Microbial Ecology

Microbial Exopolymers Link Predator and Prey in a Model Yeast Biofilm System

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Abstract

Protistan grazing on biofilms is potentially an important conduit enabling energy flow between microbial trophic levels. Contrary to the widely held assumption that protistan feeding primarily involves ingestion of biofilm cells, with negative consequences for the biofilm, this study demonstrated preferential grazing on the noncellular biofilm matrix by a ciliate, with selective ingestion of yeast and bacterial cells of planktonic origin over attached and biofilm-derived planktonic cells. Introducing a ciliate to two biofilm-forming *Cryptococcus* species, as well as two bacterial species in a model biofilm system, fluorescent probes were applied to determine ingestion of cellular and noncellular biofilm fractions. Fluoromicroscopy, as well as photometric quantification, confirmed that protistan grazing enhanced yeast biofilm metabolism, and an increase in biofilm biomass and viability. We propose that the extracellular polymeric matrix of biofilms may act as an interface regulating interaction between predator and prey, while serving as source of nutrients and energy for protists.

Introduction

The current revolution in microbial ecology has highlighted the role of interaction and exchange in microbial communities [40]. Recognition of the importance of biofilms as a form of microbial existence, with attached matrix-enclosed organisms being phenotypically distinct from their planktonic counterparts, has resulted in interdisciplinary endeavors toward resolution and application of this phenomenon [12, 40]. Although protistan grazing on biofilm communities has been suggested as potentially a major conduit enabling energy flow between microbial trophic levels [1], and a pathway whereby biomass produced by other microorganisms may reenter the food web, its importance for controlling biofilm dynamics has mostly been overlooked [45]. Studies on microbial predation have primarily concerned planktonic cells, with limited and conflicting evidence of protist–prey interactions in biofilms [20, 33, 42]. The potential role of extracellular polymeric substances (EPS) as interface between the various trophic levels has received even less attention, with only recent suggestion that biofilm structural components should be investigated regarding protection from protistan grazing [33]. The ubiquity of biofilms as predominant microbial phenotype despite the presence of predators may indicate the existence of more complex adaptive mechanisms among microbial consortia. A recent call for reconsideration of fundamental questions regarding microbial predation [36] exemplifies the need for new perspectives on predation as selective force, both from the adaptive microbial and exploitive predatory viewpoints.

Feeding associations link organisms in ecological communities [7]. As a major component of this network, predation plays an important role in energy flow between trophic levels. Despite the contention that EPS provide physical protection against predation, it is possible that the noncellular fraction of biofilms may be ingested, thereby providing a carbon source to grazers [18, 56]. As the bacterial marine biomass does not provide in full for the carbon requirements of associated protists [9], it was suggested that the logical nutritional substitute would be provided by ingestion of EPS, with bacteria converting as much as 70% of their ingested carbon into EPS [19]. Therefore, EPS potentially serve as an important link between the microbial and classical food webs [43] and thus play an important role in aquatic carbon cycles [14, 29, 31, 44, 55].

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In biofilms, the EPS fraction may similarly provide the interface that regulates interaction between attached cells and grazing protists, and thus a mechanism for entry of nutrients from this noncellular nutrient sink into the food web. Accordingly, EPS-related molecules that are too large for incorporation through bacterial cell membranes reenter the microbial food web via the route of protistan ingestion without extracellular enzymatic decomposition into simple compounds [39, 50]. It has also been suggested [56] that protein-containing exopolymeric particles may influence nitrogen cycling in pelagic ecosystems, but without investigation into their production and utilization. EPS include varying amounts of polysaccharides, proteins, nucleic acids, lipids, and sequestered ions, with a range of enzymatic and regulatory activities occurring within the matrix [15, 16, 41, 49]. EPS has been described as "an operational definition" that encompasses a range of microbially secreted macromolecules with various physicochemical properties as well as biological roles, being active as stabilizer, buffer, sorptive sponge, and locus for extracellular enzyme activities [10]. However, because of the inherent difficulty to perform high-resolution microscopical characterization of EPS *in situ*, and the complexity of chemical extraction and analytical procedures [27, 54], their nutritional function in biofilms is poorly described. When the energy expenditure associated with EPS production, especially in oligotrophic environments, and the thermodynamic equilibria prevalent in microbial food webs are considered, the nutritional implication of EPS seems obvious—also for biofilm-associated protists.

We focused our study on the relationships between a ciliate (*Tetrahymena*) and two biofilm-forming yeast species (*Cryptococcus laurentii* and *C. podzolicus*). These organisms were isolated from the same oligotrophic sandy-loam soil, where protistan activity is restricted to water films and water-filled pores [47]. Oligotrophic protistan grazing and yeast biofilm dynamics are both poorly described in literature, although they frequently are part of complex biofilms in clinical, industrial, and natural environmental settings [13, 17, 42]. We observed a strong feeding preference of the protist for the noncellular fraction of yeast biofilms. This, and the demonstrated function of EPS in aquatic food webs, led us to hypothesize that the EPS produced by the yeasts function as an important source of nutrients for the grazing ciliates.

We subsequently coinoculated continuous flowcells containing *C. laurentii* or *C. podzolicus* biofilms with the ciliate and utilized light and fluorescence microscopy to observe their interactions with the noncellular as well as the cellular attached and planktonic fractions. Comparisons with planktonic and attached bacteria as prey organisms were included by introduction of a *gfp*-labeled pseudomonad to ciliates in yeast biofilms. The influence of cellular and noncellular yeast and bacterial fractions on ciliate excystment was furthermore evaluated, whereas real-time photometric quantification of biofilm formation, integrated with fluoromicroscopic assessment of yeast viability, gave insight into the influence of predation on biofilm metabolism.

