Chapter 5

Grazing Effects of Ciliates on Microcolony Formation in Bacterial Biofilms

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Abstract

The attachment to surfaces and the subsequent formation of biofilms are a life strategy of bacteria offering several advantages for microorganisms, for example, a protection against toxins and antibiotics and profits due to synergistic effects in biofilm environment. Moreover, biofilm formation is thought to serve as grazing protection against predators. From pelagic systems it is known that feeding of bacterivorous protists may strongly influence the morphology, taxonomic composition and physiological status of bacterial communities and thus may be an important driving force for a change in bacterial growth and shift in morphology towards filaments and flocs. Bacteria in biofilms had to evolve several other defence strategies: production of extrapolymeric substances (EPS) or toxins, formation of specific growth forms with strong attachment, specific chemical surface properties and motility. In addition, bacteria can communicate via quorum sensing and react on grazing pressure. The results of the case study presented here showed that even microcolonies in bacterial biofilms are affected by the activity of grazers, though it may depend on the nutrient supply. Feedback effects due to remineralization of nutrients because of intensive grazing may stimulate biofilm growth and thereby enhancing grazing defence. Predator effects might be much more complex than they are currently believed to be.

Keywords: Bacterial biofilms, protozoan grazing, predator-prey interactions, defence mechanisms, colony formation

1. Defence mechanisms of biofilm bacteria—implications from plankton

Bacteria are an important food source for protozoans. The impact of protozoan grazing on bacterial communities can significantly affect bacterial biomass and may shape morphology

and taxonomic composition of bacterial communities. While this is well known for pelagic microbial communities [e.g. 1–4], bacterial communities in biofilms have mainly been viewed from a microbial perspective rather than the food web perspective [5]. However, biofilm studies have shown that bacterivorous organisms, such as protozoans, can effectively reduce the biovolume and morphology of bacterial biofilms, too [e.g. 6, 7]. Especially, amoebae may significantly influence biofilms [e.g. 8].

For pelagic habitats, a variety of defence mechanisms has been described: a widespread observation is a shift to larger cells in bacterial communities that are subject to strong protozoan grazing. In addition, several other defence strategies had been described as for example extreme reduction in cell size, certain motility patterns, specific surface properties of the bacteria, toxin production and the production of exopolymeric substances that surround bacteria (for a summary see [3]).

The change of size as response to protozoan grazing has been observed in several planktonic field studies (e.g. [9–11] as well as in laboratory experiments [12–16]). Pernthaler et al. [17] found a shift in size classes of bacteria as result of intensive protozoan grazing in oligomesotrophic lake plankton during spring and argued that small cells (<0.4 μm) and large cells (>2.4 μm) being the most resistant groups with reference to their size. Matz and Jürgens [18] found that bacteria > 0.5 and <0.1 μm^3 (the latter are called 'ultramicrobacteria') showed the highest survival rates. The shift to larger cell sizes and filaments and flocs has been reported from several laboratory studies (e.g. [15, 16, 18, 19]). These latter studies also pointed to the decrease in cell size as a potential strategy for bacteria to escape protozoan grazing. However, Boenigk et al. [20] could demonstrate that bacteria may even feed on this small prey size which implies that small-sized bacteria are not generally protected against grazing.

Furthermore, exopolymeric substances secreted by the bacteria may hinder bacterial predators from grazing. This has been found for example in a study on batch and continuous cultures of two pelagic bacterial species isolated from the field [21]. In this study, the extrapolymeric substances were shown to form an essential portion of flocs and microcolonies in suspensions at strong flagellate grazing. Grazing experiments with the flagellate *Ochronomas* and the bacterium *Pseudomonas* as prey by Matz et al. [22] revealed that thenon-mucoid-producing morph of *Pseudomonas* was severely affected and reduced in terms of abundance, whereas the primary mucoid-producing type survived due to the formation of inert suspended microcolonies stabilized by an extracellular matrix.

Moreover, it could be shown that bacteria might kill their prey by producing substances that are toxic for their potential predators. Matz et al. [23] found in a study where they analysed grazing of different common heterotrophic flagellates on violacein-producing bacterial strains, a rapid cell death of the flagellates after ingestion of these bacteria.

The production of toxins was shown to be induced by quorum sensing, which emphasizes that this kind of communication between bacterial cells plays an important role in the grazing defence.

Motility of bacteria has been identified as another defence mechanism [24]. Small bacteria, which increased in size under strong grazing pressure, were additionally much more motile.

