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Research Article

RESEARCH ARTICLE

Grazing resistance of bacterial biofilms: a matter of predators' feeding trait

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One sentence summary: Grazing resistance in bacterial phenotypes (plankton versus biofilm) depends on the feeding trait of the grazer rather than being an intrinsic attribute of biofilms.

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ABSTRACT

Biofilm formation in bacteria is considered to be one strategy to avoid protozoan grazing. However, this assumption is largely based on experiments with suspension-feeding protozoans. Here we test the hypothesis that grazing resistance depends on both the grazers' feeding trait and the bacterial phenotype, rather than being a general characteristic of bacterial biofilms. We combined batch experiments with mathematical modelling, considering the bacterium *Pseudomonas putida* and either a suspension-feeding (i.e. the ciliate *Paramecium tetraurelia*) or a surface-feeding grazer (i.e. the amoeba *Acanthamoeba castellanii*). We find that both plankton and biofilm phenotypes were consumed, when exposed to their specialised grazer, whereas the other phenotype remained grazing-resistant. This was consistently shown in two experiments (starting with either only planktonic bacteria or with additional pre-grown biofilms) and matches model predictions. In the experiments, the plankton feeder strongly stimulated the biofilm biomass. This stimulation of the resistant prey phenotype was not predicted by the model and it was not observed for the biofilm feeders, suggesting the existence of additional mechanisms that stimulate biofilm formation besides selective feeding. Overall, our results confirm our hypothesis that grazing resistance is a matter of the grazers' trait (i.e. feeding type) rather than a biofilm-specific property.

Keywords: protozoa; biofilm; plankton; predator-prey model; grazing defence; feeding trait

INTRODUCTION

Studies on planktonic systems have revealed that protozoan grazing on bacteria is a key interaction within microbial food webs (e.g. Hahn and Höfle 2001; Sherr and Sherr 2002). Protozoans control bacterial biomass (e.g. Hahn and Höfle 2000; Garcia-Chaves *et al.* 2015) and community composition (e.g. Jürgens and Matz 2002; Chow *et al.* 2014). Within the food web, they link bacterial production to higher trophic levels and are thus important players which mediate mineralisation of bacterial biomass (e.g. Sherr and Sherr 2002). Protozoan grazing on bacteria is considered the oldest predator–prey relationship. This long evolutionary history, together with the omnipresence of this interaction, supports the evolution of diverse defence strategies in bacteria. Such defence strategies include morphological changes (e.g. cell shape, Hahn and Höfle 1999;

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Simek *et al.* 2001), the production of virulence factors (Weitere *et al.* 2005; Erken, Lutz and McDougald 2013) and behavioural adaptations such as swimming speed (Matz and Jürgens 2005). In this context, biofilm formation, i.e. the formation of matrix-embedded bacteria adherent to each other and to interfaces, is also considered to be one strategy to avoid protozoan grazing (e.g. Costerton *et al.* 1999; Matz and Kjelleberg 2005; Justice *et al.* 2008). It was shown in both controlled laboratory experiments (Matz, Deines and Jürgens 2002) and in field-related flow cell experiments with natural biofilm communities (Wey *et al.* 2008) that especially heterotrophic flagellates stimulate the formation of bacterial biofilms in early stages of biofilm formation.

These studies greatly stimulate the discussion on grazing losses of biofilms but do not unambiguously allow to conclude that bacterial biofilms are resistant against predators per se. This is challenged by the fact that many specialised surface-feeding protozoans exist within benthic microbial communities, especially among some ciliates and amoebae (e.g. Arndt *et al.* 2003; Parry 2004). In fact, grazing studies which consider those grazers such as the amoebae *Acanthamoeba* sp. revealed efficient grazing of bacterial biofilms (Huws, McBain and Gilbert 2005; Weitere *et al.* 2005; Queck *et al.* 2006). This finding suggests that grazing resistance of biofilms is restricted to certain grazers being able to feed only on suspended or at least single-cell bacteria. This view is in line with field (Šimek *et al.* 2014) and lab studies (Salcher *et al.* 2005) in planktonic systems, which showed that the formation of less grazing-vulnerable phenotypes is a highly dynamic process and that different bacterial phenotypes could develop, which show only temporary resistance towards specific grazer communities. Besides these ecological sorting processes, prey organisms can rapidly evolve grazing resistance (Yoshida *et al.* 2003; Meyer *et al.* 2006; Hall, Meyer and Kassen 2008; Koch *et al.* 2014; Friman *et al.* 2016). Such results highlight the importance of the succession of different defence strategies and thus the relevance of system dynamics in understanding grazing resistance. Transferring this dynamic view to plankton–biofilm systems, we ask whether grazing resistance is a dynamic trait of prey bacteria depending on the predator's feeding type. The trait-based approach (e.g. Litchman and Klausmeier 2008; Litchman *et al.* 2012) helps to develop a differentiated view on grazing interactions within microbial food webs. When considering specialised predators, one feeding on suspended prey and the other feeding on biofilm prey, the trait 'feeding type' has different values which results in a feeding trait variation in the predator guild. We test the hypothesis that grazing resistance in bacterial phenotypes (plankton versus biofilm) is dynamic and depends on the feeding trait variation of the grazer. We assume that when a single grazing type is present, the non-preferred prey phenotype (i.e. either biofilm or plankton) is stimulated, and that in the presence of two grazing types (i.e. biofilm and plankton feeders) efficient control of the total bacterial biomass is possible. In batch experiments, we considered two different feeding trait values of protozoans a suspension feeder (the ciliate *Paramecium;* Foissner Berger and Kohmann 1994; Weerman *et al.* 2011) and a surface feeder (the amoebae *Acanthamoeba* sp.; Huws, McBain and Gilbert 2005; Weitere *et al.* 2005). Additionally, two different scenarios were considered: an early stage of biofilm formation (start with pure planktonic bacteria) and a later stage of biofilm formation (significant amount of bacterial biofilm already formed). To obtain further insight into the effects of the feeding interactions on prey and predator biomass dynamics, we derived a mathematical model that accounts for the mechanisms (predation, resource competition of prey and migration of bacteria cells between plankton and biofilm phenotype) considered as relevant based on our pre-knowledge. With this, we attempt to reproduce the observed dynamics through numerical simulations.

MATERIALS AND METHODS

We conducted grazing experiments with bacterial prey and protozoan predators and simulated the experiments in a mathematical model using Wolfram Mathematica 10.0.2.0. During the experiments, the development of predators was monitored by light microscopy, and the prey biovolume in the plankton and biofilm were analysed by confocal laser scanning microscopy (CLSM).

