasuta* with *V. cholerae* under laboratory conditions and could show that the wildtype strain *V. cholerae* A1552 El Tor is toxic for the grazers. Interestingly, a mutant strain, *V. cholerae hapR* did not prevent the flagellates from growing and was grazed by the flagellates. The *hapR* gene is the QS response regulator, which represses CT expression (Zhu *et al*. 2002). This indicates that a CT independent toxin prevents potential predators from grazing the bacteria. Certainly, this is interesting since it might be a protection mechanism for the bacteria in the natural environment.

Chapter 2

[1]Impact of *Tetrahymena* Grazing on *Acinetobacter* sp. Strain C6 Biofilms

[1] Ein Teil der Experimente (mit dem gfp-markierten *Acinetobacter* sp. Stamm) wurde von Dr. Anja Scherwaß an der Technischen Universität Dänemark, Lyngby, Dänemark durchgeführt. Alle weiteren Arbeiten (Experimente, Auswertung, Anfertigung des Textes) wurden von mir, Martina Erken, durchgeführt.

Abstract

In aquatic systems formation of bacterial clusters such as filaments and microcolonies help to protect individual cells from protozoan grazing. In the present study the influence of the browsing ciliate *Tetrahymena pyriformis* on a mono-species biofilm built by the microcolony-forming *Acinetobacter* sp. strain C6 variant was investigated using a continuous flow channel system. We hypothesized that early microcolony formation protects bacteria from protozoan grazing. Two different substrate supply rates and two different carbon sources (sodium benzoate and citrate) were used to investigate bacterial growth under optimal and less optimal growth conditions for bacteria. Biofilms supplied with the less optimal carbon source were strongly affected by protozoan grazing, resulting in the almost complete elimination of the formed microcolonies after only four days of growth. Lowering the supply rate of medium also showed a clear reduction in biomass as well as a change in microcolony shape under grazing influence. However, even under optimal growth conditions, the presence of the grazer had an apparent influence on the morphology of biofilms as the microcolony size increased significantly compared to the non-grazed control biofilms. Thus, results of the present study revealed that microcolony formation of *Acinetobacter* sp. strain C6 variant did not generally protect bacteria from protozoan grazing.

Introduction

Bacterial biofilms can cause severe problems in industrial systems, food processing equipment and the medical sector (e.g. biocorrosion and if occurring in catheters, implants, respectively). For the bacteria themselves, the attachment to surfaces and the subsequent formation of biofilms is an opportunistic life strategy which offers several advantages for the microorganisms: better protection against toxins and antibiotics and profit due to synergistic effects in biofilm environments (e.g. Costerton *et al.* 1995, Hall-Stoodley *et al.* 2004). Grazing of bacterivorous organisms like protozoa can reduce the biovolume and the expansion of bacterial biofilms (e.g. Huws *et al.* 2005, Weitere *et al.* 2005). From aquatic pelagic systems, it is known that feeding of bacterivorous protists may strongly influence the morphology, taxonomic composition and physiological status of bacterial communities (Hahn and Höfle 2001) and thus may be an important driving force for a change in bacterial growth and morphology (Posch *et al.* 1999, Jürgens and Matz 2002). Initial studies on the grazing influence by protozoans on bacterial biofilms suggested that microcolony formation plays an important role in grazing defence. Matz *et al.* (2004) showed that a strain of *Pseudomonas aeruginosa* formed microcolonies if faced with protozoan grazing by the benthic flagellate *Rhynchomonas nasuta*. In another study Weitere *et al*. (2005) saw grazing protection through microcolony formation in bacterial biofilms to be dependent on the protozoans' feeding mode and the phase of succession of the biofilm. Early formation of microcolonies in *P. aeruginosa* biofilms resulted in a grazing protection against early biofilm colonizers (e.g. the flagellate *Bodo saltans*). Contrary to this, grazing of late biofilm colonizers such as the browsing ciliate *Tetrahymena* sp. caused high losses of bacterial biomass.

The aim of the present study was to investigate if biofilms built by the microcolony forming *Acinetobacter* sp. strain C6 variant are affected by grazing of the ciliate *Tetrahymena pyriformis* and whether the vulnerability might be changed by variation in biofilm growth conditions.

Material and Methods

Biofilms of *Acinetobacter* sp. strain C6 variant were grown in flow chambers using two different carbon sources and two different flow rates in the presence or absence of the ciliate *T. pyriformis.* Citrate was used as an alternative carbon source to sodium benzoate (Christensen *et al.* 1999, Heydorn *et al.* 2000). Similarly, the delivery rate of medium was lowered. The influence of protozoan grazing on the single-species biofilm was tested in a continuous flow chamber system (Sternberg *et al.* 1999) supplemented with minimal media containing either 0.5 mM sodium benzoate or 4 mM citrate. Experiments were run at two different flow rates: 0.3mm sec^{-1} (hereafter: high flow) and 0.1mm sec^{-1} (low flow). The development of biofilms was monitored by confocal laser scanning microscopy (CLSM) and subsequently analyzed regarding structural and quantitative characteristics.

Organisms and culture conditions

Acinetobacter sp. strain C6 variant isolated by J. Haagensen was used as the biofilm forming bacterium, which forms round microcolonies (Haagensen et al., submitted). Due to laboratory restrictions two different *Acinetobacter* sp. C6 variant strains were used, one being a genetic modified organism (gmo) and tagged with green fluorescent protein (gfp), the other without gfp. The gfp-tagged strain was used for the high flow experiments supplemented with sodium benzoate as carbon source. The non-tagged *Acinetobacter sp* strain was used for the experiments under low flow conditions and high flow with citrate as carbon source. Bacteria were grown in lysogenic broth (LB medium: tryptone 10g L^{-1}, NaCl 10g L^{-1}, yeast 5g L^{-1}) at 30°C prior to the experiments. The ciliate *Tetrahymena pyriformis* (Culture Collection of Algae and Protozoa, Windermere, UK, CCAP 1630/1W) was used as grazer. Ciliate stocks were cultured axenically in organic-rich medium (PPY: proteose peptone 20g L^{-1}, yeast 2.5 g L^{-1}) at 10°C and transferred weekly to fresh medium. Cultures were adapted to the experimental temperature of 20°C prior to experiments. For all experiments a 3-day-old suspension of ciliates was diluted four times by a bacterial suspension of an overnight *Acinetobacter* sp. culture. Abundances of *T. pyriformis* were counted daily. Abundances doubled in the sodium benzoate experiments over the course of the first four days. Although ciliate numbers declined in the citrate

experiments grazers were active and feeding on the biofilms. All experiments ran for 8 days.

Laboratory setup

Biofilms were cultivated in three-channel flow chambers with individual channel dimensions of 1 x 4 x 40 mm (Christensen *et al.* 1999) and in FAB-medium (15.1 mM $(NH_4)_2SO$, 33.7mM Na_2HPO_4 x $2H_2O$, 22mM KH_2PO_4, 0.05mMNaCl, 1 mM $MgCl_2$, 0.1mM $CaCl_2$, 0.1mM Fe-EDTA), supplemented with either 0.5mM sodium benzoate or 4mM citrate as a carbon source. All tubes and media were autoclaved before use. Prior to the start of the experiment, the system was sterilized with 0.5% NaClO for 4h, followed by a overnight washing step with sterile distilled water using a Watson Marlow peristaltic pump (model 205S). Subsequently, media was pumped through the system for one hour prior to the addition of bacteria and ciliates. For inoculation of bacteria and ciliates, the flow was stopped for one hour and the bacteria-/ciliate-suspensions were added using a 1-ml syringe to the flow channels as follows: a suspension of bacteria four times diluted with PPY medium for the control without ciliate grazing. For the grazed treatment, a mixture of *Acinetobacter* sp. strain C6 variant and *Tetrahymena* (1:4) was added. Experiments were run for 8 days, scanning of the biofilms was proceeded on days 4 and 8.Biofilms were stained with propidium iodide (100 µg mL^{-1}, Sigma Aldrich) after fixation with 2% buffered formalin and subsequent washing with PBS (phosphate buffered saline; 37mM NaCl, 2.7mM KCl, 100mM Na_2HPO_4, 2mM KH_2PO_4) prior to microscopic investigation. For experiments run with the gfp-tagged strain under high flow conditions supplied with sodium benzoate, biofilms were scanned without prior fixation and staining. All experiments presented here were run in duplicates.

Confocal laser scanning microscopy and quantitative analysis

Biofilm structure was analyzed by confocal laser scanning microscopy (using LSM 510 Meta Zeiss, Germany). Variability was estimated from different flow channels run as replicates (n=3-4). To minimize potential disturbances, which might influence the biofilms, all experiments were handled in exactly the same way. Biofilms were scanned at three randomly selected areas in the first half of the individual flow channels to consider inner-cell variability due to possible spatial heterogeneity. Two different software programs were used for the quantitative analysis of the biofilms:

"3D for LSM" (Zeiss, Germany) to analyze the biovolume of the biofilm and "Image J" (NIH, US, http://rsb.info.nih.gov/ij) to determine the abundances and the average size of microcolonies (a microcolony was defined to be at least 10 μm^2). A two-factorial ANOVA was used for statistical analysis of all acquired parameters with time and treatment being the independent variables and the analyzed parameters as dependent variables. All statistical analyses were carried out using WinStat (Microsoft, Version 2003.1).

Results

Grazing and different growth conditions revealed structural differences in the biofilms during the present study. The quantitative analysis of the biofilms (biovolume, number of and size of microcolonies) pointed to a major impact of *T. pyriformis* grazing on biofilm structure.

Under high flow conditions with sodium benzoate as carbon source, microcolonies dominated the biofilm structure in experiments with and without grazing of *T. pyriformis* (Fig. 2.1.A, 1-4). While in the non-grazed control the number of microcolonies decreased nearly by half from day 4 to day 8 (2398 to 1361microcolonies mm^{-2}, Fig. 2.1.A5), average microcolony size (day 4:80μm^2, day 8: 181μm^2, Fig. 2.1.A6) as well as biovolume of the biofilm (4.2μm^3 μm^{-2} to10.7μm^3 μm^{-2}, Fig. 2.1.A7) more than doubled in the same time course.

Under grazing influence microcolonies were elongated and not round shaped as in the non-grazed controls (Fig. 2.1.A, 2+4). Also, the abundance of microcolonies was less than in the non-grazed control and did not increase over time (day 4 and 8: 1022 microcolonies mm^{-2}, respectively, Fig. 2.1.A5). Moreover, grazed microcolonies were larger than in the non-grazed biofilms (day 4: 372μm^2, day 8: 268 μm^2, Fig. 2.1.A6). Biovolume increased only slightly from 5.9μm^3 μm^{-2} to 7.5μm^3 μm^{-2}. While time and treatment both had a significant effect on the number of microcolonies of the biofilm (time: $p=0.03$, treatment: $p=0.007$), the statistical analysis also showed that biofilm maturation influenced the average size of microcolonies significantly in the non-grazed as well as the grazed treatments ($p=0.02$ and $p=0.002$, respectively). Additionally, the biovolume was significantly influenced by the maturation state of the biofilm ($p=0.001$).

Biofilms grown under high flow conditions with citrate as carbon source showed a less-structured biofilm development compared to the biofilms grown on benzoate (Fig. 2.1.B1-4). In the control treatments without ciliates, microcolonies could be seen at the beginning of biofilm development (day 4) although they were of a different shape than in the benzoate treatments (Fig 2.1.B1 and 2.1.A1, respectively). Microcolony abundance decreased from day 4 to day 8 nearly by the half (807 to 473 microcolonies mm^{-2}, Fig. 2.1.B5). At the same time these microcolonies grew markedly with further biofilm development and the average size of the microcolonies doubled (day 4: 647μm^2, day 8:1204 μm^2, Fig. 2.1.B6). Correspondingly, the biovolume stayed nearly constant over the four days (Fig. 2.1.B7).

In the grazed biofilms, microcolonies were severely affected and almost eliminated (Fig. 2.1.B3+4). The data of the grazed biofilms showed a higher variability in microcolony abundance and size. The number of microcolonies increased from 735 microcolonies mm^{-2} on day 4 to 1112 microcolonies mm^{-2} on day 8 (Fig. 2.1.B5), while microcolony size strongly declined from 1713μm^2 (day 4) to 464μm^2 (day 8). Consequently, the biovolume of the biofilms stayed on the same level (Fig. 2.1.B6+7).

Biofilms grown under low flow conditions were strongly affected by both the lower flow rate and the protozoan grazer. Although the non-grazed biofilms showed clearly visible microcolonies on day 4, the structure was heavily changed on day 8 (Fig. 2.1.C1+3). While microcolony size (day 4: 325 μm^2, day 8: 348μm^2, Fig. 2.1.C5) and the abundance of microcolonies (day 4: 861 microcoloniesmm^{-2}, day 8:1002 microcolonies mm^{-2}, Fig. 2.1.C6) stayed the same throughout the experiments, the biovolume decreased from day 4 to day 8 and showed a high variability (day 4: 28μm^3 μm^{-2}, day 8: 19μm^3 μm^{-2}, Fig. 2.1.C7). The structure of the grazed biofilms was distinctively different from the non-grazed biofilm, showing irregular shaped microcolonies instead of round shaped microcolonies seen in the non-grazed controls (Fig. 2.1.C 2+4).

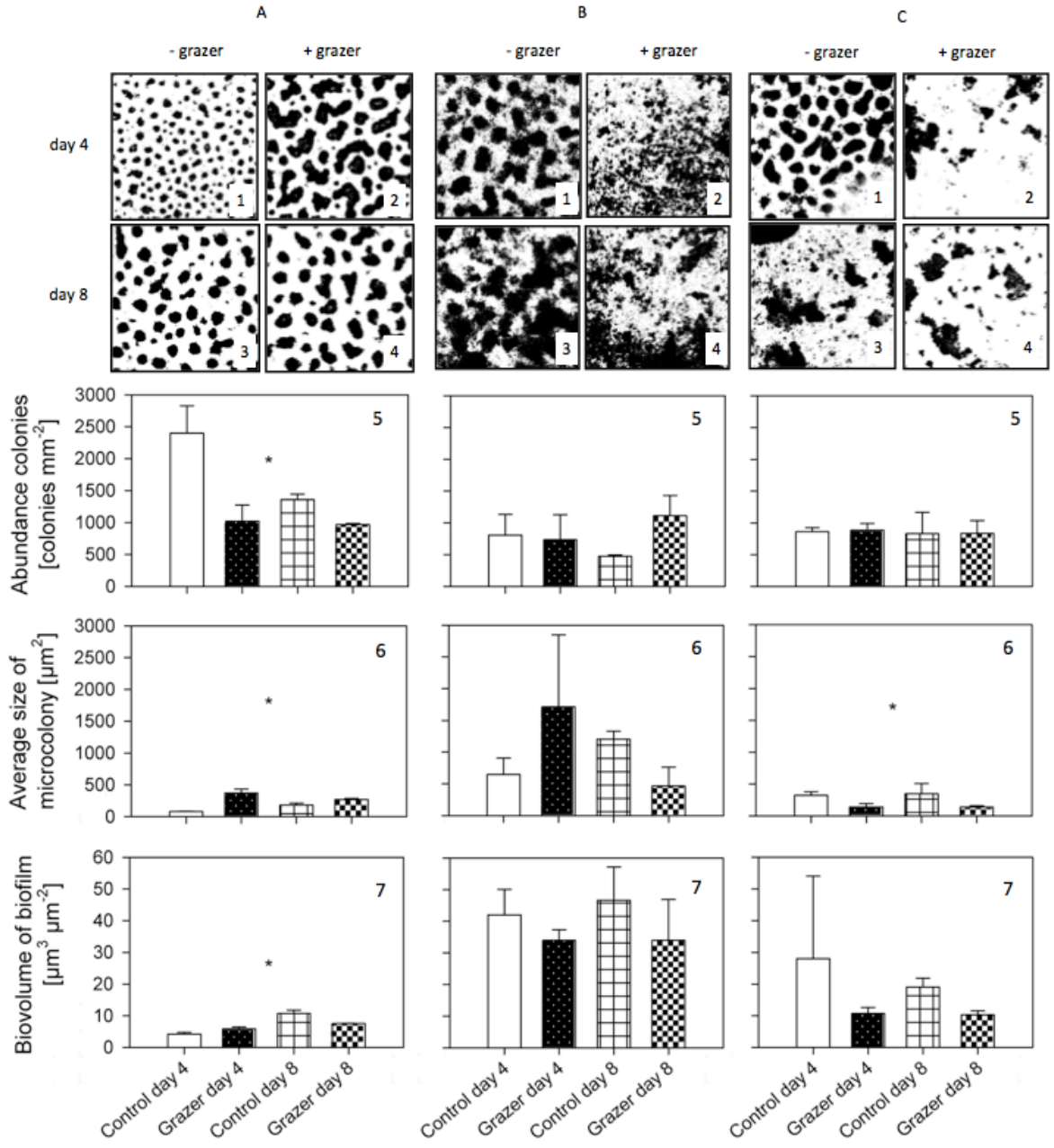

Figure 2.1. LSM images of Acinetobacter sp. strain C6 variant. Biofilms grown under different conditions.

A) Biofilms grown under 'high flow' rate and supplied with 0.5 mM sodium benzoate as a carbon source. B) Biofilms grown under 'high flow' conditions and 4 mM citrate as a carbon source. C) Biofilms grown under 'low flow' conditions and sodium benzoate as a carbon source. Shown are biofilm xy-images of day 4 (A1-2, B1-2, C1-2) and day 8 (A3-4, B3-4, C3-4), non-grazed and grazed biofilms (1,3 and 2,4, resp.). 5) Abundance of µm-2, 6) average size of microcolony μm^2 µm-2 and 7) biovolume of biofilm μm^3 µm-2. Shown are mean values (+ SD). * $p < 0.05$ ANOVA.

The average size was significantly smaller in the grazed experiments than in the non-grazed control (day 4: 142 μm^2, day 8: 138 μm^2; p=0.01), but same as in the non-grazed treatments, microcolony abundance and biovolume both remained on an equal level but were less variable than in the control.

Discussion

It has previously been shown for planktonic environments that formation of microcolonies can protect bacterial cells against grazing (Simek *et al.* 1997, Hahn and Höfle 1999). Therefore we hypothesized in this communication that the microcolony forming variant of *Acinetobacter* sp. strain C6 would be resistant to grazing by *T. pyriformis* as long as conditions for optimal microcolony formation exist. However, the results of the present study revealed that protozoans can have a considerable impact on the formation of microcolonies even under optimal growth conditions, which could be observed for the structure as well as for the biovolume of biofilms.

This vulnerability could be enhanced when conditions for microcolony formation were reduced, supporting our hypothesis that grazing by protozoa on biofilms has a stronger effect under less optimal conditions for formation of microcolonies and thereby the supposed defence against grazing.