Materials and Methods

Flowcell Experiments, Staining, and Visualization. Yeast biofilms were prepared by inoculating multiple channel flowcells [57] with the yeasts *C. laurentii* or *C. podzolicus*, previously isolated from oligotrophic sandy-loam soil [5]. With the peristaltic pump (Watson Marlow 205S) turned off, each flowcell channel was inoculated with 200 μL of a 24-h batch culture of the respective yeasts. Flow was resumed after 3 h, and the resulting yeast biofilms were maintained on oligotrophic growth medium consisting of 2.5 mg L^{-1} sucrose and 6.7 mg L^{-1} Yeast Nitrogen Base (Difco) at a flow rate of 6 mL h^{-1} (linear fluid velocity 680 mm h^{-1}).

To visualize yeast viability, feeding preferences of protists, and partial characterization of EPS, replicate channels containing *Cryptococcus* biofilms were flooded overnight with 40 μM FUN-1™ yeast viability probe (Molecular Probes), or for 3 h with either 25–50 μM Calcofluor™ White M2R (Molecular Probes), or 200 μg mL^{-1} Concanavalin A–fluorescein isothiocyanate (FITC, Sigma) to stain cells, cell-wall polysaccharides, and/or EPS, respectively. Flow was resumed for 1 h to rinse the channels, after which biofilms were visualized with a Nikon Eclipse E400 epifluorescence microscope, equipped with a multipass filter set appropriate for viewing 4′,6-diamidino-2-phenylindole (DAPI), as well as excitation/barrier filter sets of 465–495/515–555 and 540–580/600–660 nm. Images were captured with a Nikon Coolpix 990 digital camera mounted on the microscope. This equipment was also used to collect video footage of protist behavior in the presence of either planktonic or attached cells with EPS. Proteins were stained for 1 h in flowcell-cultivated biofilms with a 1:4 dilution of the Coomassie® brilliant blue-based protein assay dye reagent (Bio-Rad) and visualized with light microscopy.

For scanning electron microscopic (SEM) investigation, *Cryptococcus* biofilms were cultivated on sterile sand grains in standard flowcells using oligotrophic growth medium. Sand grains were removed from the flowcells after 3 days and viewed unfixed and hydrated with a LEO 1430 VP SEM operated at 7 kV, after gold coating with an Edwards S150A sputter coater.

Selective Feeding of Protists in Flowcells

Isolation. Protists were isolated from the same sandy loam soil as the yeasts by selective feeding with overnight cultures of either *C. laurentii* or *C. podzolicus* and

addition of 0.2 mg mL^{-1} streptomycin. Initial incubation in the dark (23°C; 7 days) of a 10% soil suspension in an autoclaved soil-water extract contained the protists in the top suspension, of which 5-mL aliquots were sampled for selective feeding with the yeasts. Evaluation of various antibiotics and experimental procedures revealed that pure culture ciliates free of bacterial contamination could be obtained by repeated transfer and reinoculation with streptomycin-containing prey solutions. Ciliates were maintained as cysts in sterile flasks containing the same soil-water extract and periodically fed with streptomycin-containing (0.2 mg mL^{-1}) cell suspensions of either one of the yeast strains to obtain active organisms. After recording the results on ciliate excystment (see Fig. 2), axenic cultures were obtained by feeding with cell-free suspensions of either of the yeasts.

Predation on Yeast Biofilms. Ciliates were introduced to replicate flowcell channels containing stained, 2-day-old yeast biofilms and viewed hourly with epifluorescence microscopy over a 12-h period, and at regular intervals for the following 36 h, to assess whether sessile yeast cells were ingested. Biofilm cells were stained with either Calcofluor White and FUN-1 or Concanavalin A-FITC.

Predation on Planktonically Grown Yeast Cells. Calcofluor-stained batch-grown yeast cells were introduced to 2-day-old unstained yeast biofilms, containing noticeable ciliate populations. Staining before inoculation enabled differentiation between biofilm-associated cells and freshly added stained planktonic cells and their respective interactions with the ciliates.

Predation on Sessile and Planktonically Grown Bacterial Cells. A *gfp*-tagged pseudomonad (*Pseudomonas* CT07) [2] was introduced to 2-day-old yeast-ciliate biofilms by direct inoculation with 200 μL of an overnight batch culture [cultivated in Luria-Bertani broth (LB, Merck)] into the flowcell following the same procedures as when the yeasts were introduced. Gfp tagging was previously performed with a mini-Tn-7 transposon inserted into the chromosome of the pseudomonad [21]. The inserted sequence was stably maintained and did not affect bacterial growth. The fate of the planktonic and attached bacterial cells was followed continuously for 6 h by direct visualization using epifluorescence microscopy and thereafter at regular intervals for an additional 66 h.