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Increased swimming speed of bacteria enhanced the probability of capturing of bacteria by flagellates, but the ingestion rates dropped down with increasing swimming speed [25]. Increased motility clearly increased the survival rate of bacteria under protozoan predation.

The surface properties of bacteria have also been shown to influence the rate of their ingestion by protozoa. In two studies, it has been shown that gram-positive bacteria were grazed to a lower extant than gram-negative bacteria by flagellates and ciliates [26, 27].

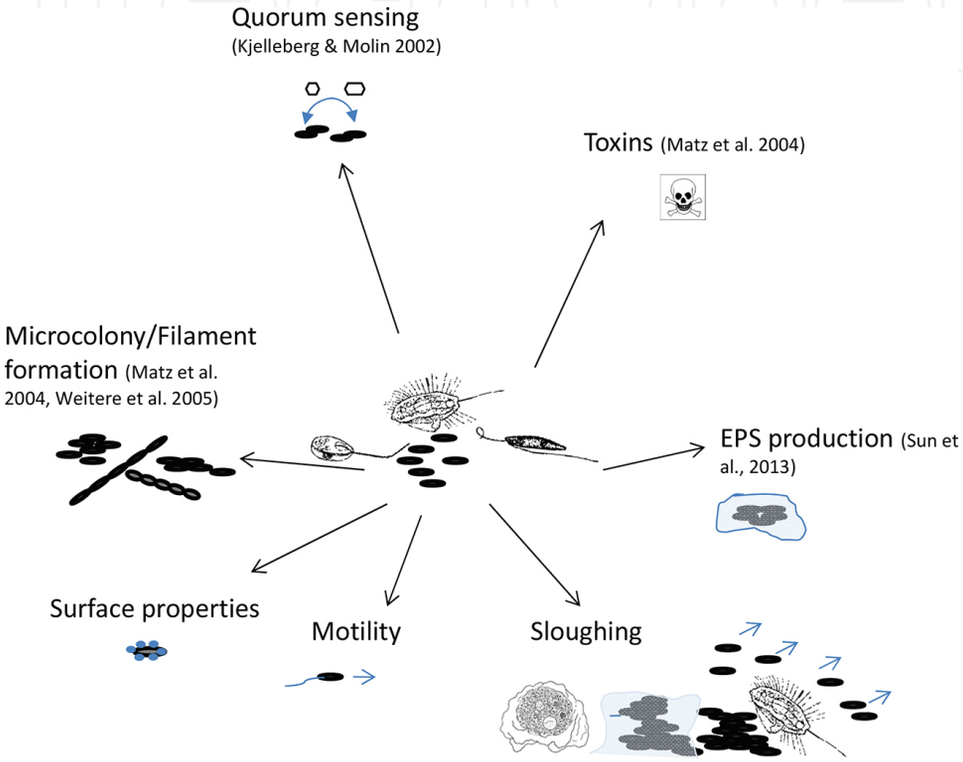

Figure 1. Defence mechanism of bacteria against grazing in biofilm communities.

Defence strategies of bacteria in biofilms are much less understood than those for the pelagial. We summarized the potential defence phenomena that could be derived from studies of planktonic communities (**Figure 1**). The importance of protozoans on biofilms has been reviewed by Arndt et al. [28] and Ackermann et al. [29]. In biofilms, a very common phenomenon is the increase in size due to the formation of microcolonies and filaments. Matz et al. [30] reported that a wild-type strain of *Pseudomonas aeruginosa* formed microcolonies if faced with protozoan grazing by the benthic flagellate *Rhynchomonas*. They showed that type-IV pili of bacteria creating the so-called 'twitching motiliy' (a certain way of movement over the substrate) are important for microcolony formation, as bacteria lacking these pili could form only a considerably lower number in microcolonies than the wild type. Weitere et al. [31] showed that grazing protection due to microcolony formation in bacterial biofilms is dependent on the protozoans' feeding mode. This was underlined by a consecutive study by Erken et al. [32] who analysed the influence of grazing of three gliding flagellates (*Neobodo*, *Rhynchomonas* and *Planomonas*) differing in feeding modes, that is, regarding the contactrates, handling