Organisms and culture media

We used *Pseudomonas putida* KT2440 as the bacterial prey organism in this study. This strain is chromosomally tagged with a consistently expressed gene coding for a yellow florescent protein (YFP) (Seoane *et al.* 2011). As predators, the planktivorous ciliate *Paramecium tetraurelia* 7S (Sonneborn 1975) (plankton predator) and the biofilm grazer *Acanthamoeba castellanii* (biofilm predator) were used. Protozoa and bacteria were routinely cultured at 28°C in Neff's amoeba saline (Page and Siemensma 1991) supplemented with 300 mg L^{-1} glucose and 0.5 mg L^{-1} stigmasterol (a steroid essential for *Paramecium* cultivation). Axenic cultures of *P. tetraurelia* and *A. castellanii* were diluted stepwise and fed with the prey strain *Ps. putida* KT2440, in order to obtain monoxenic protozoan cultures for the experiments. The culture medium and growth conditions for experiments and routine cultivation were identical.

Grazing experiments: set-up and microscopic analysis

We conducted two types of batch experiments in which monoclonal plankton and biofilm prey were introduced to plankton and/or biofilm predators; only the developmental stage of biofilm prey differed between both experiments (early-stage or matured biofilms). Four treatments with different predator guild compositions were applied: (i) protozoan-free control, (ii) plankton predator only (P.tet), (iii) biofilm predator only (A.cast.) and (iv) plankton and biofilm predator (P.tet. + A.cast.). In the early biofilm set-up (experiment 1), planktonic bacteria and protozoa were simultaneously inoculated and biofilm could establish during the experiment. This approach was applied in order to test the grazing effect on young biofilms within the establishment phase. In the late biofilm set-up (experiment 2), an overnight bacterial culture (10^8 cells mL^{-1}) was introduced into the wells and the set-up was left for 4 days for biofilms to establish. After that, the overlaying medium was replaced with fresh bacterial overnight culture, and either predator culture or protozoan-free filtrate was added. The predator-free filtrates were prepared by filtering the culture through a 0.5-μm pore-size syringe filter (Satorius). This addition of grazer-free filtrate was done to have equal contributions of the different culture mediums in each treatment. In order to introduce comparable quantities of both predators to the experiments, the biovolume of amoebae and ciliates were determined beforehand as described by Hillebrand *et al.* (1999) (for starting densities of both predators, see Table 1). The bacterial inoculum originated from an overnight culture and was adjusted to an initial concentration of 6×10^6 cells mL^{-1} in each well. All treatments were set up in five replicates and experiments were run in

Table 1. Early and late biofilm experiments: protozoan abundances at the beginning and the end of the experiments.

Predator	Experiment	Treatment	Start abundance (cells mL^{-1})	End abundance (cells mL^{-1})
Paramecium tetraurelia	Early biofilm experiment	P.tet.	27 (2)	66 (25)
		P.tet. + *A.cast.*	24 (1)	26 (5)
Acanthamoebae castellanii	Early biofilm experiment	*A.cast.*	3160 (303)	2896 (133)
		P.tet. + *A.cast.*	2127 (36)	1798 (112)
Paramecium tetraurelia	Late biofilm experiment	*P.tet.*	40 (10)	36 (8)
		P.tet. + *A.cast.*	41 (5)	27 (6)
Acanthamoebae castellanii	Late biofilm experiment	*A.cast.*	1847 (300)	6825 (309)
		P.tet. + *A.cast.*	1853 (236)	4372 (210)

The values represent are the mean of five independent experiments and their standard deviation in brackets.

Table 2. Model parameters, their biological interpretations and units, and the values used in the simulations.

Parameter	Description	Value	Units	Reference
e_B	Conversion efficiency bacteria	0.2	-	Fuhrman and Azam (1982)
r_{PL}	Growth rate plankton	0.21	h^{-1}	Own data
r_{BF}	Growth rate biofilm	0.007	h^{-1}	Own data
H_{PL}	Half-saturation constant plankton growth	1	$\mu g\ C\ mL^{-1}$	Monteiro, Boaventura and Rodrigues (2000)
H_{BF}	Half-saturation constant biofilm growth	1	$\mu g\ C\ mL^{-1}$	Monteiro, Boaventura and Rodrigues (2000)
g_{PA}	Maximum grazing rate *Paramecium*	0.12	h^{-1}	Own data
g_{AM}	Maximum grazing rate *Acanthamoeba*	0.09	h^{-1}	Huws, McBain and Gilbert (2005)
H_{PA}	Half-saturation constant *Paramecium*	1	$\mu g\ C\ mL^{-1}$	Own data
H_{AM}	Half-saturation constant *Acanthamoeba*	0.1	$\mu g\ C\ cm^{-2}$	Huws, McBain and Gilbert (2005)
e_{PA}	Conversion efficiency *Paramecium*	0.5	-	Own data
e_{AM}	Conversion efficiency *Acanthamoeba*	0.33	-	Huws, McBain and Gilbert (2005)
χ_{BF}	Exchange rate $BF \rightarrow PL$	0.005	h^{-1}	Own data
χ_{max}	Maximum exchange rate $PL \rightarrow BF$	0.05	$cm\ h^{-1}$	Own data
χ_{min}	Minimum exchange rate $PL \rightarrow BF$	0.0005	$cm\ h^{-1}$	Own data
a	Strength of density dependence in migration	0.01	$\mu g\ C\ cm^{-2}$	Own data
S	Surface area for biofilm	8	cm^2	Own data
V	Total volume of medium	3	ml	Own data

Parameter values obtained from own experiments are labelled with 'own data'.

24-well tissue culture plates with a final volume of 2.5 mL. After inoculation, the experiments were incubated on a shaker (170 r.p.m.) at 28°C for 3 or 4 days for experiment 1 or experiment 2, respectively.

During the experiment, the development of predator abundance and prey biovolume was analysed daily. The fluorescence of YFP-producing planktonic and biofilm prey bacteria was monitored by CLSM. For this purpose, a TCS SP1 (Leica Wetzlar, Germany) equipped with an inverted microscope and an Ar, DPSS and He/Ne laser was used. The system was controlled by the Confocal software version 2.6.1. Image data sets were recorded at an excitation of 514 nm and an emission range of 525–600 nm. Samples were examined using a 63x NA 1.2 water immersion lens and a step size of 0.5 μm. Bacterial biofilms established at the surface of the wells were scanned at five randomly chosen spots. To analyse bacterial plankton biovolume, a 0.1 mL sample was taken from every well and filtered through a 0.2-μm pore-size, 25-mm-diameter Anodisc filter (Whatman). The cells collected on the filter were analysed by CLSM as described above for biofilm monitoring. Subsequently, planktonic and biofilm prey biovolume was calculated from the xyz scans with the ImageJ software (Rasband WS, ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/1997–2014) and a self-developed plug-in (Staudt *et al.* 2004). Pixels with a lower intensity than 33 were subtracted from the 8-bit image stacks to remove the background.