Impact of EPS on Predator Behavior. Microtiter wells were filled with 100-μL aliquots of a suspension containing ciliate cysts, but no active ciliates. Added to these were 150-μL aliquots of suspensions prepared from potential yeast and bacterial prey organisms. *C. laurentii* and *C. podzolicus*, and two bacterial species (*Dyadobacter* and *Pseudomonas* spp.), were grown on 100% yeast malt extract (YME) and 3 g L^{-1} tryptone soy broth (TSB, Merck), respectively, to logarithmic as well as stationary phase. From each one of these, a cell suspension, cell-free supernatant, and washed cells were prepared in triplicate, and fractions added to the respective microtiter wells. Sterile water, YME, and TSB were used in control wells. Plates were incubated at 23°C in the dark for 24 h. Active ciliates were enumerated by repeated sampling of 5-μL aliquots from triplicate wells to evaluate the degree to which the respective fractions stimulated excystment and subsequent proliferation. To immobilize protists, glutaraldehyde was added as fixative to individual samples to a final concentration of 1%. Quantification in individual microscope fields was carried out until a 95% probability had been reached (n typically 20–40) that there was ≤10% deviation from the mean.

Application of an Optical Large Area Photometer for Real-time Monitoring of Biofilm Biomass in the Presence and Absence of Protists. The variation in biofilm biomass in the presence of predation was quantified in real-time on the inner surfaces of an adapted parallel plate flowcell. The optical large area photometer (OLAPH) measures visible light scattered by small particles that are distributed across a relatively large rectangular plane. The apparatus was set up with a 10-mL hexagonal Plexiglas flowcell covered on both sides with glass coverslips (75 × 50 mm) [2, 48]. Following inoculation of the flowcell with 5 mL of an overnight batch culture of *C. laurentii*, flow of the oligotrophic medium was resumed from top to bottom of the vertically positioned flowcell. After 3 days, the pump was switched off for 1 h, and ciliates were added (2-mL culture, ~5 ciliates μL^{-1} inoculum) without interruption of data capturing. Biofilm biomass was subsequently monitored for 9 days. Captured data were analyzed using LabView-based software [2, 48].

Influence of Predation on Planktonic Yeast Abundance and Yeast Biofilm Viability. Aliquots (100 μL) of ciliate suspensions were introduced to replicate flowcell channels (n = 4) containing 2-day-old unstained yeast biofilms. Using spread plates, planktonic yeast cells in the effluent were then enumerated after 6, 24, 30, 48, 54, and 72 h. At the same sampling intervals, protists were counted in individual flowcell channels. Two of the four channels were sacrificed after 1 week and the remaining two channels after 2 weeks to determine yeast viability by staining overnight (10–12 h) with 200 μL FUN-1 and visualized as described before. Live yeast cells containing red intravacuolar structures were quantified under 400× magnification in randomly selected microscope fields (n = 20–40) in every channel, ensuring a 95% confidence level that the deviation from the mean was ≤10%. Viable yeasts were expressed as percentage of total yeast number in each microscope field.

Results and Discussion

Cryptococcus biofilms formed extensive EPS covering and enveloping the cells, as observed with SEM (Fig. 1a) and epifluorescence microscopy (Fig. 1b). We used Calcofluor™ White M2R (indicating 1–3 and 1–4 β-D-glucans) and FITC-labeled Concanavalin A (indicating α-glucopyranosyl and α-mannopyranosyl units) to evaluate