times and relative predation success of each species. A longer handling time, as found for *Planomonas*, was shown to result in a significantly higher success in ingestion rates. However, the lower ingestion rates of the other two species were compensated by higher contact rates. Microscopic observations revealed that heterotrophic flagellates contacted microcolonies, but in no case bacterial cells of these bacterial aggregations were ingested. Another study on stream biofilms indicated that various protozoans may differently affect microcolony formation [7]. The ciliate *Dexiostoma* did not change biofilm volume and porosity but stimulated the formation of larger microcolonies. In contrast to this, the heterotrophic flagellate *Spumella* and the ciliate *Chilodonella* did not stimulate microcolony formation; however, the biofilm volume was decreased 2.5–6.3-fold compared to ungrazed biofilms. Contrary to this, grazing of the raptorial feeding amoebae *Vannella* reduced microcolony size clearly. On the other hand, the porosity and the ratio of biofilm surface area to biofilm volume were 1.5–3.7 and 1.2–1.8 times higher under grazing pressure. This points to possible stimulating effects as grazing might improve the exchange of nutrients and gases in deeper biofilm layers and enhance microbial growth.

These examples clearly show that the formation of microcolonies may serve as defence strategy for protozoans. However, in contrast to the pelagial, for biofilms, the reduction in individual cell size seems not to play a role in biofilms.

The secretion of exopolymeric substances (EPS) is a typical characteristic of biofilms and is believed to be a clue for grazing defence, as it has been shown for pelagic bacteria. Weitere et al. [31] showed that alginate-mediated microcolony formation served as effective defence mechanism against grazing on *Pseudomonas aeruginosa* biofilms by flagellates of the following two different feeding types: the suspension feeding *Bodo saltans* and the surface feeding *Rhynchomonas nasut*a. In a parallel study with an alginate-overproducing mutant strain of *P. aeruginosa,* the bacteria built significantly larger microcolonies under grazing pressure of a surface-feeding flagellate (*Rhynchomonas nasuta*) compared to the wild-type strain [30]. Hence, the production of EPS might provide a sufficient grazing defence. Moreover, this production was shown to be quorum-sensing regulated, which underlines the importance of communication between bacteria for their defence against grazing. This is supported by Sun et al. [33], who emphasized the importance of EPS-production and quorum sensing. Biofilms with mutants of the pathogenic bacteria *Vibrio cholera,* which expressed less polysaccharides, were also less resistant against grazing. The same was true for mutants with a deficiency in quorum-sensing ability.

An additional defence factor is the production of inhibitors. Weitere [31] found flagellate growth to be affected in *Pseudomonas aeruginosa* biofilms in a late phase of biofilm development.

To summarize the knowledge of defence strategies of bacteria in biofilms, the most well-known phenomenon is the increase in size or the shift to a more grazing-resistant morphology by the formation of microcolonies and filaments. However, this mechanism depends on quorum-sensing-mediated communication among bacteria. The production of exopolymeric substances or toxic substances additionally may strongly affect protozoan predators or kill them, respectively.

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2. Bacteria defence from grazing in the course of biofilm aging

Within the process of maturing, bacterial biofilms have shown to undergo certain morphological changes (for an overview see e.g. [34]). From a scattered distribution of bacteria, this changes to clustered microcolonies and increases in height, followed by the establishment of mushroom-like structures. With an increase in height, an increase in the detachment of single bacteria or bacterial flocs into the pelagial occurs due to increasing shear stress in running waters affecting the biofilm thickness [5, 34]. This effect is called 'sloughing' and may also decrease the probability of being captured by protists (**Figure 2**). Ammendola et al. [35] found that *Serratia liquefaciens* exposed to certain surfaces formed elongated, highly motile swarm cells which were grazing-resistant provided their length exceeded 15 μm.

Figure 2. Different phases of biofilm development including bacterial settlement (1), aggregation (2), EPS formation (3) and sloughing (4).

The grazing pressure by protozoans changes with the ongoing process of biofilm maturation. Weitere et al. [31] showed that the early formation of microcolonies in *Pseudomonas aeruginosa* biofilms resulted in a grazing protection against early biofilm colonizers (e.g. the kinetoplastid flagellate *Bodo saltans*). In contrast to this, grazing by late biofilm colonizers such as the browsing ciliate *Tetrahymena pyriformis* or the amoeba *Acanthamoeba polyphaga* caused high losses of bacterial biomass. A different result was obtained by Chavez-Dozal et al. [36] for *Vibrio fischerii* biofilms. In late biofilms, the expression of antiprotozoan substances affected the late biofilm colonizers and grazers (*Tetrahymena pyriformis)*, whereas the flagellates *Rhynchomonas nasuta* and *Neobodo designis* were able to graze and show significant growth in early biofilms.