Table 3. State variables and their units used for model simulations.

State variable	Description	Units
AM	Biomass *Acanthamoeba*	$\mu g\ C\ cm^{-2}$
BF	Biomass biofilm bacteria	$\mu g\ C\ cm^{-2}$
C	Carbon concentration of the medium	$\mu g\ C\ mL^{-1}$
PA	Biomass *Paramecium*	$\mu g\ C\ mL^{-1}$
PL	Biomass planktonic bacteria	$\mu g\ C\ mL^{-1}$
χ_{PL}	Exchange rate $PL \rightarrow BF$	$cm\ h^{-1}$

Additionally, the abundance of protozoa was determined with a stereo microscope (Nikon SMZ 1000) (*P. tetraurelia*) and inverse light microscopy (Zeiss Axio Observer Z1) (*A. castellanii*).

Model structure and simulations

We developed a model describing the dynamics of the organic carbon in the medium (i.e. the food source for the bacteria), the prey and the predators. Model parameterisation was based on own measurements as far as possible; otherwise literature values were used (see Table 2 for values and units).

The temporal changes in carbon concentrations (*C*), biomass of planktonic and biofilm bacteria (*PL* and *BF*) and biomass of *P. tetraurelia* and *A. castellanii* (*PA* and *AM*) are described by the following set of differential equations and the units are given in Table 3:

$$\frac{dC}{dt} = -\frac{1}{e_B}\left(\underbrace{r_{PL}\frac{C}{C+H_{PL}}PL}_{\text{growth plankton bacteria}} + \underbrace{r_{BF}\frac{S}{V}\frac{C}{C+H_{BF}}BF}_{\text{growth biofilm bacteria}}\right) \quad (1)$$

$$\frac{dPL}{dt} = \underbrace{r_{PL}\frac{C}{C+H_{PL}}PL}_{\text{growth plankton}} - \underbrace{g_{PA}\cdot PA\frac{PL}{PL+H_{PA}}}_{\text{grazing by P. tetraurelia}} - \underbrace{\chi_{PL}\cdot\frac{S}{V}\cdot PL}_{\text{migration PL->BF}} + \underbrace{\chi_{BF}BF\cdot\frac{S}{V}}_{\text{migration BF->PL}} \quad (2)$$

$$\frac{dBF}{dt} = \underbrace{r_{BF}\frac{C}{C+H_{BF}}BF}_{\text{growth biofilm}} - \underbrace{g_{AM}\cdot AM\frac{BF}{BF+H_{AM}}}_{\text{grazing by A. castellanii}} + \underbrace{\chi_{PL}PL}_{\text{migration PL->BF}} - \underbrace{\chi_{BF}BF}_{\text{migration BF->PL}} \quad (3)$$

$$\frac{dPA}{dt} = \underbrace{e_{PA}g_{PA}\cdot PA\frac{PL}{PL+H_{PA}}}_{\text{grazing by P. tetraurelia}} \quad (4)$$

$$\frac{dAM}{dt} = \underbrace{e_{AM}g_{AM}\cdot AM\frac{BF}{BF+H_{AM}}}_{\text{grazing by A. castellanii}} \quad (5)$$

Growth of both prey phenotypes (i.e. biofilm and plankton) is limited by the carbon concentration in the medium, C. It is modeled as a type II functional response, with maximum growth rates r_{PL} and r_{BF} and half-saturation constants H_{PL} and H_{BF}, respectively. Biofilm growth is typically much slower than plankton growth (see Table 2). The conversion efficiency of C into bacterial biomass is given by e_B. S/V is the surface-to-volume ratio of the wells, reflecting that biofilm density is measured (in the model) in μg C cm^{-2}, while plankton density and carbon concentrations are measured in μg C mL^{-1}.

Both planktonic and biofilm prey are consumed by specialist grazers: *P. tetraurelia* feeds exclusively on plankton and *A. castellanii* exclusively on biofilm prey. Their type II functional responses are described by the maximum grazing rates g_{PA} and g_{AM}, and half-saturation constants H_{PA} and H_{AM} for *P. tetraurelia* and *A. castellanii*, respectively. The efficiency of conversion of prey into predator biomass is given by e_{PA} and e_{AM}. Non-grazing mortality in the prey and starvation mortality in the predators are ignored because the experiments are not expected to run long enough for significant mortality to take place.

Finally, migration between plankton and biofilm is characterised by the per capita migration rates χ_{PL} (migration from plankton to biofilm) and χ_{BF} (migration from biofilm to plankton). The per capita detachment rate of biofilm χ_{BF} is assumed to be constant. In contrast, a constant attachment rate does not well capture the colonisation of empty surface to form biofilms (Fig. S1, Supporting Information). Based on migration experiments, we assume that the migration rate into biofilm declines with biofilm density:

$$\chi_{PL} = \frac{a\cdot\chi_{max} + \chi_{min}\cdot BF}{a + BF} \quad (6)$$

where χ_{max} is the maximum migration rate at a completely empty surface, and the function declines asymptotically to the minimum migration rate χ_{min}; a describes the steepness of this decline; χ_{max} and a were estimated from our migration data; χ_{min} is assumed to be small (1% of χ_{max}).

For the initial values of the simulations, the starting concentrations of the four experimental treatments were converted into carbon concentrations according to Romanova and Sazhin (2010) (per mL for C, PL and PA, and per cm^2 for BF and AM). In order to compare empirical and modelling results, simulations were run for 72 h (early biofilm experiment) or 96 h (late biofilm experiment) according to the endpoints of the respective experiment. Finally, the total carbon accumulated in PL and BF was converted into total bacterial biovolume for comparison with experimental data (Romanova and Sazhin 2010).