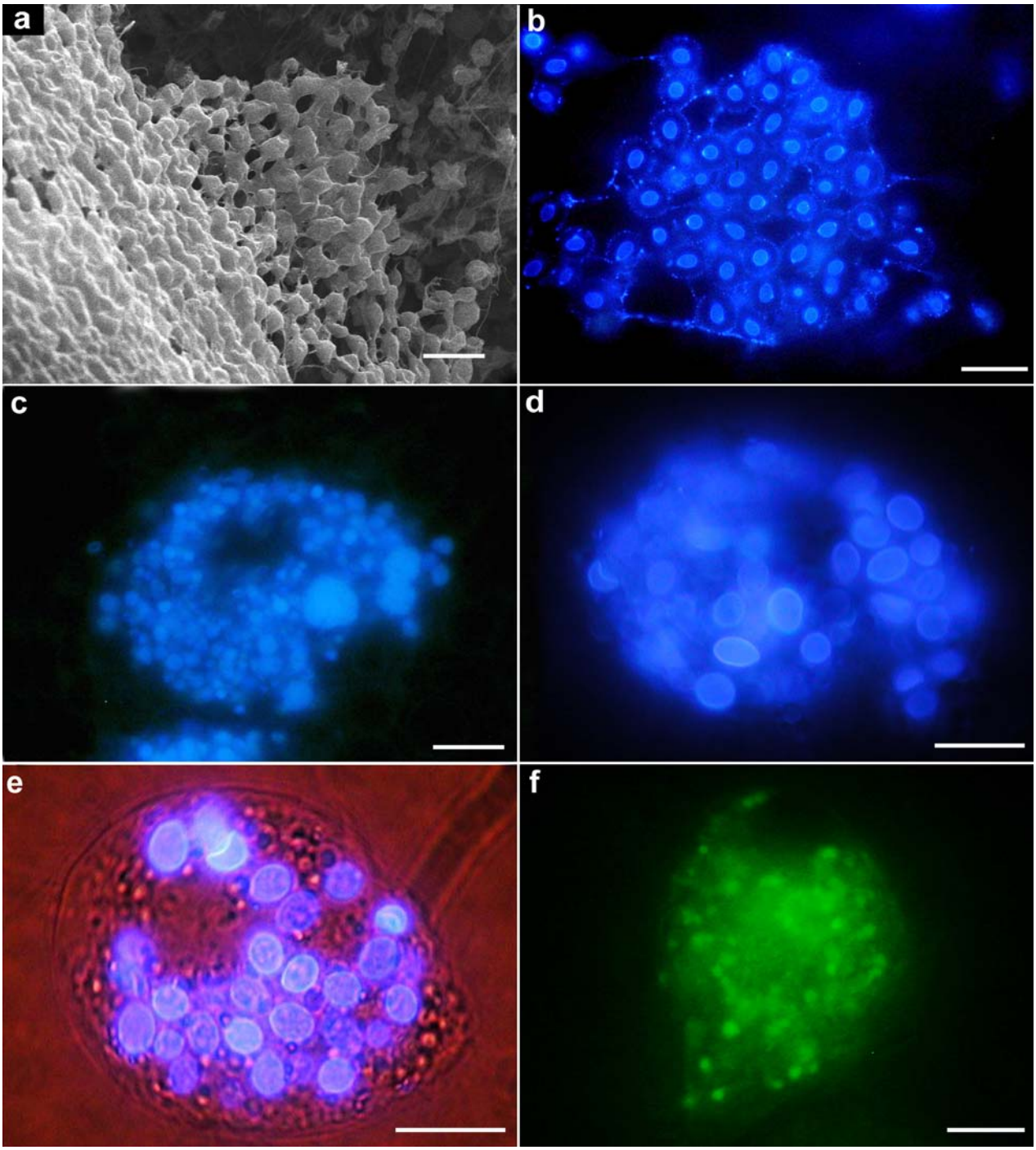

Figure 1. Photomicrographs of 3-day-old *Cryptococcus laurentii* biofilms, viewed with (a) SEM to show extensive EPS and (b) epifluorescence microscopy to show the cells and surrounding EPS stained with Calcofluor White. (c–f) Ciliate feeding preferences. In (c), there was increased fluorescence in the ciliate food vacuoles from ingestion of Calcofluor White-stained EPS while feeding on established yeast biofilms. When planktonically grown Calcofluor White-stained yeast cells were introduced into flowcells with ciliates feeding on unstained biofilms, the planktonic yeast cells were rapidly ingested (within 10 min), as seen in (d) (epifluorescence microscopy) and (e) (light epifluorescence microscopy). (f) A similar preference of the ciliate for *gfp*-labeled bacterial cells of planktonic origin. Scale bar = 10 μm.

the polysaccharide composition of the EPS. A protein assay dye (Bio-Rad) used here for *in situ* staining of yeast biofilms provided positive evidence (micrographs not shown) for the presence of extracellular proteins, which may have either a structural or regulatory function in the matrix.

To enable microscopic tracking of ingested cells and/or EPS, yeast biofilms were stained with either FUN-1 yeast viability probe, or Calcofluor White, before introduction of ciliates. FUN-1 indicates active cells, as metabolism of the stain results in fluorescent red intravacuolar structures, whereas nonviable yeast cells exhibit a yellow-green fluorescence of the cytoplasm. FUN-1 not only indicates viability, but also metabolic activity under aerobic and unaerobic conditions, with the formation of cylindrical intravacuolar structures being adenosine triphosphate dependent [38]. After introduction of the ciliate to the biofilms, there was a rapid increase in its numbers (up to 10 highly mobile ciliates per microscope field at 400× magnification after 6 h). However, there was no evidence of ingestion of whole yeast cells by the ciliates. The ciliates showed a strong preference for the noncellular fraction of the biofilms, as indicated by the increased blue fluorescence in their food vacuoles while feeding on biofilms stained with Calcofluor White (Fig. 1c). Staining of biofilm EPS with Concanavalin A resulted in corresponding green fluorescence inside the ciliate food vacuoles, whereas no intact yeast cells were found in the ciliates feeding on mature biofilms.

Planktonically grown yeasts stained with either Calcofluor White or FUN-1 [38] to allow differentiation from unstained biofilm-associated cells were subsequently introduced to unstained flowcell channels containing ciliate-yeast biofilms. Within 4–6 h, fluorescent yeast cells were taken up by the ciliates (Figs. 1d, e), demonstrating that whereas the predators preferred EPS to sessile cells, high numbers of planktonic cells were ingested. Interestingly, when fluorescently stained planktonic yeast cells were introduced to ciliates not exposed to the biofilm matrix, but fed on yeasts from batch culture and lacking the associated extracellular matrix, ingestion of the added planktonic cells occurred within 10 min, i.e., even more rapidly than when sessile cells and associated EPS were present. It seems apparent that whereas ciliates preferred grazing on the biofilm matrix, planktonic cells from batch culture are the preferred alternative food source. Sessile cells and planktonic cells derived from biofilms seem to be the least favored. Matz *et al.* [37] recently reported on planktonic cells that were eliminated by predators, whereas attached cells were afforded grazing resistance by exopolymer production (in addition to a secreted antiprotozoal factor). This implies a selective force by protozoan predation, resulting in an adaptive advantage of biofilm-forming strains. Apart from the reported consumption of transparent exopolymer particles and other oceanic gel particles [31, 43, 44, 55], limited reference has been made of the preference for biofilm exopolymers by protozoa.