The change of growth conditions for *Acinetobacter* sp. due to carbon source exchange and lowering of the flow rate showed strong effects on the biofilm morphology over time. While in the experiments with optimal growth conditions microcolonies were round shaped and increased in size and biovolume over time, the morphology of biofilms grown under low flow and with citrate as carbon sources showed a higher structural heterogeneity and did not show the typical round shaped microcolonies for the whole course of the experiments. Such a high heterogeneity could also be seen with biofilms of *Pseudomonas aeruginosa* where the structure showed a high variability (if grown with citrate as carbon source Heydorn *et al.* 2000). However, the biovolume of biofilms grown with citrate was higher than those grown with sodium benzoate. Thus, speaking of optimal and less-optimal growth conditions in general might be wrong. However, if applied to being optimal and less-optimal growth conditions for the typical round-shaped microcolony morphology of

the biofilm it has its eligibility. Here, it is also important to mention the difference in analysis between gfp-tagged bacterial biofilms (used in the first experiments with high flow and sodium benzoate) and propidium iodide stained biofilms (which were used in all other experiments of this study). While gfp-tagged biofilms did not require staining biofilms of the non-tagged strain was stained for visualization by confocal laser microscopy. The green-fluorescent protein (gfp) is expressed by the ribosomes of the bacteria and thus, each single cell expresses the fluorescent signal. Propidium iodide is a nucleic acid stain and might under certain conditions also stain nucleic acids present in the extrapolymeric matrix of the biofilms (eDNA) that surrounds the bacteria. However, the settings used in the present study did not allow the detection of the very low signal of stained eDNA. Thus, qualitative comparability of biofilm morphology is capable.

Under conditions, which were optimal for biofilm microcolony development by *Acinetobacter sp*. strain C6, we observed formation of distinct microcolonies. When introducing *T. pyriformis* to this array of biofilm microcolonies at the beginning of the experiment, the shape of microcolonies was changed: single microcolonies were larger and connected to each other. This enlargement of microcolonies could also be observed when *Tetrahymena* was added at day two of biofilm formation (data not shown). This morphological change of the shape of microcolonies probably served as an enhanced protection against protozoan grazing. A similar phenomenon was found for planktonic bacteria in which filaments and microcolonies are formed as a response to grazing (Simek *et al.* 1997, Hahn *et al.* 1999, Jürgens and Sala 2000). In addition, the comparison of bacterial biovolumes in the control and in presence of *T. pyriformis* revealed that the ciliate reduced bacterial biovolume clearly. This confirms previous studies showing that grazing by browsing ciliates (*Colpoda*, *Tetrahymena*) caused a clear reduction in bacterial biomass in biofilms (Huws *et al.* 2005, Weitere *et al.* 2005).

In a study of Tanasescu (2006) feeding rates of *T. pyriformis* were gained by the use of the same experimental setup as in the present study. We used these data to perform calculations of the carbon demand for the observed *T. pyriformis* and *Acinteobacter* sp. growth using published carbon conversion factors (0.11 pgC μm^{-3} for ciliates (Turley *et al.* 1986) and 220 fgC μm^{-3} for bacteria (Bratbak 1985)). These calculations revealed that the mean growth rate of *Acinetobacter* of 0.4 pgC μm^{-3}

day^{-1} can match the mean demand of 0.29 pgC μm^{-3} day^{-1} for *T. pyriformis*. This indicates that grazing losses can be compensated by growth of *Acinetobacter* sp. (see Fig. 1A-C 7). Grazers have a massive impact on the physiology of bacteria. Due to sloppy feeding and excretion of nutrients grazers release bacteria from nutrient limitation (Wang *et al.* 2009). Movements within the biofilms (especially of browsing ciliates as *T. pyriformis*) create free patches and ventilate the bacterial biofilm base. Thus, bacteria at the base of the biofilm might get increased nutrient and oxygen supply who might otherwise starve or become inactive. Additionally, substances produced either by grazers or bacteria (chemical cues or quorum-sensing signals) might have additional growth-stimulating effects at this point (Matz *et al.* 2005, Queck *et al.* 2006). These feedback effects between grazers and bacteria might have had a significant influence on the observed structural and quantitative changes.

Our results showed that even microcolony forming bacteria in biofilms are affected by the activity of grazers and that the interactions between biofilm bacteria and its predators might be much more complex than currently believed.

Chapter 3

Predation of *Tetrahymena pyriformis* enhances *Vibrio cholerae* biofilm formation

Abstract

Protozoan grazing is considered one of the most important mortality factors of bacteria in aquatic habitats. Here, we tested effects of an efficient surface and plankton feeding ciliate grazer (*Tetrahymena pyriformis*) on *Vibrio cholerae* biofilms. *V. cholerae* A1552 wild type biofilms inhibited ciliate growth, in contrast to enhanced growth on biofilms of a quorum sensing response regulator (*hapR*) mutant strain. The grazing resistant wild type A1552 biofilms showed a 'reverse grazer effect', i.e. the biofilm biomass increased in the presence of the ciliate grazers, with increasing proportions of live versus dead bacteria. Stimulation of the biofilm biomass was also observed when *T. pyriformis* was physically separated from the biofilm by filter inserts. When heat-killed A1552 bacteria were fed to the grazer within the insert, a higher ratio of A1552 biofilm bacteria versus planktonic bacteria was detected in comparison to inserts with the grazer fed on a non-*Vibrio* species, which indicates specificity of the chemical interaction. The results suggest that the formation of grazing resistant *V. cholerae* biofilms is stimulated by chemical cues released by both grazer and prey and that biofilm biomass benefits from grazing probably due to nutrient recycling.

Introduction

Cholera, the disease caused by the bacterium *Vibrio cholerae*, continues to pose a threat to human health, especially in developing countries where outbreaks occur in a seasonal pattern with high peaks in spring and autumn (e.g. Faruque *et al.* 1998), but the factors that trigger outbreaks remain unknown (Huq *et al.* 2005). In order to predict cholera outbreaks, it is important to understand the mechanisms affecting persistence and growth of the bacterium in the environment. Grazing by protozoans is one of the most important mortality factors for free-living bacteria in the environment (Hahn and Höfle 2001). One important environmental refuge for *V. cholerae* is growth in biofilms (e.g. Alam *et al.* 2007, Islam *et al.* 2007) and our recent studies showed that the *V. cholerae* biofilms produce an antiprotozoal factor that kills the heterotrophic flagellate *Rhynchomonas nasuta* and thus prevents grazing losses (Matz *et al.* 2005). We also demonstrated that the production of the antiprotozoan factor is regulated by the quorum sensing (QS) response regulator, *hapR* (Matz *et al.* 2005).

The interactions between bacteria and protozoa are considered to be the oldest predator-prey interactions in nature (Jürgens and Güde 1994). Predation gives rise not only to the evolution of defence strategies in bacteria but also to the co-evolution of diverse grazing strategies among protists, allowing for efficient grazing on bacteria (e.g. Parry 2004, Matz and Kjelleberg 2005). In recent years an increasing number of grazers have been shown to have a significant impact on bacterial phenotypes, such as biofilms that before were considered grazing resistant (Huws *et al.* 2005, Weitere *et al.* 2005, Queck *et al.* 2006). One very efficient grazer on both biofilms and suspended bacteria is the ciliate *Tetrahymena* spp. (Eisenmann *et al.* 1998). Weitere *et al.* (2005) showed that *Tetrahymena* sp. strongly reduced the biomass of matrix-encased biofilms. Furthermore, the ciliate was able to graze on biofilms of the virulent *Pseudomonas aeruginosa* due to frequent retreat of the grazers into the plankton.

In addition to direct negative effects of protozoa feeding on bacteria, indirect effects such as nutrient recycling, can stimulate bacterial activity and growth (Caron 1994, Sherr and Sherr 2002). Furthermore, chemical cross-talk between predator and prey

can induce the formation of grazing resistance morphotypes (Corno and Jürgens 2006). In order to thoroughly evaluate the effects of predation on bacteria, various direct and indirect effects on bacterial abundance and activity need to be considered. In the present study we analysed the grazing impact of *T. pyriformis* on biofilms of the toxigenic *V. cholerae* wild type strain A1552. After confirming that *hapR* also regulates grazing resistance (e.g. toxin production) against the ciliate *T. pyriformis* (as shown earlier for flagellates), we investigated how grazing affects the status (live versus dead) of the bacteria within the biofilm. Grazing assays were performed in microtitre plates containing simulated marine medium. Subsequently, we separated different grazing-associated factors (physical presence of grazers vs. chemical cues as released by grazer and prey) by means of filter-insert experiments to distinguish the roles of direct versus indirect grazer effects.

Material and Methods

Strains and culture conditions

The isogenic *V. cholerae* wild type A1552 El Tor and the *hapR* mutant strains were routinely cultured on lysogeny broth (LB) agar containing 2 % NaCl or grown in LB broth overnight at 37°C with shaking (200 rpm). The browsing ciliate *Tetrahymena pyriformis* (Hymenostomatia, Culture Collection of Algae and Protozoa, Windmere, UK, CCAP 1630/1W) was kept axenically in organic rich medium and 0.5 × NSS (marine minimal medium, Väätänen 1976). The ciliate was originally held in PPY medium and was acclimated to higher salinities gradually by increasing the salinity by 10% every week for 5 weeks. Prior to the experiments, *T. pyriformis* was transferred to 0.5 × NSS (free of organic resources) and fed with heat-killed *P. aeruginosa* PAO1, in order to acclimate the ciliates to the phagotrophic mode of feeding and to eliminate dissolved organic carbon from the culture medium of the ciliates. The ciliates were transferred to fresh medium two additional times for further cleaning. Protozoan cultures were maintained by transferring to fresh medium every 2 weeks.

Grazing assays

Overnight cultures of *V. cholerae* A1552 wild type and hapR mutant strains were inoculated into 0.5 × NSS + 0.1 % glucose in 24-well tissue culture plates (Sarstedt, Newton, USA) at a final concentration of 10^5 cells ml^{-1} and incubated at 20°C with shaking (60 rpm). Two hours after inoculation, the grazers were added at a final concentration of 10^3 Ind ml^{-1}. Grazer abundance on the biofilm was followed over 3 days by means of inverted microscopy (Olympus, Japan). After three days biofilm biomass was quantified using the crystal violet assay as previously described (O'Toole *et al.* 1999). Briefly, the supernatant was removed, biofilms washed 3 × with PBS, stained for 10 min with 0.3 % crystal violet and then washed with PBS 3 times. Biofilms were destained with 96% ethanol and absorbance read at 490 nm (Wallac 1420 Multilabel Counter, Perkin Elmer Life Sciences). Each treatment was done in triplicate.

Grazing assays investigating effect of the physical presence of the grazer on biofilms

To distinguish between direct and indirect effects of *T. pyriformis* grazing on *V. cholerae* biofilms, filter insert experiments were performed in 24-well tissue culture plates (Multiwell™, Falcon®). The grazer was separated from the bacterial biofilm in Millicell® Hanging Cell Culture Inserts (pore size 0.4 µm, PET). Seven different treatments were performed in the experiment: 1) A grazer free control (*V. cholerae* only, no insert) and 2) a no insert grazing treatment (co-inoculation of *V. cholera* and *T. pyriformis*, no insert) served as references. To test the effects of chemical cues released by the grazer, 3) only the sterile (0.2 µm filtered) culture medium of *T. pyriformis* was added into the inserts. To investigate the effects of nutrient recycling and chemical cues released by the different bacteria and the grazer, *T. pyriformis* was co-inoculated with heat-killed bacteria, 4) *V. cholerae* A1552 in one treatment and 5) *P. aeruginosa* PAO1 in the other, within the insert. To determine effects of heat-killed bacteria only, heat-killed 6) *V. cholerae* A1552 or 7) *P. aeruginosa* PAO1 was added to inserts without grazers.

V. cholerae A1552 (overnight culture) was inoculated into each well of a microtitre plate in 0.5 × NSS + 0.1 % glucose at a final concentration of 10^5 cells ml^{-1}. Protozoa

(10^3 Ind. ml^{-1}) and heat-killed bacteria were inoculated two hours later into the inserts (hanging above the biofilm) or wells, respectively, of the respective treatments. Experiments were run for three days at room temperature with shaking (60 rpm). Planktonic samples (from the well or the inserts) were fixed in Lugols solution and protozoan numbers determined. Dilution drop plates of the suspended phase were performed to determine suspended bacterial numbers. Subsequently, the biofilm biomass was determined by crystal violet staining (see above).

Quantification of live vs. dead bacterial cells in V. cholerae biofilms

The effect of the grazer on the ratio of live to dead biofilm biomass was determined using confocal laser scanning microscopy (CLSM) of biofilms stained with the Live/dead stain (LIVE/DEAD® BacLight™ Bacterial Viability Kit L7012, Invitrogen, Eugene, USA). Assays were performed in 6-well tissue culture plates (Cellstar®, greiner bio-one, Monroe, USA). In each well, a sterile cover slip (18 × 18 mm, ESCO, Portsmouth, USA) was added. The *V. cholerae* overnight culture was diluted with 0.5 × NSS + 0.1 % glucose to a final concentration of 10^5 cells ml^{-1} and 5 ml added to each well. *T. pyriformis* was inoculated two hours later at a concentration of 10^3 Ind ml^{-1}, to allow for bacterial attachment to the cover slip. Experiments were run for three days at room temperature with shaking (60 rpm). Protozoan abundances on the biofilms were followed over three days with an inverted microscope (Olympus, Japan). On days 1, 2 and 3 a cover slip from three individual wells was removed, washed in PBS, stained for 10 min in the dark and washed again in PBS. Images were taken using a CLSM (Leica DMRB, BioRad MRC 1024, Software LaserSharp 2000) and analysed with 'Image J' (version 1.41, National Institutes of Health, USA [http://rsbweb.nih.gov/ij/]). Live and dead biomass was analysed for total surface coverage, average microcolony size and average number of microcolonies. All treatments were performed in triplicate.

Statistical analyses

The statistical analyses were performed using SPSS 17.0. The two-factorial designs were analysed with the help of two-factorial analyses of variance (ANOVA). Subsequent pair-wise comparisons were performed with the help of t-tests. The significance level was Bonferroni-adjusted to account for multiple comparisons. The

data from the live-dead stain were analysed with the help of three-factorial ANOVAs with time (days 1, 2 and 3), stain (live vs. dead) and grazer (present vs. absent) as independent factors. The data from the filter insert experiments were analysed with the help of a one-factorial ANOVA and (after the ANOVA revealed significant treatment effects) subsequent Tukey-HSD post hoc test for pair wise comparisons.

Results

Grazing by T. pyriformis on V. cholerae A1552 wild type and hapR mutant biofilms

Grazing by *T. pyriformis* on *V. cholerae* A1552 wild type and *hapR* mutant biofilms revealed significant differences in protozoan abundances and biofilm biomass between the two strains. Protozoan abundances on the A1552 wild type biofilms were reduced by approximately 33 % in relation to abundances on the *hapR* mutant biofilms for all three days of the experiment (Fig. 3.1, ANOVA: $p < 0.001$).

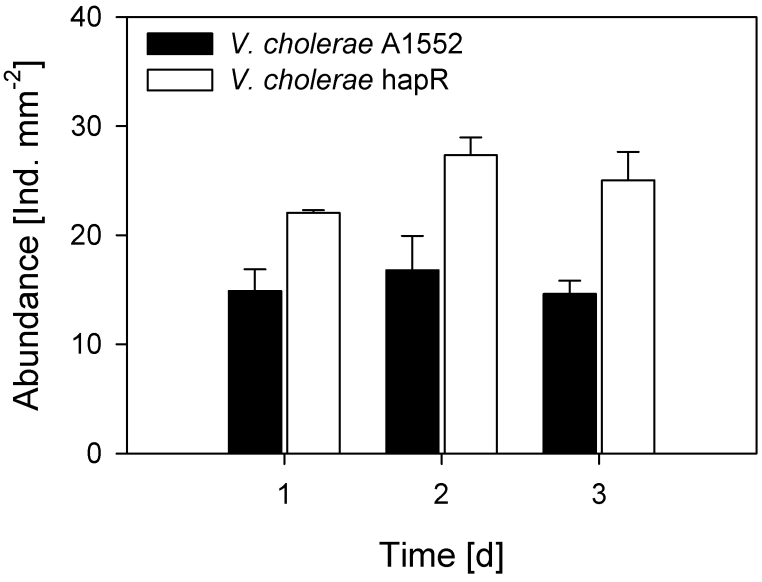

Figure 3.1. Abundance *T. pyriformis* on *V. cholerae* A1552 wild type and *hapR* mutant biofilms. The two-factorial ANOVA revealed significant differences between the strains ($p < 0.001$). Shown are mean values (± SD). Experiments were done in triplicate.

The reduced protozoan abundance in the wells with the A1552 wild type corresponds to a significant increase in the biofilm biomass (to 143 %, Fig. 3.2) compared to the protozoan free control (t-test: p = 0.01). In contrast, the biofilm biomass of the *V. cholerae hapR* mutant exposed to grazing was significantly reduced (84 % of the non-grazed control, t-test p = 0.012). The two factorial ANOVA revealed a highly significant interaction of strain (*hapR* vs. wild type) and treatment (grazed vs. non-grazed) on the biofilm biomass (p < 0.001, Fig. 3.2), supporting the differential response of the two strains towards grazing.

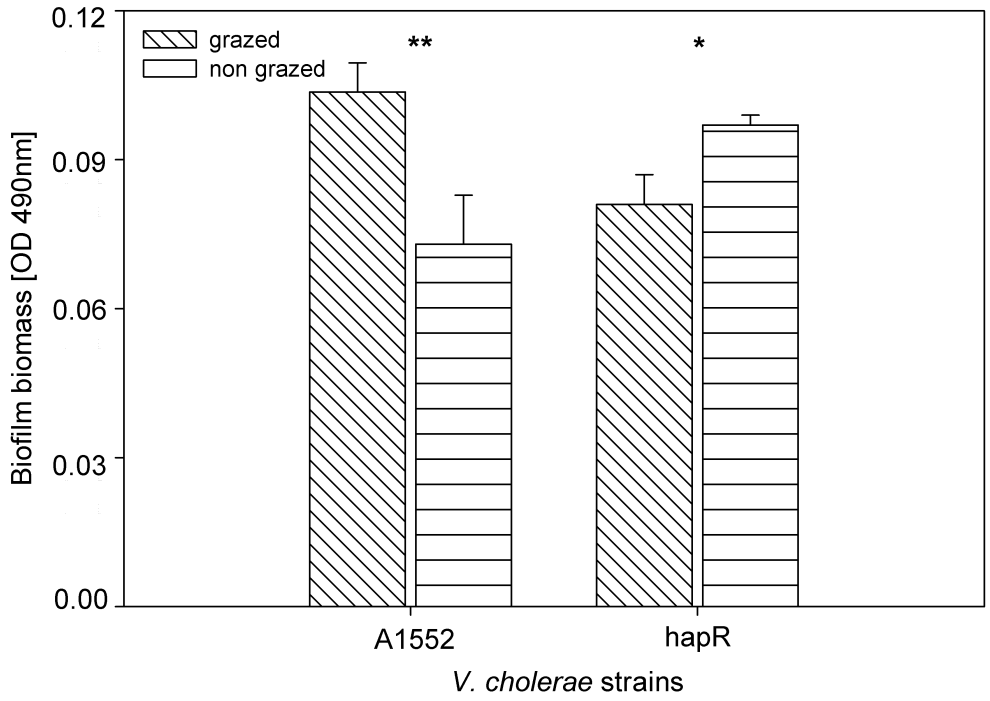

Figure 3.2. Biomass (measured as absorbance of crystal violet at 490 nm) of grazed and non-grazed control biofilms of *V. cholerae* A1552 wild type and the *hapR* mutant after 3 days. The two-factorial ANOVA revealed significant interactions of treatment and strain (p < 0.001), indicating that the effects of grazing differ between the two strains. Significant differences as revealed by pair wise comparisons are indicated: ** p < 0.01; * p < 0.05. Experiments were done in triplicate.

