Università degli Studi di Parma
Dottorato in Ecologia – XVII Ciclo

Bacteria-protozoan interactions and the underlying mechanisms of grazing-resistance in aquatic bacteria

Tesi di dottorato di

Gianluca Corno

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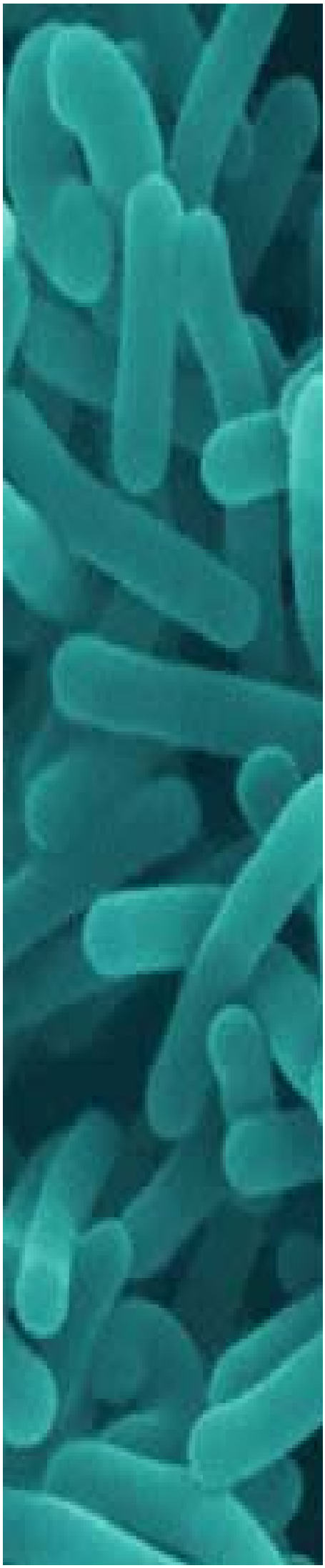

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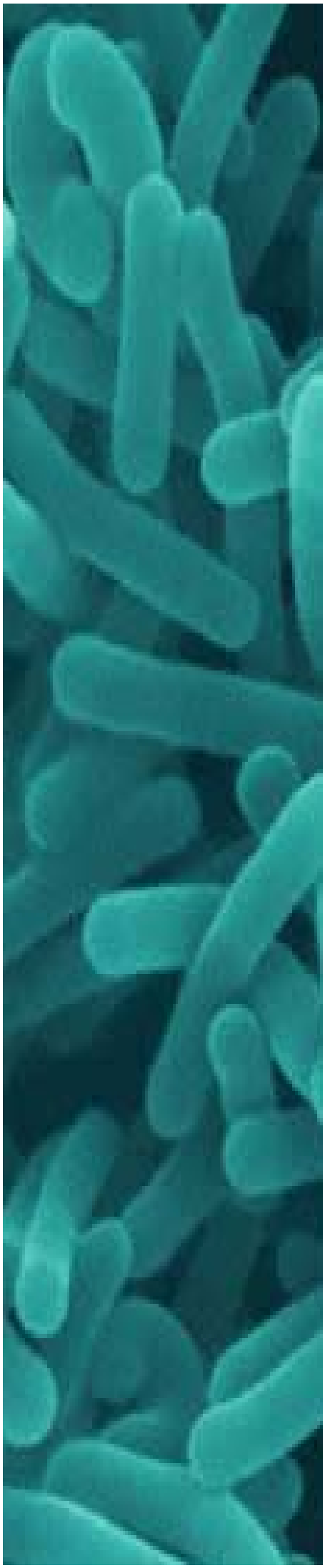
Everything is everywhere, the environment selects.

M.W. Beijerinck (1920)



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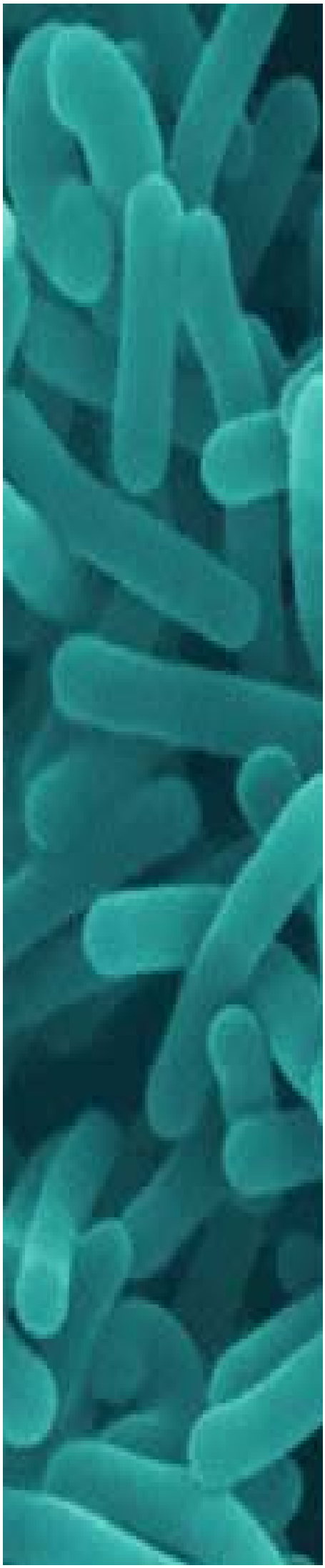


Abstract - Riassunto

Abstract

This thesis was prepared in the laboratories of the Max Planck Institute for Limnology in Plön (Germany). It is focused on bacteria-protozoan interactions and the underlying mechanisms of grazing-resistance in aquatic bacteria: phenotypic properties of aquatic bacteria with respect to their potential effect on bacterial vulnerability and survival in the presence of bacterivorous protists (such as heterotrophic nanoflagellates, HNF).

Interactions were studied mainly as impact of the presence of predators on both single bacterial strains and bacterial communities, considering variations of phenotype, genotype and fitness in the first case, and variations in diversity, productivity and distributions in the second part of the work. Chemostat systems with bacteria and bacterivorous protists were used to enrich and isolate bacterial species which had a potentially reduced vulnerability towards grazers. These strains were analysed with respect to their anti-grazer phenotypic properties, phenotypic plasticity and regulating mechanisms in the expression of these properties. Bacterial communities composed by strain isolated with the same methodology were tested in chemostat systems to analyze the impact of predation by HNF and of inter-specific competition for resources on their diversity and on their genetic and phenotypic composition.

One principal aim was to elucidate the diversity and effectiveness of the underlying mechanisms of grazing resistance in aquatic bacteria: cell-size and shape are important and well-documented features which determine bacterial vulnerability to grazers. However, other characters which might also impact upon the predator-prey interactions with protozoans were still poorly understood.

Of particular interest was the question as to whether some of the resistance mechanisms found (e.g., morphology, toxins, exopolymers) can be induced by predator chemicals, or whether their development is mainly related to substrate supply and elimination of competitors by grazing. A cost-benefit analysis for selected polymorphic bacterial strains (with resistant and vulnerable phenotypes) should reveal further insight into the evolution and the ecological consequences of grazing-resistant bacteria.

In the first part of the thesis it was studied the impact of grazing and substrate supply on the population size structure of a freshwater bacterial strain (*Flectobacillus* sp.) which showed a high morphological plasticity, with cell lengths in the range 2 - 40 µm, encompassing rods, curved cells and long filaments. Without grazers and with sufficient

substrate supply bacteria grew mainly in form of free-living medium-sized rods (4-7 µm) with a smaller proportion of filaments.

Batch grazing experiments with the bacterivorous flagellate *Ochromonas* sp. showed that cells < 7 µm were highly vulnerable to grazers and became eliminated whereas resistant, filamentous forms become enriched. Comparing long-term growth in carbon-limited chemostats with and without grazers revealed that bacterial biomass was on a similar level but the morphological composition strikingly different, with >80 % filaments in the grazer treatments. These morphological differences did not seem to have a strong impact on the physiological capabilities as revealed from substrate uptake and utilisation measurements. Carbon starvation resulted in a low degree of morphological plasticity and a fast increase of small rods which were highly vulnerable to grazing.

Dialysis bag experiments were used to test for chemical induction of bacterial morphological changes. They revealed that the excretion of organic substrates by flagellates stimulates bacterial growth. When these experiments were combined with continuous cultivation they gave strong evidence for chemical induction of resistant bacterial morphologies: The development of filamentous forms occurred even without direct contact with predators when bacteria were exposed for several generations to predator-released substances.

The second part of the thesis was focused on HNF predation and competition for resources can effect on bacterial populations. The effects of limiting factors on ecological systems and their possibility to influence community's structure and composition represent one of the most debated topics in modern ecology.

The understanding of processes related to biological communities is extremely difficult, because of their extraordinary complexity. For these reasons, the most fruitful approaches have been the reduction of the complexity of the systems, and the development of related mathematical models. Because of their qualities, microbial communities are the most commonly used for these studies.

Using carbon-limited chemostats with differing substrate input, the influence of system productivity on predator-prey interactions in classical artificial communities of planktonic bacteria and bacterivores, was examined for two different experiments.

Analysing population dynamics, diversity and functional attributes of the mixed bacterial assemblages and of bacterivorous nanoflagellates it was assessed the relative importance of the classes of edible and inedible bacteria. In accordance with existing models on microbial predator-prey interactions, the relative importance of grazing-resistant prey increased with increasing productivity.

With a detailed analysis on the morphology and functionality of bacterial cells it was possible to recognize different mechanisms of resistance along the productivity gradient.

Performing a T-RFLP analysis of bacterial populations was noticed that predator-mediated increases in phenotypic diversity were not necessarily reflected in changes of the genetic diversity of our bacterial populations.
Starting from the huge background from former studies and models on simple communities, this approach tries to clarify the validity of the most debated ecological theories about relations between prey-predator interactions, productivity of the system and biodiversity on larger scale.

Riassunto

Questa tesi è stata svolta presso i laboratori del Max Planck Institute for Limnology di Plön (Germania). Il tema principale attorno al quale si sviluppa il lavoro sono le interazioni tra batteri e protozoi ed i meccanismi fondamentali di resistenza al grazing da parte dei batteri acquatici: le loro proprietà fenotipiche possono potenzialmente influenzare la vulnerabilità dei batteri stessi e quindi la loro sopravvivenza in presenza di protisti batterivori (per esempio i nanoflagellati eterotrofi, HNF, comuni predatori di batteri).
Le interazioni tra batteri e predatori sono state studiate sia a livello di impatto del grazing su single specie, sia a livello di impatto su comunità batteriche, considerando, nel primo caso, variazioni di fenotipo genotipo e di competitività, nel secondo caso invece, variazioni in diversità, produttività dei popolamenti e di distribuzione delle diverse specie e dei diversi morfotipi. Diversi sistemi di chemostati con batteri e protisti batterivori sono stati approntati per arricchire ed isolare delle specie batteriche che presentavano una vulnerabilità al grazing potenzialmente ridotta. Queste specie sono state analizzate dal punto di vista dello sviluppo di proprietà fenotipiche che le preservassero dalla predazione, della plasticità fenotipica e dei meccanismi che regolavano l'espressione di tali caratteristiche. Alcune comunità batteriche isolate con identiche metodologie sono state sviluppate in analoghi chemostati con l'intento di analizzare gli effetti provocati sulla loro diversità e sulla loro composizione geno- e fenotipica, dall'impatto della predazione da parte di HNF e della competizione interspecifica per l'approvvigionamento di nutrienti provocata da limitazioni nell'arricchimento dei substrati nei reattori dei chemostati.
Uno degli obiettivi principali di questo lavoro consisteva nell'elucidare la diversità e l'effettiva efficacia dei meccanismi di fondo che permettono la resistenza al grazing da

parte dei batteri acquatici: è ben noto che le dimensioni cellulari e la forma del singolo batterio o della colonia sono tratti importanti nella determinazione del grado di vulnerabilità del ceppo batterico alla predazione. Erano però ancora molti i caratteri, ed i processi che ne regolano lo sviluppo, in grado di influenzare i rapporti tra preda e predatore, e per i quali la nostra conoscenza era ancora fortemente limitata.

Di particolare interesse era la domanda "*se e come*" alcuni meccanismi di resistenza (per esempio quelli concernenti la morfologia, lo sviluppo di tossine o di exopolimeri) potessero essere indotti dal rilascio, da parte dei predatori, di particolari agenti chimici, oppure se lo sviluppo di queste forme fosse fondamentalmente legato all'aumento di substrato disponibile in seguito all'eliminazione di altre forme (competitori) non resistenti alla predazione. Un'analisi dettagliata dei costi e dei benefici riguardanti le diverse forme appartenenti ad un ceppo batterico in grado di esprimere alta plasticità polimorfica ha rivelato l'importanza evolutiva e le conseguenze ecologiche legate alla presenza di batteri resistenti alla predazione.

Quest'ultima analisi è stata affrontata ed elaborata nella prima parte della tesi dove è stato studiato nel dettaglio l'impatto della predazione di HNF eventualmente mediato dall'effetto della disponibilità di nutrienti sulla struttura e sulla ricchezza di una popolazione di batteri d'acqua dolce della specie *Flectobacillus* (ceppo GC5). Questa specie, dalla tipica forma di bacillo, aveva dimostrato alta plasticità morfotipica, con un diametro cellulare massimo che può o variare da 2 a 40 µm, allungandosi in filamenti e catene di bacilli. In assenza di predatori e con sufficiente substrato disponible questi batteri si sviluppano principalmente in bacilli liberi da aggregazioni di diametro compreso tra 4 e 7 µm, con una piccola percentuale di lunghi filamenti.

Esperimenti di valutazione del grazing da parte del flagellato batterivoro *Ochromonas* sp., effettuati in colture in batch, hanno dimostrato che le cellule batteriche < 7 µm sono decisamente vulnerabili al grazing e tendevano ad essere eliminate in presenza di predatori mentre le cellule più lunghe, filamentose, tendevano ad aumentare in proporzione.

Comparando la crescita delle popolazioni, in chemostati dove l'approvvigionamento di carbonio era controllato durante la sperimentazione (durata circa 30 giorni), in presenza ed in assenza di predatori, è stato possibile apprezzarne la fondamentale differenza in composizione morfotipica (>80 % di filamenti in presenza di predatori, e <20% in loro assenza) mentre la biomassa dei diversi popolamenti non rilevava particolare impatto dovuto al grazing. Queste impressionanti differenze nella distribuzione morfotipica non hanno dimostrato avere alcun importante impatto sulle caratteristiche fisiologiche dei diversi popolamenti come confermato dalle analisi sull'utilizzo del substrato e sulle diverse attività dei popolamenti batterici testati.