These studies suggest that the vulnerability of biofilms to grazing by protists may significantly change in the course of biofilm aging. The production of toxins and extrapolymeric substances may play an important role. Biofilm communities are complex systems and we are just at the beginning to understand the interactions occurring on biofilms.

The occurrence of macroinvertebrates on biofilms and their influence on the different trophic levels have to be considered. Ackermann et al. [29] showed that increases in macrofauna populations increased the surface and biovolume of biofilms in a river. Multifactorial field studies by Haglund and Hillebrand [37] found that the presence of grazers tended to increase bacterial biomass at ambient nutrient conditions but tended to decrease bacterial biomass under enrichment nutrient conditions. Remineralization of nutrients due to the feeding process of macroinvertebrates may play a significant role. And there may also be another indirect effect of metazoans by reducing bacterivorous protozoans [29].

3. Defence mechanisms of biofilm bacteria may change with substrate supply

From pelagic studies, it is known that the response of bacteria to grazing is dependent on the availability of nutrients. Matz and Jürgens [24] could demonstrate in their study on grazing of two flagellates (*Ochromonas* and *Spumella*) on a natural bacterial community that the nutrient quality decides how the bacterial community reacts. Small and motile bacteria dominated under carbon limitation, whereas large and elongated bacteria occurred if phosphorous was limited. On the other hand, Simek et al. [38] demonstrated that the portion of grazing resistant forms (flocs and filaments) increased when bacteria were exposed to protozoan grazing at limiting nutrient concentrations. However, up to now, this has not been analysed in detail for biofilm communities, but a comparable influence is likely.

As it has been pointed out in the first paragraph, bacterial biofilms may show microcolony formation as a defence mechanism against grazing by protozoans. Hence, we conducted an experiment with a bacterium, a variant of the genus *Acinetobacter*, which generally forms microcolonies during biofilm growth. We investigated whether this bacterium was affected by grazing of the ciliate *Tetrahymena pyriformis* under different nutrient supply for bacteria. We hypothesized that the microcolony-forming *Acinetobacter* sp. strain C6 would be resistant to grazing by *T. pyriformis* as long as microcolony formation is not affected due to limiting nutrients, and we assumed that less optimal substrate supply will weaken this defence mechanism. The experiments were run in flow chambers in a mineral medium [39] which was supplemented either with sodium benzoate or with citrate as a carbon source. Citrate is known to be a less optimal carbon source for the microcolony formation *Acinetobacter*[40, 41]. For the analysis of biofilms under the laser-scanning microscope, *Acinetobacter* was either tagged with green fluorescent protein or stained with propidium iodide.

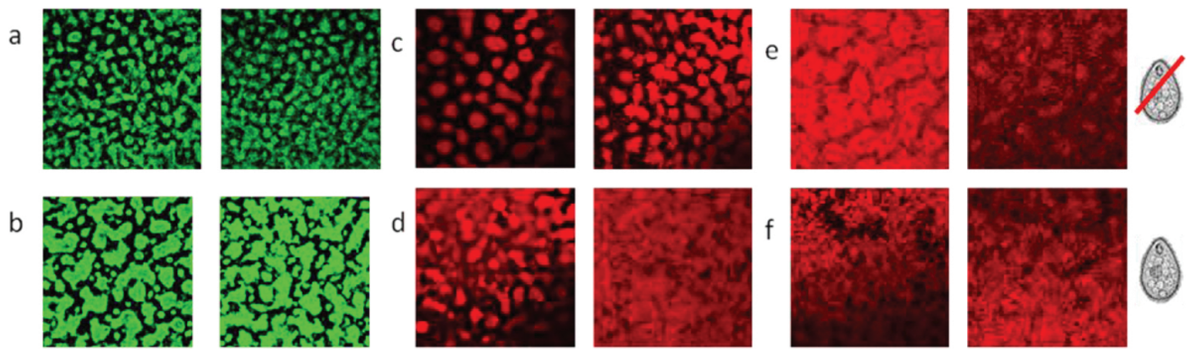

Figure 3. Structural changes of biofilms of *Acinetobacter* with and without grazing pressure by *Tetrahymena pyriformis* (confocal laser-scanning-microscope pictures in *x-y* direction; size: 230 μm × 230 μm; a and b: GFP-tagged bacteria, c–e: propidiumiodide stained bacteria). Biofilms are shown for day 4 and day 8. (a) Sodium benzoate as medium, high medium supply rate, no *Tetrahymena*. (b) Sodium benzoate as medium, high medium supply rate, *Tetrahymena* present. (c) Sodium benzoate as medium, low medium supply rate, no *Tetrahymena*. (d) Sodium benzoate as medium, low medium supply rate, *Tetrahymena* present. (e) Citrate as medium, high medium supply rate, no *Tetrahymena*. (f) Citrate as medium, high medium supply rate, *Tetrahymena* present.