The role of the physical presence of grazers versus chemical cues

To separate direct physical and indirect (possible chemical cues) grazer effects, *T. pyriformis* was physically separated from the *V. cholerae* biofilms by the use of filter inserts. In these experiments, the final abundance of *T. pyriformis* reached $10^3 \pm 577$ individuals (Ind) ml^{-1} when grown directly on the biofilms (no inserts), whereas a higher abundance of $1.3 \times 10^5 \pm 2 \times 10^4$ Ind ml^{-1} and $5.5 \times 10^4 \pm 8 \times 10^3$ Ind ml^{-1} was obtained when grown within the inserts supplied with heat-killed *P. aeruginosa* PAO1 and *V. cholerae* A1552, respectively.

The number of suspended bacterial cells was significantly reduced when the grazer was physically present on the biofilm, in relation to the grazer-free control (Fig. 3.3A), whereas the biofilm biomass increased significantly (Fig. 3.3B). Importantly, when *T. pyriformis* was physically separated from the biofilms by the inserts, the biofilm biomass significantly increased in comparison to the non-grazed control, regardless of the food source within the inserts (heat-killed *P. aeruginosa* PAO1 or *V. cholerae* A1552; Fig. 3.3B). The abundances of planktonic cells, however, showed a significant difference between the two food sources (Fig. 3.3A). When the grazer was fed with heat-killed *P. aeruginosa* PAO1 within the inserts, abundances of planktonic *V. cholerae* were highest, whereas numbers were significantly lower when the grazer was fed with heat-killed *V. cholerae* A1552 within the inserts. The ratio of biofilm to planktonic cells thus increased when *Vibrio* cells were used as food source in comparison to non-*Vibrio* cells, indicating that the chemical cues released by *V. cholerae* specifically stimulate biofilm growth. Supplementing inserts with only heat-killed bacteria (no grazer) did not result in significant effects on the biofilm biomass or on suspended bacteria in comparison to the grazer free control. In contrast, supplementation with cell-free, filter sterilised *T. pyriformis* culture medium (fed with heat-killed PAO1) resulted in an increase of biofilm biomass, but not of the planktonic biomass in comparison to the grazer-free control.

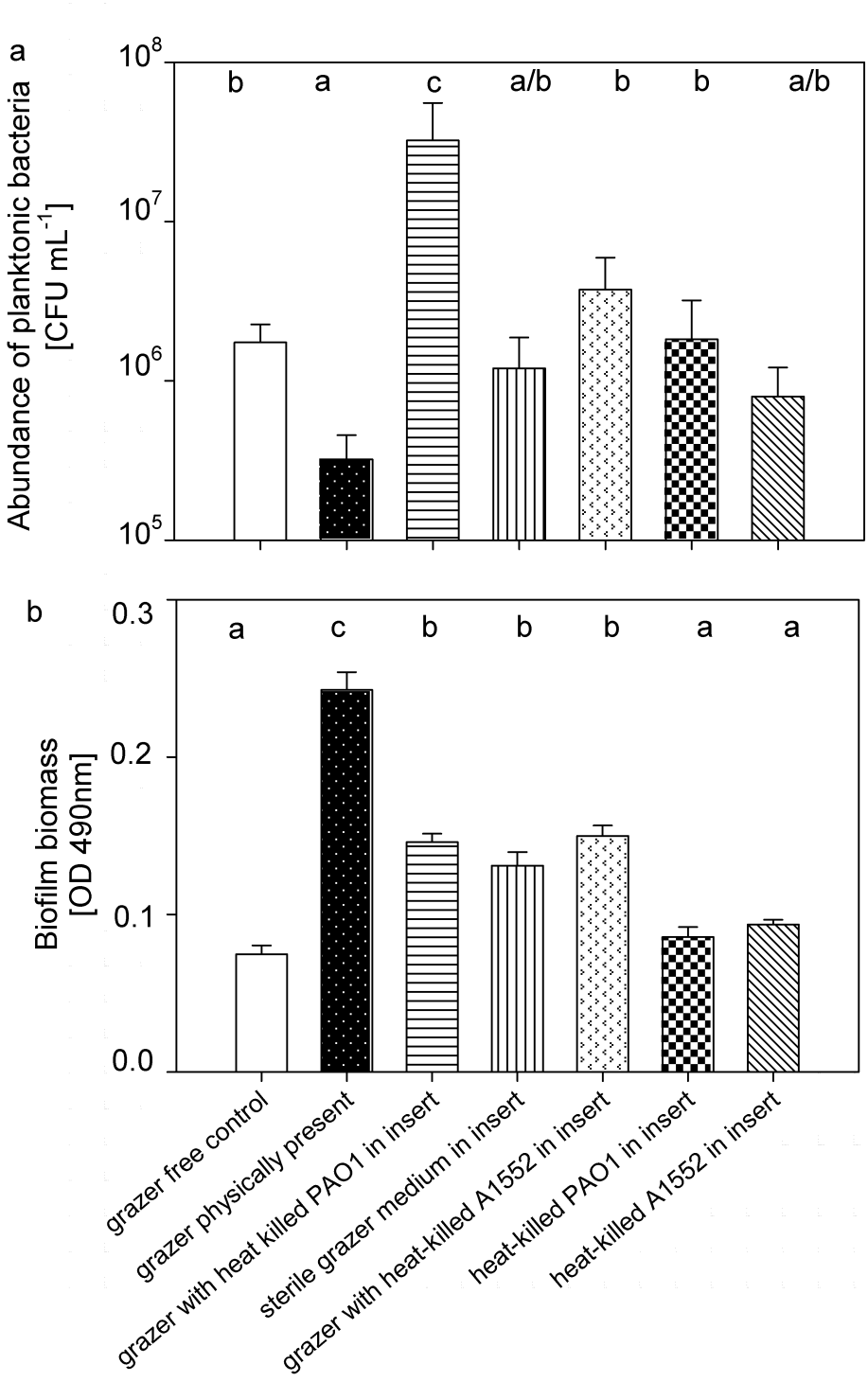

Figure 3.3. Effects of the physical presence/absence of *T. pyrifomis* on (a) planktonic bacteria and (b) biofilm biomass (insert experiments) on day 3. The *V. cholerae* A1552 strain was exposed to different treatments: grazer-fee control, grazer physically present, grazer with heat-killed PAO1 in insert, filtered spent medium from the protozoan culture, grazer with heat-killed A1552 in insert, heat-killed PAO1 in insert, heat-killed A1552 in insert. Letters above bars indicate grouping in Tukey-HSD test. Shown are mean values (±SD).

Grazing by T. pyriformis increases the ratio of live versus dead V. cholerae cells

In order to examine the viability of the bacteria in the grazed biofilm as well as the biofilm structure, we compared grazed and non-grazed biofilms stained with Live/Dead stain using confocal laser scanning microscopy (CLSM). Although the predator abundance declined throughout the experiment, grazers were continuously present and active within the biofilm (2h: 3.2 ± 1.9 Ind mm^{-2}, day 3: 0.8 ± 0 Ind mm^{-2}).

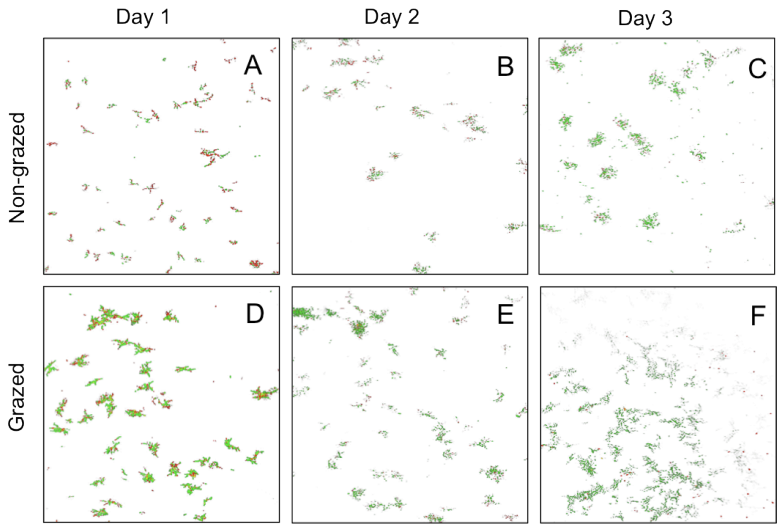

Figure 3.4. Randomly chosen CLSM Images of *V. cholerae* A1552 wild type biofilms grown in absence (A-C) and presence (D-F) of *T. pyriformis* over three days. Red: dead cells stained with propidium iodide, green: living cells stained with SYTO 9. Image size: 230 × 230 µm.

The CLSM images indicated that small microcolonies were visible after day 1 in all biofilms (Fig. 3.4). In the non-grazed control, live (SYTO 9 stained) and dead (propidium iodide stained) bacteria were abundant in equal amounts on day 1. In contrast, a higher number of living bacteria were observed with greater substratum coverage in the grazed than in the non-grazed biofilms. This trend was consistent throughout the 3 days of the experiment (Fig. 3.5A) and the differences in live vs. dead bacterial abundance were significant (Table 3.1A). Analysis of these data using ANOVA revealed significant interactions between the viability and grazer effects. In the X – Y scans of the biofilm, the ratio of live to dead cells was 4:1 in the non-grazed treatment and 11.5:1 in the grazed treatment on day 3. The size of the colonies generally decreased from day 1 to day 3 and was only marginally affected by grazing

(Fig. 3.5B, Table 3.1B). In contrast, the abundance of bacterial colonies increased in both grazed and non-grazed biofilms over time. Significantly more live colonies could be seen in the grazed set-up, whereas no grazer effects were found on the dead cells (Fig. 3.5C, Table 3.1C).

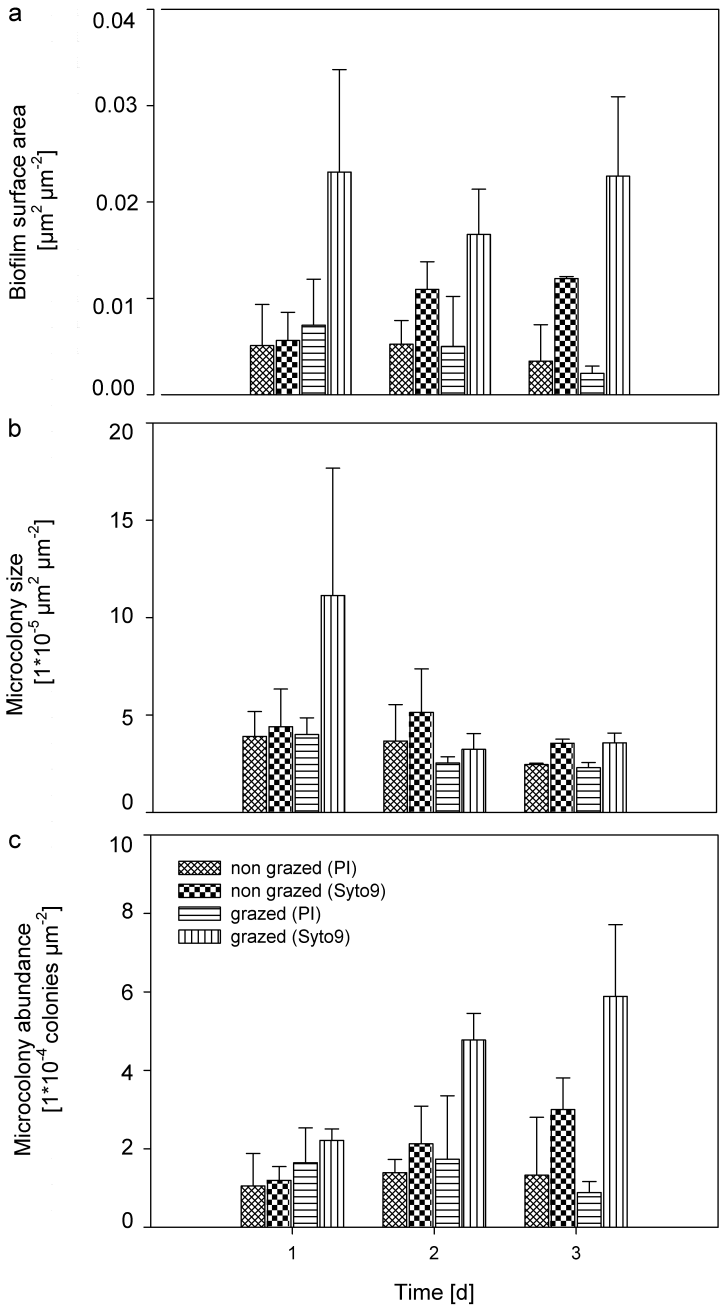

Figure 3.5. Effects of grazing on live versus dead cells of *V. cholerae* A1552 biofilms over three days: Non-grazed biofilm dead cells, non-grazed biofilm live cells, grazed biofilm dead cells and grazed biofilm live cells. (A) Surface coverage (μm^2 μm^{-2}) of biofilm. (B) Average size of microcolonies (μm^2 μm^{-2}). (C) Average abundance of colonies. Shown are mean values (± SD). See Table 1 for results of ANOVAs.

Table 3.1. Results of the three-factorial ANOVAs on the effects of *T. pyriformis* ('grazer') and time on live versus dead cells ('stain') of *V. cholerae* A1552 biofilms. (A) Biofilm area. (B) Colony size. (C) Abundance of colonies. See Fig. 4 for data. SS: Sum of squares; df: degrees of freedom; F: F-ration; P: significance.

	SS	df	F	P
A) Biofilm covered area				
Grazer	$2.74*10^{-4}$	1	9.993	0.005
Time	$4.34*10^{-6}$	2	0.079	0.924
Stain	$9.09*10^{-4}$	1	33.182	< 0.001
Grazer x Time	$7.93*10^{-5}$	2	1.447	0.257
Grazer x Stain	$2.54*10^{-4}$	1	9.286	0.006
Time x Stain	$6.39*10^{-5}$	2	1.166	0.330
Grazer x Time x Stain	$3.40*10^{-5}$	2	0.621	0.547
Error	0.001	22		
B) Colony size				
Grazer	$3.21*10^{-10}$	1	0.599	0.447
Time	$5.15*10^{-9}$	2	4.811	0.018
Stain	$3.49*10^{-9}$	1	6.528	0.018
Grazer x Time	$3.79*10^{-9}$	2	3.543	0.046
Grazer x Stain	$8.62*10^{-10}$	1	1.610	0.218
Time x Stain	$1.38*10^{-9}$	2	1.288	0.296
Grazer x Time x Stain	$2.39*10^{-9}$	2	2.235	0.131
Error	$1.18*10^{-8}$	22		
C) Colony abundance				
Grazer	$1.14*10^{-7}$	1	11.876	0.002
Time	$9.71*10^{-8}$	2	5.039	0.016
Stain	$2.88*10^{-7}$	1	29.859	< 0.001
Grazer x Time	$7.2*10^{-9}$	2	0.374	0.693
Grazer x Stain	$8.47*10^{-8}$	1	8.789	0.007
Time x Stain	$1.19*10^{-7}$	2	6.197	0.007
Grazer x Time x Stain	$2.97*10^{-8}$	2	1.541	0.236
Error	$2.12*10^{-7}$	22		

Discussion

Here we present a series of experiments, which demonstrate the grazing resistance of *V. cholerae* A1552 wild type against an efficient grazer and reveal mechanisms on how biofilms of this species benefits from the presence of the grazer. After confirming that the cell density dependent regulator HapR is involved in grazing resistance against *T. pyriformis*, as has been shown previously against *R. nasuta* (Matz *et al.* 2005), we showed that the presence of the grazer enhances the densities of living bacteria in the wild type biofilm while having no significant effect on the number of dead cells. We compared the contributions of physical and chemical factors and discuss potential mechanisms below.

Involvement of hapR in the grazing resistance of V. cholerae biofilms

Previous results have shown that the *V. cholerae* A1552 strain produces a QS-regulated antiprotozoal factor that inhibits growth of the flagellate *R. nasuta* (Matz *et al.* 2005). The data presented here support the role of HapR in grazing resistance against the ciliate *T. pyriformis,* suggesting that *V. cholerae* produces a general antiprotozoal compound. However, in contrast to the findings of Matz et al. (2005) with *R. nasuta* where the flagellate was completely eliminated, growth of the ciliate *T. pyriformis* was less than that observed when grazing on the *hapR* mutant biofilms or growth on plain media, but was not completely eliminated (Fig. 3.1). This demonstrates the broad relevance of the *hapR*-regulated antiprotozoan factor(s). In contrast to previous findings showing killing of the flagellate grazer, the survival of the ciliate suggests that different grazers are affected in different ways by the *V. cholerae* antiprotozoal factor(s). In contrast to the strictly benthic grazer *R. nasuta,* the ciliate *T. pyriformis* is able to feed on attached as well as suspended prey (Eisenmann *et al.* 1998). It has been shown in an earlier study that *Tetrahymena* is able to survive exposure to biofilms of virulent *P. aeruginosa* PAO1, whereas strictly surface attached protists were killed (Weitere *et al.* 2005). This survival is likely due to the ability of the ciliate to escape the toxicity of *P. aeruginosa* PAO1 by migrating out of the biofilm to feed on suspended bacteria (Eisenmann *et al.* 1998).

Grazing by T. pyriformis on V. cholerae biofilms results in a 'reverse grazer effect'

We consistently saw a 'reverse grazer effect', i.e. a stimulation of the biofilm biomass of *V. cholerae* A1552 wild type when *T. pyriformis* was present in all experiments performed during the present study. This is in contrast to the reduced biofilm biomass in the *hapR* mutant strain. Furthermore, we could show that in contrast to the efficient utilization of both planktonic and biofilm cells of the virulent bacterium *P. aeruginosa* PAO1 by *Tetrahymena* (Eisenmann *et al.* 1998), *V. cholerae* biofilms are not only able to resist grazing but also benefited from the presence of the grazer.