L'effetto limitante dovuto alla carenza di carbonio disponibile nel substrato ha causato una riduzione della plasticità fenotipica e di conseguenza un rapido incremento percentuale di piccoli bacilli completamente vulnerabili all'azione dei predatori.

Attraverso esperimenti in effettuati utilizzando apposite sacche da dialisi sono stati studiati i meccanismi di induzione chimica dei cambiamenti morfologici. Si è dimostrato che l'escrezione di sostenze organiche da parte dei flagellati durante l'attività di grazing stimola un aumento della crescita batterica. Combinando le sacche da dialisi ai chemostati si è potuto dimostrare che, dopo alcuni giorni di adattamento, la sola presenza di flaggelati, anche senza una predazione diretta, stimolava chimicamente lo sviluppo di nuove morfologie di batteri della specie *Flectobacillus* (ceppo GC5) resistenti al grazing. Lo sviluppo di queste forme filamentose, dopo un'esposizione alla presenza dei predatori per alcune generazioni, era perciò completamente indipendente dalla predazione ma dipendeva completamente dall'esposizione alle sostanze escrete dai predatori stessi.

La seconda parte della tesi ha studiato il modo in cui gli effetti della predazione da parte di HNF e contemporaneamente della competizione interspecifica per le risorse possono influire su dei popolamenti batterici. Gli effetti dei fattori limitanti sui sistemi ecologici e la possibilità di influenzare la struttura e la composizione delle comunità naturali rappresentano uno dei temi più dibattuti nell'ecologia moderna.

La comprensione dei processi relativi alle comunità biologiche è molto difficile, a causa loro straordinaria complessità. Per queste ragioni, gli approcci più efficaci hanno teso a ridurre la complessità dei sistemi naturali, accoppiando le osservazioni allo sviluppo di modelli matematici che permettessero eventuali previsioni. Le comunità microbiche, grazie alle loro straordinarie caratteristiche, possono essere considerate comunità modello in questo genere di ricerche.

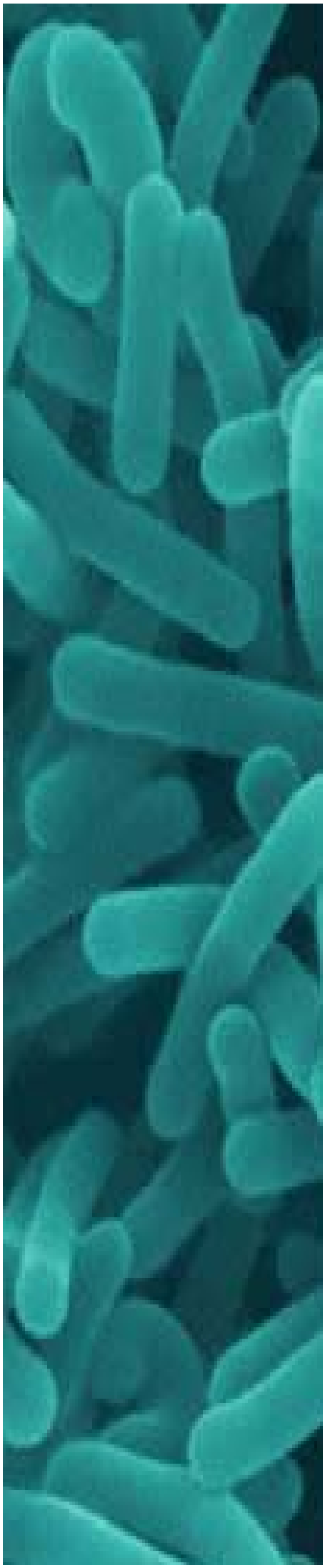
Utilizzando chemostati nei quali la disponibilità di carbonio potesse essere controllata e mediata, è stata esaminatai, in due diversi esperimenti, l'influenza della produttività del sistema sulle interazioni preda-predatore in classiche comunità di batteri e flagellati batterivori, artificialmente costruite in laboratorio.

L'analisi delle dinamiche e della biodiversità dei popolamenti batterici è stata valutata la relativa importanza delle classi di batteri edibili ed inedibili. Sulla traccia di modelli matematici di analisi delle dinamiche preda-predatore a livello microbico preesistenti si è dimostrato che la relativa importanza delle "prede invulnerabili" aumenta con l'accrescere della produttività del sistema.

Attraverso una dettagliata analisi della morfologia e della funzionalità delle cellule batteriche è stato possibile riconoscere diversi meccanismi di resistenza alla predazione al variare del gradiente di produttività.

Attraverso un'analisi molecolare di classico *fingerprinting* (T-RFLP) si è riconosciuto che l'incremento di fenotipi di prede legato alla presenza di predatori non è necessariamente legato ad un relativo incremento in ricchezza genetica delle popolazioni batteriche.

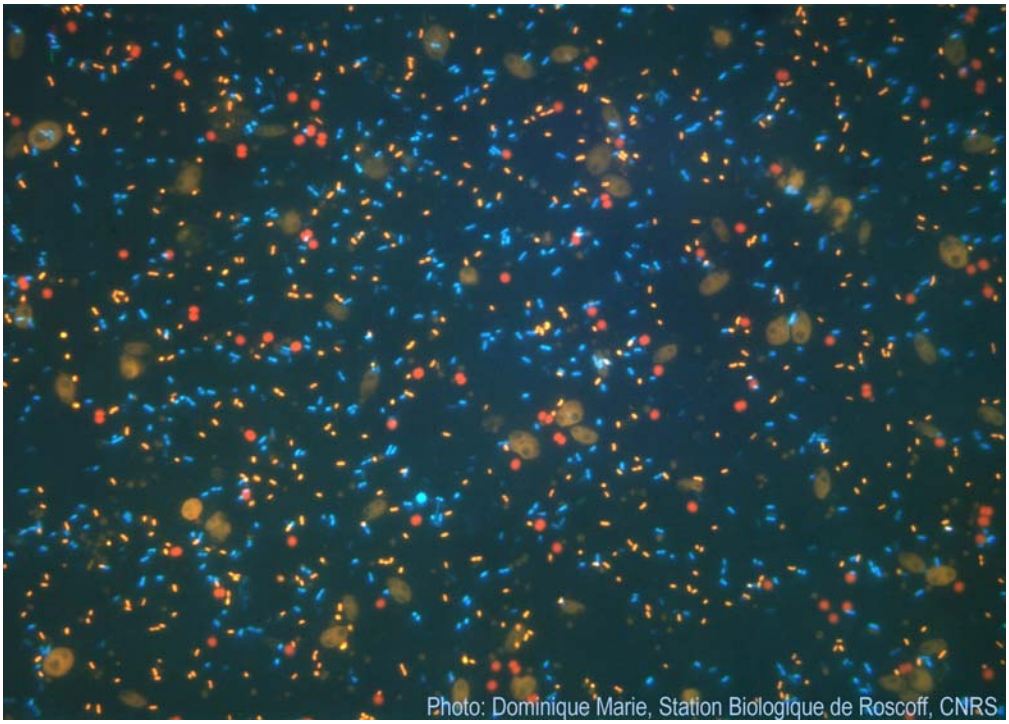
Partendo dall'ampio *background* assicurato dai molti studi portati a termine negli ultimi decenni su semplici comunità, questo lavoro vuole essere un apporto nel dibattito sulle più discusse teorie ecologiche a proposito di relazioni tra preda e predatore, produttività del sistema e biodiversità, viste su una scala più ampia.



Chapter I

Introduction and outlines

Introduction and outlines



Microorganisms, and especially bacteria, play a fundamental role in aquatic ecosystems (**93**). In planktonic food web bacteria can contribute a significant fraction to total biomass, in oligotrophic systems even exceeding that of phytoplankton (e.g., Simon *et al.*, **94**; Del Giorgio and Gasol, **95**; Gasol et al. **96**). Bacteria convert dissolved organic matter (DOM) into particulate biomass, making it available as a potential food source for other trophic levels. Bacterial production is in the range of 20-30 % of primary production (**97**) and most of planktonic respiration is carried out by prokaryotes (**96**).

Bacterial productivity, biomass regulation, community structure and biodiversity are thus essential issues in aquatic ecology, because only a proper understanding of these processes allows more accurate predictions of ecosystem processes such as food web dynamics and biogeochemical cycling. Bacterial growth and biomass in aquatic systems are controlled by several factors, such as organic and inorganic nutrient availability (bottom-up regulation), predation and viral lysis (top-down regulation) (**98**). Predation by bacterivorous protists (grazing) has been identified as the main factor in the removal of bacterial biomass (**99**), balancing in many systems bacterial production. Grazing activity of bacterivorous protists is essential for nutrient regeneration (**4**) and stimulates bacterial decomposition of organic matter (**77**).

Since Pomeroy (**100**) established the potential importance of microbes in water biogeochemical cycling and Azam *et al.* (**101**) formulated the concept of "microbial loop", our understanding of microbially mediated processes has been deepened, and many of the complex interactions within the microbial food web have been elucidated. Many other interactions are still far from a complete understanding and, in recent years, they were investigated by several studies using very different approaches. One of the most debated topics is the regulation and the role of bacterioplankton in respect of the higher trophic levels of the food web. The traditional approach considering heterotrophic bacteria as one functional unit (the classical "black-box approach") was helpful for a first analysis and produced a knowledge of the basic processes regulating aquatic bacteria. With progresses in our understanding of the mechanisms, including a more detailed analysis of functions, adaptations and structural organization of bacterial communities, the knowledge about the functions and controlling factors is improving.

Studies on predator-prey interactions between bacteria and their most important grazers became more successful when bacteria as prey were recognized as a community of different organisms with different characteristics. Feeding selectivity of grazers (**102**) and prey anti-predators adaptations, sometimes mediated by chemical signals (**103**), was studied much more for zooplankton-phytoplankton (**104**) than for protists-bacteria interactions (**33**). Predation effects on bacteria may be compensated by and increase in bacterial growth rates of some resistant strains, determining not only a higher number of bacteria from those strains, and thus, lower predation impact per cell, but also an increase in bacterial cells dimension, making them more resistant against predation (**13**).

Substrate availability is assumed to be influential for many bacterial defence strategies against predation (summarized in **19**) as fitness costs are expected to be involved. This fact

seems to implicate trade-offs between the impact of predation and the degree of competition (**73**; **75**). The interacting effects of predation and competition for the structuring of communities in ecological systems are an intensively debated topic in community ecology (studies summarized by Bohannan *et al.*, **92**). Several studies tried to clarify the relative impact of these associated factors on community structure, diversity and evolution (**63**; **71**). For microbial communities only in the last years the establishment of molecular techniques allowed to use microbial assemblages as microorganisms' model systems for studying predator-prey dynamics. Even before the introduction of molecular techniques few studies have been successfully performed, mainly on very simple microbial systems (e.g., one bacterial strain, one bacteriophage; **105**; **106**; **107**; **108**; **109**; **80**).

Continuous cultures performed in laboratory. The advantages acquired performing these experiments are fundamental for the results obtained and explained in the thesis. (Photo G. Corno, 2002).

The use of microbial laboratory model communities has several advantages. It is possible to use organisms with short generation times, so that steady state responses to enrichment are achieved relatively quickly. It is also easy to quantify unambiguously trophic-level population densities, and experimental variables such as resource input are simple to manipulate (e.g., in chemostats, etc.). Variables other than resource input can be controlled, and the experiments can be replicated with relative ease as well. It is also feasible to measure population parameters such as prey edibility and the trade-off between edibility and competitiveness in laboratory model communities (**60**). Ecological experiments with model laboratory systems

bridge the gap between ecological theory and natural communities. Such studies allow theoretical predictions to be examined rigorously in a biological system that is easily manipulated, replicated, controlled, and monitored in ways that would be difficult or impossible with natural communities (**110**).

I.I Role of protists as predators of aquatic bacteria

Predation by small protists (<10 µm, *sensu* Sherr and Sherr, **78**) is known to be as well selective force in bacterial communities (**111**; **112**; **113**) despite several organisms such as ciliates (e.g., Sherr and Sherr, **114**) and planktonic (cladocerans, **18**; gelatinous zooplankton, **115**) or benthic (**116**) filter-feeders are known to be consumers of bacteria. With terms like "heterotrophic nanoplankton" (HNAN) or "heterotrophic nanoflagellates" (HNF) scientists are indicating functional groups of small protists which comprise phylogenetically very different organisms but with a very important common trait: they are the only organisms able to handle and process individual bacterial cells that are representing their only source of nutrients (**117**).

Experiments with natural bacterial communities have demonstrated mainly size-related impacts of protist and also revealed how phenotypic selection results in altered bacterial community composition. Heterogeneity in prey vulnerability should result in community shifts during increased grazing pressure (**20**). Significant effects of predators on bacterial community structure have been detected mainly in meso- and eutrophic systems (e.g., **21**; **119**; **47**), in oligotrophic systems only when bacterial growth had been stimulated with the addition of supplementary nutrients (**119**). This suggests that, to enable changes in bacterial community composition due to protozoan impact, sufficient nutrients have to be available.

Bottom-up factors for bacterial growth are the availability of organic and inorganic nutrients, including not only organic matter, N, and P, but also Fe (**120**; **121**; **122**; **123**), and even other micronutrients such as Se (**124**). Bottom-up factors are often separated into inorganic versus organic, but I considered here all resources needed for bacterial growth as one conceptual unit.

It is well known that protists have the potential for reducing bacterial abundance and to create Lotka-Volterra-like oscillations between bacterial and predator abundance (**125**; **101**; **126**). Mechanistic models predict that bacterial production is controlled by the rate of nutrient supply while final abundance and specific growth rates are determined by predation pressure, by substrate supply or by both control modes (**127**; **128**; **98**).

Thus, more nutrient availability would mean more biomass and production, and more predators could mean less biomass or activity. This simple framework has been routinely used to infer the main mode of control of the different microbial populations in whole-system manipulations (**129**) and in empirical analyses of data bases (**130**; **128**). However no clear-cut conclusions about the predominant mode of control, top-down or bottom-up, audits importance for determining bacterial abundance, production and growth rate have been reached (**131**).

I.II Impact of predation on bacterial community structure and phenotypic composition

A possible co-evolution between HNF and bacteria would result in adaptations for more effective predation by the bacterivores and a in the development of anti-predators traits in bacteria. Many different mechanisms and strategies were discovered in the last years (earliest summarized by Jürgens and Güde, **19**, and lately by Jürgens and Matz, **20**), but morphological features (size and shape), resulting from size-selective bacterivory, seems to be a major shaping factor for planktonic bacteria if compared to non-morphological characters, such as motility, exopolymers formation, aggregation and cell surface features (reviewed in Jürgens and Matz, **20**).