Statistical analysis

The statistical data analysis was performed with R and the R add-on package MASS (R Core Team 2015). Plankton data was log-transformed and biofilm data was cube root transformed to achieve homogenous variances. A one-factorial analysis of variance (ANOVA) was conducted in order to test for significant differences in plankton and biofilm biovolume between the treatments (control, P.tet, A.cast. and P.tet. + A.cast.). If the ANOVA indicated significant treatment effects, a multiple treatment contrast analysis between the four treatments was conducted to determine statistical differences between treatments. Treatment effects on total prey guild biovolume in the predator-free control and the *P. tetraurelia* treatment (P.tet.) were tested with the Wilcoxon test.

RESULTS

The two grazers, i.e. *Paramecium tetraurelia* and *Acanthamoeba castellanii*, had contrasting effects on planktonic and biofilm bacteria with consistent trends in both experiments (Fig. 1A and B). *A. castellanii* strongly and significantly reduced the early-stage biofilms to 5% (exp. 1; Fig. 1A) and mature biofilms to 2% compared to the control (exp. 2; Fig. 1B). As a result, biofilm fractions in the total prey guild dropped down to 0.4% and 2.9% in experiment 1 and 2, respectively (Fig. 2C and D). In contrast to this, the surface-feeding amoeba had no significant impact on plankton prey biovolume in both experiments (Fig. 1A and B). Experiments supplemented with the suspension feeder *P. tetraurelia* showed the opposite effect: plankton prey biovolume decreased to 38% in experiment 1 (Fig. 1A) and to 35% in experiment 2 (Fig. 1B). Furthermore, we found a significant increase of biofilm biovolume when the ciliate *P. tetraurelia* was present (to 304% in early biofilms and to 191% in late biofilms, relative to the control; Fig. 1A and B). The co-cultivation of *A. castellanii* and *P. tetraurelia* led to a strong reduction of both planktonic and biofilm prey (Fig. 1A and B). Thus, plankton biovolume was reduced to 17% in experiment 1 and to 10% in experiment 2 and biofilm biovolume decreased to 1% in both experiments (Fig. 1A and B).

Early biofilm experiments were dominated by plankton prey and only 5.6% biofilm prey was found after 3 days in the predator-free control (Fig. 2C). In contrast, the late biofilm experiment was set up with a pre-grown biofilm; consequently,

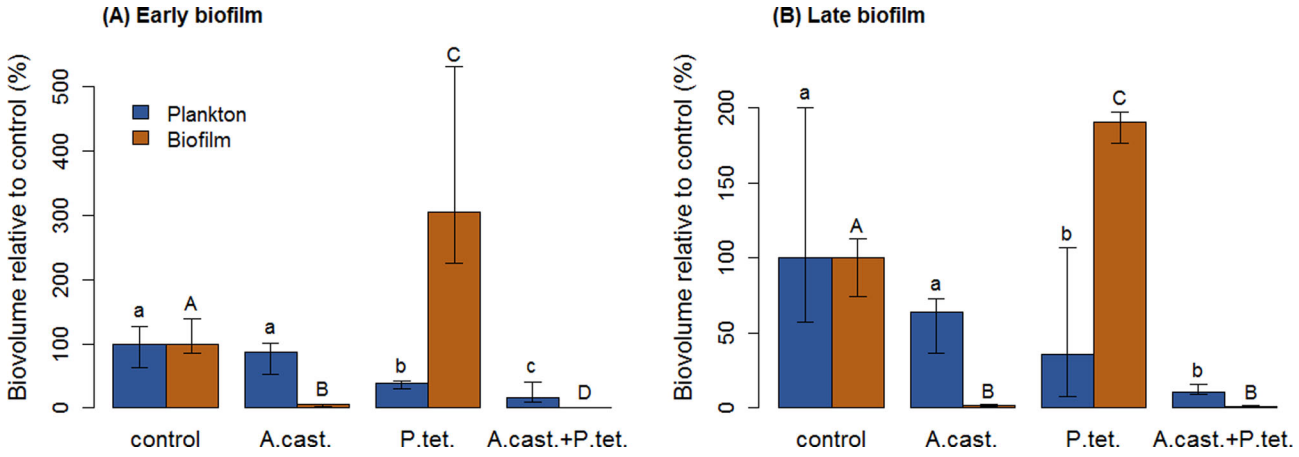

Figure 1. Treatment effects on the biovolume of planktonic and biofilm prey of (**A**) the early biofilm experiment and (**B**) the late biofilm experiment after 3 or 4 days, respectively. Note the different scaling of the y-axes. The biovolume is given in percentage relative to the biovolume of the predator-free control. Significant differences between all treatments (predator-free control—'control', *Acanthamoeba castellanii* only—'A.cast', *Paramecium tetraurelia* only—'P.tet.', and *Acanthamoeba castellanii* and *Paramecium tetraurelia*—'A.cast. + P.tet.') in a one-factorial ANOVA with subsequent treatment contrast analysis are indicated with small letters for planktonic prey and capital letters for biofilm prey. Depicted are medians and 0.25 and 0.75 quantiles (error bars).

the biofilm fraction increased to 56% in the predator-free control (Fig. 2D). Due to the strong stimulation of biofilm biovolume in the presence of *P. tetraurelia*, we observed the highest total prey guild biovolume in the *P. tetraurelia* treatment (P.tet.) of the late biofilm experiment (0.31 mm^3 $batch^{-1}$; Fig. 2B), which was similar to the total biovolume in the predator-free control (0.26 mm^3 $batch^{-1}$; *P*-value > 0.05). This increase of total bacterial biovolume as a consequence of plankton predation was less strong in young biofilms which resulted in significantly lower total prey biovolume in *P. tetraurelia* treatments (0.16 mm^3 $batch^{-1}$) compared to the control (0.31 mm^3 $batch^{-1}$; *P*-value < 0.01). The co-cultivation of *A. castellanii* and *P. tetraurelia* led to the strongest observed reduction of total prey biovolume (0.05 mm^3 $batch^{-1}$, exp.1, Fig. 2A and 0.01 mm^3 $batch^{-1}$, exp.2, Fig. 2B). Predator densities were monitored in order to determine grazing susceptibility of bacterial prey (Table 1). Abundance of *A. castellanii* decreased in the early biofilm experiment (with less biofilm prey, Fig. 2A) and increased in the late biofilm experiment (higher fraction of biofilm prey, Fig. 2B). The density of the plankton predator *P. tetraurelia* decreased in the treatment with both predators and increased or remained constant in single predator treatments independent of the age of the biofilm.