The interactions between predator and prey are complex. Besides the direct negative effects of predation pressure (removal of cells by grazing), bacteria may also experience positive effects due to possible enhanced nutrient recycling and subsequent growth stimulation (Corno and Jürgens 2006). Usually, the direct negative effects are stronger than indirect positive effects. In the present study we observed that the grazer fed on the biofilms and had densely packed food vacuoles. However, by inhibiting the growth of *T. pyriformis* (but not completely eliminating the grazer) and by responding to the presence of the grazer by retreating into the biofilm mode, *V. cholerae* grew better potentially by simultaneously inhibiting activity of the ciliate and from nutrient recycling by the ciliate. Partial inhibition of the predators' growth can thus lead to a greater benefit to the bacterial population than that experienced by killing the grazer.

Our data further show that the positive grazer effect on the biofilm biomass is exclusively due to a stimulation of living cells, while the densities of dead cells remained unaffected by the grazer. Grazing pressure resulted in a 3-fold increase in the ratio of live to dead bacteria compared to the non-grazed control biofilms and thus in an increase in biofilm activity (Table 3.1). Several mechanisms can affect the proportion of active versus non-active bacteria cells. It is known for microbial communities in planktonic habitats that protozoan grazing increases bacterial activity due to nutrient recycling and enhanced growth (see above). It is also known that grazers usually prefer to feed on active rather than inactive cells (Gasol *et al.* 1995, del Giorgio *et al.* 1996). The strong increase in living cells and thus the overcompensation of grazing losses further support the predominance of indirect

positive grazing effects on the bacterial biofilms. Evidence for increased bacterial activity under grazing pressure of *Acanthamoeba castellani* has also been reported in soil biofilms (Rosenberg *et al.* 2009).

Nutrient recycling, chemical communication and physical presence of T. pyriformis facilitate the formation of V. cholerae biofilms

We have shown previously that grazing pressure induces biofilm formation by *V. cholerae* (Matz *et al*. 2005). Here we explored possible mechanisms that lead to the stimulation of the *V. cholerae* biofilms in the presence of grazers. When *T. pyriformis* was physically separated from the biofilm, stimulation of the biofilm biomass was observed. This stimulation occurred when the grazer was separated from the biofilm by inserts containing the predator and heat-killed bacteria, as well as when filtered spent medium from the protozoan culture was added. This indicates that chemical cues released by the grazer can result in induction of biofilm formation. However, an increase in the number of bacteria in suspension only occurred when *T. pyriformis* was fed with heat-killed bacteria. This increase was small when heat-killed *V. cholerae* A1552 was provided, and large when *P. aeruginosa* PAO1 was supplied. Controls with heat-killed bacteria only had no effect on planktonic or biofilm biomass when compared to the grazer free control. These results suggest that nutrient recycling by the feeding activity of protozoan predators has a positive effect on both planktonic and biofilm phases and that chemical components released by *T. pyriformis* when grazing on *V. cholerae* result in enhanced ratios of biofilm to plankton biomass in comparison to chemical components released by *T. pyriformis* grazing on non-*Vibrio* species (*P. aeruginosa*). Such induced defences by chemical cross talk of bacteria and protozoa have been shown for bacterial communities in the formation of grazing resistant morphotypes, in the presence of grazers (Hahn and Höfle 1998).

In addition to the effects described above, the physical presence of the grazers on the biofilms resulted in the highest stimulation of biofilm biomass and strongest reduction of suspended bacteria. The nutrient recycling that occurs due to grazing on living planktonic cells might be more efficient than the nutrient recycling when *T. pyriformis* is fed with heat killed bacteria, which would favour enhanced biofilm

growth. In addition, the physical presence of the predator on the biofilm may have other indirect positive effects. Glud and Fenchel (1999) demonstrated enhanced ventilation of biofilms by grazing activity, and altered biofilm structure with the introduction of higher porosity and surface area to volume ratios would result in increased nutrient supply (Weitere *et al.* 2005, Böhme *et al.* 2009). Taken together, the results of the insert experiments show that nutrient recycling, chemical crosstalk and other factors linked to the direct presence of the grazers on the biofilms stimulate the formation of *V. cholerae* A1552 biofilms.

Conclusions

The results of the present study establish the efficiency of *hapR*-regulated grazing resistance against the ciliate *Tetrahymena*, a grazer that was shown to significantly reduce virulent *P. aeruginosa* PAO1 biofilms in earlier studies. However, unlike strictly surface feeding protists, the ciliate was not completely eliminated by the wild type. This survival, in low numbers, is of benefit for *V. cholerae* due to indirect grazing effects such as nutrient recycling. Importantly, our results give support for the existence of an induced defence by *V. cholerae,* i.e. that of biofilm formation, and highlights the interplay of different factors, including chemical cues as released by both grazers and prey. Such mechanisms support the formation of grazing resistant biofilms and thus the persistence and growth of *V. cholerae* in the environment (Huq *et al.* 1983, Kierek and Watnick 2003, Islam *et al.* 2007).

Chapter 4

In situ grazing resistance of *Vibrio cholerae* in the marine environment

Abstract

Previous laboratory experiments revealed *Vibrio cholerae* A1552 biofilms secrete an antiprotozoal factor that prevents *Rhynchomonas nasuta* from growing and thus prevents grazing losses. The antiprotozoal factor is regulated by the quorum sensing response regulator, HapR. Here we investigate whether the antiprotozoal activity is ecologically relevant. Experiments were conducted in the field as well as under field-like conditions in the laboratory to assess grazing resistance of *V. cholerae* A1552 and N16961 (natural frameshift mutation in *hapR*) biofilms to *R. nasuta* and *Cafeteria roenbergensis*. In laboratory experiments exposing the predators to *V. cholerae* grown in seawater containing high and low glucose concentrations, we determined that *V. cholerae* biofilms showed increased resistance towards grazing by both predators as glucose levels decreased. The relative resistance of the *V. cholerae* strains to the grazers under semi-field conditions was similar to that observed *in situ*. Therefore, the antipredator defense is environmentally relevant and not lost when biofilms are grown in an open system in the marine environment. The *hapR* mutant still exhibited some resistance to both predators and this suggests that *V. cholerae* may co-ordinate antipredator defenses by a combination of density dependent regulation and environmental sensing to protect itself from predators in its natural habitat.

Introduction

In the last 20 years, cholera has occurred in areas that have been free from outbreaks for almost a century (for review see Tauxe*, et al.*, 1994). The recent increases in occurrences may be due to the fact the *Vibrio cholerae* El Tor biotype (cause of the seventh and current pandemics), may be more environmentally fit than the Classical biotype (etiological agent for the first six pandemics), and thus has replaced the Classical biotype in the environment. This highlights the need to better understand what factors affect the occurrence and survival of *V. cholerae* in the environment. Researchers have begun to use remote sensing to determine if they can identify correlations between cholera outbreaks and ocean parameters (e. g. phytoplankton and zooplankton blooms, seawater temperature, nutrient concentration) in an attempt to predict outbreaks (Lobitz, et al., 2000). Many of the studies monitoring *V. cholerae* in the marine environment have focused on the effect of nutrient availability (Singleton*, et al.*, 1982) and on interactions of *V. cholerae* with copepods (Huq*, et al.*, 1983, Pruzzo*, et al.*, 2008). Several studies have shown that *V. cholerae* attaches preferentially to biotic surfaces such as copepods in the marine environment (Heidelberg*, et al.*, 2002, Mueller*, et al.*, 2007), while others have demonstrated a preference for planktonic growth of *V. cholerae* in the water column (Worden, 2006) in which case the bacterial cells experienced heavy grazing pressure by protozoa.

The interactions of bacteria and protozoa are considered to be one of the oldest predator-prey interactions in nature (Cavalier-Smith, 2002). Grazing by phagotrophic protists is one of the main mortality factors of bacteria in marine and freshwater systems (Azam*, et al.*, 1983, Hahn & Höfle, 2001, Matz & Jürgens, 2001) and a major selective force for evolution of bacterial defense strategies (Matz & Kjelleberg, 2005). Predation can alter bacterial morphology and community structure through direct (predation (Hahn & Höfle, 1999, Jürgens*, et al.*, 1999)) and indirect (nutrient recycling (Sherr*, et al.*, 1982, Pernthaler*, et al.*, 1997)) interactions. Bacteria have evolved different defense strategies including general avoidance (e.g. motility) and direct consumer effects (e.g. digestional resistance, toxin production) (Matz & Kjelleberg, 2005).

The majority of microbes in natural habitats occur as surface-attached communities called biofilms (Davey & O'Toole, 2000), which function to protect cells in the community from a variety of stresses. The biofilm architecture and bacterial-produced extracellular polymeric substances (EPS) offer important protection against various stresses such as antimicrobial agents (Gilbert*, et al.*, 1997) and grazing (Parry, 2004, Weitere*, et al.*, 2005). Biofilm formation as well as toxin production are controlled by density dependent bacterial gene regulation, or quorum sensing (QS) in many bacterial species (Hammer & Bassler, 2003, Turovskiy*, et al.*, 2007). For example, in the pathogens *Pseudomonas aeruginosa* and *V. cholerae*, QS regulates the production of toxins that have been shown to kill predators resulting in grazing resistance (Matz*, et al.*, 2004, Matz*, et al.*, 2008). While several studies have assessed the interactions of protozoa and *V. cholerae* in the suspended state and planktonically in mesocosms (Macek*, et al.*, 1997, Worden, 2006), surprisingly little is known about the impact of protozoa on the occurrence of attached *V. cholerae.*

In laboratory studies, we have shown that microcolony formation in biofilms of *V. cholerae* (Matz*, et al.*, 2005) and *P. aeruginosa* (Matz*, et al.*, 2004) protected against grazing losses. Further, it has been demonstrated that biofilms have antiprotozoal activity (Matz*, et al.*, 2004, Weitere*, et al.*, 2005). Matz *et al.* (2005) demonstrated that biofilms of *V. cholerae* A1552 wild type strain could prevent the benthic grazer *Rhynchomonas nasuta* from growing, while biofilms of a QS mutant (lacking the response regulator, HapR) were grazed. In this study, we investigate the efficacy of the grazing resistance of biofilms observed in laboratory experiments, in an ecologically relevant context. The survival of *V. cholerae* biofilms under grazing pressure was tested *in situ* in environmental diffusion chambers (McFeters & Stuart, 1972), where massive dilution effects occur from the surrounding seawater. The grazing assays were performed in the marine environment over a period of 10 days and survival and persistence in the presence of two marine flagellates, *R. nasuta* and *Cafeteria roenbergensis* was assessed.

Material and Methods

Strains and culture conditions

V. cholerae A1552 wild type, *V. cholerae* N16961 (natural *hapR* frameshift mutant), *V. cholerae hapR* (isogenic genetically modified organism (GMO) lacking the *hapR* gene encoding the QS response regulator) and *E. coli* B were routinely cultured on Luria Bertani agar containing 2 % NaCl (LB20) or grown in LB20 broth overnight at 37°C with shaking (200 rpm). The benthic grazer, *R. nasuta,* was isolated from the field site at the Sydney Institute for Marine Science (SIMS), treated with an antibiotic cocktail (streptomycin, spectomycin, gentamycin, tobramycin, ampicillin and kanamycin at 150 µg mL^{-1}) and serially diluted for many generations to remove the natural contaminating bacterial community. *R. nasuta* and the predominately planktonic flagellate, *C. roenbergensis* (Bicosoecida, Baltic sea, isolated by A. P. Mylnikov), were maintained axenically in 0.5 × nine salts solution (NSS, Väätänen, 1976) supplemented with heat-killed *P. aeruginosa* PAO1 as prey at room temperature, and transferred to fresh medium every two weeks.

Environmental chamber set-up

Four replicate experiments were performed during the period of January 2008 to May 2009. Environmental diffusion chambers (McFeters & Stuart, 1972) were suspended in the marine environment at SIMS (see Table 4.1 for details). The chambers (volume 28.3 mL) were sealed with membranes (Supor® -100 membrane filters, 0.1 µm pore size, 90 mm, PALL Life Science) which were permeable to seawater but retained bacteria and protozoans inside the chambers. These were further suspended in a mesh-lined crate to prevent puncture from larger marine animals. The chambers were modified to hold a glass slide as a substratum for biofilm formation. Cover slips were attached to the slide with silicone glue and one cover slip was removed for protozoan counting and one for staining and imaging by confocal laser scanning microscopy (CLSM; Leica DMRB, Leica, Switzerland). For each treatment, at least three, in one case four autoclaved chambers, were inoculated with 28.5 mL of bacteria- (overnight cultures diluted to 10^7 cells mL^{-1}) and protozoa-suspension (10^4 cells mL^{-1}) in 0.5 × NSS and incubated in the marine environment for 10 days (see Table 4.1 for details). Following incubation in the

marine environment, chambers were collected and immediately transferred to the laboratory in seawater. Although a thin biofilm was sometimes detectable on the outside of the chambers, diffusion of seawater was not impeded as evidenced by the rapid exchange of seawater observed when the chambers were removed. Protozoan numbers inside chambers were determined by microscopy (Leica DMLB, Leica, Switzerland) and suspended bacterial numbers determined by dilution drop plates (Hoben & Somasegaran, 1982). The abundance of *V. cholerae* and absence of contamination was verified by plating on selective CPC agar (Massad & Oliver, 1987) as well as LB20 agar. Microscopy was used to verify absence of protozoan contamination at the end of experiments. Glass slides with the cover slips were stored in 2% glutaraldehyde at 4°C until staining and imaging. For staining, the cover slips were detached from the glass slides, washed 3 times in sterile PBS and stained with propidium iodide (100 μg mL^{-1}). Stained biofilms were incubated for 10 min in the dark followed by washing with PBS (three times). The cover slips were inverted on a drop of PBS and "clay feet" on a glass slide and the sides were sealed with liquid candle wax. CLSM images were analysed with Image J (http://rsbweb.nih.gov/ij/).

Table 4.1. Environmental chamber field experiments

	Exp. I	Exp. II	Exp. III	Exp. IV
Date	11-21/01/08	02-12/03/09	08-18/05/09	04-14/05/08
Bacterial strains	*V.c*. A1552[a]	*V.c*. A1552	*V.c*. A1552	*V.c*. A1552
	V.c. N16961	*V.c*. N16961	*V.c*. N16961	*E.c*. B[b]
Grazers	*C. roenbergensis*	*C. roenbergensis*	*C. roenbergensis*	*C. roenbergensis*
	R. nasuta		*R. nasuta*	*R. nasuta*
No. replicates[c]	3 each	4 each	3 each	3 each
Exposure time	10 days	10 days	10 days	10 days

[a]*V.c.* represents *V. cholerae* strains, [b]*E.c.* represents *E. coli,* [c]Number of chambers containing individual bacterial strains and grazers. Each chamber held 1 bacterial strain and 1 grazer.

Grazing assays performed under field-like conditions in the laboratory

To quantify protozoan dynamics on the *V.cholerae* biofilms in a non-destructive environment under conditions similar to those in the field, we performed experiments in the laboratory where the diffusion chambers were place in a circuit with traditional biofilm flow cells (Christensen*, et al.*, 1999) connected with silicon

tubing. A peristaltic pump circulated water from the chambers into the flow cells in which protozoan succession on the *V.cholerae* biofilms could be quantified, and back into the chambers. The chambers were held in large plastic containers (25 L) in fresh seawater which was changed twice daily. Four chambers for each experiment were inoculated with 28.5 mL of a suspension of 10^7 cells mL^{-1} of an overnight culture of *V. cholerae* strains in 0.5 × NSS and 10^5 cells mL^{-1} of *R. nasuta* or *C. roenbergensis*. Protozoan abundance was determined by microscopic observation of flow cells over 5 days.

Effect of glucose concentration on persistence of V. cholerae under grazing pressure

Due to seasonal differences in the results of our experiments we investigated the influence of different carbon concentrations on the persistence of *V. cholerae* biofilms under grazing pressure, as nutrient levels would be expected to differ between these seasons. Overnight cultures of *V. cholerae* strains were inoculated (10^5 cells mL^{-1}) in triplicate into 24-well microtiter plates (Sarstedt, Newton, USA) in sterile filtered seawater containing 0.1% or 0.001 % sterile filtered glucose as a carbon source. *R. nasuta* (10^5 cells mL^{-1}) was inoculated 2 hours later to give the bacteria time to attach. As a control flagellates were inoculated into the same medium but with heat-killed *P. aeruginosa* as a food source. Experiments were run for 3 days at room temperature with shaking (60 rpm). Protozoan numbers were determined by microscopy and the biofilm biomass measured as previously described (O'Toole*, et al.*, 1999). Briefly, biofilms were washed 3 × with PBS, stained with 0.3 % crystal violet for 10 minutes and washed 3 times with PBS. The biofilm was destained with 96% ethanol and absorbance read at 490 nm (Wallac 1420 Multilabel Counter, Perkin Elmer Life Sciences).

Statistical analyses

Statistical analyses were performed using SPSS 17.0 software. Pair-wise comparisons were performed using t-tests. Multiple comparisons were done using one- or two-factorial ANOVAs. Tukey-HSD-test was used as post hoc test after significant group effects were detected by ANOVA. In the case of non-homogenous variances, data were log(x+1) transformed prior the analyses. If the variances were

still non-homogenous after transformation, non-parametric Kruscal-Wallis H-test for comparing multiple groups or Mann-Whitney U-test for pair-wise comparisons was applied.

Results

Protozoan abundance on V. cholerae and E. coli biofilms in the marine environment

After 10 days in the field, protozoa could be detected on all biofilms grown in the environmental chambers, with the abundance being dependent on the time of year (generally abundance of protozoa was higher in autumn than in summer). Surprisingly, in midsummer (Fig. 4.1A) the number of the surface-feeder, *R. nasuta,* was significantly higher on the biofilms of the A1552 wild type strain than on the biofilms of the N16961 natural *hapR* mutant strain (t-test $p < 0.01$).