Moreover, size-structured predator-prey interactions are known to be important in planktonic systems (**26**) and thus it was possible to demonstrate that bacteria obtain a refuge against protist grazing at the lower end of the size classes as well as at the upper end, when cells become too large to become ingested. This bimodal size distribution of planktonic bacteria during high protist grazing has been found in freshwater plankton (**11**; **84**): bacteria can acquire a complete phenotypic controlled grazing-resistance with respect to heterotrophic

nanoflagellates (HNF) just modifying their shape and their size in a proper way, not only as result of grazing pressure but as a real active adaptation.

In fact, the development of complex bacterial morphologies such as filamentous, spiral-shaped or aggregated cells during high protists grazing seems to be a common phenomenon in more productive environments. First observations came from activated sludge systems (**10**; **132**), later also from meso-eutrophic lakes (**11**; **18**). Molecular analysis (FISH) of bacterial populations revealed that grazing-resistant morphotypes can be found in the major phylogenetic groups such as the alpha- and beta-Proteobacteria and the Cytophaga-Flavobacterium phylum (**21**; **47**; **48**).

In Chapter II the morphological resistance against nanoflagellates of a single, phenotypically highly plastic bacterial strain was examined in laboratory continuous cultures systems, dialysis bags and batch cultures.

I.III Detailed studies on bacteria-protist interactions: mechanisms of grazing resistance

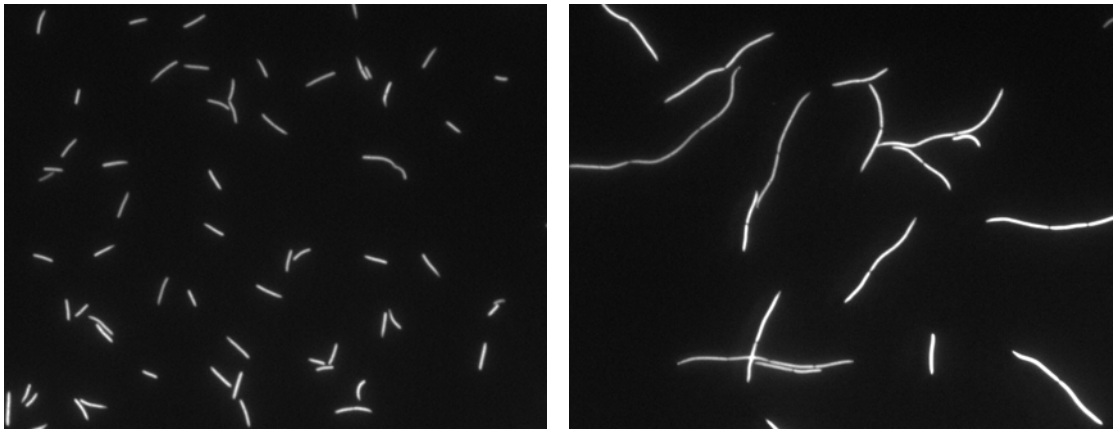
From the observations of lab and natural systems three possible regulatory mechanisms which might result in the dominance of grazing-resistant bacterial morphologies within mixed bacterial assemblages were described (**20**):

- Grazing-mediated selection of permanently resistant morphologies
- Increase of cell size and development of complex morphologies due to enhanced substrate supply, which is followed by grazing and elimination of freely suspended cells
- Shift of certain bacteria towards resistant morphologies due to chemical cues.

The first, simplest, case is when certain bacterial taxa are permanently resistant to ingestion by nano-protists because of their morphology. Although generally not abundant, resistant cells, would increase in number when edible (and presumably more competitive) bacteria are eliminated by an increase in grazing pressure. Many bacterial taxa with a permanent complex morphology, (stalked and filamentous cells) have been observed and isolated from freshwater and marine plankton (**133**; **134**). The most common phenotypic adaptations in bacteria, such

as cells surface structures (e.g. flagella, pili, fimbriae), exopolysaccharides and pigments were reviewed by Rainey et al. (**135**) and recently by Jürgens and Matz (**20**).

This thesis was focused on morphological adaptations of bacteria against grazing by hetero- and mixotrophic nanoflagellates and, in Chapter II, on phenotypic adaptations of a single polymorphic strain (*Flectobacillus* sp.). However, when complex bacterial communities were used, other resistance strategies could be involved as well. Bacterial motility, if performed by relatively small cells and with sufficient speed, was recognized as a possible strategy of grazing resistance (Matz and Jürgens, in press). In contrast, moderately motile bacteria can be favourite prey for flagellates (**136**; **137**) due to increased encounter rates. In the bacterial communities which were used for this study, a slight number of fast motile bacteria were present and their impact on bacterial dynamics was examined as well (outlined in Chapters III and IV).



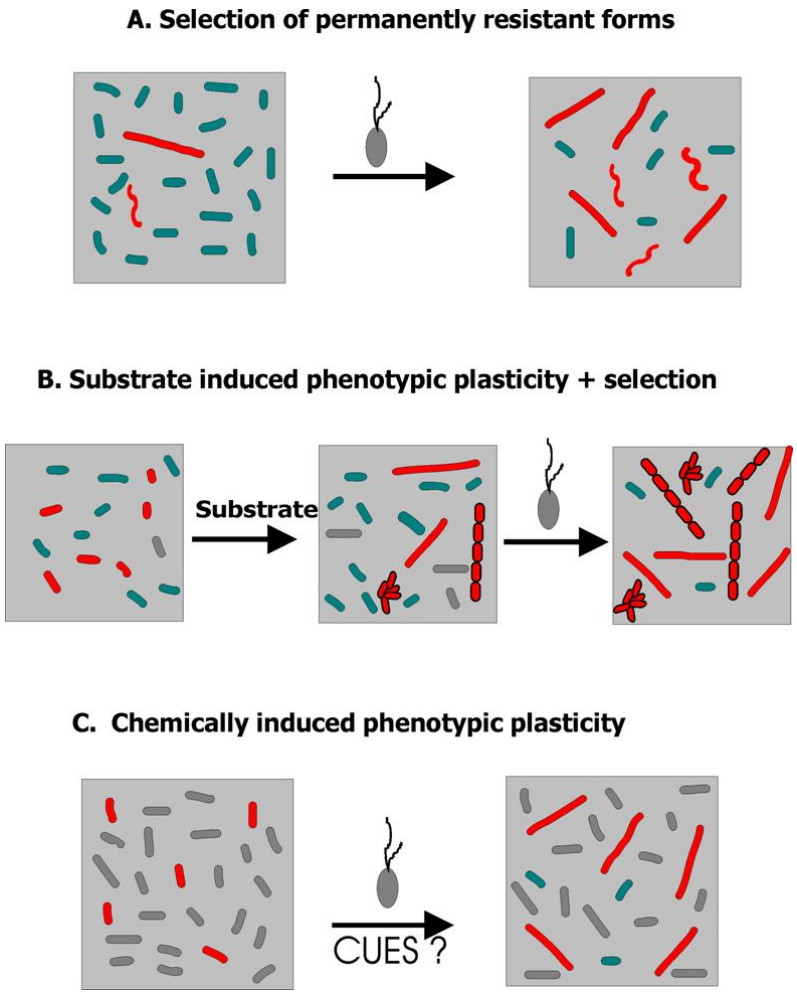
Microphotographs (1250x, G. Corno, 2002) of *Flectobacillus* sp. GC5 grown in absence of predators (left photo) and in presence of predators (right photo). Under grazing pressure, this strain, developed a classical morphological mechanism of defence.

Another hypothesis considers the dominance of large, resistant, bacterial morphologies simply as a response to enriched substrate supply, and thus, to higher growth rates (**17**). Enrichments in the presence of grazers nearly always resulted in an increased proportion of bacteria with elongated cell forms and with bacterial clumps or aggregates (**138**) that can be recognized as grazing-resistant. These morphotypes dominate the community after suspended, single edible cells have been reduced by grazers. In some bacterial strains an increase in mean cell length is indeed correlated with the growth rate (**17**), while others show enhanced production of exopolymers (**139**) which makes cells more sticky and favours aggregation. Interestingly, phenotypic plasticity and a shift towards resistant morphotypes in certain strains can be directly related to increased protists grazing pressure: the bacterial strains *Comamonas acidovorans* and *Flectobacillus* sp. both showed a shift towards inedible filaments when

grown in chemostat cultures in the presence of bacterivorous flagellates (**15**; **17**). Because this increase in cell length is similar to an increase which can be observed at higher growth rates, it was concluded that the grazers enhanced the specific bacterial growth rate due to recycling of nutrients and elimination of competitors (**17**).

The third possible mechanism for the development of bacterial grazing-resistance (exposed in the figure below) suggests the induction of phenotypic changes due to specific signals released by the predators (kairomones).

Chemical predator-induced plasticity has been demonstrated for many other plankton organisms from fish to protists and comprises alterations in morphology, chemistry, behaviour and life history (**53**).



Suggested mechanisms of selection of grazing resistant bacterial forms: red bacteria are supposed to be sensitive to the pressure due to grazing activity by nanoflagellates, green cells are cells supposed to be completely vulnerable to grazers. Picture is taken from Jürgens and Matz, 2002 (**20**).

Chemically induced plasticity could not be convincingly demonstrated yet for bacteria. There were some indications that the phenotypic plasticity which was involved in the formation of microcolonies by different *Pseudomonas* strains (**16**; **33**) during protist grazing was triggered by some unknown external factors related to grazing activity.

I.IV Grazing impact can be avoided through phenotypic plasticity (e.g. Flectobacillus sp. strain GC5)

Phenotypic plasticity can be described as the ability of an organism to express different phenotypes in response to environmental changes. This ability is peculiar for some particular organisms and makes them more adaptable to changes: their phenotype switch to a different one when it would be better adapted to the changed environmental conditions, for example reducing costs of cell maintaining, making it more competitive in substrate uptake or preserving the organism to predation. Due to phenotypic plasticity single genotypes can change their chemistry, physiology, development, morphology or behaviour in response to environmental cues. This mechanism of coordinate gene regulation involves alterations in genes expression in response to environmental signals. In the case of bacteria, phenotypic plasticity and a shift towards resistant morphotypes can be directly related to increased protists grazing pressure (**15**; **17**, **66**) as was explained in the previous paragraph. Another hypothesis, proposed in a study by Matz and Jürgens (**35**), and indirectly confirmed by the results of this study, suggests that chemical signals (CUES) released by predators during grazing activity can results in a shift in bacterial population size-composition independent of direct grazing impact and bacterial growth rate.

This was supported by an experimental study with the strain *Pseudomonas* sp. CM10, which produces two different morphotypes (**33**). Intraclonal polymorphism means that a small fraction of cells in the natural population is pre-adapted to different environmental forcing (grazing, nutrient conditions, etc.) before it actually occurs (**140**). Such a mechanism would facilitate rapid adaptation and survival of environmental changes. In all the bacterial populations which were analysed in the experimental part of this thesis, either in monocultures or in complex bacterial communities, a bacterial population was never represented by a single morphotype. Even under severe selection pressure (high grazing

pressure, complete starvation) bacterial morphologies were diverse, and a small number of cells with non-optimal morphologies were present.

Although resistant morphologies might be the most obvious resistance mechanisms against predation by protists, the regulating factors for its development by phenotypic plasticity are still unknown. Some recent studies (**76**; **88**) tried highlighted the general importance of morphologically peculiar bacterial cells in natural systems and obtained data on abundance, dynamics and biotic interactions such as competition and predation.

Rainey and Travisano (**141**) described the ability of the common bacteria *Pseudomonas fluorescens* to rapidly adapt to a spatially heterogeneous environment by morphological diversification through mutations and to maintain phenotypic diversity because of natural selection.

However, every change in phenotype is related to some alteration of its gene expression and thus not necessarily to mutations. It is possible to distinguish in two different mechanisms providing changes in bacterial size and shapes:

- Phenotypic plasticity
- Mutations and evolutionary changes

These two mechanisms provide in fact really different ways to acquire resistance against predation. Mutations are the most direct way to provide changes in phenotype, and the importance of them in providing grazing resistance was well shown in **59**. Nevertheless, reliability and repeatability of morphological adaptations in response of environmental changes, allowed scientists to propose different theories about the nature of the adaptation: the most common is the occurrence of phenotypic plasticity. The developing of grazing-resistant morphologies can be observed using aquatic bacteria in continuous culture lab systems as shown in **35** and in **16**.

Considering that protist grazing is an old evolutionary selective force for bacteria, was worth to look for mechanisms providing grazing resistance in bacteria, through adaptations connected to phenotypic plasticity, implementing the knowledge about the feed-back mechanisms between predators and prey, during the activity of grazing. Experiments using dialysis-bags and continuous cultures systems were performed to test our expectations and the outcomes of the study are exposed in detail in Chapter II.

In brief, our results demonstrate that indirect effects of grazer activities can significantly stimulate changes in bacterial morphology even without direct predation, e.g., probably by .a kind of "info-chemical" released during bacterial grazing.

These findings were related with changes in cells dimensions for the same strain (*Flectobacillus* sp. strain GC5), tested in presence (with or without direct predation) and in absence of predators. The experiments tested the exponential phase of bacterial growth in batch cultures and low growth rates at steady-state continuous cultures. We demonstrated that for the dominance of filaments in *Flectobacillus* sp. strain GC5, sufficient substrate supply is necessary, but not as only factor as proposed by Hahn *et al.* (**17**) because the presence of active predators is absolutely necessary.

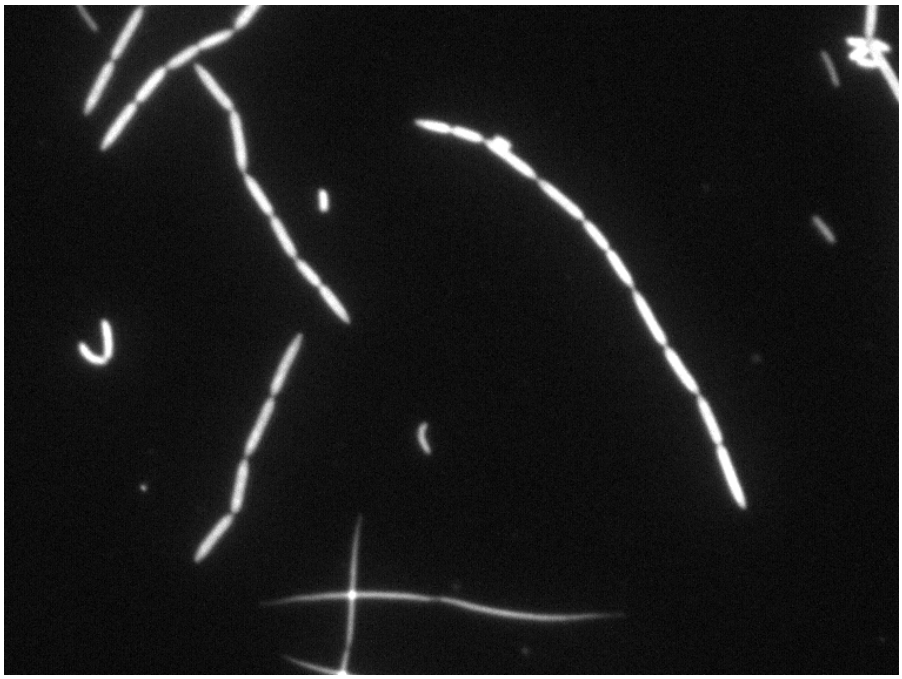
I.V Predation and productivity as factors which determine population dynamics, biomass levels and composition of complex bacterial communities

The relative importance of population regulation by resources and predation is determined by several factors (**142**; **143**; **144**; **145**, **71**). It was already demonstrated by mathematical models that the relative importance of competition and predation will change with the productivity of the environment, the latter becoming more important in productive systems (**63; 76**). Such a general pattern has been found also in experimental laboratory systems for bacteria-bacteriophage interactions (**60**) and would be also consistent with the frequent observations of morphologically resistant bacteria in more productive aquatic environments (**33**; **35**).