The simulations captured the qualitative and quantitative pattern of the experimental data reasonably well (Fig. 2A–D) and reproduced the essential features of the experiments. This refers to central results such as (i) the selective feeding of the predators either on plankton prey (*P. tetraurelia*) or on biofilm prey (*A. castellanii*), (ii) the effective control of prey biomass when both predators were present, (iii) the increase of the proportion of the inedible prey on total prey biomass while the edible prey phenotype decreased and (iv) the slight decrease of planktonic prey biovolume in presence of *A. castellanii*. The non-significant (*P* > 0.05) effect of the biofilm feeder on planktonic prey biovolume in the experiment is explained in the simulation by the migration of plankton cells into the biofilm. The reduction of biofilm biomass leads to an increased migration rate χ_{PL} (Fig. S1, Supporting Information). However, in contrast to the experimental results, simulations of biofilm biomass in the *Paramecium* treatments were at the control level, and the stimulation of the biofilm as found in the experiments was not observed in the model. Furthermore, plankton biomass is overestimated by the model in the late biofilm experiment (Fig. 2B and D) and biofilm biomass is underestimated. In this context, we observed that the model does capture the experimental data of experiment 2 when the minimum migration rate (χ_{min}) from plankton to biofilm would be twice as high in matured biofilms (experiment 2) compared to early-stage biofilms (experiment 1). However, we are lacking the experimental proof of this simulation result and thus used the same minimum migration rates from plankton to biofilm for the simulation of both experiments.

DISCUSSION

Neither biofilm nor plankton prey is generally resistant to grazing

It is often argued that bacterial biofilms are resistant against protozoan grazing (e.g. Costerton *et al.* 1999; Matz and Kjelleberg 2005; Justice *et al.* 2008). Here we show consistently, in two independent experiments conducted with either early-stage or matured biofilms, that the presence of the biofilm feeder *Acanthamoeba castellanii* leads to a strong reduction of biofilm biomass, while the plankton prey was much less vulnerable to the biofilm feeder. In contrast, in the presence of the filter feeder *Paramecium tetraurelia* the planktonic bacteria were reduced. These patterns were accurately reproduced by the model, supporting the conclusion that the dominant mechanism is specialised grazing on either of the two bacterial phenotypes (plankton or biofilm). It matches expectations when considering the feeding preferences of the grazers, i.e. exclusively surface feeding in *A. castellanii* (Huws, McBain and Gilbert 2005) and suspension feeding in *Paramecium* (Foissner, Berger and Kohmann 1994; Weerman *et al.* 2011). The results thus show that neither the biofilm nor the plankton phenotype is generally grazing resistant. Instead, grazing resistance depends on the feeding trait variation between the protozoans. Our experiments also show that suspended bacteria can be considered grazing resistant if exposed to a specialised surface feeders. Free-living protozoans are extremely diverse, covering many different feeding strategies (e.g. Fenchel 1980; Arndt *et al.* 2003; Erken *et al.* 2012). This diversity must be considered when transfering

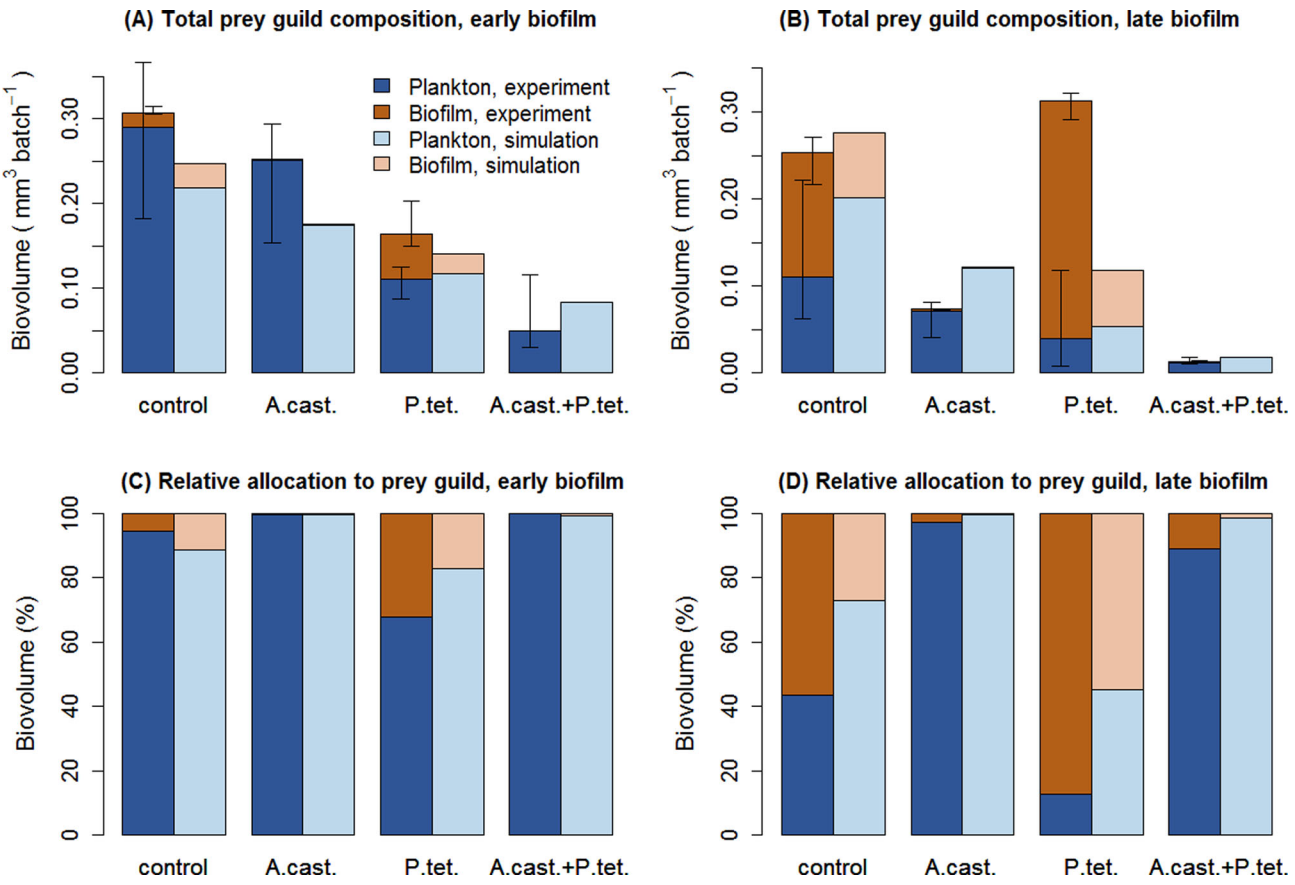

Figure 2. Treatment effects on the total prey guild composition (upper panels, **A** and **B**) and on the relative biovolume allocation to the prey guild (lower panels, **C** and **D**) in the early biofilm experiment (left panels, A and C) and the late biofilm experiment (right panels, B and D) after 3 or 4 days, respectively. Presented are experimental and simulation results for all treatments (predator-free control—'control', *Acanthamoeba castellanii* only—'A.cast', *Paramecium tetraurelia* only—'P.tet.', and *Acanthamoeba castellanii* and *Paramecium tetraurelia*—'A.cast. + P.tet.'). Depicted are medians and 0.25 and 0.75 quantiles (error bars).

results of laboratory experiments with selected grazers to the field situation.