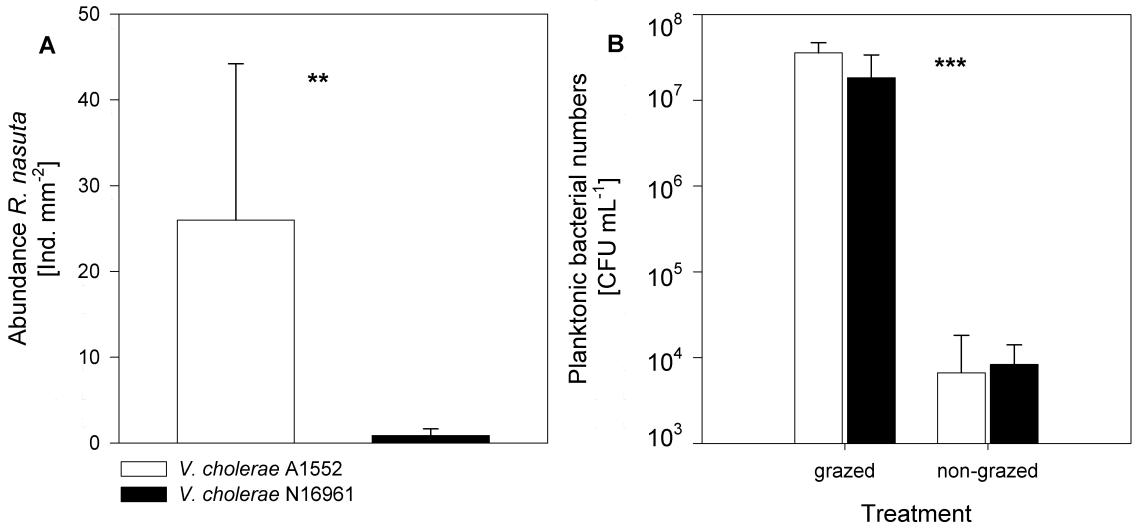

Figure 4.1. Abundance of (A) *R. nasuta* on *V. cholerae* A1552 and N16961 biofilms and (B) abundance (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar) and N16961 (black bar) from environmental chambers after 10 days co-culture in the marine environment. Chambers were co-inoculated with the flagellate *R. nasuta* (grazed) or not (non-grazed). Shown are mean values (± SD, n=3). Note the logarithmic y-scale. (A) ** T-test $p < 0.01$, (B) *** 2-way ANOVA $p < 0.001$.

Since this strain carries a frameshift mutation in the QS response regulatory gene, *hapR*, it was expected that this strain would support a higher abundance of flagellates than the wild type A1552 strain, as has been shown with the isogenic A1552 *hapR* mutant in laboratory studies (Matz*, et al.*, 2005) even though these two

strains are not isogenic. Counts of suspended *V. cholerae* A1552 and N16961 were similar to each other (Fig. 4.1B), but were significantly higher ($p < 0.001$) in the grazed chambers when compared to the grazer free controls.

Figure 4.2A shows the abundance of the suspension feeder, *C. roenbergensis,* on *V. cholerae* A1552 and N16961 biofilms after 10 days exposure in the field (experiments were performed middle to end of summer). The abundance of *C. roenbergensis* was 10 times higher on the *V. cholerae* N16961 biofilms, in contrast to *R. nasuta* abundances which were 20-fold higher on the A1552 wild type biofilms (Fig. 4.1A). The abundance of suspended *V. cholerae* A1552 in the chambers was higher in the grazed chamber than in the non-grazed control (Fig. 4.2B), while the opposite was observed for N16961 where the number of suspended cells was higher in the non-grazed chamber (Fig. 4.2B). The differences in planktonic cell numbers were significant ($p < 0.05$). These results are similar to those observed in laboratory experiments where the isogenic *hapR* mutant strain supported strong growth of the flagellate resulting in reduced bacterial cell numbers, while the opposite was true for the wild type strain. Biofilm biomass did not differ significantly between the grazed and ungrazed treatments or between strains (data not shown).

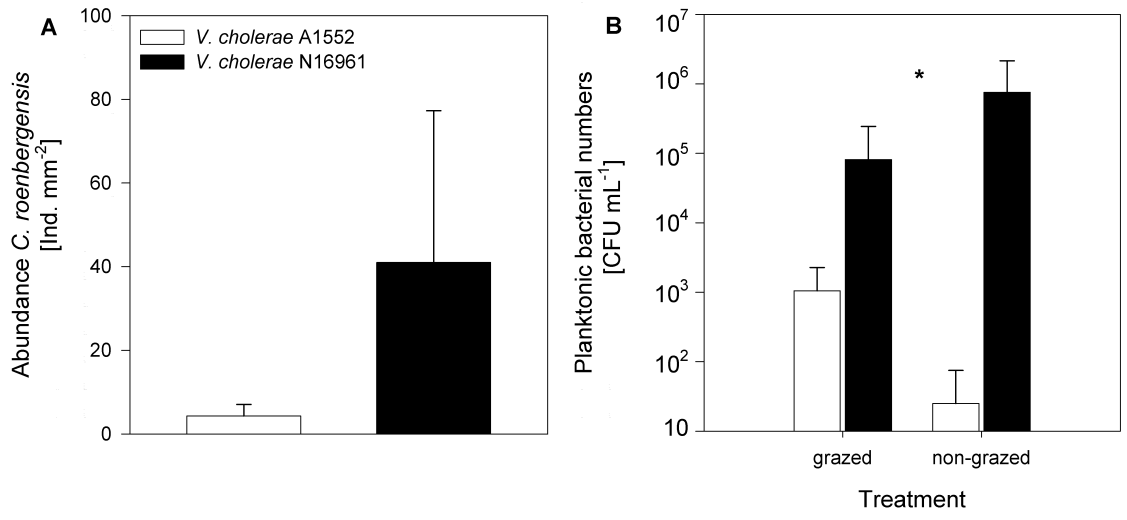

Figure 4.2. Abundance of (A) *C. roenbergensis* on *V. cholerae* A1552 and N16961 biofilms and (B) abundance (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar) and N16961 (black bar) from environmental chambers after 10 days co-culture with the protozoan in the marine environment. Chambers were co-inoculated with the flagellate *C. roenbergensis* (grazed) or not (non-grazed). Shown are mean values (± SD, n=4). Note the logarithmic y-scale, (B) * 2-way ANOVA $p < 0.05$.

Experiments with *R. nasuta* or *C. roenbergensis* inoculated in diffusion chambers in the marine environment with either *V. cholerae* A1552 or N16961 biofilms were also performed at the end of autumn (Fig. 4.3). In the chambers containing *C. roenbergensis,* the trend is the same as for the experiments performed at the end of summer (Fig. 4.2) with a higher abundance of grazers on the N16961 biofilms. For chambers containing *R. nasuta*, there was a lower abundance of grazers on the N16961 strain compared to the A1552 strain biofilms (Fig. 4.3A) but the difference was not as pronounced as in the experiments performed in mid summer (Fig 4.1A). Counts of suspended *V. cholerae* A1552 exposed to *R. nasuta* in the chambers at the end of autumn (Fig. 4.3B) differed from the previous series of experiments performed in midsummer (Fig. 4.1B). The abundance of suspended *V. cholerae* A1552 in the chambers containing *R. nasuta* was higher than the abundance of N16961 (9.2×10^5 and 5.5×10^4, respectively; Fig. 4.3B), while in the earlier experiments they were similar (3.6×10^7 and 1.8×10^7, respectively; Fig. 4.1B). In the chambers with *C. roenbergensis*, the number of suspended N16961 was higher than that of A1552 (Fig. 4.3B), similar to the previous experiment (Fig. 4.2B). Again, biofilm biomass did not differ significantly between treatments or strains indicating that the biofilms were not significantly grazed (data not shown).

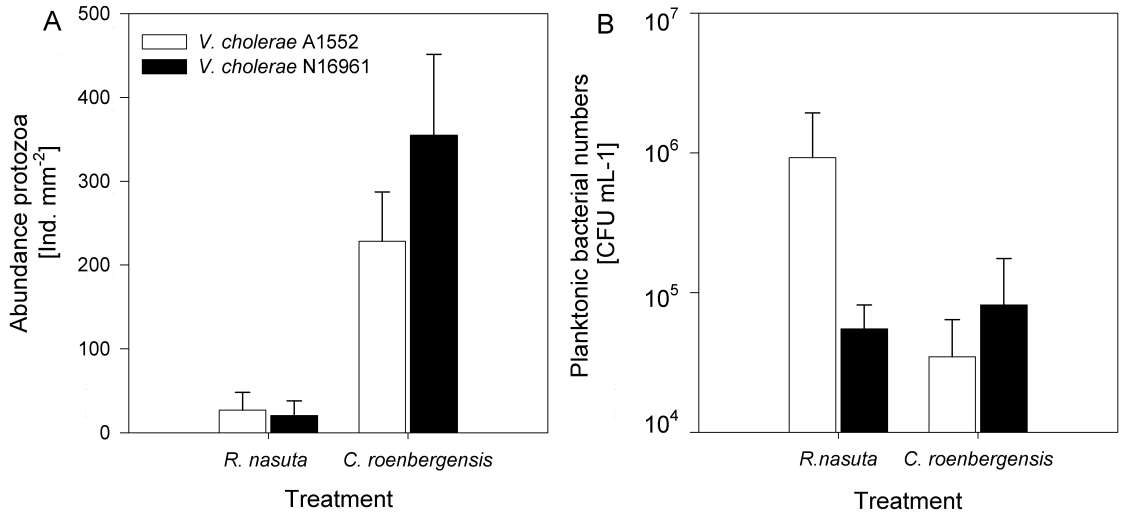

Figure 4.3. Abundance of (A) the flagellates *R. nasuta* and *C. roenbergensis* on *V. cholerae* A1552 (white bar) and N16961 (black bar) biofilms and (B) the abundance (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar) and N16961 (black bar) from environmental chambers after 10 days co-culture with the protozoan in the marine environment. Shown are mean values (± SD, n=3). Note the logarithmic y-scale. 2-factorial ANOVA revealed no significant differences.

To compare flagellate growth on *Vibrio* biofilms to a non-*Vibrio* biofilm which was previously shown to support growth of the protozoa, we exposed *E. coli* B and *V. cholerae* A1552 to both grazers in the field in autumn (Fig. 4.4). Both *R. nasuta* and *C. roenbergensis* numbers were higher on the *E. coli* B biofilms compared to the *V. cholerae* biofilms after 10 days in the field (Fig. 4.4A). While 0.32 ± 0.29 *R. nasuta* mm^{-2} could be detected on the *V. cholerae* A1552 biofilms, *E. coli* biofilms harboured 50 times more. The abundance of *C. roenbergensis* was 2.5 times higher on the *E. coli* biofilms than the *V. cholerae* A1552 biofilms (Fig. 4.4A). Two-factorial ANOVA revealed that the differences in abundance of *C. roenbergensis* compared to *R. nasuta* were significant ($p < 0.001$). The numbers of suspended *E. coli* were higher than the abundance of planktonic *V. cholerae* in chambers containing *R. nasuta* but lower than the abundance of planktonic *V. cholerae* in chambers with *C. roenbergensis* (Fig. 4.4B). In this series of experiments, the numbers of planktonic *V. cholerae* were higher in the chambers with *C. roenbergensis* than in those with *R. nasuta*, which is opposite to what was previously observed at the end of autumn (Fig. 4.3B).

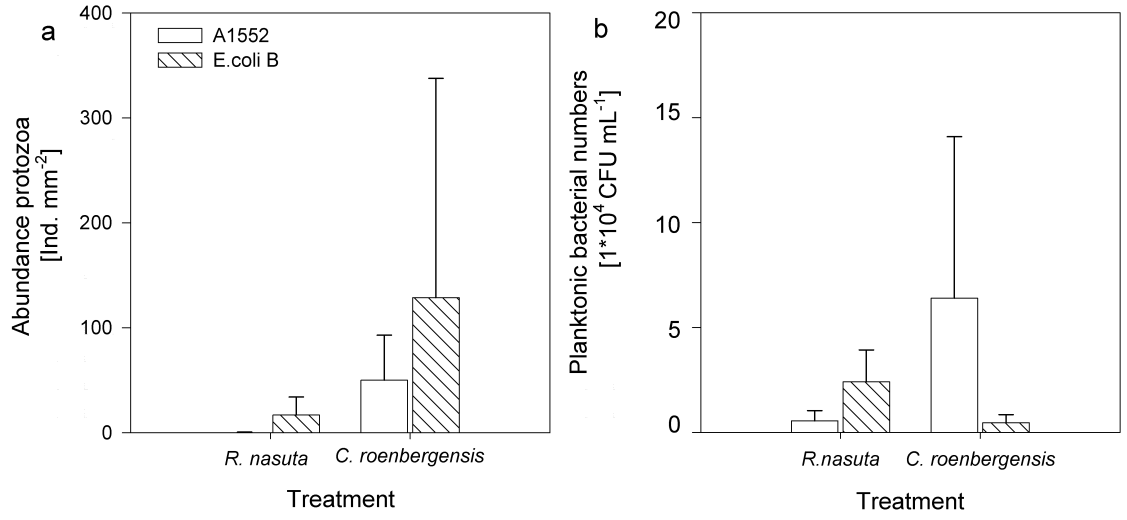

Figure 4.4. Abundance of (A) the flagellates *R. nasuta* and *C. roenbergensis* on *V. cholerae* A1552 (white bars) and *E. coli* B (striped bars) biofilms in environmental chambers after 10 days in the marine environment. *R. nasuta* appeared on the *V.cholerae* A1552 biofilms in very low abundances (0.32 $\pm$ 0.29 Ind mm^{-2}). 2-factorial ANOVA revealed highly significant differences between the grazers *** $p < 0.001$. (B) Abundance (CFU mL^{-1}) of suspended *V. cholerae* A1552 (white bar) and *E. coli* B (black bar) in environmental chambers after 10 days in the marine environment. Shown are mean values (± SD, n=3).

Abundance of R. nasuta and C. roenbergensis on V. cholerae biofilms under semi-field conditions

The isogenic *V. cholerae* A1552 *hapR* mutant strain is a GMO and cannot be exposed to the natural marine environment, thus, in order to compare all three *Vibrio* strains, we designed grazing assays in the laboratory under conditions that simulated the field experiments as closely as possible. The abundance of *R. nasuta* and *C. roenbergensis* on the three *V. cholerae* biofilms was monitored for 5 days in the flow cells (Fig. 4.5). While *R. nasuta* appears on the biofilm in the flow cells in low numbers from day 2 onwards and slowly increases thereafter (Fig. 4.5A), *C. roenbergensis* is abundant in high numbers from day 1 onwards (Fig. 4.5B). The difference in abundance of *R. nasuta* on the three different *V. cholerae* strains was significant (H-test p = 0.02). *R. nasuta* was, expectedly, most abundant on the *V. cholerae* A1552 *hapR* mutant biofilms as seen previously in laboratory experiments (Matz*, et al.*, 2005) and was not detected in high numbers on either the *V. cholerae* N16961 or the A1552 biofilms after day 2 (Fig. 4.5A). For *C. roenbergensis,* there were differences in the abundance of the grazer on the *Vibrio* strains for the first 3 days, but abundances thereafter were similar on all strains (Fig. 4.5B).

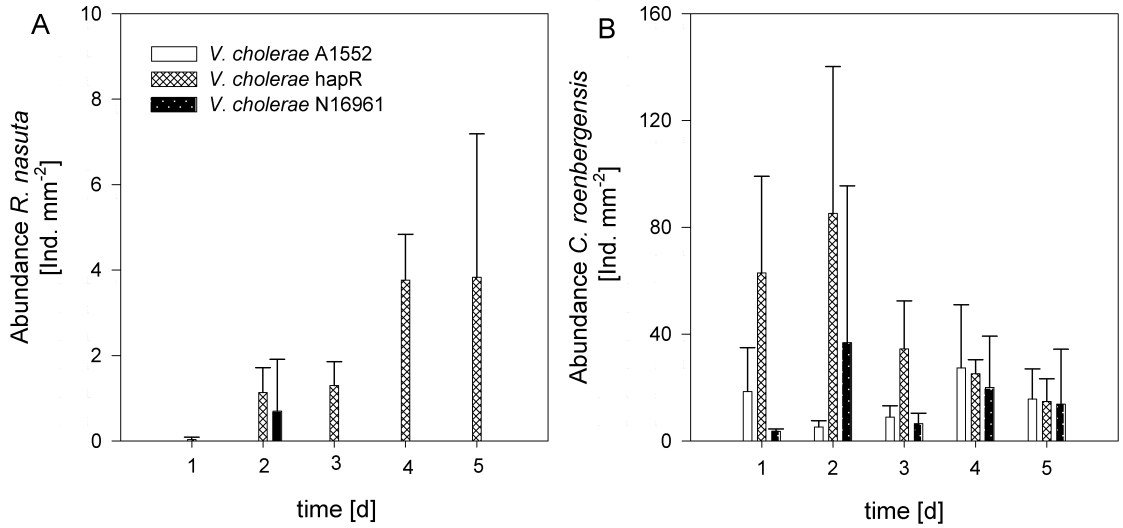

Figure 4.5. Abundance of the flagellates *R. nasuta* (A) and *C. roenbergensis* (B) *on V. cholerae* A1552 (white bar), the A1552 *hapR* mutant strain (checked bar) and N16961 (black bar) biofilms in the flow cells of the semi-field set-up over 5 days. Shown are mean values (± SD, n=4). One-factorial ANOVA revealed no significant differences for *C. roenbergensis* on the different *V. cholerae* strains on day 5. H-test (Kruskal-Wallis-Test) showed significant differences for *R. nasuta* on day 5 (p = 0.02). A U-test (Mann-Whitney) revealed significant differences between the strains A1552 and *hapR* (p < 0.04) and N16961 and A1552 *hapR* (p < 0.04) and no significant difference between the A1552 wild type and N16961 strains (p = 1).

Effect of glucose concentration on persistence of V. cholerae biofilms under grazing by R. nasuta

Growth of *R. nasuta* on *V. cholerae* biofilms supplemented with high and low carbon concentrations revealed that biofilms grown under low carbon concentrations (0.001 %) supported a lower abundance of grazers than those grown at a higher glucose concentration (0.1 %) regardless of strain (Fig. 4.6). Growth of *R. nasuta* on the A1552 biofilms was 2.8 times higher if the biofilms were grown on the higher glucose concentration while for the *hapR* mutant strain, the increase was 1.5 times more and for N16961, 1.6 times higher. A two factorial ANOVA revealed significant influences of the strain ($p = 0.003$) and the glucose concentration ($p = 0.002$) on the growth of *R. nasuta*. A posthoc test revealed significant differences between the growth of *R. nasuta* on A1552 compared to growth on the isogenic *hapR* mutant biofilm ($p = 0.004$) and growth on N16961 compared to A1552 *hapR* biofilms ($p = 0.014$). No significant differences in growth on A1552 and N16961 were found. Growth of the flagellates on medium with either high or low glucose supplied with heat-killed bacteria was not significantly different (data not shown). The biofilm biomass was not significantly different on the two glucose concentrations (data not shown).

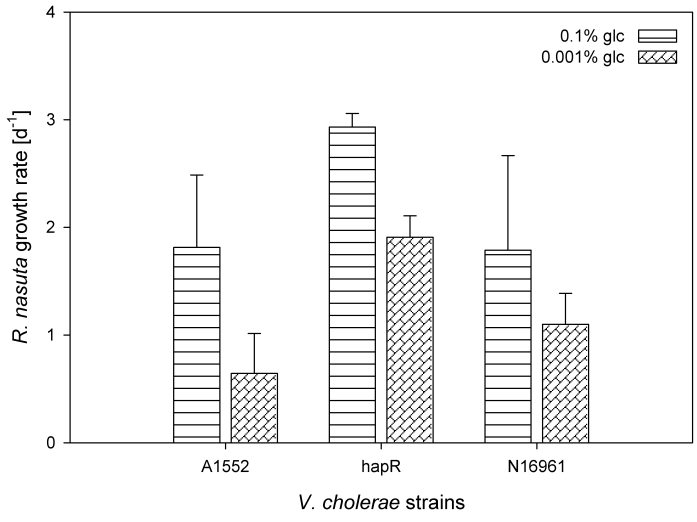

Figure 4.6. Growth rates of *R. nasuta* on *V. cholerae* A1552 wild type and *hapR* mutant, and N16961 biofilms grown in seawater with two different glucose concentrations for 3 days. Sterile filtered seawater was supplemented with 0.1% glucose (striped bar) or 0.001% glucose (bricked bar) as a carbon source, respectively. Shown are mean values (± SD, n=3). A 2-factorial ANOVA showed significant differences in strain ($p = 0.03$) and glucose concentrations ($p = 0.02$). A Tukey-HSD posthoc-test revealed significant differences between A1552 wild type and *hapR* mutant ($p = 0.004$) and N16961 and A1552 *hapR* biofilms ($p = 0.014$).