The presence of heterogeneity in prey edibility has been recognized as a fundamental factor that could shift the balance between top-down and bottom-up control (**146**; **63**; **86**; **147**; **64**; **148**). The presence of inedible individuals in a prey population has been shown theoretically to result in the reduction of top-down forces and an increase in the relative importance of bottom-up control (**63**; **86**; **64**). This shift occurs because non-edibility of some prey (refuge from predation), allows the prey population to respond numerically to resource increases.

This refuge can have a profound effect on the regulation of the prey populations. Bohannan and Lenski (**59**) proposed the following example. Compare the response of the

following two prey populations to an enrichment of their resources, in the presence of predators: a homogeneous and highly edible population and a population that consists of both inedible and highly edible individuals. The equilibrium density of the homogeneous population would be unaffected by enrichment, because the prey population is highly edible and thus regulated completely by predators. The homogeneous prey population would grow faster in response to enrichment, but at equilibrium this additional growth would be converted into predator biomass (**149**; **86**). In contrast, the equilibrium density of the heterogeneous prey population would increase in response to enrichment. This would occur because the inedible component of the population would increase (because it is not limited by predators and thus can respond to enrichment), whereas the edible component would remain unchanged (because it is limited by predators), with the net effect being an increase in the prey population in response to resource enrichment (**150**; **86**; **64**). Adding heterogeneity in edibility to the prey population has thus shifted the balance of forces regulating the prey population from predominantly top-down to predominantly bottom-up.



Microphotograph of heterogeneous freshwater bacteria isolated from natural communities (G. Corno 2003). Bacterial shape and size are decisive factors for the degree of edibility of a prey cells for heterotrophic nanoflagellates.

Performing continuous cultures experiments, Bohannan and Lenski (**76**; **59**) demonstrated that heterogeneity in prey edibility can alter the balance between bottom-up and top-down forces. They described the responses of model predator and prey populations (bacteriophages, *Escherichia coli*) to an enrichment in substrate supply, and compared the responses of

systems with and without heterogeneity in prey edibility. They showed that the presence of heterogeneous prey (more or less vulnerable to predators) can results in alterations of the trophic chain due to a reduced impact of predators on the prey population: the negative impact of the predators is concentrated just on a part, the more vulnerable, of the prey population, while the other part get an advantage by this sort of "selective predation" eliminating their competitors for resources.

In the present study the effect of heterogeneity of a bacterial community was tested for the first time through a gradient of substrate supply in continuous cultures systems, using bacterivorous flagellates as predators.

Following the results from the model communities observed by Lenski (**73**), Simms (**79**), Grover (**74**), Kraaijeveld and Godfray (**75**), Bohannan-Lenski (**59**), Horner-Devine *et al*. (**88**) and also from the data of the experiments shown in this thesis, a trade-off between competitive ability and edibility is revealed: the more resistant an individual is to predation, the less able it is to compete for resources.

If this assumption is not made, then the least edible prey individuals would always exclude those of higher edibility (**63**; **64**; **59**). Finally, resource availability is essential in supporting a heterogeneous prey population. If the amount of provided resources is too low, then the less edible prey may not be able to coexist with the more edible prey, because of the trade-off between edibility and competitiveness (**74**; **64**).

I.VI Existing models on predator-prey interactions include vulnerable and resistant prey

A model of prey-predator (in that case bacteria-bacteriophage) interactions presented by Levin *et al.* (**151**) was matching perfectly with most of the dynamics of the prey populations, but was lacking in the definition of prey. The model was improved by Leibold (**64**) and then by Bohannan and Lenski (**76**) by enlarging and adapting to this topic the concept of "heterogeneity of the prey population". Their prey population was defined by the degree of resistance against the phage predation.

Bohannan and Lenski were not only successfully emphasizing the importance of heterogeneity in prey populations. Always evolving the ideas and the suggestions of Leibold

(**64**) they also developed and tested (**59**), in simple laboratory continuous cultures system, an interesting theory relating the impact of productivity on predation and competition: at low levels of productivity, it predicts that the better competitor for substrate uptake will exclude the more resistant prey species, through a combination of resource and *apparent competition* (*sensu* Holt, **52**). At high levels of productivity, theory predicts that the more resistant prey will exclude the superior competitor, again through a combination of predation and *apparent competition*.

At intermediate levels of productivity, theory predicts that both prey species may coexist due to the trade-off between exploitation ability and predator resistance. General patterns predicted by Bohannan and Lenski (**60**) have been observed in natural communities, particularly freshwater communities. For example, positive correlations between biomass at adjacent trophic levels in response to changes in productivity have often been observed (**63**; **64**). The biomass of predator-resistant species (in phytoplankton) has also been observed to increase with productivity (**90**).

It is possible to summarize three general patterns, tested by Bohannan and Lenski, that are predicted even if the models are expanded to include multiple prey types (heterogeneity) and spatial patchiness (**64**):

- The biomass of total prey and predator will both increase, in a constant manner, in response to increased productivity
- The relative biomass of predator-resistant species will increase as productivity is increased
- The diversity of prey species will first increase then decrease as productivity is increased

These patterns were also tested by Bohannan and Lenski (**60**) on simple communities formed by *E.coli* B strain REL6584 as heterogeneous prey, and the phage T2 as predator. They tested their model communities developed at two different productivity levels, using a mathematical model designed on the basis of the models of Levin *et al.* (**151**) and Leibold (**64**) with the incorporation of prey heterogeneity.

In the present study the set-up of the experiments was complicated by an increased diversity of the prey and of the predators (heterotrophic nanoflagellates instead of phages). Additionally, instead of only two substrate level, a gradient of productivity was simulated (outlined in Chapter III).

Diversity of prey and predators in our communities increased not only the number of interactions between the two trophic levels but also interactions between the two groups of prey, and especially between different bacterial morphotypes belonging from different bacterial strains.

For a correct evaluation of biodiversity changes in this study, both genetic and morphological properties of the prey populations were assessed. The richness in operational taxonomic units (OTUs) was revealed from terminal restriction fragment length polymorphism (T-RFLP) fingerprints, and the richness in operational morphological units (OMUs) was estimated by visual identification and quantification of the different bacterial morphologies. Results show a clear discrepancy in OMU-OTU relation between communities subjected to grazing and communities without grazers. In the absence of predators, biodiversity is clearly related to morphological richness, whereas in the presence of predators, especially in very productive systems, a high OMU richness is combined with only few OUTs. An explanation could be that these taxa might constitute the highest plasticity and, in these extreme conditions, are able to dominate the community by developing adaptive morphologies (outlined in Chapter IV).

I.VII Aims of the study

Purpose of my thesis was to improve our understanding on the predator impact on a partially resistant and plastic bacterial strain, and then to elucidate the underlying mechanisms that are involved in the development of grazing resistance. Going from a simple, well-controlled single prey and single predator model system to more natural-like communities I studied the impact of predation on a bacterial community, and I tried to evaluate the relative importance of predation and competition for resources. More specifically I intended to answer the following questions:

In the one prey – one predator system:

- What are the underlying mechanisms for the development of morphologically resistant forms such as filamentous bacteria?
- Are fitness costs involved in morphological alterations of bacterial populations?

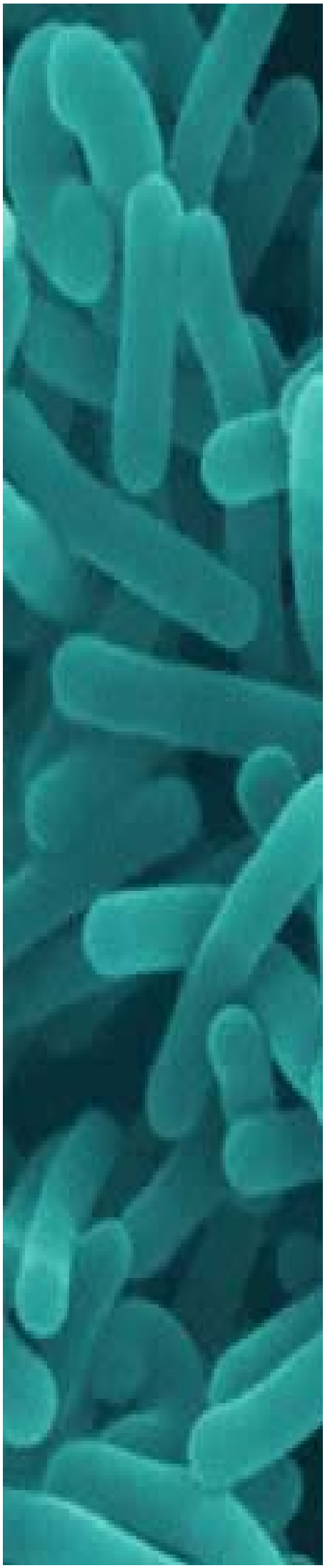
- How do substrate supply and grazing interact in the development of resistant bacterial morphologies?
- Is phenotypic plasticity and grazing-resistance chemically induced (e.g., by “info-chemicals“ released by the predators)?

In the complex communities of bacteria and bacterivores, exposed to a productivity gradient:

- Can the presence of invulnerable prey result in a shift in the balance between top-down and bottom-up control?
- Does equilibrium density of the heterogeneous prey population increase in response to enrichment in resources and how does grazing modify this pattern?
- How do predation and competition affect abundance and diversity of bacterial prey assemblages?

I.VIII A note on the structure of this thesis

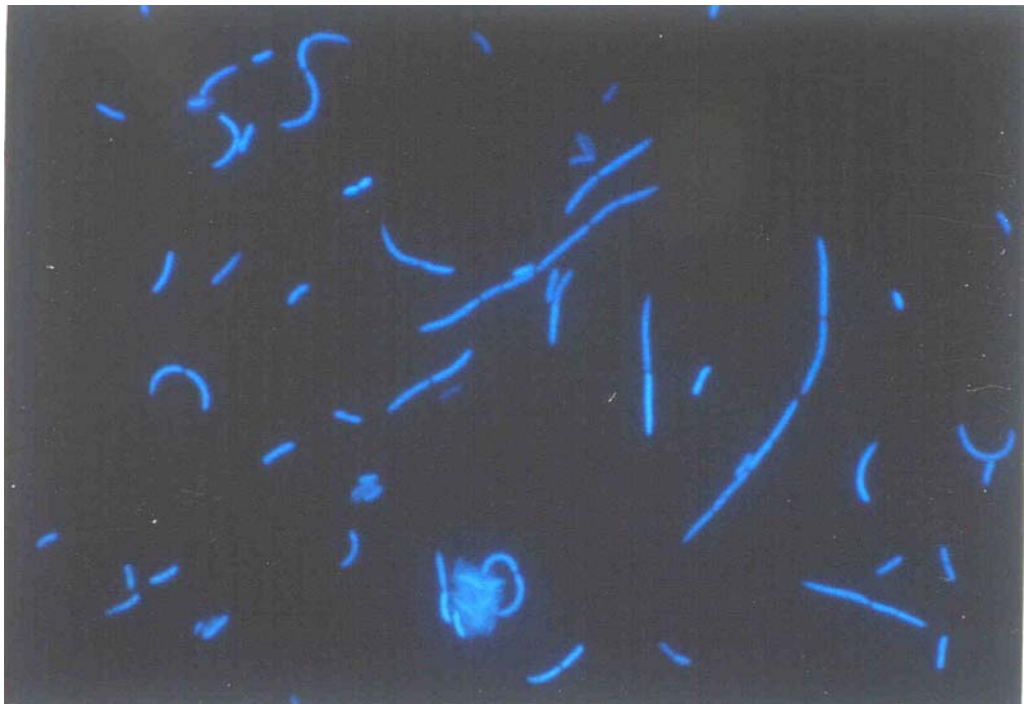
The studies made for this thesis are presented as discrete chapters which encapsulate a specific stage or goal of the overall objective. Each chapter contains an introduction, and is then broken down into descriptions of individual experiments containing results and discussion. In each chapter, an overview of the methods used is given.



Chapter II

Substrate supply and grazing as determining factors for the population size structure of a bacterial strain (*Flectobacillus* sp.) with high phenotypic plasticity

Substrate supply and grazing as determining factors for the population size structure of a bacterial strain (*Flectobacillus* sp.) with high phenotypic plasticity



Abstract

We studied the impact of grazing and substrate supply on the size structure of a freshwater bacterial strain (*Flectobacillus* sp.) which showed a pronounced morphological plasticity. Cell length varied from 2 → 40 µm and encompassed rods, curved cells and long filaments. Without grazers and with sufficient substrate supply bacteria grew mainly in form of medium-sized rods (4-7 µm) with a smaller proportion (< 10%) of filamentous

forms. Grazing experiments with the bacterivorous flagellate *Ochromonas* sp. showed that freely suspended cells < 7 µm were highly vulnerable to grazers whereas filamentous cells were grazing-resistant and became enriched during predation. Comparing long-term growth in carbon-limited chemostats with and without grazers revealed that strikingly different bacterial populations developed: treatments with flagellates were composed by more than 80 % of filamentous cells. These attained a biomass comparable to that of the populations in chemostats without grazers, which were composed of medium-sized rods. Carbon starvation resulted in a fast decrease of cell length and a shift towards small rods, highly vulnerable to grazing. Dialysis bag experiments were used to test for chemical induction of bacterial morphological changes. When performed in batch cultures, they revealed that the excretion of organic substrates by flagellates significantly stimulates bacterial growth but does not induce morphological alterations. In contrast, filament formation was stimulated by grazer excretory products when dialysis experiments were performed in continuous cultivation. This indicates that, besides active bacterial growth, prolonged exposition to grazer-released substances is necessary to trigger the phenotypic, adaptive response towards resistant morphotypes.