Two relevant processes in microbial communities need to be considered that were not in the focus of our experiments. First, resistance of biofilms could also be due to chemical defence. The production of virulence factors in biofilms was especially observed in opportunistic pathogenic bacteria such as *Pseudomonas aeruginosa* and *Vibrio cholerae* (e.g. Weitere *et al.* 2005; Sun *et al.* 2015). Expression of such virulence factors is density dependent (under control of quorum-sensing systems) and thus common under high cell densities within biofilms. This phenomenon was not addressed in our study as we used a non-virulent prey organism. It remains questionable how relevant chemical defence is in nature as (i) some specialised grazers are still able to feed on virulent strains (Weitere *et al.* 2005) and (ii) the role of virulent strains in the field might be smaller than suggested from the focus of laboratory experiments (Sun *et al.* 2015). A second potentially important mechanism in microbial communities is rapid prey evolution including the development of grazing-resistant phenotypes (Yoshida *et al.* 2003; Meyer *et al.* 2006; Hall, Meyer and Kassen 2008; Koch *et al.* 2014; Friman *et al.* 2016). However, rapid prey evolution is defined in different manners in the literature. Even though mutation is an essential process in evolution, some authors consider the selection of clones and resulting changes in genotype frequencies that lead to eco-evolutionary predator–prey dynamics (i.e. the selection of existing genotypes; see Yoshida *et al.* 2003; Meyer *et al.* 2006) as rapid prey evolution. Others additionally consider mutations that lead to the formation of new genotypes in their experiments to investigate rapid evolution (e.g. Hiltunen, Ayan and Becks 2015). Even though both processes (mutation followed by selection of favourable prey traits and clonal sorting) can occur on short ('ecological') time scales, our experiments were probably too short (~7 generations) to detect evolutionary effects. Particularly, we found consistent patterns in five replicates per treatment and the occurrence of mutations appears unlikely in the short time frame. Also clonal selection is probably of minor importance, as we used monoclonal cultures for our experiments.

The strongest top–down control was detected in treatments where both predators were present; here prey biovolume was effectively reduced. Even though the two feeding trait values considered here do not reflect natural feeding trait diversity by far, the results suggest that protozoan communities with diverse feeding strategies are able to control the bacterial biomass regardless of phenotypic plasticity in the prey. In such systems, grazing resistance must thus be considered in a spatiotemporal context depending on the population dynamics of the different feeding traits of the grazers (see also Salcher *et al.* 2005; Šimek *et al.* 2014). Additionally, evolutionary processes can take place over longer time scales and thus fluctuations in predator densities and their feeding traits may cause eco-evolutionary dynamics in prey communities and their defence traits (e.g. Friman *et al.* 2016).

Stimulation of resistant phenotype only for the suspension feeder

In both experiments, we found that the presence of *P. tetraurelia* led to a strong increase in biofilm biovolume over time. Thus, biofilmswere not only resistant against grazing by the suspension feeder, they were even stimulated in both early and matured biofilms. In contrast to that, planktonic prey biovolume was not stimulated in the presence of the surface-feeding amoeba. However, other authors found indications that the densities of planktonic bacteria increased as a result of grazing by surface-feeding protozoans (e.g. Risse-Buhl *et al.* 2012).

The increase in biofilm biomass was also observed in various other experiments. For example, Wey *et al.* (2008) observed increasing numbers of initial biofilm cells attached to the surface of a flow cell in the presence of heterotrophic flagellates. Furthermore, flagellates such as *Ochromonas* sp. or *Bodo saltans* and the ciliate *Tetrahymena* sp. favour grazing defended and biofilm-forming morphotypes or stimulated the formation of microcolonies in *Pseudomonas* sp. CM10, *Ps. aeruginosa*, *Ps. fluorescens* or *Serratia marcescens* in controlled laboratory experiments (Matz, Deines and Jürgens 2002; Weitere *et al.* 2005; Queck *et al.* 2006; Hall, Meyer and Kassen 2008, Friman *et al.* 2016). Similar results were obtained for the flagellates *Rhynchomonas nasuta* (Matz *et al.* 2005), which feeds on single bacterial cells which are loosely associated with surfaces (Erken *et al.* 2012). Matured biofilm growth was also stimulated in the presence of suspension-feeding predators (Weitere *et al.* 2005; Scherwass, Erken and Arndt 2016). Overall, both our experiment and recent findings show that initial stages of biofilm formation and matured biofilms are stimulated in the presence of ciliates or heterotrophic flagellates.

In contrast to the experimental results, our model simulations revealed no increase in biofilm biomass in the presence of the suspension-feeding predator (Fig. 2A and D). This mismatch between experimental and model results indicates that a mechanism not included in the model is responsible for the biofilm stimulation. Four main possible mechanisms for protist-induced biofilm formation are discussed in the literature. First, Queck *et al.* (2006) suggested that the mechanical stimulation of bacteria by the movement of the flagella of the flagellate *Bodo saltans* causes biofilm growth. Further findings indicated that the feeding currents caused by the flagella and cilia of filter feeders such as ciliates promote the transport of nutrients and oxygen into the biofilm (Glud and Fenchel 1999; Vopel *et al.* 2005; Böhme, Risse-Buhl and Küsel 2009). This could result in increased access to dissolved resources, increasing growth. Second, signalling molecules might stimulate the formation of grazing-resistant phenotypes. It was shown that chemical substances excreted by the flagellate *Poterioochromonas* sp. strain DS act as signal molecules and induce biofilm formation in *Sphingobium* sp. strain Z007 (Blom *et al.* 2010). Third, protists contribute to nutrient recycling in aquatic systems because they release nutrients that bacteria use as substrate for growth (e.g. Johannes 1965; Taylor 1982; Risse-Buhl *et al.* 2012). Fourth, rapid prey evolution has the potential to cause the selection of traits in prey populations within ecological time scales, which can lead to the enrichment of grazing-resistant geno- and phenotypes compared to edible prey (Yoshida *et al.* 2003; Meyer *et al.* 2006; Koch *et al.* 2014; Hiltunen, Ayan and Becks 2015; Friman *et al.* 2016).