Discussion

Most studies investigating predator-prey interactions in biofilms have been performed in laboratory settings under strictly controlled conditions. Our previous results indicated that *V. cholerae* A1552 wild type biofilms grown in 24 well microtiter plates were toxic to the benthic grazer, *R. nasuta* and resulted in flagellate death, while feeding on the *hapR* QS mutant biofilm resulted in positive growth (Matz, *et al.*, 2005), indicating that a factor regulated by HapR is responsible for protozoan killing. The current study was designed to investigate whether *V. cholerae* biofilms grown *in situ* where there is a large dilution effect due to surrounding seawater could inhibit protozoan growth, i.e. whether the toxicity seen in the laboratory was an artefact due to concentration effects of the microtiter experiments. In these field experiments, we used the biofilm feeder *R. nasuta* as well as *C. roenbergensis,* which is primarily a suspension feeder but also attaches to the biofilm. Results presented here reveal that *V. cholerae* A1552 wild type biofilms prevent predation associated loss of biofilm biomass in the marine environment where there is a large dilution effect due to surrounding seawater and thus support the concept that the antipredator activity observed in the microtiter experiments is ecologically relevant.

Protozoan abundance varied on biofilms of different V. cholerae strains in the field as well as under semi-field conditions

The abundances of *R. nasuta* and *C. roenbergensis* varied on different *V. cholerae* and non-*Vibrio* strains and at different times of the year. While the abundance of *R. nasuta* was significantly lower on *V. cholerae* N16961 than on the wild type strain A1552 in summer (Fig. 4.1A, $p < 0.01$)), the difference in abundance on the two strains in autumn was not significant (Fig. 4.3A). The predominately planktonic grazer, *C. roenbergensis,* appeared on the N16961 biofilms in higher numbers in midsummer and late autumn (Fig. 4.2A and 4.3A). In general, the numbers of *C. roenbergensis* on biofilms of all strains was higher than the numbers of *R. nasuta*, indicating that the surface grazing flagellate *R. nasuta* might be more negatively affected by *V. cholerae* than the suspension feeding *C. roenbergensis*. This may be due

in part to the fact that *C. roenbergensis* can escape the biofilm and feed on planktonic bacteria but may also indicate that the biofilms affects the grazers differently.

The fact that *V. cholerae* N16961 supported lower abundances of the obligatory benthic-feeding *R. nasuta* than A1552 was surprising as N16961 is a QS mutant and thus, it was expected that the QS-regulated antiprotozoal activity (seen in A1552 in previous laboratory studies) would not be expressed. This suggests that there may be a QS-independent pathway for expression of traits that lead to biofilm persistence, however other differences between the strains or nutritional quality may also account for this difference in grazer abundance, as these strains are not isogenic. It has been previously shown that *P. aeruginosa* expresses QS-regulated lethal factors which play a key role in grazing protection of late biofilms, while QS-independent upregulation of the type III secretion system is important as an immediate response to predation (Matz *et al.*, 2008).

Due to the unexpected result of higher numbers of *R. nasuta* on the wild type *V. cholerae* strain A1552 than on the QS mutant strain, we compared growth of the grazer on a non-*Vibrio* strain, *E. coli* B, in the field. Both *R. nasuta* and *C. roenbergensis* occurred on the *E. coli* biofilms in higher abundances than on the *V. cholerae* A1552 strain (Fig. 4.4A) but the differences in abundance were not significant.

Under semi-natural conditions, the same trend for low grazer abundances on the *V. cholerae* A1552 and N16961 strains when compared to the A1552 *hapR* mutant strain was observed (Fig. 4.5). While abundances on the A1552 *hapR* biofilm were higher for both *R. nasuta* and *C. roenbergensis*, the number of grazers on biofilms of the N16961 strain was similar to the A1552 strain. Thus the results obtained in the field and under semi-field conditions were similar. The N16961 strain has a frameshift mutation in the *hapR* gene and thus was considered to be more susceptible to losses to predation than the wild type A1552. Matz *et al.* (2005) showed that the QS dependent *hapR* gene controls antiprotozoal factor(s) that when secreted prevented flagellate grazing. The field experiments reported here indicate that that this activity might be ecologically relevant and that there may be *hapR* independent defensive mechanisms expressed *in situ.* Previous results have shown that *P. aeruginosa* uses both QS-dependent and QS-independent mechanisms for predation resistance and that these mechanisms operate under different

physiological and environmental conditions (Matz*, et al.*, 2008), therefore, the same types of responses may be expressed by *V. cholerae* during grazing.

Grazing resistance increases as carbon levels decrease

The differences in protozoan growth on the biofilms in mid summer and autumn may be due to differences in DOC levels in the seawater. Thus, we investigated the influence of prey grown on different nutrient concentrations on grazer numbers. When the glucose concentration was higher, the flagellate growth rates were at least 1.5 times higher for all three *V. cholerae* strains, while in controls (flagellates with heat killed bacteria) there was no effect of glucose concentration on flagellate growth rate. The greatest difference in growth rates between the high and low glucose biofilms was for the A1552 strain. Interestingly, there was a difference in numbers of grazers on the *hapR* mutant biofilms grown under different nutrient concentrations indicating that under low nutrient conditions, QS-independent grazing resistance occurs.

It is known from planktonic studies that in nutrient rich environments with a high abundance of metazooplankton, the phagotrophic protists experience a high grazing pressure (e.g. Jürgens, 1994, Corno*, et al.*, 2008) allowing the bacterial community to increase in numbers. This is in part due to sloppy feeding and from the excretion of the recycled zooplankton nutrients, which then become available for the bacterial community (e.g. Lampert, 1978, Pernthaler*, et al.*, 1997, Corno & Jürgens, 2006). Thus, when nutrient levels are high in the environment, grazing pressure on the bacterioplankton decreases while available nutrient increases resulting in rapid growth of the bacterioplankton (Cole*, et al.*, 1988, Simek*, et al.*, 2003).

In contrast, in nutrient poor environments, the impact of grazers on the bacterial community is greater, resulting in significant changes in the composition of the bacterial community (Jürgens*, et al.*, 1999, Hahn & Höfle, 2001). The greater effectiveness of prevention of grazing losses of *V. cholerae* grown under low nutrient concentrations suggests an adaptation to higher grazing pressure in natural food webs with less available nutrients. In fact, top-down control (of the grazers) is generally more pronounced in resource-limited areas (Simek*, et al.*, 2003). This pattern of defense metabolite production fits with the resource availability hypothesis which states that in environments with low resource availability, plants

with low growth rates and high levels of defense will be favored, while in environments with high resource availability, plants with fast growth and lower defense levels will be favored (Coley*, et al.*, 1985). Thus, when nutrients are available, *V. cholerae* is able to grow more quickly than it is eliminated by predation, but when nutrients are limited, resources may be shifted from growth to defense metabolites.

The results presented here show that *V. cholerae* biofilms are protected from grazing losses *in situ* where there are large dilution effects due to the surrounding seawater environment. While the biofilm biomass remained stable under grazing pressure, the planktonic biomass increased for both strains when the benthic feeder, *R. nasuta* was present on the biofilm. This may be due to cells leaving the biofilm for the planktonic phase to 'escape' predation and to increased nutrient availability due to nutrient recycling by predator feeding. The lower abundances of flagellates on N16961 biofilms was unexpected as this strain has a frameshift mutation in *hapR*, which has been shown to be required for the antiprotozoal activity of the A1552 strain expressed in the laboratory (Matz, *et al.* 2005). These data indicated that there is potentially QS-independent antipredation activity exhibited by this strain, but does not rule out the possibility that there may also be other strain differences that account for the grazing protection. This work is the first to show that the protection against grazing losses expressed by *V. cholerae* is ecologically relevant and further highlights the advantages of surface-associated growth in environmentally relevant contexts. The data also clearly show that *V. cholerae* expresses traits which prevent grazing induced loss of biomass that are regulated by the cell-cell signaling pathway, *hapR*, as well as in response to environmental conditions (e.g. nutrients) and thus, such traits could play important roles in the persistence of *V. cholerae* in the environment within predator resistant biofilms.

Chapter 5

[2] Quantification of individual protozoa - bacteria interactions within semi-natural biofilm

[2] In der Durchführung der Experimente waren zwei von mir co-betreute Examensstudentinnen, N. Farrenschon und S. Speckmann, involviert. Die hier vorliegenden Auswertungen und Darstellungen wurden von mir erarbeitet.

Abstract

Protozoan grazing is considered the major control of bacteria abundances in natural environments. However, most data addressing these microbial interactions came from studies within planktonic communities, whereas appropriate methods to quantify food web interactions within biofilms are lacking. Here we present a new approach to quantify food-web interactions within semi-natural biofilms by combining the establishment of biofilms from natural rivers in flow cells with video microscopy to quantify feeding behaviour. We focused on the typical vagile surface associated heterotrophic flagellates (HF) *Neobodo designis, Rhynchomonas nasuta* and *Planomonas* sp.. We could show that the three benthic HF generally ingested single biofilm associated bacteria whereas bacteria within microcolonies were contacted but not ingested. A size preference for larger cells could be verified for all three species. However, grazing strategies differed considerably, especially between the two kinetoplastids *N. designis* and *R. nasuta*, and *Planomonas* sp.. While the kinetoplastids had long search and short handling times, *Planomonas* sp. showed the opposite grazing characteristics. Our results provide direct evidence that microcolony formation in biofilms protects bacteria from grazing by heterotrophic flagellates *in situ*. Apparently similar HF species showed different feeding strategies and by this probable niche separations (i.e. grazing on different physiological stages of bacteria). Our new approach to combine video microscopy with experimental flow cell systems provides direct insights into the complex food web interactions within biofilms.

Introduction

Since the description of the microbial loop (Azam *et al.* 1983), it is widely accepted that protozoa have a major influence on the bacterial community in aquatic systems (e.g. Hahn and Höfle 2001, Jürgens and Matz 2002, Sherr and Sherr 2002). They can control the bacterial density, influence the taxonomic composition and alter the morphology of the bacterial community in the plankton (Hahn and Höfle 2001). The interplay of bacteria and protozoa is considered to be the oldest predator prey interaction in nature. Most likely this gave rise to the co-evolution of different defence mechanisms in bacteria and feeding strategies in protozoa, which are mirrored in these complex interactions. Motility, toxin production and size shifts (e.g. filament formation) in the presence of grazers (esp. heterotrophic flagellates (HF)), might be evolved as grazing protection (Matz and Kjelleberg 2005). However, grazing protection is not absolute, as different protozoa possess different feeding types that allow certain species to graze on bacteria that other grazers are not capable of ingesting (Weitere *et al.* 2005).

While in planktonic systems bacteria-protozoa food webs interactions are well studied, only little knowledge exists about surface associated communities, so called biofilms, even though surface-attachment is considered the dominant live-form of microbes (Costerton *et al.* 1987). Furthermore, densities of bacteria and protozoa on surfaces can reach up to 4 orders of magnitude compared to suspended communities (Artolozaga *et al.* 1997). Grazing rates of predominantly attached living heterotrophic flagellates as well as ciliates have been studied in the laboratory mostly on fluorescently labelled beads or fluorescently labelled bacteria. Mean grazing rates ranging from 4.6 bacteria flagellate^{-1} h^{-1} (Artolozaga *et al.* 2002) up to 73 bacteria flagellate^{-1} h^{-1} (Starink *et al.* 1994) were measured, depending on the experimental set-up, the protozoan species and the prey characters used. (e.g. Caron 1987, Böhme *et al.* 2009). However, such data needs to be treated with care, as the labelling of the bacteria heavily alters the three-dimensional biofilm structure as well as the structure of the extracellular matrix, which is a major reason for grazing resistance (Matz and Kjelleberg 2005).

From laboratory studies it is known that microcolony formation can be an efficient grazing protection depending on the stage of biofilm development and the feeding mode of the grazer (Matz *et al.* 2004, Weitere *et al.* 2005). While biofilms of an alginate-overproducing, non-virulent strain of *Pseudomonas aeruginosa* (PDO300) were protected against grazing by the heterotrophic flagellates (early biofilm coloniser), the amoebae *Acanthamoeba sp.* (late biofilm coloniser) was able to decrease biofilm biomass compared to non-grazed control biofilms (Weitere *et al.* 2005). Nevertheless, HF can profoundly alter the early biofilm formation by (1) reducing attached bacteria (single cells) and (2) stimulating the formation of microcolonies (Matz *et al.* 2004, Wey *et al.* 2008). However, up to now, no data on the ingestion rates in non-disturbed biofilms exists. Furthermore, it is not clear whether or not microcolony formation provides full grazing resistances against HFs and at which level the grazers select against microcolonies. These open questions challenge the development of new tools to quantify grazing interactions within biofilms.

Here, we combine two techniques, i.e. videomicroscopy (Boenigk and Arndt 2000b) and the establishment of field-related biofilms in flow cells running as river bypass (Norf *et al.* 2007), to quantify bacteria-HF grazing interactions within semi-natural biofilms. By using this new tool, we quantified for the first time the grazing behaviour of three common surface-feeding HFs.

Material and Methods

Study site and facilities

Experiments were run at the Ecological Rhine Station of the University of Cologne, a ship that anchors permanently in the main flow of the river (50°54'25''N, 6°58'43''E; Rhine-km 684.5). The station is equipped with laboratories where fresh river water is continuously pumped from the river on board the ship and used for bypass experiments. Before reaching the flow cells, the water is sieved though a gauze with a mesh size of 300µm and subsequently through a 20µm filter (Sartopure Capsules, PP2, Sartorius, Goettingen, Germany) (Wey *et al.* 2008). The biofims were established in flow cells, which allowed non-intrusive cultivation and observation of

natural biofilm communities (Norf *et al.* 2007). Protozoan taxonomic composition could be studied *in situ* within a two-week time course and the behaviour of individuals of typical biofilm grazer species could be followed and video recorded under microscopic observation.

Experimental set-up

To quantify individual protozoan grazer effects on bacterial biofilm communities we set up two experiments in November to December 2009 (table 5.1). The experimental flow cell system previously described by Wey et al. (2008) was used for both experiments. All tubes and flow cells were autoclaved before use. An impeller pump (Watson Marlow, model 205S) was used to pump water (flow velocity 2.8 mL min^{-1}) from a water basin on board the station into flow cells where a semi-natural biofilm community could develop. To avoid possible air-bubble interruption on biofilm development the flow cells were kept vertical in a small basin in which natural river water was guided to keep the flow cells at field temperature during the experiments. The flow cells (40x19x4.9mm) consisted of plexiglas frames that are sealed on either side by a microscope slide and a cover slip (see Norf *et al.* 2007). Bacteria and protozoa attached to the glass slide and cover slip and thus allowed microscopic observation without removing the flow cell from the system. The flow cells had a volume of 3.4 mL and an observation area of 7.6 cm^2. Microscopes were equipped with digital camcorders and screens were attached to the camcorders to allow viewing and recording of the biofilm community.

Table 5.1: Overview on the experiments I and II. Water temperature (°C) is the mean ± SD from the cooling basin in which the flow cells were held, which corresponded the natural water temperature of the Rhine. The ranges for temperatures and water levels are given in brackets.

	Exp I	Exp II
Date	02/11 – 11/11/2009	30/12 – 11/12/2009
Water temperature (°C)	11.4 + 0.4 (10.7-11.8)	9.3 + 0.4 (8.8 – 10.0)
Water level at Cologne (cm)	202 + 34 (132-236)	331 + 46 (298-462)
Species studied	*Planomonas* sp. *Neobodo designis* *Rhynchomonas nasuta*	*Planomonas* sp. *Neobodo designis* *Rhynchomonas nasuta*

Quantification of the protozoan and bacterial communities

The protozoan community was analysed on a daily basis with the help of light microscopy (Axiostar, Zeiss Jena, Germany). Starting on day one of the experiments 60 flagellates and ciliates were mapped on a defined and randomly distributed area in every flow cell to measure protozoan abundance. The taxonomic classification followed the system of Adl et al. (2005) and was conducted with the help of specific keys (Patterson and Hedley 1992). For the quantification of the bacterial community 10 to 15 randomly distributed spots of 50x50µm for young, sparsely populated biofilms and 25x25µm for mature, densely populated biofilms, respectively, were analysed. Bacterial cells were measured and quantified by considering single cells, microcolonies or filaments. A colony was defined as bacterial assemblages with more than three cells in direct proximity, a filament was defined as at least two cells in a row.

Organisms and video microscopic analysis

We concentrated on three dominant raptorial feeding flagellate species in our investigation: the heterotrophic flagellates (i) *Neobodo designis,* (ii) *Rhynchomonas nasuta* (both Kinetoplastida) and (iii) *Planomonas* sp*.* (Planomonadida). These three species are well-known bacterial biofilm grazers and belong to the so-called ”weed-species”. All species can reach high abundances in the river Rhine (Weitere and Arndt 2003).

All microscopes were equipped with digital camcorders (Sony HDR-SR7, HDR-SR8 and HDR-XR 200) and videos were taken in HD quality. Every day from day 1 until day 9 (Experiment I) and day 12 (Experiment II), respectively, after the start of the experiments individuals of all species were followed for between 5 and 180 minutes. The videos were analysed regarding different parameters. Searchtime, handlingtime, ingestion time, success rate, prey size, average size of the bacteria of the biofilm community, flagellate size and food preferences were analysed. The definitions of the analysed characteristics are described in table 5.2. Organism sizes were measured on a calibrated screen.

Table 5.2. Definitions of the analysed feeding characteristics.

Handling time	time spend with prey from first contact until (i) ingestion or (ii) predator leaves the prey
Search time	time spend searching for prey, i.e. time without prey contact.
Ingestion rate	number of ingested prey items per unit time
Success rate	percentage successful ingestion in relation to total prey contact
Prey size	length prey cell µm

Statistical analysis

Statistical analysis was performed using Sigma Plot for Windows Version 11.0. For comparison of feeding characteristics a one-factorial ANOVA was applied with a Tukey test for pairwise multiple comparison. Correlation between feeding characteristics and biofilm density was analysed according to Spearman. For the prey selection a non-parametric Kruskal-Wallis H-test for multiple comparison was performed. In case of significant effects groups were compared pairwise using the Mann-Whitney rank test.