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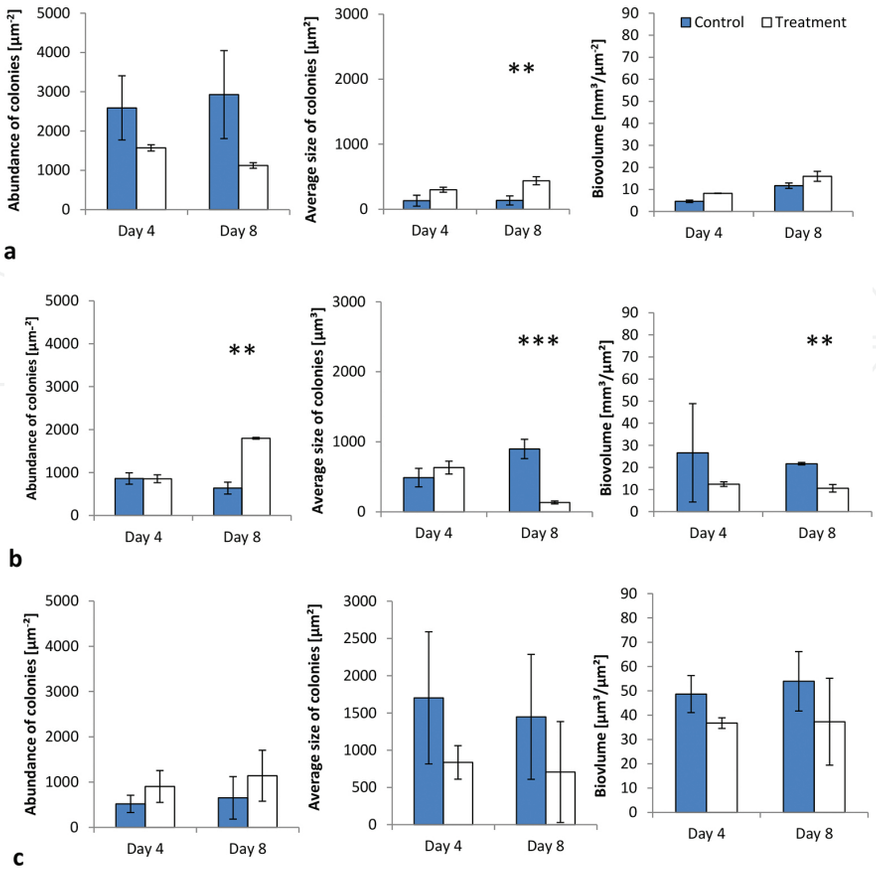

Figure 4. Quantitative analysis of biofilms (abundance of colonies, average size of colonies and biovolume) of *Acinetobacter* with and without grazing pressure by *Tetrahymena pyriformis* (average +/- standard deviation) after 4 and 8 days. Quantification was made with 3D for LSM (Zeiss, Germany) and Image J (NIH, Maryland, USA). (a) Sodium benzoate as medium, high-medium supply rate, (b) sodium benzoate as medium, low-medium supply rate, (c) citrate as medium, high-medium supply rate.

Grazing of *Tetrahymena* led to different microcolony formation of *Acinetobacter* biofilms depending on different growth conditions for bacteria and under grazing pressure of *Tetrahymena*. Under high supply rate of medium and by the use of an optimal carbon source (sodium benzoate as carbon source), round microcolonies dominated the biofilm, which were regularly distributed over the substrate (**Figure 3a**). In contrast, microcolonies showed a more irregular, elongated shape in the presence of the grazer (**Figure 3b**) and the size of the colonies was significantly larger (**Figure 4a**). Interestingly, the biovolume of the biofilm increased under grazing influence of *Tetrahymena* during the whole experiment which points to a stimulating effect of protzoan grazing to bacterial growth (**Figure 4a**). In contrast, biofilms grown with low medium supply (and sodium benzoate as carbon source) were affected by the presence of the protozoan grazer in terms of size and biovolume. The nongrazed biofilms showed similar, clearly visible round microcolonies as found with high medium supply (**Figure 3c**), being evenly distributed over the substrate during the whole experiment. In the presence of *Tetrahymena*, the microcolonies showed always an irregular shape (**Figure 3d**). Moreover, a significant decrease in microcolony size and biovolume in the course of the experiment was recorded (**Figure 4b**). The effect of weakening the potential to form microcolonies was even