Introduction

Main environmental factors which regulate the growth and abundance of planktonic bacteria are substrate supply (bottom-up control) and predation (top-down control). The capacity of bacteria to adapt to different growth conditions, limiting nutrients and abiotic factors has been extensively examined. For many strains a high phenotypic plasticity with respect to physiology, macromolecular composition and morphology has been documented (**52**). This flexibility is a prerequisite of bacterial populations to cope with temporarily changing environmental conditions and to survive under hostile environmental conditions (**42**).

Much less evidence exists for adaptations towards biotic selective factors such as predation by bacterivores and viral lysis. Grazing mortality, mainly by small protists, is considered a major loss factor and therefore an important selective force for aquatic bacteria (44). Studies from recent years have demonstrated that different bacterial strains are not equally vulnerable towards grazers and have evolved different mechanisms to resist capture, ingestion or digestion by bacterivores (reviewed by **13**, **20**). Phenotypic bacterial properties which have been identified to influence grazing mortality are size and morphology (**9**, **46**),

swimming speed (Matz et al. submitted for publication), toxic pigments (**32**), and the physico-chemical surface structure (**36**, **34**). In planktonic environments, size-structured predator-prey interactions are of particular importance (**26**), and bacterial cell size must also be considered as a major feature which impacts vulnerability towards different grazers (**11**). Size-selective grazing by bacterivorous nanoflagellates exerts the strongest grazing pressure on medium-sized cells whereas bacteria obtain a predation refuge at the lower end of the prey size spectrum as well as at the upper end (**11**). For the smallest bacteria this is partly due to a reduced encounter and partly due to the inability of certain grazers to capture small cells (**19**). Elongated or filamentous cells and complex morphologies might be grazing-resistant as they are too large to be ingested by the smallest bacterivores (**10**). The interplay between size-selective grazing and differences in the cell size distribution of different bacterial strains, which finally results in an altered bacterial community composition, has also been demonstrated in chemostat experiments with mixed bacterial assemblages and bacterivorous nanoflagellates (**49**, **38**, **14**).

Grazing-resistant, filamentous bacteria are frequently observed in freshwater lakes, where they can temporarily attain significant concentrations (**50**, **22**, **51**, **39**). The appearance of filamentous, suspended bacteria has, less frequently, also been observed, in brackish (**8**) and marine (**5**) systems. The enhanced development of filamentous morphotypes has often been correlated to an increased abundance of small bacterivores (heterotrophic nanoflagellates), and this link could also be demonstrated in food web manipulation experiments (**18**, **21**, **47**, **48**). It is assumed that the appearance of grazing-resistant bacteria has important ecological implications such as the stabilization of bacterial biomass and a decreased trophic transfer efficiency (**19**).

Although these observations point towards grazing as a causative factor for the development of morphologically predation-resistant cells in natural bacterial assemblages, little is known regarding the underlying regulating mechanisms. Many bacterial taxa possess a considerable degree of phenotypic plasticity with respect to cell size and morphology e.g., (**25**). In studies with pure bacterial cultures and single predator species it has been shown that selective grazing and morphological plasticity of bacteria results in a shift towards resistant morphotypes, which could be filamentous forms (**45**, **15**) or bacteria growing in microcolonies (**16**, **33**). A simple but effective mechanism for the morphological shift towards filamentous growth, which involves phenotypic plasticity, was suggested by the studies of Hahn *et al.* (**15**, **17**). In continuous culture experiments the authors demonstrated that in the case of three bacterial species with moderate to high phenotypic plasticity, filament formation

was not necessarily a direct response of the bacteria to the presence of predators but was also observed when cells were grown at higher growth rates without grazing. Thus, the appearance of filaments in response to grazing was interpreted to be indirectly caused by an increase in specific growth rate, probably due to substrate excretion and elimination of non-resistant competitors by the grazers. With such a mechanism the development of resistant bacterial morphotypes would be independent of chemical cues released by the bacterivores, which have also been assumed to be involved (**38**, **19**), and it would constitute a non-adaptive response towards growth-accelerating substrate conditions.

However, the paucity of studies which have examined bacterial adaptations towards grazers does not allow further generalisations at present. The diversity of bacterial resistance mechanisms, which act at different stages of the predator-prey interactions (**20**) and which vary between different bacterial grazer species (**1**), seems to be quite large. Similarly as in previous studies (**15**, **33**) we used a bacteria-flagellate chemostat system to select and enrich for grazing-resistant bacterial strains. Interestingly, by this approach we isolated a facultative filamentous bacterium belonging to the same genus (*Flectobacillus*) which has been examined in previous studies on bacteria-protist interactions (**17**). We examined how the interaction of grazing pressure and substrate supply determined the size structure and vulnerability of this highly phenotypic plastic bacterial strain. In order to test for chemical induction of phenotypic changes we performed different types of dialysis experiments in batch and continuous culture. In several aspects our study confirmed the results of Hahn et al. (**17**) but with respect to the underlying mechanism of filament formation our isolate did not follow the suggested growth-rate dependency. Additionally, we present evidence that excretory products of bacterial grazers seem to be involved in the observed morphological shift towards resistant filaments.

Materials and Methods

Isolation, cultivation, and identification of microorganisms

Bacterial and flagellate strains were isolated from the euphotic zone of a mesotrophic lake in North Germany (Schöhsee). A culture of the mixotrophic flagellate *Ochromonas* sp. was obtained from a 10 µm-filtered water sample, inoculated on artificial mineral medium (WC,

12) which was supplemented with heat-killed bacteria (*Pseudomonas putida,* strain MM1, 6) but without organic carbon substrates. The chrysophyte genus *Ochromonas* is characterized by mixotrophic nutrition, i.e. photosynthesis and bacterivory. Our isolate (4-7 µm diameter) was able to grow also purely heterotrophically in the dark. Stock cultures of *Ochromonas* sp. were kept in WC mineral medium supplemented with a wheat grain to promote bacterial growth.

For axenisation of the *Ochromonas* culture a similar procedure as described by Hahn *et al.* (**15**) was used. From a dense flagellate culture, growing on a suspension of heat-killed bacteria, aliquots were inoculated into 24-well cell culture plates containing WC mineral medium, heat-killed bacteria (10^7 ml^{-1}) and a mixture of bacterial antibiotics (streptomycin, chloramphenicol and gentamycin, weight ratio 1:1:0.5). To account for flagellate sensitivity against antibiotics, a gradient of different concentrations of the antibiotic mixture (range 20-100 mg l^{-1}) was used. After 12 hours the culture plates were inspected by light microscopy and samples from wells which supported growth of flagellates were inoculated in new cell culture plates enriched with heat-killed bacteria but without antibiotics. After four days the presence of bacteria was examined microscopically, and by plating subsamples on NB agar plates. After repeating this procedure twice we obtained subcultures of *Ochromonas* sp. which did not contain live bacteria. These axenic cultures were maintained in Erlenmeyer flasks in the light, supplemented with heat-killed bacteria. For bacterial contamination subsamples were regularly checked microscopically and by plating subsamples on NB agar.

For enrichment and isolation of potentially grazing-resistant bacteria a one-stage chemostat was operated which was inoculated with a natural mixture of planktonic bacteria from Schöhsee and axenic *Ochromonas* sp. The chemostat reactor was fed with WC medium supplemented with 10 mg L^{-1} glucose as organic substrate source. The dilution rate was held at 0.5 d^{-1}. The system was installed in a climate room at 16°C with a 12:12 light-dark cycle. The microbial populations were quantified in the epifluorescence microscope after staining formalin-fixed samples with DAPI (4',6-diamidino-2-phenylindole) (**40**). After 4 weeks of continuous cultivation the bacterial community was dominated by filamentous bacteria which were obviously too large to be ingested by *Ochromonas* sp. For isolation of bacterial strains, culture suspension was plated on different agar plates (either NB or WC plus 1 g glucose L^{-1}). Isolated bacterial strains were kept in glycerol medium at -80° C until further examination.

From the isolated bacteria one strain was chosen for further experiments. This strain grew on agar plates in small, well-defined red colonies (diameter 0.5-2 mm) and developed elongated and variable cell morphologies (C-shape, long rods, cell chains etc.)

when grown in liquid media. None of the examined morphotypes showed any motility. For the taxonomic affiliation almost the full-length 16S ribosomal DNA was amplified by PCR with the primers 27f and 1492r (**28**). PCR products were sequenced by Taq cycle sequencing and universal 16S rRNA-specific primers using an ABI377 (Applied Biosystems, Inc.) sequencer. Sequence data were aligned using the online gene bank BLAST of NCBI. There was a 98 % similarity with *Flectobacillus mayor*, the type species of the genus *Flectobacillus* (**30**). Therefore, we will use the name *Flectobacillus* sp. strain GC-5 for this isolate. The accession number of the nearly full-length 16S rRNA gene sequence is AK00000.

Grazing exper. with *Flectobacillus* sp. strain GC-5 in batch and continuous cultures

For a first analysis of the morphological and size structure in response to flagellate grazing, and to obtain information about the vulnerability of the strain, bacteria were grown with and without *Ochromonas* sp. (starting concentration ca. 130 cells ml^{-1}) in short-term (3-4 d) batch cultures on WC medium supplemented with either glucose (10 mg L^{-1}) or, to test for higher growth rates and cell densities, with yeast extract (20 mg L^{-1}). Subsamples were taken regularly, fixed with formalin (2 % final conc.) and stored at 4° C until DAPI staining.

In order to compare the morphology and size structure of *Flectobacillus* sp. strain GC-5 growing under prolonged protist grazing pressure versus carbon-limited growth without predators, we performed a chemostat experiment. It consisted of eight 250 ml cylindrical chemostat reactors (filling volume) which were continuously aerated and mixed by fine bubbling with sterile air from below. WC mineral medium supplemented with glucose (10 mg l^{-1}) served as growth medium and was pumped by peristaltic pumps into the reactors to achieve a dilution rate of 1.0 d^{-1}. The chemostat system was assembled in a climate chamber at 16 ± 1°C at dim light.

Flectobacillus sp. strain GC-5 cells from a pre-culture were inoculated (100 µl, about 7 x 10^5 bacterial cells) into all eight reactors. After three days, when bacteria reached relatively stable numbers, axenic *Ochromonas* sp. cells were inoculated in four of the reactors to achieve an initial density of approximately 250 cells ml^{-1}. Subsamples were taken daily from all reactors and fixed with formalin (2 % final conc.) for counts of bacteria and protists, and bacterial size and volume measurements. Total chemostat runtime was four weeks.

Purity of the chemostat cultures was checked by immunofluorescence microscopy, using strain-specific polyclonal antibodies which were produced from rabbits immunized with *Flectobacillus* sp. strain GC-5 (Eurogentec, Herstal, Belgium). Staining with primary and

secondary antibodies and assessment by epifluorescence microscopy was done with a modification of the procedure described in (**6**).

Transfer of chemostat bacteria into batch cultures

In order to obtain information about the conservation of bacterial population size structures, and for a comparison of the growth dynamics of the differently pre-adapted subpopulations, bacteria from the different chemostat treatments were transferred to batch growth cultures at the end of the chemostat experiment. For this, the *Ochromonas* population inside the chemostats was killed by the addition of a eukaryotic inhibitor (Cycloheximide, final concentration 40 mg L^{-1}), which was added directly into the reactors after stopping the flow-through. After an exposition time of 6 hours, which proved to be sufficient to kill all flagellates, bacteria from the reactors were inoculated (500 µl) into batch cultures (100 ml flask, filled with 50 ml WC medium), which contained different concentrations of glucose (2, 10, and 20 mg L^{-1}, each in triplicate).The flasks were incubated for 60 h in the same climate chamber as the chemostat systems. Subsamples for determination of cell numbers and morphological analysis of bacteria were taken every 4 to 12 h.

In order to examine the morphological alterations of *Flectobacillus* sp. strain GC-5 when transferred from continuous cultivation to starvation conditions, 50 ml-samples collected from reactors with and without flagellates were transferred to batch cultures (100 ml flasks) without any substrate addition and incubated for 60 hours in the dark at16 ± 1°C. Subsamples for determination of cell numbers of bacteria and flagellates and for morphological analysis of bacteria were taken every 4 to 12 h.

Dialysis bags experiments

In order to examine the bacterial response to the excretory products of grazers without any direct predation impact we performed different types of dialysis bag experiments. First, batch experiments were performed in 250 ml bottles filled with WC medium, either without organic carbon or supplemented with 20 mg glucose L^{-1}. A dialysis bag (regenerated cellulose, 15,000 MWCO, ZelluTrans/Roth, Karlsruhe) with a volume of 100 ml was inserted in each bottle, thereby reducing the external volume to approximately 150 ml. *Flectobacillus* sp. strain GC-5 cells from a clonal pre-culture (100 µL, about $5x10^5$ cells) were inoculated into the bottles outside of the dialysis bags (final conc. approximately 3×10^3 cells ml^{-1}). Inside the bags were inoculated either *Ochromonas* (final conc. ca. 120 cells ml^{-1}) and live *Pseudomonas putida* MM1 (2.3×10^6 cells ml^{-1}) as prey, or, as controls, the same quantity of *P. putida* without

grazers. For each treatment we had three replicate trials. 2 ml subsamples were taken with syringes every 24 hours from inside and outside the dialysis bags and fixed with formalin (2 % final conc.) for cell counts and bacterial size measurements.