Following these results, we evaluated feeding preferences when the ciliate was supplied with bacterial cells. Yeast biofilms containing ciliates were cultivated for 3 days before a *gfp*-labeled *Pseudomonas* species was introduced. Using epifluorescence microscopy and video imaging, the fate of the bacterial cells was then followed over a 72-h period. There was a rapid ingestion of planktonic *gfp*-labeled bacterial cells introduced to flowcell channels (within 10 min; Fig. 1f). However, after 12 h, the ciliates did not contain any further bacteria, although the bacteria were at this point widely distributed and proliferating throughout the biofilm, whereas the ciliates were still actively grazing. Therefore, similar to the yeast biofilm, the ciliates ingested planktonic but not sessile bacteria.

Despite the occasional observation that cells were ingested from biofilms, yeast and bacterial cells, dislodged and resuspended by grazing activity from older biofilms, were generally not consumed. This lack of ingestion of detached and resuspended biofilm cells may be the result of size-selective feeding in the case of yeasts, as previously reported [28, 34, 47], or may be evidence of a typical feast–famine phenomenon [22]. A plausible explanation that needs further investigation is that the EPS produced by mature sessile cells have a composition and nutritional value different from those produced by planktonic and recently detached cells. Surface-associated compounds signaling chemical cues for rejection to predators have previously been recognized in planktonic protists [4, 51, 58]. Large numbers of biofilm-released cells were detected in the bulk liquid phase by microscopy and standard plate counts. We demonstrated a notable yield of bacterial cells from biofilms to the planktonic phase ($>10^6$ cells mL^{-1}) in a parallel study under similar conditions where dilution rate greatly exceeded maximum specific growth rate (>30×) [2]. In this study, the cell yield from the yeast biofilms typically ranged between 5.2×10^5 and 5.7×10^6 cells mL^{-1}.

Our next objective was to determine whether the ciliates similarly differentiate between the cellular and noncellular fractions of yeast and bacterial cultures during excystment and subsequent proliferation. We hypothesized that EPS, including the type and age of EPS, may impact on ciliate excystment by serving both as stimulant for excystment and food source to active ciliates.

The yeasts *C. laurentii* and *C. podzolicus*, and two bacterial strains, a *Dyadobacter* and a *Pseudomonas* sp., were grown to logarithmic as well as stationary phase. Washed cells, cell suspensions, and cell-free supernatant fractions were prepared from these cultures. Aliquots of a suspension containing only ciliate cysts were subsequently dispensed in microtiter plates with these different

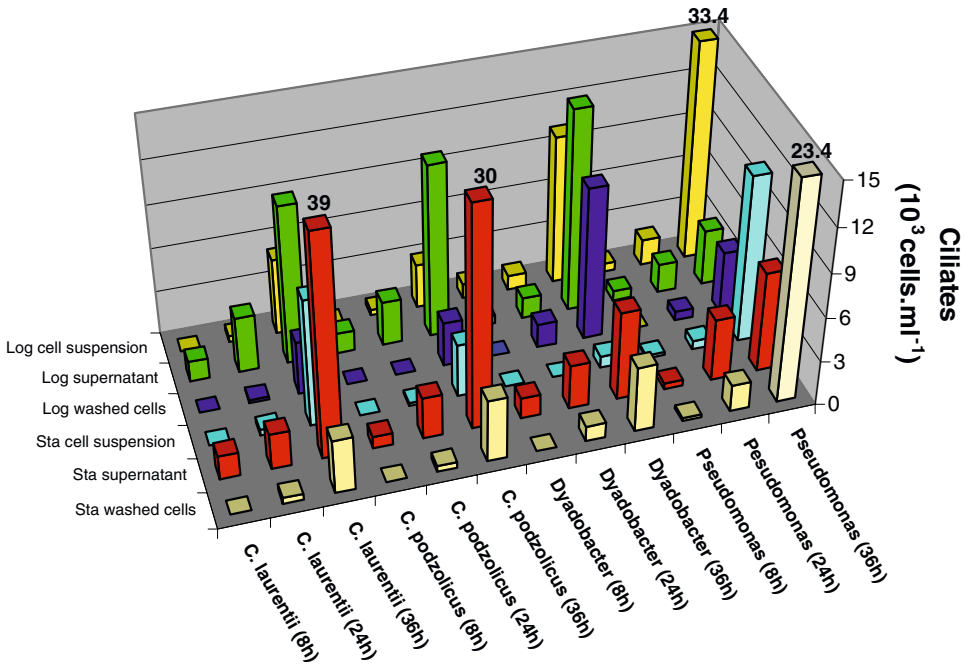

Figure 2. Effect of prey type, growth phase, and the fraction of yeast and bacterial cultures on ciliate excystment. Washed cells, cell suspensions, and cell-free supernatant were prepared in triplicate from logarithmic (Log) and stationary (Sta) phase cultures of the yeasts *C. laurentii* and *C. podzolicus*, and two bacteria, namely, a *Dyadobacter* and *Pseudomonas* species. Excystment of ciliate cysts was quantified in triplicate samples after 8, 24, and 36 h. Enumeration was carried out in individual microscope fields (n = 20–40) until a 95% probability had been reached that there was ≤10% deviation from the mean.

fractions, and the resulting excystment and protistan proliferation quantified microscopically after 8, 24, and 36 h.