Results

Feeding strategies on single bacteria

The videos revealed differences in contact rate, handling time, success rate and ingestion rate between the two kinetoplastids *R. nasuta* and *N. designis,* and *Planomonas* sp. (Fig. 5.1). *N. designis* and *R. nasuta* contacted 2.6$\pm$ 1 and 1.6$\pm$ 1 bacterial cells flagellate^{-1} min^{-1}, respectively. Those differences were already significant ($p<0.01$). Nevertheless, *Planomonas* sp. contacted only 0.6$\pm$ 0.3 bacterial cells flagellate^{-1} min^{-1} (Fig. 5.1A) and this rate differed significantly from that of *N. designis* ($p<0.001$) as well as *R. nasuta* ($p<0.01$). Correspondingly, the handling time (i.e. the time a individual spend on one bacterial cell) was by far the highest in *Planomonas* sp. (26$\pm$ 16 seconds prey^{-1}, Fig. 5.1B). This flagellate handled the prey more than 10 times longer than the kinetoplastids (2.1 $\pm$ 0.7 seconds prey^{-1} for *N. designis* and 1.9$\pm$ 1.2 seconds prey^{-1} for *R. nasuta*). The differences between *Planomonas* and either of the two kinetoplastids were highly significant ($p<0.001$), whereas no significant differences were found between the handling times of the two kinetoplastids ($p>0.05$). The relatively long handling time of *Planomonas* sp.

was accompanied by success: These flagellates were able to ingest 80± 26% of the attacked prey, whereas *R. nasuta* as well as *N. designis* were only able to ingest 21± 15% and 20± 9% of the attacked prey, respectively (Fig. 5.1C). Again, the differences between *Planomonas* sp. and either of the two kinetoplastids was highly significant ($p<0.001$), whereas no significant differences were found between the success rates of the two kinetoplastids ($p>0.05$). Taking the ingestion rate as overall success rate (i.e. the number of prey items ingested per unit time), both *Planomonas* sp. (0.5± 0.3 ingestions flagellate^{-1} min^{-1}) and *N. designis* (0.6± 0.3 ingestions flagellate^{-1} min^{-1}) showed no significant differences, even though the strategies of the two species contrasted. *R. nasuta*, which had a similar feeding strategy than *N. designis* (short handling time, long search time), showed lowest ingestion rates (0.3± 0.1 ingestions flagellate^{-1} min^{-1}). This ingestion rate differed significantly from the ingestion rate of both *Planomonas* sp. ($p<0.05$) and *N. designis* ($p<0.001$; Fig. 5.1D)

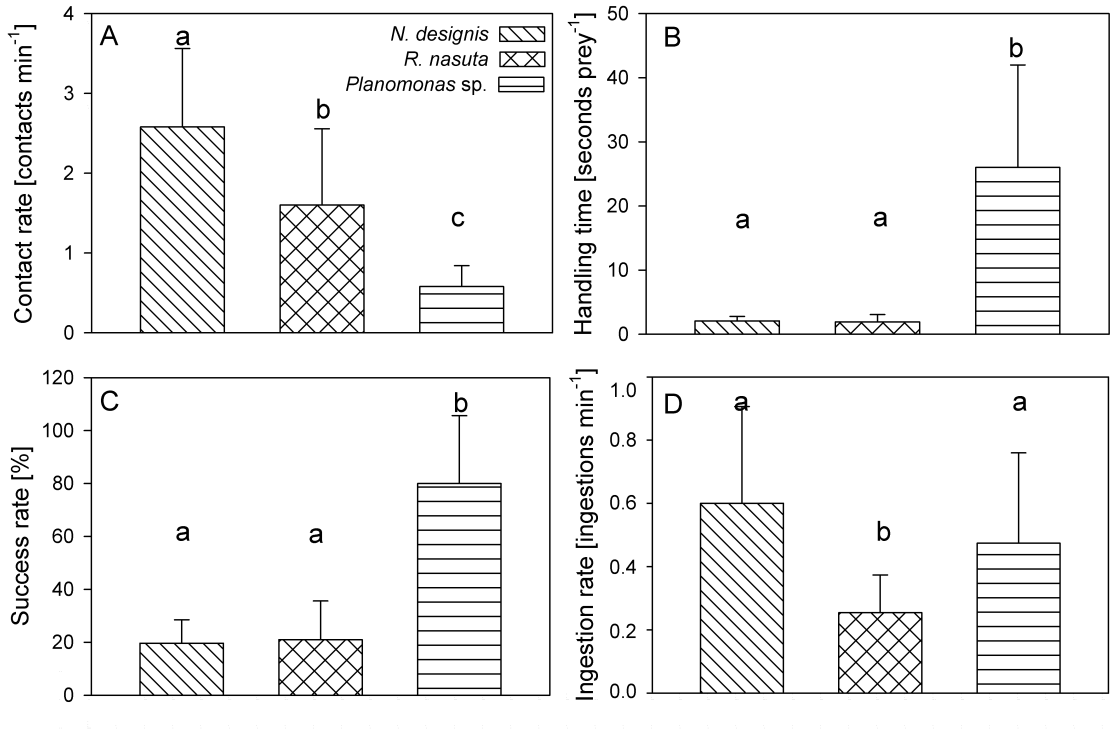

Figure 5.1. Feeding characteristics of three bacterivorous HFs *N. designis*, *R. nasuta* and *Planomonas* sp.. A) Contact rate, B) handling time, C) success rate (as proportion of ingested prey items to total attacked prey) and D) ingestion rate. Shown are mean values ± SD. One-factorial ANOVA showed significant effects of the grazer species for all parameters ($p<0.001$ in all cases). Different letters indicate significantly different groups as indicated by Tukey-HSD-test.

Functional response

A higher bacterial abundance on the biofilms was accompanied by a higher contact rate for all three species (Fig. 5.2A-C). While *R. nasuta* showed a significant increase of prey contacts with increasing bacteria density (r= 0.573, p<0.05), *Planomonas* sp. (r= 0.444) as well as *N. designis* (r= 0.285) showed positive but non-significant correlations. Although a significant increase of contacts on more densely packed biofilm would suggest more, the ingestion rate of *R. nasuta* did increase only slightly (r=0.165, Fig. 5.2E), and for *N. designis* the ingestion rate was independent of the bacterial biofilm density (r= -0.002, Fi. 5.2D).

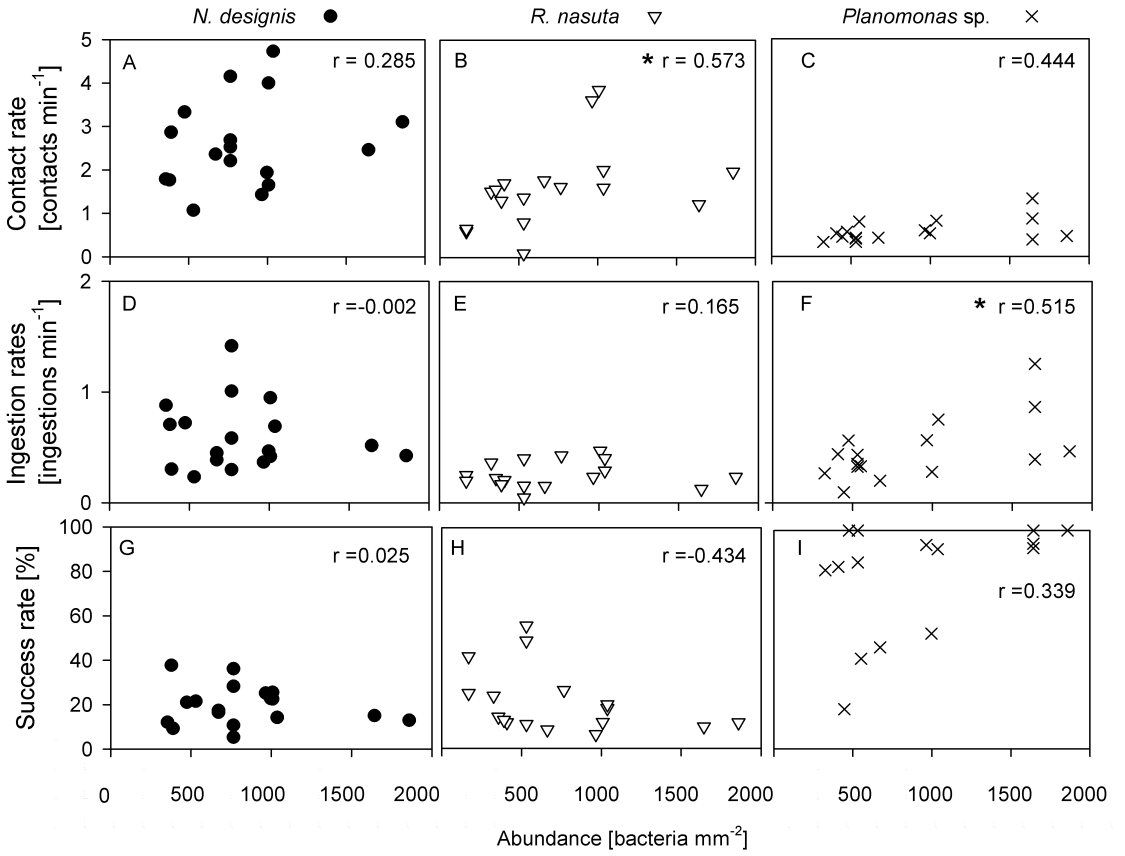

Figure 5.2. Functional response for the three HF species. Every data point represents one individual of *N. designis* (A, D, G), *R. nasuta* (B, E, H) and *Planomonas* sp. (C, F, I), respectively. (A, B, C) Contact rate, (D, E, F) ingestion rate and (G, H, I) success rate. Spearman correlation coefficient R is indicated. * p<0.05.

In contrast, for *Planomonas* sp. the higher contact rate was accompanied with a significant increase of ingestions (r= 0.515, p< 0.05, Fig 5.2F). This trend was mirrored in the success rate. While *N. designis* showed near independence of success and biofilm density (r=0.025, Fig 5.2G), *R. nasuta* showed a strong decrease in success rate on more densely packed biofilms (r= -0.434, Fig. 5.2H). Contrary to that, *Planomonas* sp. showed an increasing success rate with high success already on less packed biofilms up to very abundant biofilms (r= 0.339, Fig. 5.2I).

The differences in ingestion and success rate of the three species are reflected in the correlations of success rate with handling time and search time, respectively (Fig. 5.3). While the success of ingesting prey cells in correlation to the time handling a prey cell was nearly independent for *N. designis* (r= 0.048, Fig. 5.3A) and *R. nasuta* (r= 0.066, Fig. 5.3B), *Planomonas* sp. was increasingly successful the longer individuals of this species handled a prey cell (r= 0.636, p<0.01, Fig. 5.3C) showing a significant positive interrelation.

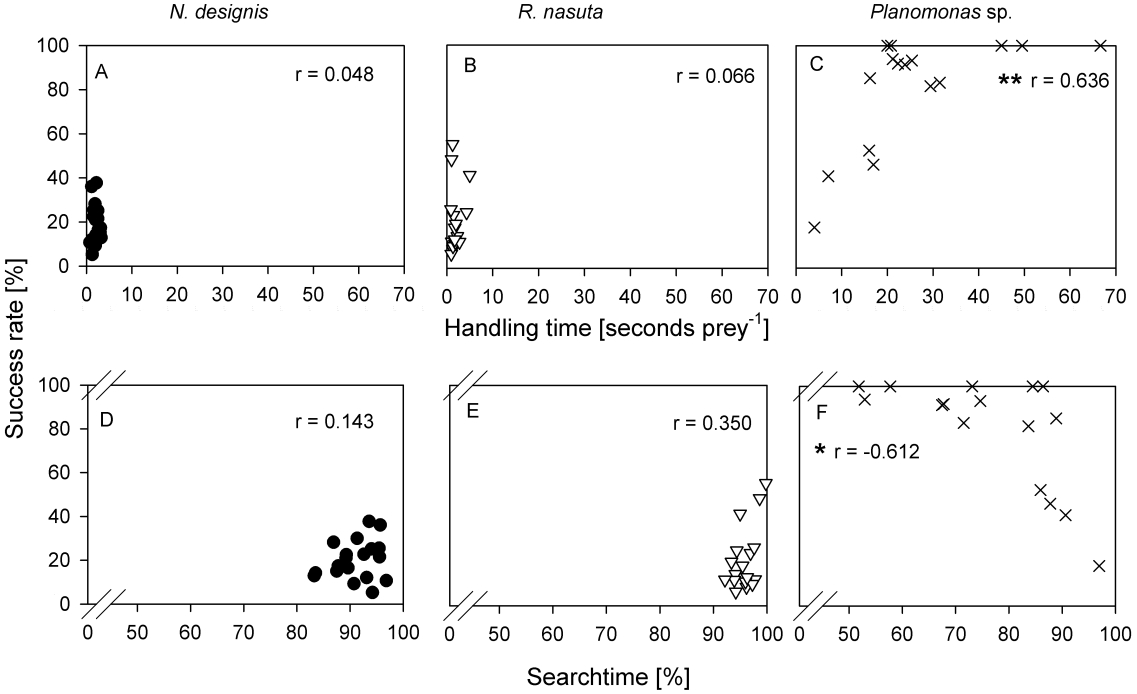

Figure 5.3. The success rate dependent on (A, B, C) handling and (D, E, F) search time, respectively, for *N. designis* (A, D), *R. nasuta* (B, E) and *Planomonas* sp. (C, F). Note the different scales of the x-axis in A, B and C. Every data point represents one individual of *N. designis*, *R. nasuta* and *Planomonas* sp.. Spearman correlation coefficient R is indicated. * p<0.05, ** p<0.01.

Correspondingly, *Planomonas* sp. success decreased significantly the longer individuals spent searching for prey cells (r= -0.612, p<0.05, Fig. 5.3D). Also accordingly, *N. designis* and *R. nasuta* showed the opposite trend. The success rate increased for *N. designis* (r= 0.143, Fig. 5.3E) as well as *R. nasuta* (r= 0.350, Fig. 5.3F) the longer individuals of these species searched for prey cells.

Bacterial colonies, filaments and prey preferences

Differences in prey morphology (regarding single celled prey, microcolonies or filament formation) had an influence on the grazing success of all three species (Fig. 5.4). Single bacterial cells contributed to more than 90% to the bacterial biofilm community. While more than 85% of the contacted bacteria prey cells were single cells, microcolonies and filaments were also contacted by all three species. However, individuals of the three species were only able to ingest single bacteria cells.

The size distribution of the cells in the biofilm community, of the contacted as well as ingested prey cells for the three HF species showed a clear trend (Figure 5.5). Although the size range of the bacterial cells of the biofilm community was expansive, the median was similar for the bacterial communities around the different grazers (bacterial cell size median: community around *N. designis* (Fig. 5.5A): 0.26 μm^3, n= 3199; community around *R. nasuta* (Fig 5.5B): 0.33 μm^3 n= 3745; and community around *Planomonas* sp. (Fig. 5.5C): 0.26 μm^3, n= 3310).

While individuals of all three species contacted bacteria of the same size (median 0.44μm^3, *N. designis*. n= 779; *R.nasuta* n= 702; and *Planomonas*.sp. n= 248), only *Planomonas* sp. and *R. nasuta* also ingested bacteria with the same volume of 0.44μm^3 (*Planomonas* .sp. n= 191, *R.nasuta.* n=120; Fig 5.5B and C). *N. designis* ingested bacteria slightly smaller (median 0.3μm^3, n= 182, Fig. 5.5A). However, no significant difference between the ingested and contacted prey sizes could be detected for any species. Nevertheless, individuals of all three species contacted and ingested prey cells that were significantly larger than the bacterial community of the biofilm (p<0.001).

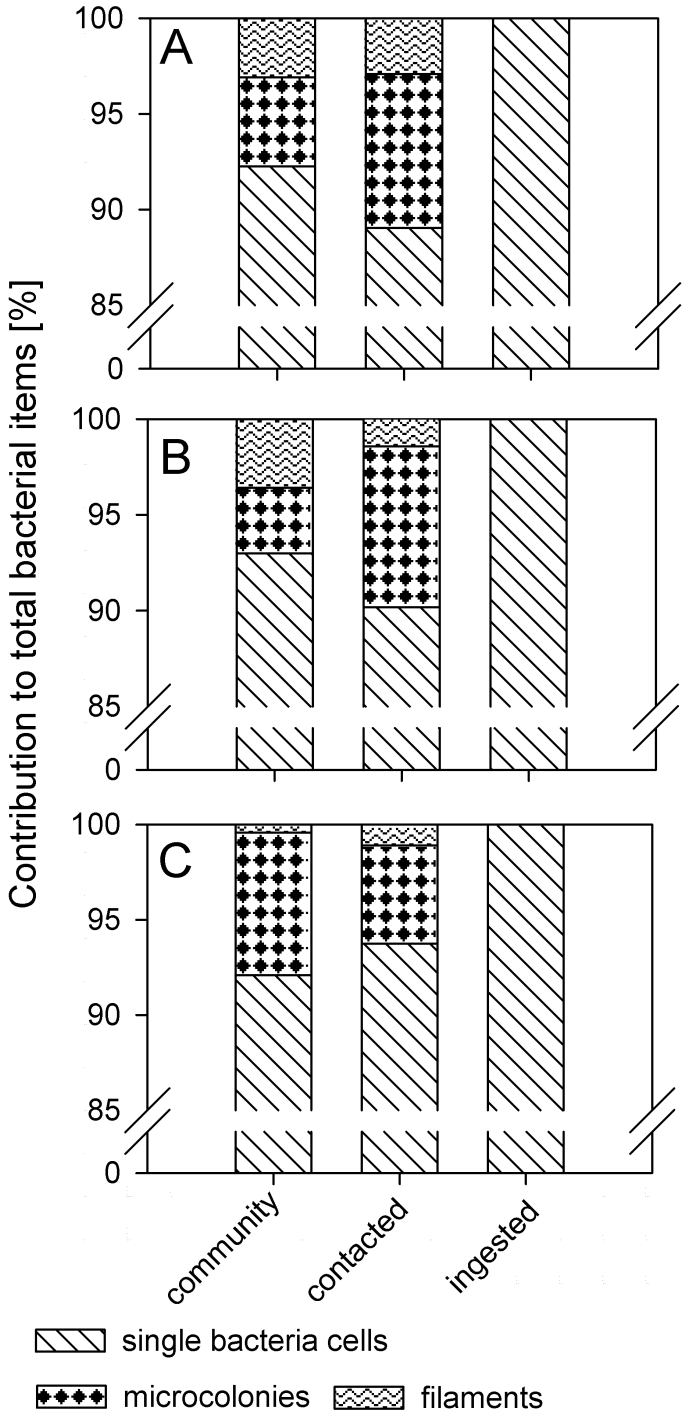

Figure 5.4. Selection for bacterial morphotypes on the level of contact and ingestion for A) *N. designis*, B) *R. nasuta* and C) *Planomonas* sp.. The bars respresent the contribution of bacterial single cells, microcononies and filaments to the total number of bacterial items. Note the y-axis break at 85%.