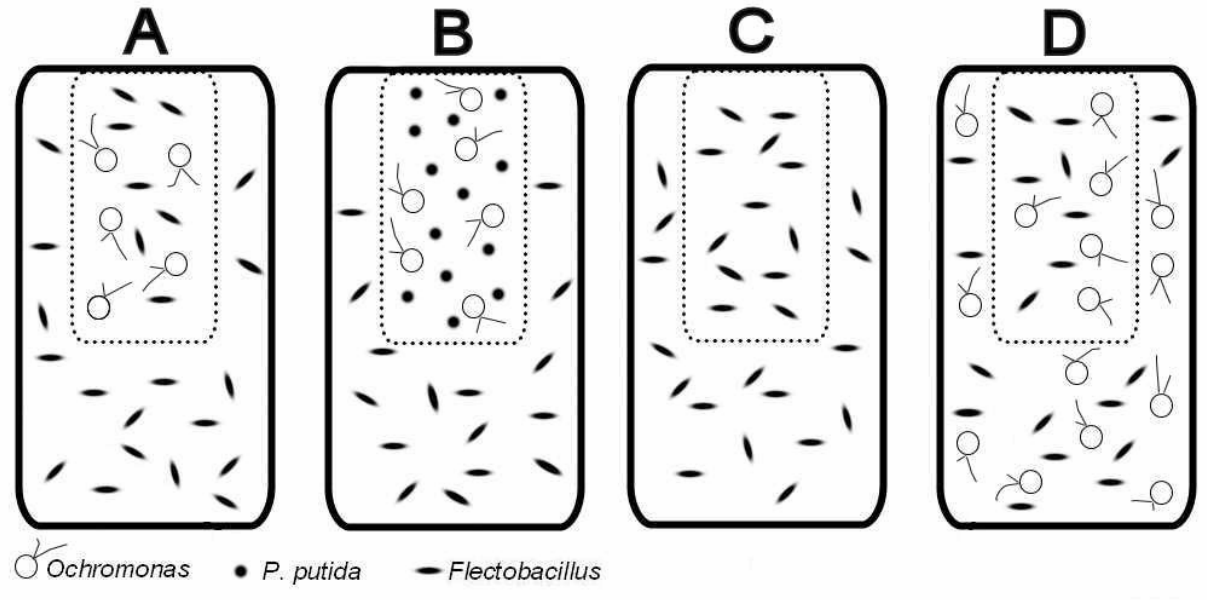


Figure 1: Scheme of the experimental set up in the dialysis bag chemostat experiment, showing microbial populations inside and outside of the dialysis bag (dashed line). A: grazing treatment with *Ochromonas* sp. and *Flectobacillus* GC-5 as prey bacteria; B: grazing treatment with *Ochromonas* sp. and *Pseudomonas putida* MM1 as prey bacteria; C: control treatment with *Flectobacillus* GC-5 only; D: control treatment with *Ochromonas* sp. and *Flectobacillus* GC-5 inside and outside of the dialysis bag.

For dialysis bag experiments under continuous substrate supply we used a chemostat system with the same type of dialysis bags as in the batch experiments, attached inside the reactors. The sterilized continuous-flow system was comprised of twelve cylindrical vessels continuously aerated, mixed by bubbling and filled with 500 ml WC medium (+20 mg glucose L^{-1}), set up at the same conditions as described above. A scheme of the inoculation with bacteria and flagellates inside and outside of the dialysis bags is shown in Fig. 1. *Flectobacillus* sp. strain GC-5 cells from a single clone pre-culture were inoculated (100 µL, about 7 x 10^5 bacterial cells) in all twelve reactors outside the bags. Into the bags were inoculated either *Flectobacillus* sp. (9 chemostats) or *P. putida* (3 chemostats). After 3 days, when bacterial growth in the reactors approached steady state, *Ochromonas* sp. was inoculated inside 3 of the dialysis bags with *Flectobacillus* sp. (Fig. 1A) and in the 3 bags with *P. putida* (Fig. 1B) (final conc. approximately 150 cells ml^{-1}). Three replicates of the remaining chemostats with *Flectobacillus* sp.-GC-5 inside the dialysis bags served as flagellate-free controls (Fig. 1C). In three other replicates *Ochromonas* sp. was inoculated

inside and outside the dialysis bags (Fig. 1D). These served as controls to compare for direct predation on *Flectobacillus* sp. The dilution rates of all reactors were adjusted to 1 d^{-1} after inoculation of the flagellates. Subsamples for determination of bacterial and protozoan abundance, bacterial size, shape and biovolume were taken from all reactors every day for a period of 20 days.

Cell numbers and bacterial cell size

Bacterial and flagellate cell numbers were determined from formalin (2 % final concentration) fixed and DAPI-stained samples, filtered onto 0.2 µm pore-size polycarbonate filters and counted by epifluorescence microscopy. At least 400 bacteria and 100 flagellates were counted per sample. We did not enumerate the abundance of bacterial cells but rather of bacterial ”morphological units” which comprised, besides free single cells, also filaments which consisted of several connected cells. This procedure yields correct estimates of bacterial biovolume but it does not reflect true cell numbers. However, for simplicity we further include all morphological units in the terms “bacterial cells” or “bacterial abundance”.

Bacterial cell size measurements were taken from DAPI stained samples. For some samples with mainly small morphotypes an automated image analysis system (SIS GmbH, Münster, Germany) was used which measures area and perimeter of 300-500 cells, which are used to calculate cell dimensions (length, width, volume) according to the algorithms given in (**31**). Since morphological changes (e.g., cell elongation) were mainly reflected in a change of the cell length, whereas cell width stayed relatively constant, for most samples only cell length was assessed. For this, the length of at least 100 randomly selected cells per filter was directly measured with the help of an ocular grid.

Statistical analysis

Statistical analyses were carried out using the software Statistica (version 5.1, StatSoft Inc.). Differences in development of bacterial numbers, biomass and cell length were tested for all chemostat experiments using analysis of variance with repeated measures (rm ANOVA). A multivariate analysis of variance (MANOVA) was used to test for differences in cell length distribution in the chemostat dialysis experiments. Differences in the daily values of the bacterial parameters were tested with one-way ANOVA with comparison of the means by Tukey’s post-hoc test. When necessary, bacterial number, biomass, cell length, and uptake rates were log-transformed to obtain a normal distribution of the data.

Results

Growth of *Flectobacillus* sp. strain GC-5 in batch culture

The polymorphic phenotypic appearance of *Flectobacillus* sp. strain GC-5 and the grazing-mediated shift in population size structure became apparent during short-term growth in batch cultures with either glucose or yeast extract as a carbon source. Without flagellates and supplemented with 10 mg glucose l^{-1}, bacteria grew exponentially (doubling time about 3.5 h) to about 5 x 10^6 cells ml^{-1} and remained afterwards in the stationary phase at more or less constant cell numbers for 3-4 days (Fig. 2). When flagellates were present, grazing reduced bacterial numbers to 1.3 ± 0.2 x 10^6 cells ml^{-1} (Fig. 2).

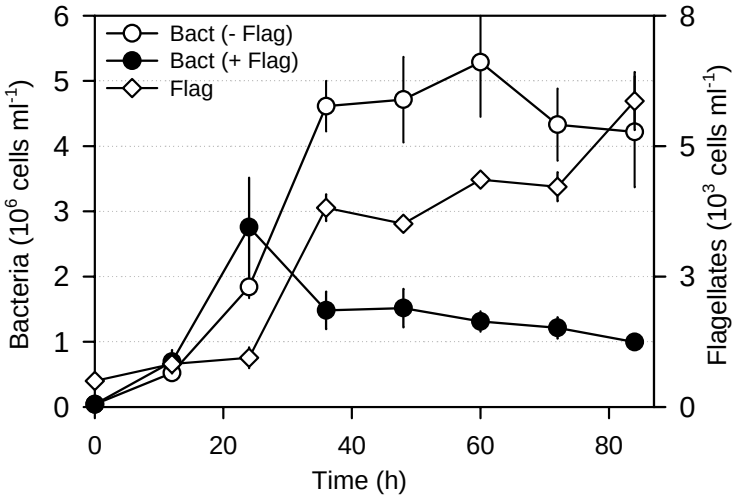


Figure 2: Growth of *Flectobacillus* GC-5 in batch culture on WC medium (+10 mg glucose l^{-1}) in the presence and absence of the bacterivorous flagellate *Ochromonas* sp.. Values are expressed as means of three replicates ± standard deviation.

There was always a mixture of differently sized and shaped cells present, in a range of cell length between 2 µm (single straight or c-shaped rods) and up to 40 µm long filaments (chains of 3-10 elongated rods). Without predation, the mean bacterial cell length at the end of the exponential phase was 5.0 ± 0.7 µm and the population was composed to about 80 % of single cells shorter than 7 µm, increasing to more than 90 % in stationary phase (Fig. 3B). Increasing the substrate concentration to 20 mg glucose l-1 approximately doubled the bacterial concentration in grazer-free trials but did not change the morphological composition

(data not shown). Using yeast extract instead of glucose as carbon source, the average cell length was slightly larger (6.3 ± 0.9 µm) but still around 80 % of the population consisted of single cells < 7 µm at the end of the exponential phase (data not shown).

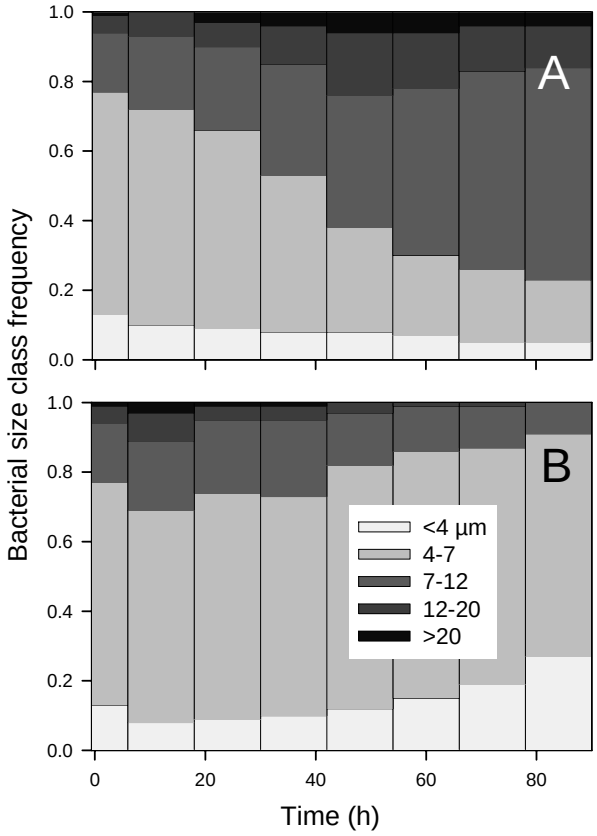


Figure 3: Development of different cell length classes of *Flectobacillus* GC-5 when grown in batch cultures in the presence (A) or the absence (B) of *Ochromonas* sp. Data were collected from the experiment shown in Figure 1.

Strong shifts in population size structure became apparent when *Flectobacillus* sp. strain GC-5 was grown in batch culture together with *Ochromonas* (Fig. 3A). Freely suspended cells < 7 µm appeared to be completely edible for the flagellates, as judged from the continuous decline of this size class parallel to the growth of *Ochromonas* sp. In contrast, bacterial cells > 7 µm (mainly chains of 2-5 elongated cells) seemed to be too large to become ingested and increased in relative proportions in the grazing treatments, constituting 75-80 % of the bacteria at the end of the batch experiment shown in Figure 2. Based on these results we therefore used an operational size limit of 7 µm to distinguish *Flectobacillus* sp. GC-5 cells into edible and inedible (resistant) morphotypes for *Ochromonas* sp. The cell width of the bacteria remained fairly constant (0.5-0.7 µm) independent of the impact of grazing and

the realised cell length. Estimates of total bacterial biovolume were therefore based on cell length measurements and an assumed constant cell width.

Long term bacterial growth in continuous cultivation

Flectobacillus sp. strain GC-5 was grown during four weeks in carbon-limited chemostats with and without grazers in order to examine long-term adaptations during continuous grazing pressure. Immunofluorescence microscopy revealed that both populations contained no contamination by other bacteria and were composed until the end of the experiment to 100 % by our target strain. After the dilution rate was lowered from 1.9 to 1.0 d^{-1} at day 8, both bacteria and flagellates maintained rather stable populations until the end of the experiment (Fig. 4).

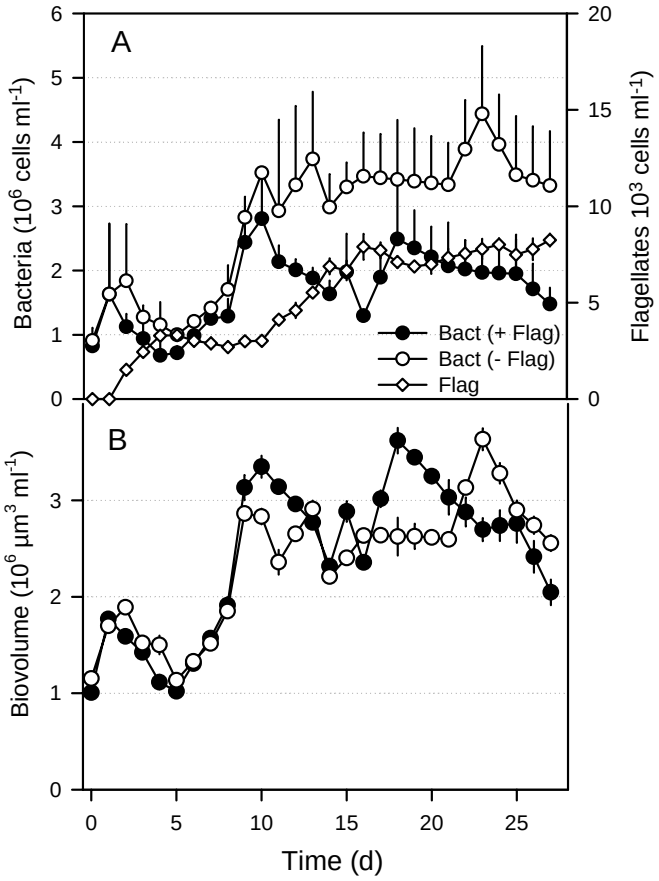


Figure 4: Temporal development of bacterial and flagellates abundance (A) and bacterial biovolume (B) in one-stage continuous cultures of *Flectobacillus* GC-5 in the presence and absence of the bacterivorous flagellate *Ochromonas* sp.. Abundance and biovolume data are given as means ± standard deviation of four replicate reactors.

The bacterial concentration in the flagellate-free chemostats stabilised between days 10-28 at 3.5 ± 0.3 x 10^6 cells ml^{-1}. Bacterial numbers fluctuated slightly more in the chemostats with

Ochromonas and achieved an average concentration of 1.9 ± 0.3 x 10^6 cells ml^{-1}. Flagellate numbers between day 10 and 28 had an average concentration of 6.8 ± 3.1 x 10^3 cells ml^{-1}. The significant reduction of bacterial numbers in the grazer chemostats ($p<0.01$, rm ANOVA) was not reflected in differences of total bacterial biovolume between chemostats with and without grazers ($p>0.1$, rm ANOVA, Fig. 4B).

The mean bacterial biovolume values for the period day 10-28 were identical for the two treatments (3.6 ± 0.6 x 10^6 μm^3 ml^{-1}). Testing for differences independently for each date, only 2 of the 18 days had significantly different biovolumes between the two treatment groups ($p<0.05$, Tukey´s post-hoc-test).