Similar to active ciliate cells, the cysts had a varied response to different fractions of the yeast and bacterial cultures (Fig. 2). The highest occurrence of excystment was observed when cysts were provided with cell-free supernatant of either stationary or log-phase cultures of the prey suspensions. Treatment with washed cells resulted in the lowest incidence of excystment. This suggests that the stimulant for excystment is located extracellularly of the yeast and bacterial cells, probably accumulating in the EPS, which also serve as a carbon source for ciliates grazing mainly on the biofilm matrix once the planktonic cells have become immobilized at a surface. These results also suggest that EPS act as an interface between different trophic levels. With yeast cells, this effect continued throughout the 36-h observation period. In the case of bacterial cultures, the prominent effect of the cell-free supernatant was more varied and did not differ from the effect caused by washed cells after 36 h, suggesting a more prominent role of bacterial cells as carbon source once excystment had occurred. It is also possible that differences between exudates of the yeasts and bacteria applied in this study, and accumulated in the EPS, may have different long-term effects on excystment. *Cryptococcus* is known to produce biologically active metabolites [5], whereas pseudomonal exoproducts have been indicated as virulence factors targeting eukaryotic pathways with probable ecological function [36]. Studies on the influence of dissolved and cell surface-associated chemical clues on predation [53], as well as on microenvironments such as the rhizosphere [4], referred exclusively to planktonic organisms. Nevertheless, these studies exemplified the complexity of predator–prey interactions in various environmental niches.

Following the demonstration of preferential feeding on biofilm EPS and batch-derived planktonic cells, we investigated the effect of ciliate grazing on biofilm formation and activity to evaluate the assumption that grazing reduces biofilm biomass through dislodging and ingestion of cells. For this purpose, we applied a photometer measuring the intensity of light scattered by *C. laurentii* biofilms in a large-area flowcell. After inoculation, initial washout of unattached yeast cells was followed by an exponential increase in biofilm biomass until a quasi steady state was reached (~40 h; Fig. 3). The minor fluctuations evident during periods of general increase can be attributed to continuous attachment and detachment of individual yeast cells during biofilm development, leading to architectural changes. Addition of protists typically resulted in a more variable biofilm structure with continuous fluctuation in biomass. This variation was probably caused by active dislodging of cells by the ciliates feeding on the adhesive EPS matrix, followed by increased biofilm growth. It was indeed found that no sustained decrease in biofilm biomass occurred, as would have been expected if the ciliates obtained their nutrients primarily from ingesting biofilm

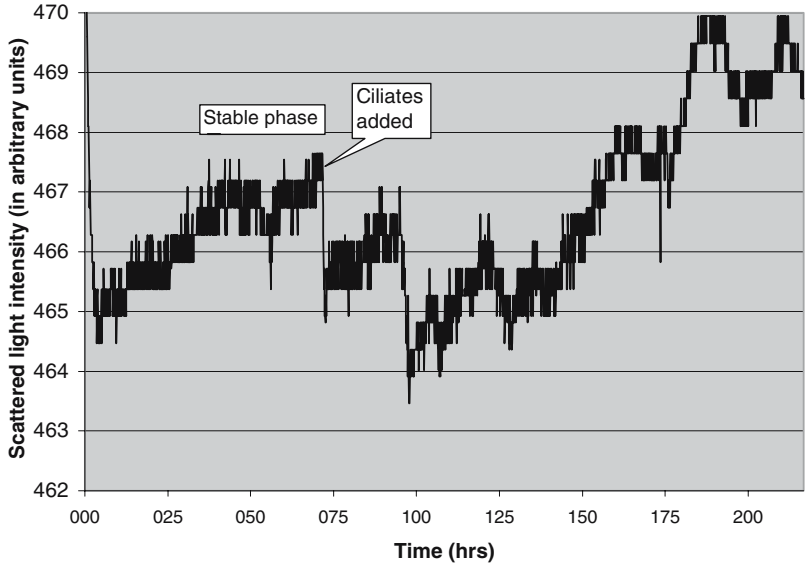

Figure 3. Typical example to show the effect of predation on sessile biomass of the yeast *C. laurentii*. After introduction of the ciliates, the extent of biofilm biomass, measured photometrically in an adapted parallel plate flowcell, generally became more variable. There was an overall increase in biofilm biomass following the introduction of predators in all cases.

cells. In fact, a general increase (>150 h in Fig. 3) in biofilm biomass was repeatedly observed in replicate experiments after introduction of protists.

To address the questions whether there was a concomitant increase in the number of viable cells within the biofilms, and whether predation would influence the number of cells released from biofilms, we introduced aliquots containing ciliate suspensions into triplicate flowcell channels 2 days after inoculation of the yeasts and cultivated the biofilms for two more weeks. Planktonic yeast cells in the effluent were quantified using spread plates, whereas protists were enumerated microscopically in individual flowcell channels. Metabolic activities of yeast biofilms were subsequently evaluated by staining with FUN-1 yeast viability probe. There was a positive correlation ($r^2 = 0.85$) between the number of protists in the flowcells and the number of yeast cells in the effluent (Fig. 4), providing support for earlier observations that protists cause release of cells from biofilm communities [24]. However, our data showed that such