more pronounced in the biofilms grown under high medium supply with citrate in comparison to the biofilms with low medium supply of sodium benzoate (**Figure 3e**). In the control treatments without ciliates, round microcolonies dominated the biofilm though with a more irregular, larger shape compared to the sodium benzoate treatments. In the grazed biofilms, microcolonies were severely affected and did not show any round shape, but irregular flocs and after 8 days, the microcolony formation nearly disappeared completely. The remaining microcolonies were smaller in size and had a lower biovolume (**Figure 4c**).

We hypothesized that the microcolony-forming *Acinetobacter* sp. strain C6 would be resistant to grazing by *T. pyriformis* as long as microcolony formation is not affected by substrate supply. However, we found that the presence of protozoans had a considerable impact on the structure of microcolonies of *Acinetobacter* sp. strain C6 in every treatment tested and thereby affect this defence mechanisms of bacteria regardless of the available nutrient source. Without *T. pyriformis* being present, we observed formation of round microcolonies in the *Acinetobacter* sc. strain C6 biofilm with sodium benzoate as carbon source. After introduction of *T. pyriformis* to this array at the beginning of the experiment, the shape of microcolonies was changed, as microcolony size increased and single microcolonies connected to each other. This enlargement of microcolonies could also be observed when *Tetrahymena* was added later (e.g. at day two of biofilm formation, data not shown). This morphological change of the shape of microcolonies probably serves as a further enhanced protection against protozoan grazing. This structure was also found in the study of Dopheide et al. [42], who examined the effect of *Tetrahymena* grazing on biofilms built by the bacterium *Serratia plymuthica.* This points to the fact that the browsing feeding mechanism of this protozoan may stimulate this kind of microcolony formation in biofilms, which is also underlined by the fact that the biovolume of the biofilm increased in the present study with sodium benzoate as nutrient source. Here, nutrient remineralization may be facilitated by the grazing activities of the protozoa. We compared the loss due to grazing with the bacterial production to check whether *Acinetobacter* may be able to grow fast enough to compensate feeding losses to predators. For this, we considered data of grazing of *Tetrahymena* (Tanasescu, pers. comm.) and used published carbon conversion factors for ciliates and bacteria [43, 44]. The calculations revealed that the mean growth rate of *Acinetobacter* of 0.4 pg C μm^{-3} day^{-1} can match the average demand of 0.29 pg C μm^{-3} day^{-1} for *T. pyriformis.* This supports the idea that grazing losses at least can be compensated by the growth of *Acinetobacter* sp. Due to sloppy feeding and excretion of nutrients, grazers release bacteria from nutrient limitation [45]. Movements of bacterivores within the biofilms (e.g. ciliates as *T. pyriformis*) may create free patches and ventilate the bacterial biofilm. Thus, bacteria at the base of the biofilm that might otherwise starve or become inactive might receive increased nutrient and oxygen supply. Additionally, substances produced by either grazers or bacteria (chemical cues or quorum-sensing signals) might have additional growth-stimulating effects [25, 46]. These feedback effects between grazers and bacteria might have had a significant influence on the observed structural and quantitative changes and thereby might result in an increased bacterial growth. The reduction in medium supply to the bacteria enhanced the competition for substrate between the biofilm bacteria. As a consequence, bacteria could not maintain the regular structure and distribution of microcolonies, and furthermore, the biovolume of the biofilm was reduced significantly under

grazing pressure. This supports our hypothesis that less optimal growth conditions in bacteria biofilms may affect the ability to defend against grazing by microcolony formation. If citrate as alternative carbon source was used, a high structural heterogeneity occurred in the presence of *Tetrahymena* for the whole course of the experiments. Such a high heterogeneity could also be seen with biofilms of *Pseudomonas aeruginosa*, if grown with citrate as carbon source [40]. The results of this study thereby showed that even microcolonies in bacterial 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.

Biofilms might serve as grazing defence, though it may differ between different species and moreover depend on the nutrient supply. Additionally, feedback effects due to remineralization of nutrients as result of intensive grazing may stimulate biofilm growth and thereby enhance grazing defence.

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