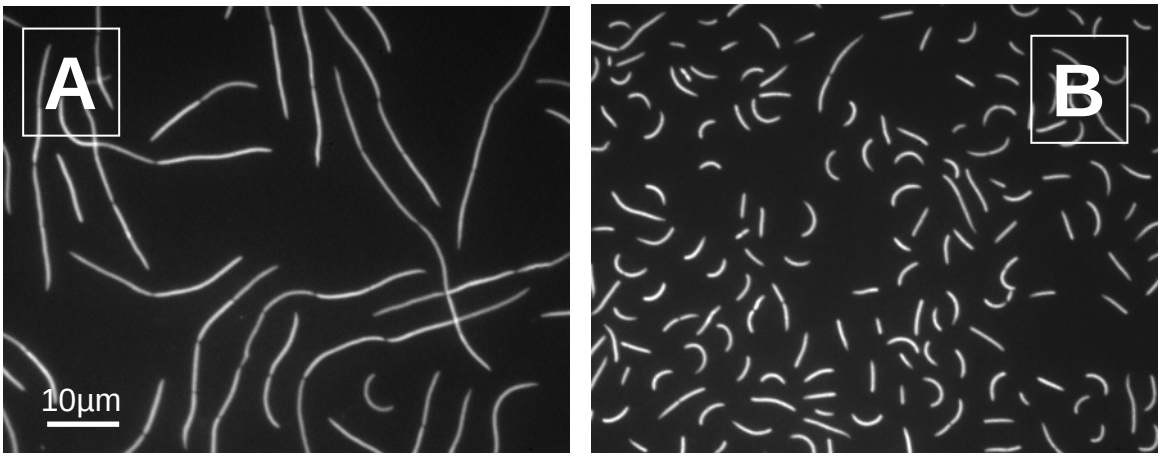


Figure 5: Microphotographs of DAPI-stained cells of *Flectobacillus* GC-5, grown in chemostat cultures in the presence (A) or in the absence (B) of *Ochromonas* sp. Samples were taken at day 28 of the chemostat run. Scale bar in A refers to both photographs.

The reason for different cell numbers but approximately similar total biovolume could easily be deduced when looking at the bacterial morphologies and size distribution in the treatments with and without grazers (Fig. 5).

After one month in the different reactors we obtained two *Flectobacillus* populations with completely different morphologies and size structure: chemostats without grazers were dominated by curved rods and c-shaped morphologies consisting of one or two cells, mostly < 7 µm in length, whereas only few filamentous cells occurred (Fig. 5B). In contrast, in the chemostats with *Ochromonas* single cells < 7 µm were nearly eliminated and the population of *Flectobacillus* sp. strain GC-5 was entirely dominated by filamentous cells, mostly 10-25 µm in length, and chains of 3-10 cells reaching up to 40 µm in length (Fig. 5A).

The detailed morphometric analysis of the two bacterial populations revealed that the mean cell width had not significantly changed and only shifts in mean cell length affected the total biovolume (Tab. 1). The mean cell length and the length to width ratio were about three times higher in the chemostats with *Ochromonas* sp. (Tab. 1). Considering our size limit of 7 µm for edibility of the bacteria, on average 76 % of the bacterial population were grazing-resistant in the chemostats with flagellates.

		With flagellates	Without flagellates
Cell volume (μm^2)	mean ± sd	2.55 ± 1.88	1.06 ± 0.38
	min.	0.280	0.337
	max.	7.772	2.999
Cell width (W) (µm)	mean ± sd	0.47 ± 0.07	0.51 ± 0.05
	min.	0.36	0.40
	max.	0.59	0.62
Cell length (L) (µm)	mean ± sd	14.50 ± 9.72	4.31 ± 1.66
	min.	2.21	2.17
	max.	48.56	12.47
L / W ratio	mean ± sd	30.4 ± 21.2	10.5 ± 3.8
	min.	5.3	4.7
	max.	84.1	23.3
% inedible morphotypes	mean	76 ± 11	17± 4
	min.	69	15
	max.	86	21

Table 1: Comparison of cell size characters of *Flectobacillus* GC-5 during growth in chemostat culture in the presence and in the absence of the bacterivorous flagellate *Ochromonas* sp. The period between day 10 and day 28 of the chemostat run was analysed.

The temporal development of the different bacterial size classes in the chemostats (Fig. 6) shows that the proportion of the two edible cell length classes, < 4 µm and 4-7 µm, continuously declined in the chemostats with flagellates, and the major size class became the fraction of 7-12 µm (Fig. 6A).

In contrast, small cells increased in the flagellate-free reactors particularly after day 10, when the dilution rate was decreased. Afterwards the edible size fractions < 4 µm and 4-7 µm dominated with a relative stable proportion of around 80 % of the total population (Fig. 6 B).

Regrowth of chemostat bacteria in batch cultures

Bacterial inocula were derived from the two chemostat populations with contrasting size structure and morphological composition (as described above) but with about similar bacterial biovolume. The inoculum from the grazer-free chemostat consisted to > 80% of single, straight or c-shaped rods, whereas the inoculum from the grazer chemostat was dominated to > 80% by long filaments and chains.

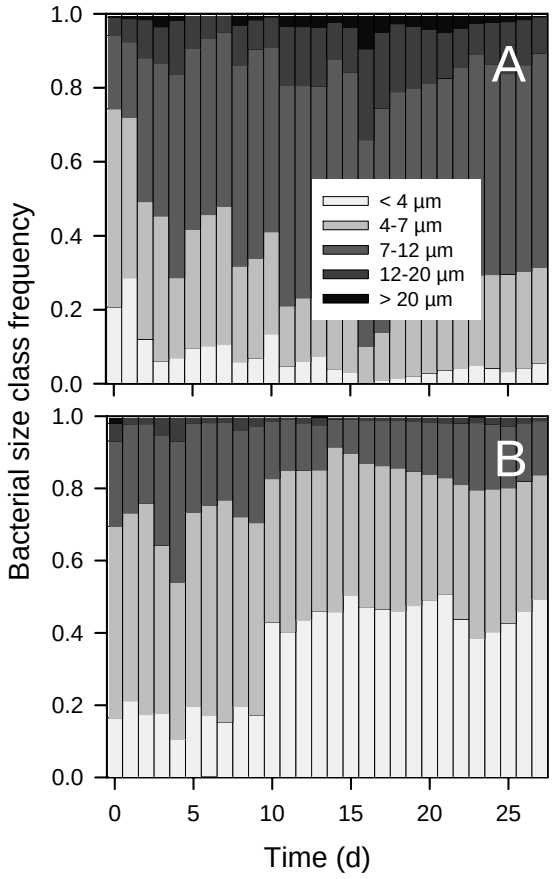


Figure 6: Development of frequency distribution of different bacterial cell length classes (relative abundance) of DAPI-stained cells of *Flectobacillus* GC-5 during growth in one-stage continuous cultures in the presence (A) and in the absence (B) of the bacterivorous flagellate *Ochromonas* sp. Data are means of three replicate chemostats. For further details see text.

Despite these differences the bacterial growth dynamics, maximal cell numbers, and final mean cell length attained in batch culture were comparable (Fig. 7). 30 - 40 h after the inoculation, bacteria reached the stationary phase in which cell numbers stayed constant and only the proportions of edible and inedible morphotypes changed. In media supplemented with 10 mg glucose L^{-1} (Figure 7C, D) both batch cultures had about similar growth rates

(doubling time during the logarithmic growth phase 3.7-3.9 h) and achieved similar maximal cell numbers (5.0 - 5.2 x 10^6 cells ml^{-1}). In media supplemented with 20 mg glucose L^{-1} bacteria inoculated from grazer-free chemostats (Fig. 7A) achieved slightly higher maximal cell numbers (10.2 ± 1.1 x 10^6 ml^{-1}) than bacteria derived from the grazer chemostats (Fig. 6B, 9.9 ± 1.2 x 10^6 ml^{-1}), and achieved these earlier due to a higher growth rate (estimated doubling time 3.4 and 4.0 h respectively in 7A and B).

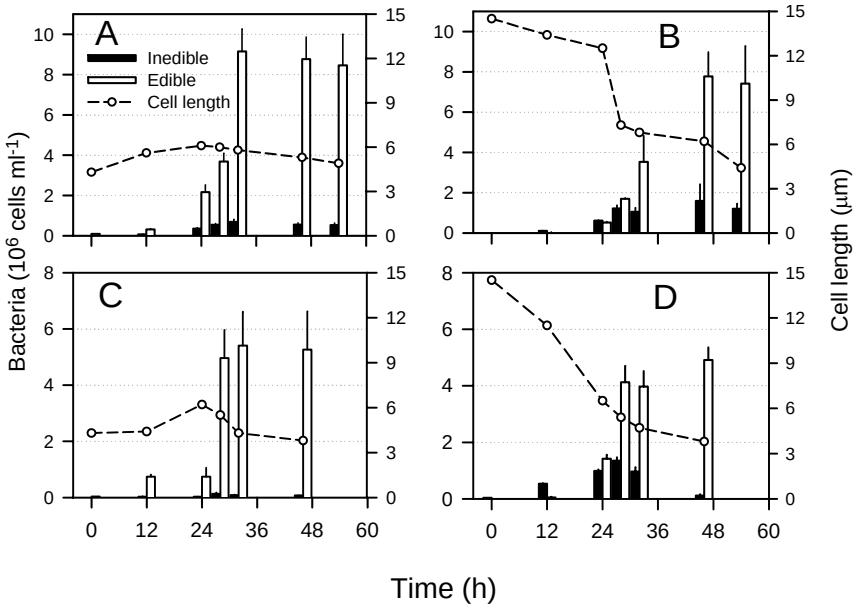


Figure 7: Regrowth of *Flectobacillus* GC-5, derived from grazer-free (A, C) and grazer chemostats (B, D), in batch cultures. Development of bacterial abundance, differentiated into edible (<7 µm cell length) and inedible (> 7 µm) cell length classes, and of mean bacterial cell length after inoculation of chemostat bacteria into batch cultures. Growth media were supplemented with 20 (A, B) or 10 mg glucose l^{-1} (C, D). Values are means ± standard deviation of three replicates.

Although in all treatments mainly edible (<7 µm) morphotypes developed, some differences between the two populations became apparent. The populations derived from grazer-free inocula were dominated by characteristic curved (c-shape) morphologies, consisting often of two connected cells. This “double C-shape”, presumably recently divided but not separated cells, was permanently present during the batch experiment. Populations derived from grazer-adapted inocula developed only few c-shaped cells (< 5%) but straight rods, roughly similar in length and volume to the c-shaped cells. Abundance of filamentous cells, dominant in the inoculum, decreased and newly developed cells seemed to consist only of short rods. Bacteria from both inocula achieved a similar mean cell length of around 6 µm during the exponential growth phase. For cells derived from the grazer chemostats this

implied a reduction in cell length from an average of 14.5 µm long filaments, for cells derived from grazer-free chemostats it involved an increase from an initial mean length of 4.3 µm.

A shift towards smaller cell sizes became apparent when chemostat bacteria were transferred to batch cultures without substrate supply (Fig. 8). Bacteria from the grazer-free chemostats (Fig. 8A) declined by about 30 % in numbers within 48 h. Mean cell length was reduced to 2.6 ± 0.3 µm and inedible size classes (> 7 µm) nearly disappeared in these starvation conditions.

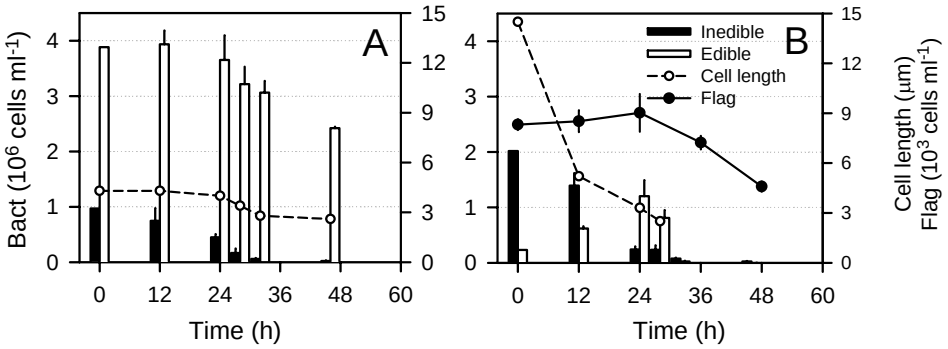


Figure 8: Transfer of chemostat bacteria, derived from grazer-free (A) and grazer chemostats (B), into starvation conditions in batch culture. Development of flagellates, bacteria, differentiated into edible (<7 µm) and inedible (> 7 µm) bacterial cell length classes, and development of mean bacterial cell length after transfer into batch culture. Values are means ± standard deviation of three replicates.

More drastic changes occurred in the populations taken from the grazer chemostats (Fig. 8B): mean cell length shifted in less than 24 h from grazing-resistant long filaments to straight rods in the edible size range (<7 µm). As flagellates were still present in this starvation culture, the size reduction and grazing pressure finally resulted in a severe decline of bacterial abundance to 0.5 ± 0.2 x 10^6 ml^{-1} after 24 hours and to less than 0.1 x 10^6 ml^{-1} after 48 hours.

Dialysis bag experiments in batch culture

Growth and morphological changes of *Flectobacillus* sp.GC-5 in the presence of flagellates, but without a direct predation impact were examined in dialysis batch experiments with different substrate concentrations. The food bacteria in the dialysis bags were nearly entirely consumed by *Ochromonas* sp. within three days. Stimulation of *Flectobacillus* GC-5 growing outside the bags by the excretory products of the flagellates became evident from the differences in bacterial growth curves between treatments with and without flagellates in the bags (Fig. 9). Bacterial cell numbers at the end of the experiment were up to 40 % higher in

the flagellate treatments ($p<0.05$, student t-test). In contrast to cell numbers, no significant effect of the flagellate excretory products on the bacterial size distribution could be observed. In all treatments the proportion of bacteria within the edible size class range < 7 µm (straight and c-shaped rods, short filaments) was between 78 to 82% of the whole population and no significant differences between the treatments occurred (data not shown). This is roughly the normal proportion of edible morphotypes observed during batch growth of *Flectobacillus* sp. GC-5 without predation.

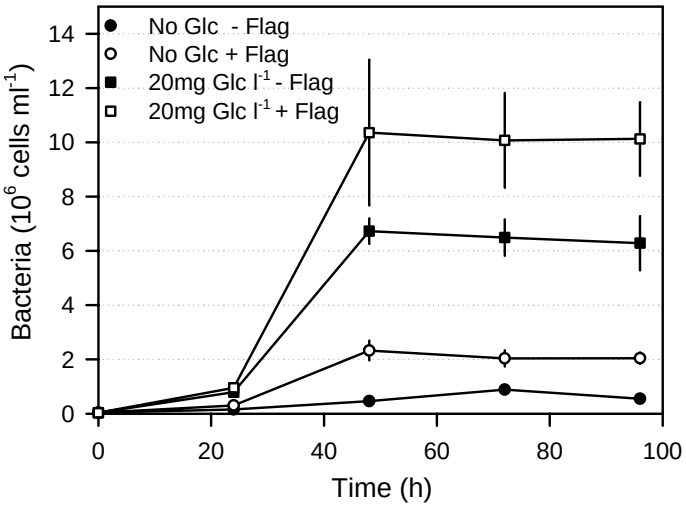


Figure 9: Growth of *Flectobacillus* GC-5 in dialysis bag batch culture experiment with and without glucose addition (shown is the bacterial growth outside the dialysis bags). Dialysis bags contained either only *Flectobacillus* GC-5 (-flag) or *Flectobacillus* GC-5 and *Ochromonas* sp. (+flag). The development of Cells numbers are given as means ± standard deviation of 3 replicates.