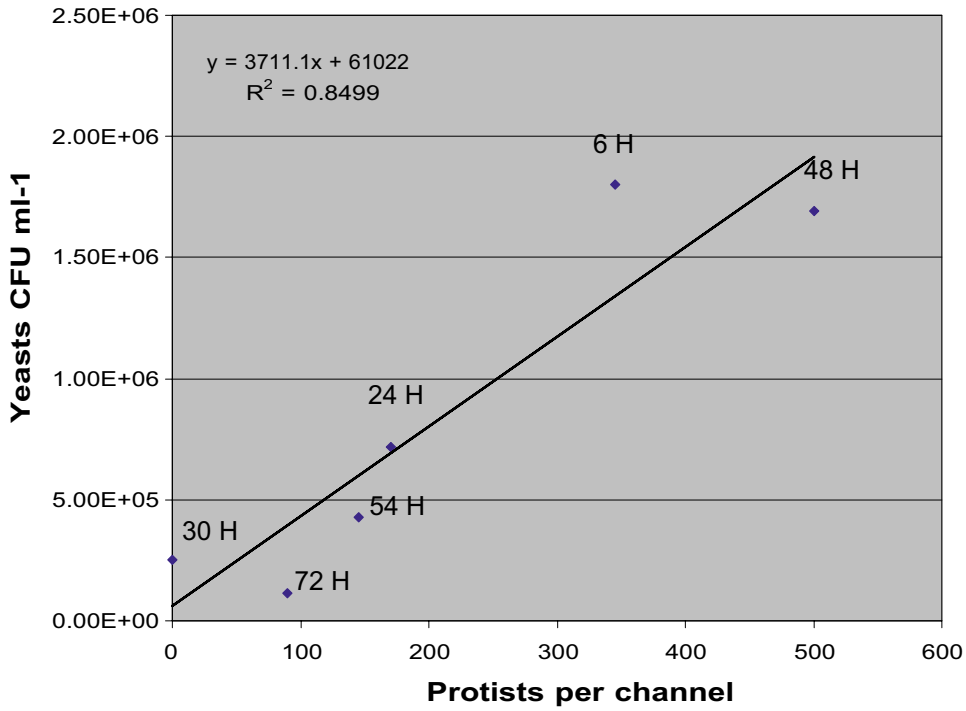

Figure 4. Typical data to show the correlation between the numbers of biofilm-associated ciliates (total number visualized in 50 fields at 400× original magnification) and planktonic cell yield by biofilms. The values at the data points represent the time (in hours) of sampling after introduction of ciliates to 2-day-old biofilms and show the dynamic nature of yeast-ciliate biofilms with no correlation between biofilm age and protist or yeast numbers. However, the positive correlation between the number of ciliates per flowcell channel and yeast cells in the respective effluent, together with the overall increase in biofilm biomass during predation as shown in Fig. 3, suggests that protistan grazing enhances biofilm productivity.

release did not cause reduction in biofilm biomass (Fig. 3), but rather enhanced metabolism and growth, and ultimately overall biofilm activity. We discussed elsewhere the role of biofilms as a cell factory where active outer biofilm layers sustain the planktonic phase under conditions of flow when the dilution rate greatly exceeds planktonic growth rate [2]. The data from the present study suggest that predation contributes to the maintenance and even enhancement of activity in biofilms. After 1 week, the number of metabolically active cells in the yeast biofilms was 1.75 times higher in the presence of protists compared to controls without predation.

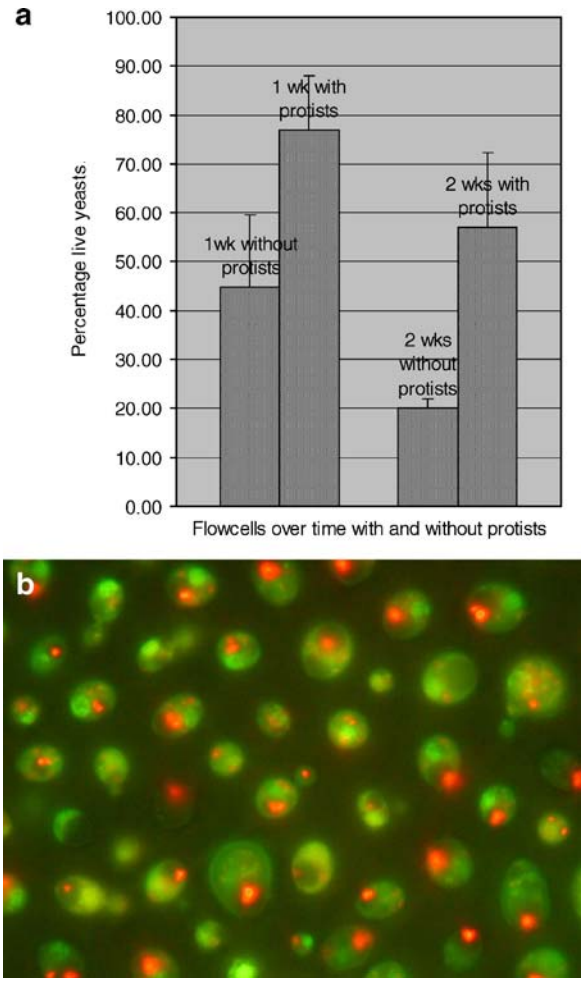

Figure 5. (a) Graph illustrating influence of protists on biofilm viability over time. Yeast biofilms were cultivated in duplicate flowcells for 2 weeks on oligotrophic medium with inclusion of protists after 3 days. Viability was evaluated after 1 and 2 weeks by staining the biofilm with FUN-1 yeast viability probe and enumeration by direct visualization with epifluorescence microscopy. Quantification in randomly selected microscope fields (n = 20–40) were carried out until a 95% confidence level was reached that the deviation from the mean was ≤10%. FUN-1 indicates active cells, as metabolism of the stain results in fluorescent red intravacuolar structures as shown in (b).