While the first point (mechanical stimulation and feeding current by flagellates and ciliates) would explain why this stimulation only occurs for biofilm bacteria in response to a flagellated or ciliated suspension feeder, the other three mechanisms (chemical signalling, nutrient recycling and rapid prey evolution) should also appear for planktonic bacteria in response to the surface-feeding amoebae. The fact that *A. castellanii* did not stimulate planktonic bacteria in our experiments suggests that signalling substances that induce the formation of the grazing-resistant phenotype do not play a role in this context and that grazing-related resource recycling and rapid prey evolution are of minor importance in our system. Moreover, evolution appears unlikely to explain patterns in our short-term study (as mentioned above).

The mass balance of plankton and biofilm prey in our experiments and corresponding model predictions suggests that active migration of plankton cells into the biofilm in presence of *P. tetraurelia* can be excluded as a possible mechanism for biofilm biovolume increase. Active migration from plankton into the biofilm should result in a biomass reduction of plankton prey (on top of the grazing loss caused by the predator) and thus lower the plankton density compared to the simulation (Fig. 2A and B). This was not observed in our experiments. Furthermore, total prey biomass increased after treating the late biofilm batch with the plankton-feeding *P. tetaurelia*. This implies stimulation in the sense of additional utilisation of resources by the activity of the ciliate *P. tetaurelia* that can cause the transport of dissolved resources and oxygen into the biofilm due to its feeding current (e.g. Glud and Fenchel 1999; see above) rather than quantitatively important stimulation of migration of plankton phenotypes into biofilm.

Implications for community dynamics and flux of matter within aquatic ecosystems

Bacteria are a key element of planktonic microbial food webs by converting dissolved resources (nutrients and organic carbon) into particulate biomass which is accessible to the food web (Fuhrman and Azam 1980). This link via edible bacterial phenotypes supports an efficient flux of matter and energy within a highly productive and dynamic microbial food web. The assumption that biofilms are resistant against grazing would lead to the consequence that bacteria within biofilms do not represent a significant link between organic resource and microbial grazers, with consequences for both the flux of matter and energy as well as the dynamics of the grazer community. In contrast, we found a very efficient control of the total (i.e. plankton and biofilm phenotype) bacterial biomass when we exposed the bacteria to a 'diverse' grazer community with different feeding trait values. This suggests, first, that in natural (usually diverse) grazer communities, an efficient flux of matter mediated by bacteria is also possible when biofilm formation occurs. Second, it suggests that both biofilm- and plankton-feeding grazer populations could develop depending on the available bacterial phenotype. Given the high growth and feeding rates in microbial systems, it is likely that both bacterial phenotypes and their corresponding grazers show a highly dynamic pattern in aquatic systems. This would be in line with Salcher *et al.* (2005), who observed dynamic patterns of different grazing resistance strategies (such as microcolonies, large aggregates and filaments) when continuously culturing an experimental microbial community including bacteria, algae and protozoan predators. We used a batch approach, which is not appropriate to follow such dynamics. Experiments in chemostat systems are a promising next step to detect consequences of trait diversity in the grazers for community dynamics in coupled plankton–biofilm systems.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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References

Arndt H, Schmidt-Denter K, Auer B *et al*. Protozoans and biofilms. In: Krumbein, EW (ed.). *Fossil and Recent Biofilms: A Natural History of Life on Earth*. London: Kluwer Academic Publishers, 2003, 161–79.

Blom JF, Zimmermann YS, Ammann T *et al*. Scent of danger: floc formation by a freshwater bacterium is induced by supernatants from a predator-prey coculture. *Appl Environ Microb* 2010;**76**:6156–63.

Böhme A, Risse-Buhl U, Küsel K. Protists with different feeding modes change biofilm morphology. *FEMS Microbiol Ecol* 2009;**69**:158–69.

Chow CET, Kim DY, Sachdeva R *et al*. Top-down controls on bacterial community structure: microbial network analysis of bacteria, T4-like viruses and protists. *ISME J* 2014;**8**:816–29.

Costerton JW, Stewart PS, Greenberg E. Bacterial biofilms: a common cause of persistent infections. *Science* 1999;**284**:1318–22.

Erken M, Farrenschon N, Speckmann S *et al*. Quantification of individual flagellate-bacteria interactions within semi-natural biofilms. *Protist* 2012;**163**:632–42.

Erken M, Lutz C, McDougald D. The rise of pathogens: predation as a factor driving the evolution of human pathogens in the environment. *Microb Ecol* 2013;**65**:860–8.

Fenchel T. Suspension feeding in ciliated protozoa: functional response and particle size selection. *Microb Ecol* 1980;**6**:1–11.

Foissner W, Berger H, Kohmann F. Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. In: *Band III: Hymenostomata, Prostomatida, Nassulida*. Bayerisches Landesamt für Wasserwirtschaft, 1994, 548.

Friman VP, Dupont A, Bass D *et al*. Relative importance of evolutionary dynamics depends on the composition of microbial predator-prey community. *ISME J* 2016;**10**:1352–62.

Fuhrman JA, Azam F. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. *Appl Environ Microb* 1980;**39**:1085–95.

Fuhrman JA, Azam F. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. *Mar Biol* 1982;**66**:109–20.

Garcia-Chaves MC, Cottrell MT, Kirchman DL *et al*. Major contribution of both zooplankton and protists to the top-down regulation of freshwater aerobic anoxygenic phototrophic bacteria. *Aquat Microb Ecol* 2015;**76**:71–83.

Glud RN, Fenchel T. The importance of ciliates for interstitial solute transport in benthic communities. *Mar Ecol Prog Ser* 1999;**186**:87–93.

Hahn MW, Höfle MG. Flagellate predation on a bacterial model community: interplay of size-selective grazing, specific bacterial cell size, and bacterial community composition. *Appl Environ Microb* 1999;**65**:4863–72.

Hahn MW, Höfle MG. Role of microcolony formation in the protistan grazing defense of the aquatic bacterium *Pseudomonas* sp. MWH1. *Microb Ecol* 2000;**39**:175–85.

Hahn MW, Höfle MG. Grazing of protozoa and its effect on populations of aquatic bacteria. *FEMS Microbiol Ecol* 2001;**35**:113–21.