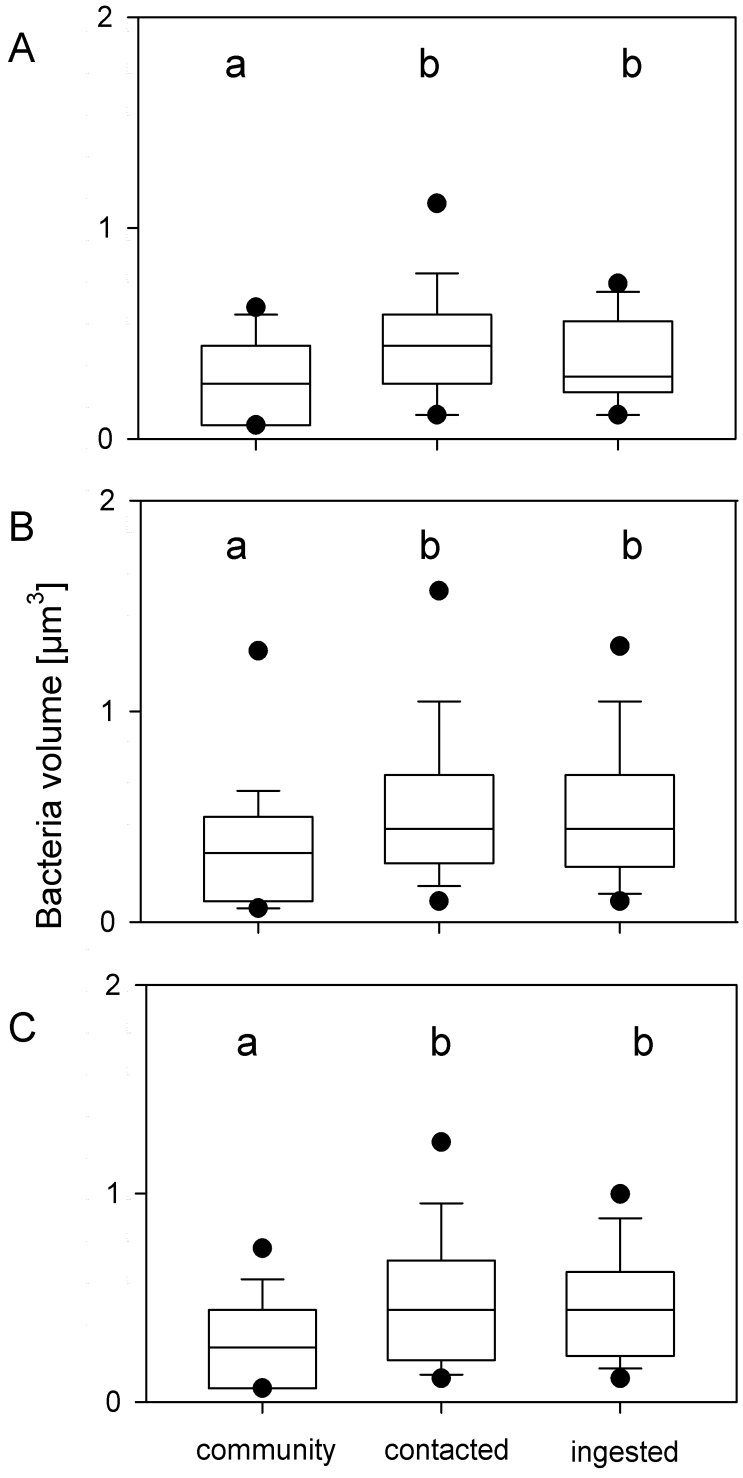

Figure 5.5. Size selectivity on the level of contact and ingestion for A) *N. designis*, B) *R. nasuta* and C) *Planomonas* sp.. Box-whisker plots represent median and quartiles as well as 5th and 95th percentile, without outliners. The non-paraletric Kruskal-Wallis test showed significant species effecets ($p < 0.001$ for all three species). Different letters indicate significant differences in the Mann-Whitney test for pariwise comparisons.

Discussion

The coupling of the river bypass system with video microscopy has proven to be a well working tool for observation and behavioural studies of food web interactions of flagellates and bacteria within semi-natural biofilms. A high number of individuals of typical phagotrophic biofilm associated flagellates could be studied regarding different feeding parameters revealing inter- as well as intraspecific variability.

Different feeding strategies among surface-feeding flagellates

Although the three HF grazers *N. designis, R. nasuta* and *Planomonas* sp. seemingly occupy a similar niche on biofilms, (being raptorial pioneer colonisers and grazing on single, surface-associated bacteria cells), different strategies of food uptake could be verified. A short handling time and a high contact rate characterized the strategy of the two kinetoplastids, i.e. *N. designis* and *R. nasuta*. Although individuals of both species were only successful with every fifth contact of prey, *N. designis* still showed ingestion rates of 36$\pm$ 18 cells flagellate^{-1} h^{-1}. These rates were similar to the ingestion rates of *Planomonas* sp.. However, the latter species showed a completely different strategy. Here, handling time was longest but the success was very high (in most individuals 100%). In other words, *N. designis* spend a long time on searching and ingested only prey items, which were detachable almost immediately, whereas *Planomonas* sp. spent a longer time in detaching bacteria and, consequently, had less time for searching. These two different behaviours also implied the presence of and handling of different physiological stages of the bacteria, i.e. loosely attached bacteria as prey for *N. designis* and firmly attached bacteria as prey for *Planomonas* sp.. Such different stages probably correlate with different stages in the biofilm formation, i.e. loosely attachment in the early biofilm formation versus firmly attachment and more densely populated in the later stage of biofilm formation (Costerton *et al.* 1987).

Even though *R. nasuta* used a similar feeding strategy as *N. designis* and their success rates are equal, it showed the lowest ingestion rates (15$\pm$ 7 cells flagellate^{-1} h^{-1}). This is explained by the lower contact rates of *R. nasuta*. By assuming similar detection rates of the two species, the contact rate is a function of moving speed, i.e. faster

species have more chances to get in contact with prey items. *N. designis* is distinctly faster than *R. nasuta*. By analysing 20 randomly chosen individuals of each species in our videos moving speeds of 0.48± 0.14 mm min^{-1} for *N. designis* and 0.25± 0.05 mm min^{-1} for *R. nasuta* were measured. The approximately two times faster speed in *N. designis* correlates with the approximately two times higher ingestion rate. This conclusion is supported by the functional response curves. In *R. nasuta*, contact rates increased significantly with prey density, whereas the contact rates of the faster *N. designis* were independent from prey density.

Although grazing rates of planktonic HF on bacteria are numerous only few studies dealt with gliding HF grazing on attached bacteria under natural conditions. Simek et al (1997) studied grazing rates of kinetoplastids (36 bacteria $flagellate^{-1}$ h^{-1}) on fluorescently labelled bacteria, which are comparable to the grazing rates of *N. designis* measured in the present study. Boenigk and Arndt (2000a), observed grazing rates of *R. nasuta* of 13 cells $flagellate^{-1}$ h^{-1}, which is also in accordance with our results. The authors also noticed that *R. nasuta* only fed on cells if they were loosely attached.

HFs select for larger cells on the biofilm

Interestingly, individuals of all three species contacted and ingested significantly larger prey cells than were present in the community and thus showed a size selection towards larger bacteria. To our knowledge there are no studies on the preferred prey size class of *Planomonas* sp. or *N. designis*. However, data for *R. nasuta* is available from laboratory investigations. In former experiments *R. nasuta* contacted and ingested on average larger prey cells than the community average (contacted: 0.29± 0.25μm^3, ingested: 0.28± 0.23μm^3 and community: 0.13± 0.12μm^3 (Boenigk and Arndt 2000a)). From planktonic communities it is known that HFs preferably graze on larger cells (Gasol *et al.* 1995, del Giorgio *et al.* 1996). The authors stated that the larger cells reflect the active part of the bacterial assemblage. Although we did not test the bacterial community for active or inactive cells the size preference might be the reason for preference of active cells.

Microcolonies are being contacted by HFs but not ingested

From planktonic environments it is known that filament and microcolony formation protect bacteria from grazing by HFs (e.g. Jürgens *et al.* 1999, Posch *et al.* 1999, Hahn

and Höfle 2001). In laboratory (Weitere *et al.* 2005, Böhme *et al.* 2009) as well as field-related experiments (Wey *et al.* 2008) microcolony formation was shown to be an efficient grazing protection of bacterial biofilms depending on the feeding mode of the predator. In the present study HFs did contact microcolonies as well as filaments. However, they were not able to ingest bacteria from colonies. The protection of microcolony formation from grazing did work during the attempt of the feeding process and not while selecting the prey. This observation argues for a morphological defence rather than a chemical repellent, which would probably prevent contact by the flagellates.

Conclusions: River bypass system and video microscopy as tools for observation of microbial food web interactions on the individual level

The combination of the river bypass system (Norf *et al.* 2007) with video microscopy (Boenigk and Arndt 2000a) allows non-invasive observation of natural biofilm communities. Interactions of predator and prey can be observed and recorded, and enable the viewer to study the behaviour of predator and prey later on. The high number of different species and individuals of the species allow for sufficient observation time to study food preferences and straighten out inter- and intra-species variability. Differences in feeding behaviour and strategies can be detected. Due to the natural protozoan and bacterial community used in the set-up, artefacts from the use of one prey species are avoided. One established method for studying protozoan feeding behaviour is the fluorescently labelling of bacteria (e.g. Gonzalez *et al.* 1990, Sherr 1993). However, prey cell characteristics are being modified by labelling with fluorescent dyes and this might influence the feeding behaviour of the protozoans. Another possibility for experiments on feeding behaviour in biofilms might be stable isotope probing. Again, the addition of probes can influence the biofilm community towards different predator-prey behaviour. One major advantage of the new method is that prey characteristics are not altered. This is especially important in biofilms where modifications of the cell might influence attachment to surfaces.

Our new approach to couple the river bypass system with video microscopy gives new insights into microbial food web interactions on biofilms. It allows investigating

selective feeding of HF on the single cell level under natural conditions and gives direct evidence for differences in feeding behaviour of HF that have seemingly the same feeding characteristics. This technique allows modifications for further studies such as feeding preferences and its applications on trophic regulations and as such proves to be a powerful tool for environmental biofilm research.

General Discussion

While protozoa-bacteria interactions are acknowledged to be an important link in the planktonic food web, their role as shaping forces within biofilms needs to be recognized. This thesis presents investigations of the mutual influence of bacteria and protozoa with special focus on bacterial defence strategies such as toxicity and microcolony formation and the power of protozoans to alter biofilm morphology notwithstanding. The challenge to study aforementioned defence mechanisms under natural conditions was accomplished by developing two new methodological tools.

Effects of protozoans on bacterial biofilms – caged in the laboratory

Laboratory experiments help to understand control mechanisms of bacterial biofilms. Here, the capillary flow cell system was used to test whether lowering nutrient quality and quantity for the bacterial biofilm might have stronger impacts in presence of a grazer than under optimal nutrient supply. The grazer *T. pyriformis* altered the morphology of *Acinetobacter* sp. biofilms independent of the nutrient supply. However, with the less optimal carbon source or flow rate the three-dimensional structure of the biofilm changed more dramatically. Thus, microcolony formation did not prevent grazing losses and reformation of the three-dimensional structure of the biofilm in general. Although biofilm morphology was altered, the biovolume of the grazed biofilms stayed the same over the course of the experiments and even increased in the set-ups with optimal nutrient supply. This strongly indicates a mutual benefit of grazer and bacterial biofilm possibly in the form of nutrient recycling as is known for planktonic communities (e.g. Caron *et al.* 1988, Hahn and Höfle 1999, Posch *et al.* 1999).

Similar results could be seen in batch experiments with the same grazer species and a toxic and non-toxic bacterial strain of *Vibrio cholerae*. Here, toxicity of *V. cholerae* A1552 and an increase of microcolony abundance on the biofilms did protect the bacterial biofilm from grazing by the ciliate *T. pyriformis*. But instead of the *V. cholerae* A1552 toxin killing the grazer as seen before in experiments with *R. nasuta* (Matz *et al.* 2005), or biomass reduction by grazing of *T. pyriformis* on toxic *P. aeruginosa* biofilms (Weitere *et al.* 2005), the ciliate was able to maintain low

abundances on the biofilm. Biofilm bacteria as well as the grazer had a mutual benefit of this. The grazer fed on planktonic cells and directly and indirectly (e.g. possible nutrient recycling, chemical cues) led to an increase in bacterial biovolume. Similar to the aforementioned experiments with *Acinetobacter* sp. this speaks for a 'reverse grazer effect', i.e. increased bacterial growth due to possible nutrient recycling and import of planktonic nutrients into the biofilm food web (Kathol *et al.* in press). This was supported by the increase of live cells on the *V. cholerae* A1552 biofilm detected by the live/dead stain and an increase of microcolony abundance in grazed treatments. The physical separation of the grazer by filter inserts highlights the positive impact on the biofilm biovolume by the physical presence of the grazer. The higher biofilm biomass to planktonic bacterial cells ratio of treatments in which *T. pyriformis* was fed with *Vibrio* than a non-*Vibrio* strain strongly suggests a *Vibrio* specific chemical cue or signal involved in the formation of biofilms. Further studies should focus on the nature of the toxin, chemical cues and possibly the nature of nutrient recycling due to grazing on bacterial biofilms by protozoan grazers.

Getting out there – development of methods to investigate protozoa effects on bacterial biofilms in the field

An important part of research focused on ecological questions is to take knowledge from the laboratory into the natural environments to see whether the information obtained is relevant in the natural habitat of the organisms involved. Thus, a new approach to take biofilm experiments into the field was presented in chapter 4. It was tested whether the anti-protozoan toxicity of the bacterial strain *V. cholerae* A1552 could be detected in the natural habitat of both the bacterial strain and the heterotrophic flagellates *R. nasuta* and *C. roenbergensis.* Diffusion chambers formerly used for suspension experiments were modified to provide a substratum for biofilm analysis and retain bacteria and protozoa but still allow the exchange with the surrounding seawater resulting in massive dilution effects. Abundances of *C. roenbergensis* were lower on biofilms of the toxigenic strain A1552 than on supposedly non-toxigenic strains, whereas the abundance of *R. nasuta* was lower on the supposedly non-toxigenic strain *V. cholerae* N16961. This was surprising as *V. cholerae* N16961 has a frameshift mutation in the hapR gene and should as such be non-toxic. Since the daily monitoring of protozoan abundances on the biofilms was

not possible on the biofilms inside the exposed chambers, protozoan numbers were monitored in a laboratory set-up. The differences in toxicity of *V. cholerae* A1552 and N16961 as seen in the field could be verified. This strongly indicates a hapR independent toxicity pathway additionally to the quorum sensing regulated hapR pathway that still remains unknown.

A possible reason for the different abundances between experiments in the field set-ups is the difference in seasons. Temperature as well as nutrient concentration in the seawater has most likely a strong influence on the interactions of protozoa and bacteria. One difficulty with the environmental set-up is the incalculability of the environmental conditions such as nutrient supply, temperature and other organisms such as viruses that can pass the 0.1µm membranes and the major consequences on the synergy of the organisms. Thus, one of these factors (nutrient supply) was taken and its impact further tested in the laboratory. The lower growth rates of *R. nasuta* on all three toxic and non-toxic *V. cholerae* biofilms, *V. cholerae* A1552, *V. cholerae* N16961 and *V. cholerae* hapR, respectively, grown under low glucose concentration also strongly indicate an independent hapR toxicity pathway and thus support the results from the field experiments.

Most experiments concerning protozoa-bacteria interactions within biofilms focused either on the bacterial community or on the protozoan community in its entirety (e.g. Böhme *et al.* 2009, Norf *et al.* 2009a/b). Experiments on the individual level are scarce and have been run in the laboratory (e.g. Boenigk and Arndt 2000a/b). With the successful approach to combine two established systems it was possible to study food web interactions on the individual level. It was possible to demonstrate that microcolony formation protects bacterial cells within these microcolonies from grazing by heterotrophic flagellates (HFs). It was shown directly that microcolonies protect bacterial cells on the mechanical level, that is to say the grazers are not able to separate the cells from the microcolony. Older studies focused primarily on summation effects, e.g. bacterial biofilms with and without grazers (Matz *et al.* 2005), impact of protozoa with different feeding modes on biofilms (Böhme *et al.* 2009), or early and late protozoan colonizer impact on biofilms (Weitere *et al.* 2005) rather than on direct mechanisms. Further studies should focus on the mechanism as to why HFs are not able to ingest bacteria from colonies, e.g. possible involvement of the biofilm matrix. Observations of individual

cells made it possible to detect inter- as well as intraspecific grazing strategies and differences in between three species that seemingly occupy a similar niche on biofilms. This strongly supports the concept to investigate protozoan bacteria interactions on the individual level to detect differences that might otherwise escape our recognition. During the examination of individual cells it was observed that some individuals repeatedly contacted cells they already tried to ingest. Occasionally flagellates even seemed to avoid certain bacterial cells on the biofilms. This suggests that chemical sensing might play an important role in food acquisition and bacterial defence.

Conclusion

Results obtained demonstrate the importance of coupling laboratory and field experiments regarding protozoa-bacteria interactions on biofilms. Especially human pathogenic strains such as *V. cholerae* that prove to be toxic towards protozoan grazers need to be further investigated. This thesis presents new insights into the defence mechanisms of bacteria, especially *V. cholerae* and the mutual benefit predator and prey might experience. The environmental methods presented in this thesis, i.e. the modified suspension chambers and the combination of video microscopy and the flow cell system with river bypass, take the laboratory a step further into the field, and with this the possibility to test defence mechanisms under natural conditions. The newly developed methods open the path for further experiments e.g. exposure of the diffusion chambers with more diverse protozoan–bacterial communities or video microscopy of grazing behaviour on toxigenic bacteria and trophic interactions within more diverse biofilm communities, respectively.

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Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen-, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlich worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahren nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir angefertigte Dissertation ist von Prof. Dr. Markus Weitere betreut worden.

Magdeburg, im Februar 2011

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Publikationen

[1] Erken, M., Weitere, M. Kjelleberg, S. and Dougald, D. (2011). *In situ* grazing resistance of *Vibrio cholerae* in the marine environment. FEMS Microbiology Ecology (published online 1. March 2011).

Eingereichte Manuskripte

[2] Erken, M., Weitere, M. Kjelleberg, S. and Dougald, D. Chemical cues produced by *Tetrahymena pyriformis* induce biofilm formation and chemical defence of *Vibrio cholera.* (submitted).

[1] Entspricht Kapitel 4 der vorliegenden Dissertation

[2] Entspricht Kapitel 3 der vorliegenden Dissertation