Although there was a decrease in overall yeast viability after 2 weeks, the ratio of live-to-dead cells was still 2.85 times higher in biofilms with protists (Fig. 5). Previous observations on aquatic bacteria suggested that planktonic cell metabolism responded similarly when challenged with predation [17]. Although the influence of ciliate, flagellate, and amoeboid predation on spatial and temporal biofilm structure has been noted by various authors [23, 24, 30, 33], this was mostly attributed to ingestion of biofilm cells, or formation of microcolonies, and not metabolic perturbations caused by either physical cell sloughing or ingestion of EPS by predators.

The significance of symbiotic relationships between protists and other microorganisms is increasingly being recognized as influential in determining environmental survival. The significance of protists as environmental reservoirs for pathogens may impact on antimicrobial resistance similar to that of biofilm inhabitants [6]. Facilitated survival and intracellular replication of capsular yeast strains in phagocytic cells have recently been described [52]. While not a focus of our study, we frequently observed whole yeast cells within cysts (data not shown). Staining with the FUN-1 viability probe, we observed that these cells were often intact and viable, suggesting that the interaction may afford the yeast protection against environmental stress conditions. A recent description of the increased degree of complexity and cooperation of protective mechanisms against predation [36] includes post-ingestional adaptations in bacteria–protist interactions. Similar exposure to acidification and enzymatic degradation within the ciliate food vacuole will have to be overcome by ingested *Cryptococcus* yeasts, probably including protective cell-wall layers, and secretion of bioactive metabolites. The two nonpathogenic Cryptococci used in this study may be useful model strains to study the intracellular survival strategies of the pathogen *Cryptococcus neoformans*. More generally, such results may provide interesting clues to the appearance of nonmotile yeasts in unexpected places and their survival under adverse conditions.

As organisms in a complex biofilm exist as different trophic levels in a food web, the phenotypic switch between planktonic and biofilm cells may have important implications for the flow of energy through the dynamic biofilm system. An important consequence of the preferential feeding on planktonic versus biofilm yeast and bacterial cells may be a resultant shift in community composition where predators are included in microbial communities. According to Jurgens and Matz [25] both genotypic and phenotypic bacterial community structure may be determined by protozoan grazing on planktonic communities. Protozoan grazing has been described as a "structuring" factor or "top-down pressure" impacting on the taxonomical, morphological, and functional composition of bacterial communities [4, 17, 26, 34].

However, it has been noted by Matz and Jurgens [35] that different microbial morphotypes survived in the presence of flagellate predators, even when community composition was not notably influenced. Community structure seems to be determined by the combined effects of predation and other factors (e.g., nutrient limitation). In the presence of attached biofilm cells, where exopolymers may be the preferred food source, as indicated by our study, the planktonic community derived from biofilm cells may therefore have a selective advantage for survival and proliferation. This advantage may furthermore depend on motility and form, as described by Matz and Kjelleberg [36], and also on size and nonmorphological secretory and surface-related cell properties [34, 37, 43]. It has recently been argued [36] that such selective advantage of bacterial consortia challenged by predation could have been imperative in the evolution of adaptive mechanisms in biofilms and multicellularity.

Inclusion of predators in biofilm communities, with the resultant effect of grazing on biofilm EPS, may furthermore imply a shift in nutrient availability as the concentration of nutrients accumulated in EPS may fluctuate in correlation to predation. Not only will the accumulated carbon and other nutrients in the noncellular matrix be ingested, providing a bypass of the microbial loop similar to marine systems [43, 44], but also the resultant stimulation in biofilm cell viability has here been indicated to result in increased biomass. Alternative feeding on the polymeric matrix and planktonic cells may also enable different fractions of the microbial ecosystem to proliferate without the diminishing effect that predation can have on their cell numbers. We propose that a feedback response to predation can lead to typical succession patterns [36], also for feeding on EPS versus planktonic microbial cells. Exopolymer particle-associated bacteria and polymer gel particles, which are ingested by euphasiids, cocepods, and other macroinvertebrates [8, 9, 11, 14, 44, 46], provide a significant trophic link between bacteria and larger consumers [18, 43, 55]. It has been postulated [43] that an aggregation web linking the microbial loop to the classical food web should be incorporated in the food-web concept. Our results support this requirement, providing data from the perspective of biofilm exopolymers.

These results are in line with those from various other studies (e.g., [3, 17, 34, 36, 47]), indicating that protistan grazing involves complex interactions. This study further suggests that associations in biofilms lead to differentiation and consequently to further complexity. It is surprising that predation is relatively seldom taken into account when evaluating biofilm metabolism. Overall, our findings suggest a reevaluation of the widely held contention that protistan predation on biofilms has predominantly negative consequences for the prey organisms (e.g., [23, 25, 36]). In fact, as shown here, predation on biofilms leads to increase in biofilm biomass, numbers of biofilm-derived cells, and cell viability.

In conclusion, considering the differential grazing on planktonic and sessile cells demonstrated in this study, it is probable that currently uncharacterized differences between the planktonic and biofilm phases may have important implications for the flow of energy through microbial communities. Through their grazing on microbial EPS, protistan predators may affect notable shifts in nutrient availability. The close association in the biofilms between ciliates and yeasts suggests an evolutionary adaptation for cooperation and survival. It appears highly probable that EPS serve as an important shared resource, or interface, that facilitates such cooperation.

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