Hall AR, Meyer JR, Kassen R. Selection for predator resistance varies with resource supply in a model adaptive radiation. *Evol Ecol Res* 2008;**10**:735–46.

Hillebrand H, Dürselen CD, Kirschtel D *et al*. Biovolume calculation for pelagic and benthic microalgae. *J Phycol* 1999;**35**:403–24.

Hiltunen T, Ayan GB, Becks L. Environmental fluctuations restrict eco-evolutionary dynamics in predator–prey system. *Proc R Soc B* 2015;**282**:20150013.

Huws S, McBain A, Gilbert P. Protozoan grazing and its impact upon population dynamics in biofilm communities. *Appl Environ Microb* 2005;**98**:238–44.

Johannes RE. Influence of marine protozoa on nutrient regeneration. *Limnol Oceanogr* 1965;**10**:434–42.

Jürgens K, Matz C. Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Anton Leeuw* 2002;**81**:413–34.

Justice S, Hunstad DA, Cegelski L *et al*. Morphological plasticity as a bacterial survival strategy. *Nat Rev Microbiol* 2008;**6**:162–8.

Koch H, Frickel J, Valiadi M *et al*. Why rapid, adaptive evolution matters for community dynamics. *Front Ecol Evol* 2014;**2**:17.

Litchman E, Edwards KF, Klausmeier CA *et al*. Phytoplankton niches, traits and eco-evolutionary responses to global environmental change. *Mar Ecol Prog Ser* 2012;**470**:235–48.

Litchman E, Klausmeier CA. Trait-based community ecology of phytoplankton. *Ann Rev Ecol Evol Syst* 2008;**39**:615–39.

Matz C, Deines P, Jürgens K. Phenotypic variation in *Pseudomonas* sp. CM10 determines microcolony formation and survival under protozoan grazing. *FEMS Microbiol Ecol* 2002;**39**:57–65.

Matz C, Jürgens K. High motility reduces grazing mortality of planktonic bacteria. *Appl Environ Microb* 2005;**71**:921–9.

Matz C, Kjelleberg S. Off the hook–how bacteria survive protozoan grazing. *Trends Microbiol* 2005;**13**:302–7.

Matz C, McDougald D, Moreno AM *et al*. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *P Natl Acad Sci USA* 2005;**102**:16819–24.

Meyer JR, Ellner SP, NGJr Hairston. *et al*. Prey evolution on the time scale of predator-prey dynamics revealed by allele-specific quantitative PCR. *P Natl Acad Sci USA* 2006;**103**:10690–5.

Monteiro ÁA, Boaventura RA, Rodrigues AE. Phenol biodegradation by *Pseudomonas putida* DSM 548 in a batch reactor. *Biochem Eng J* 2000;**6**:45–9.

Page F, Siemensma F. Nackte Rhizopoda. In: *Protozoenfauna Band 2. Nackte Rhizopoda und Heliozoea*. Stuttgart, New York: Gustav Fisher, 1991, 130–45.

Parry JD. Protozoan grazing of freshwater biofilms. *Adv Appl Microbiol* 2004;**54**:167–96.

Queck SY, Weitere M, Moreno AM *et al*. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. *Environ Microbiol* 2006;**8**:1017–25.

Core R, Team . *A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2015.

Risse-Buhl U, Trefzger N, Seifert AG *et al*. Tracking the autochthonous carbon transfer in stream biofilm food webs. *FEMS Microbiol Ecol* 2012;**79**:118–31.

Romanova ND, Sazhin AF. Relationships between the cell volume and the carbon content of bacteria. *Oceanology* 2010;**50**:522–30.

Salcher MM, Pernthaler J, Psenner R *et al*, Succession of bacterial grazing defense mechanisms against protistan predators in an experimental microbial community. *Aquat Microb Ecol* 2005;**38**:215–29.

Scherwass A, Erken M, Arndt H. Grazing effects of ciliates on microcolony formation in bacterial biofilms. In: Dhanasekaran D, Nooruddin T (eds). *Microbial Biofilms - Importance and Applications*. Rijeka: InTech, 2016, DOI: 10.5772/63516.

Seoane J, Yankelevich T, Dechesne A *et al*. An individual-based approach to explain plasmid invasion in bacterial populations. *FEMS Microbiol Ecol* 2011;**75**:17–27.

Sherr EB, Sherr BF. Significance of predation by protists in aquatic microbial food webs. *Anton Leeuw* 2002;**81**:293–308.

Šimek K, Nedoma J, Znachor P *et al*. A finely tuned symphony of factors modulates the microbial food web of a freshwater reservoir in spring. *Limnol Oceanogr* 2014;**59**:1477–92.

Šimek K, Pernthaler J, Weinbauer MG *et al*. Changes in bacterial community composition and dynamics and viral mortality rates associated with enhanced flagellate grazing in a mesoeutrophic reservoir. *Appl Environ Microb* 2001;**67**:2723–33.

Staudt C, Horn H, Hempel DC *et al*. Volumetric measurements of bacterial cells and EPS glycoconjugates in biofilms. *Biotechnol Bioeng* 2004;**88**:585–92.

Sonneborn T. The P*aramecium aurelia* complex of fourteen sibling species. *Trans Amer Microsc Soc* 1975;**94**:155–78.

Sun S, Tay QX, Kjelleberg S *et al*. Quorum sensing-regulated chitin metabolism provides grazing resistance to *Vibrio cholerae* biofilms. *ISME J* 2015;**9**:1812–20.

Taylor GT. The role of pelagic heterotrophic protozoa in nutrient cycling: a review. *Ann Inst Oceanogr* 1982;**58**:227–41.

Vopel K, Thistle D, Ott J *et al*. Wave-induced H_2S flux sustains a chemoautotrophic symbiosis. *Limnol Oceanogr* 2005;**50**:128–33.

Weerman EJ, Van Der Geest H, Van Der Meulen MD *et al*. Ciliates as engineers of phototrophic biofilms. *Freshwater Biol* 2011;**56**:1358–69.

Weitere M, Bergfeld T, Rice SA *et al*. Grazing resistance of P*seudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. *Environ Microb* 2005;**7**:1593–601.

Wey JK, Scherwass A, Norf H *et al*. Effects of protozoan grazing within river biofilms under semi-natural conditions. *Aquat Microb Ecol* 2008;**52**:283.

Yoshida T, Jones LE, Ellner SP *et al*. Rapid evolution drives ecological dynamics in a predator–prey system. *Nature* 2003;**424**:303–6.