Dialysis bag experiments in chemostat cultures

It was possible to combine the dialysis experiment with continuous cultivation of *Flectobacillus* sp. by inserting the dialysis bags with *Ochromonas* sp. and different prey bacteria (either *Flectobacillus* sp. or *P.putida*) inside the chemostat vessels. During the whole 20-day experiment, grazing by *Ochromonas* sp. occurred in the dialysis bags and their excretory products could permanently influence the *Flectobacillus* sp. population growing outside the bags.

All treatments achieved steady-state like conditions after approximately one week and had fairly stable population levels of *Flectobacillus* sp. strain GC-5 until the end of the experiment (Fig. 10).The results of the two type of control treatments, *Ochromonas* sp. inside and outside the dialysis bags and reactors without any predators (only bacteria inside the

bags), resembled the chemostat experiment shown before. The average bacterial concentrations which were achieved at steady-state like conditions were 8.0 x 10^6 ± 0.5 cells ml^{-1} without and 3.1 ± 2.1 x 10^6 cells ml^{-1} with *Ochromonas* sp. The bacterial concentrations in the reactors in which flagellate grazing inside the dialysis bags occurred achieved significantly higher mean values, 10.0 ± 0.8 x 10^6 cells ml^{-1} with *Flectobacillus* sp. and 12.2 ± 1.1 x 10^6 cells ml^{-1} with *P. putida* MM1 as prey bacterium (rm ANOVA, $p<0.01$).

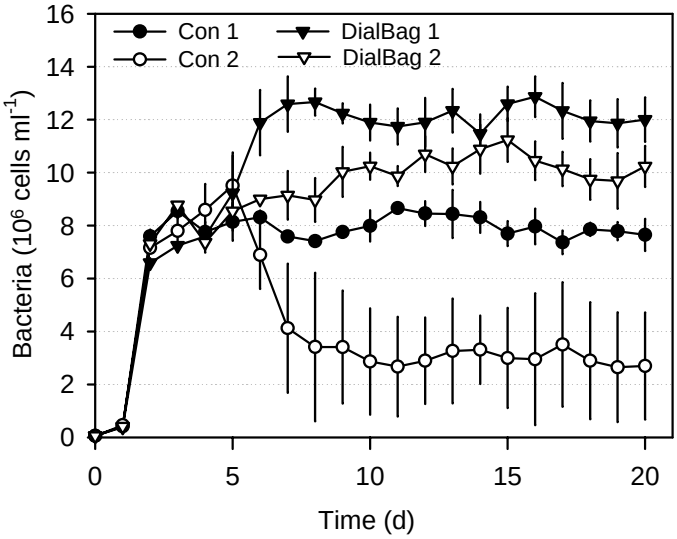


Figure 10: Growth of *Flectobacillus* GC-5 in dialysis bag continuous culture experiment. Different treatments included controls (bacteria only, bacteria + flagellates) and treatments with flagellates and bacteria (either *P. putida* MM1 or *Flectobacillus* GC5) inside the dialysis bags. Cells numbers are given as means of 3 replicates for each treatment ± standard deviation.

The size structure of *Flectobacillus* sp. strain GC-5 in the different chemostat reactors was followed for the whole experimental time (Fig. 11).

For a direct comparison of the different treatments the last six days of the experiments were chosen because here prolonged direct and indirect effects of flagellate bacterivory should have become evident. In the controls with and without flagellates the same contrasting size distribution as in the first chemostat experiment could be observed: without flagellates (Fig. 11 A) about 75% of the population was composed by cells < 7 µm (edible size range for *Ochromonas* sp.) and the median cell length was 6.2 µm. With direct grazing impact of flagellates (Fig. 11 B) on average 86 % of the cells were > 7 µm (median 18.6 µm) and thus inedible for *Ochromonas* sp..

Most interesting in this experiment was the bacterial size distribution of the populations which were not directly grazed but potentially influenced by flagellate excretory products from the dialysis bags (Fig. 11 C and D).

Here the mean cell lengths of *Flectobacillus* sp. strain GC-5 were in between the two control treatments shown in Figs. 11 A and B. With *Flectobacillus* as prey bacterium in the dialysis bag (Fig. 11C), the external bacterial population achieved a median cell length of 11.4 µm (50% between 7 to 15 µm, 80% between 5 to 24 µm) which was significantly higher than in the flagellate-free control (MANOVA, $p<0.001$). A similar effect was observed when grazers in the dialysis bags had *P. putida* as prey bacteria (Fig. 11D): 11.6 µm as the median cell length, 50% of the cells between 7 to 20 µm, and 80% between 5 to 24 µm (difference A-D, $p<0.0001$). At steady-state in both groups of reactors (C and D) with *Ochromonas* in the dialysis bags less than 30% of the cells were in the edible size range (<7 µm) for flagellates.

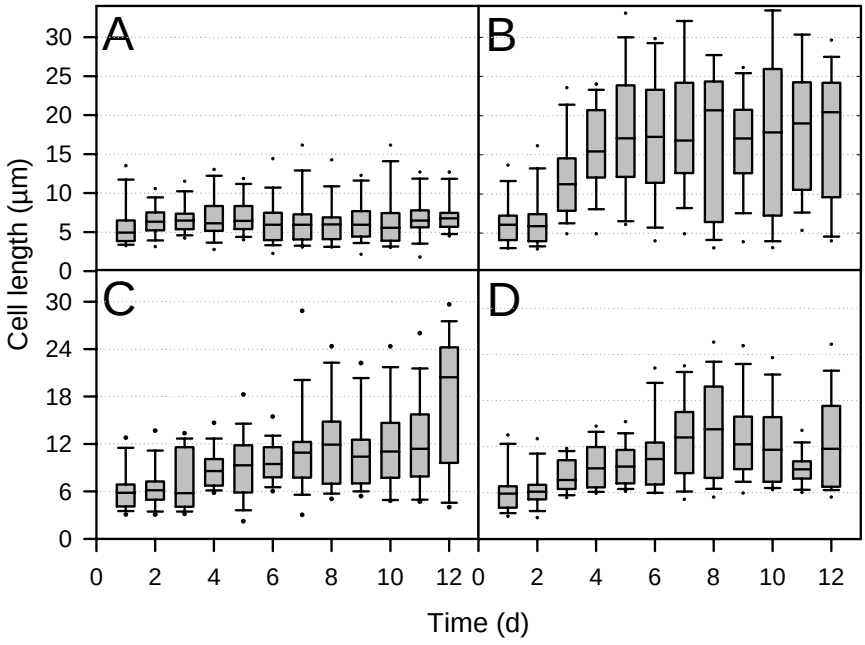


Figure 11: Development of cell length of *Flectobacillus* GC-5 in the dialysis bag continuous culture experiment. Time scale corresponds to the last 12 days of the graphs shown in Fig. 9. Treatments A-D contained different microbial populations inside and outside the bags.
A: only *Flectobacillus* GC-5 (inside and outside); B: *Flectobacillus* GC-5 and *Ochromonas* sp. (inside and outside); C: inside: *Flectobacillus* GC-5 + *Ochromonas* sp., outside: *Flectobacillus* GC-5; D: inside: *P. putida* MM1 + *Ochromonas* sp.; outside: *Flectobacillus* GC-5. Vertical boxes with error bars in the graphs represent the median, 10th, 25th, 75th, and 90th percentiles of bacterial cells length (mean of two replicates) of *Flectobacillus* GC-5.

Discussion and conclusions

Similar model systems as the one used here have been successfully used in previous studies on bacteria-protist interactions, providing insights into mechanisms of bacterial adaptations towards predation (**17**, **15**, **14**, **33**). Chemostat and batch culture experiments with bacterial monocultures and axenic (or monoxenic) bacterivores make it possible to follow phenotypic shifts in the prey bacteria and resulting alterations in their vulnerability and grazing mortality. Such simplified model systems are not designed to mimic natural conditions but to examine potential underlying mechanisms in predation-mediated changes of bacterial phenotypic properties. In these experiments, the quantitative results of the resistant and vulnerable phenotypic traits (e.g., cell size) apply only for the specific combination of bacteria and grazer organisms used. Nevertheless, by such an approach we can obtain a more detailed insight into the regulating factors for the development of resistance properties in response to protist grazing.

Effect of substrate supply and flagellate grazing on the population size structure of *Flectobacillus* GC-5

The *Flectobacillus* strain investigated in our study is obviously a highly polymorphic bacterium, covering a range of cell lengths from small rods < 2 µm to filamentous cell chains of > 40 µm. The whole range of morphotypes can be found within a clonal culture but the distribution and frequencies of the different morphologies is determined by substrate concentration (and resulting growth rate) as well as by the actual grazing pressure. The reported size limits to separate grazing-vulnerable from grazing-resistant bacterial cells range from 2.4 µm to 10 µm in different study systems (**13**). Recently it has been shown that even filamentous bacteria are not absolutely resistant against flagellate grazing but have a selective advantage due to strongly reduced ingestion efficiency by bacterivorous flagellates (**57**).

For our model system a bacterial cell length of approximately 7 µm defined the size above which grazing seemed to become too inefficient. This absolute or relative resistance of *Flectobacillus* cells > 7 µm can explain the observed shift in the population size structure when exposed to grazing by *Ochromonas* (Fig. 3), and the relatively large concentration of remaining ungrazed bacteria in batch grazing experiments (Fig. 2). This is similar in some

way to the fact that there is mostly a surviving fraction when a bacterial population is exposed to some kind of lethal stress (**3**). Phenotypic heterogeneity is a recognised property of bacterial cultures and includes morphological (**24**, **57**) and metabolic (**54**) properties. It is assumed that heterogeneity arises at different levels of cell regulation (e.g., transcription, translation) and involves some stochasticity, for example in the synthesis of different proteins (**3**). In case of strong phenotypic heterogeneity, cells surviving exposure to stress possess the same genetic potential as the original population and can generate a similar mixed population profile when returning to the previous conditions (**3**).

The *Flectobacillus* cells from chemostats with flagellates, which consisted mainly of resistant filamentous cells, returned quickly to the smaller, vulnerable phenotype when transferred to a predation-free growth medium (Fig. 7). This transient nature of phenotypic alterations in response to the environment is characteristic for phenotypic plasticity and indicates that it is not an adaptive mutational event. Phenotypic variation by mutation and selection (**41**) but also by other underlying genetic mechanisms is a well-known mechanism which can result in rapid speciation of bacteria. In the case of bacterial resistance against to *Bdellovibrio*, both processes have been demonstrated, the appearance of resistant mutants (**55**) and a phenotypic plastic response (**43**).

Flectobacillus GC-5 produced a polymorphic population which encompasses the whole range of cell lengths. Different selection pressure, realized for example in continuous cultivation, shifted the majority of the population to different optimally adapted phenotypes. Without predation, when only competition for the organic substrate was the main selective force, cells of 4-5 µm length seemed to be best adapted. This was also confirmed in the batch transfer experiments (Fig. 7). Cells in the edible size range (< 7 µm) constituted a fairly constant proportion of around 80 %, also during exponential growth on higher concentrations of organic substrates (glucose, yeast extract).

In contrast, prolonged grazing pressure shifted the *Flectobacillus* population size structure to resistant filaments with a mean length of 14-15 µm but encompassing a wide range of cell lengths (e.g., Tab. 1). A minor portion (around 20 %) of edible bacterial cells sustained a relatively small flagellate population which was able to maintain the bacterial size structure under grazing control.

This shift in population size structure resulted in a compensation of bacterial grazing mortality and nearly similar bacterial biomass in grazed and ungrazed systems (Fig. 4). A similar degree of compensation has been found in chemostats with mixed bacterial assemblages but was due to the occurrence of different resistance mechanisms (**35**). A

continuous size distribution and co-occurrence of vulnerable and resistant morphotypes has also been observed for several filament-forming bacterial strains (**17**). This polymorphism within populations should enhance the adaptability and the potential to survive environmental changes, caused by abiotic or biotic factors.

It is generally assumed that adaptive phenotypic plasticity involves fitness costs for the organisms although these might be difficult to assess (**7**).

The fact that the growth rate of both chemostat populations, with and without predators, was similar when re-inoculated into batch growth medium indicates that the fitness costs are probably rather low compared to the substrate-adapted population. Further, we could not detect a less efficient uptake of radiolabelled glucose or amino acids by the filamentous morphotypes nor a restricted substrate utilization in Biolog GN plates (Corno and Jürgens, unpublished results). The rapid shift in the size structure to medium-sized cells under carbon limited growth implies, however, that some disadvantages must be involved with the filamentous morphologies and it might need a more detailed analysis to detect these constraints.

From the starvation experiment it became obvious that it requires sufficient substrate supply for development of elongated and filamentous cell forms. When *Flectobacillus* cultures were transferred to a starvation medium, fast decline in cell size (to < 3 µm) into the edible size range for *Ochromonas* sp. occurred. Thus, when grazers were present the *Flectobacillus* population was almost completely eliminated (Fig. 8B), indicating that no other resistance mechanisms were expressed. A growth-rate dependency of the bacterial cell size and a decrease in cells size during starvation are generally known among bacteria (**23**). This differs, however, from the observations made by Hahn *et al.* (**17**) for another *Flectobacillus* strain in which high growth rates produced a strongly increased fraction of filamentous morphotypes even without predators. Even on a relatively rich medium (20 mg yeast extract l^{-1}) the *Flectobacillus* strain used in our experiments did not develop a higher proportion of filamentous cells when predators were absent.

Impact of flagellate excretory products on bacterial growth and morphology

The grazing activity of bacterivorous protists is known to be important for the remineralization of bacterially bound nutrients (**4**) but also for the release of dissolved (DOC) and colloidal organic carbon (**37**). Whereas mineral nutrients were not limited in our system, DOC excretion was presumably important and significantly increased bacterial concentrations in batch (Fig. 9) and chemostat experiments (Fig. 10). The molecular weight cut off of the

dialysis bags (15,000 Da) and the probability that the permeability of the dialysis membranes might decrease during incubations due to attached bacteria, suggest that the DOC reaching the external bacteria is only a fraction of the total carbon release. The input of extra organic carbon from the dialysis bags in the chemostat reactors is conceptually similar to a higher substrate inflow concentration and produces a higher bacterial biomass but not a higher growth rate which was similarly controlled in all reactors by the dilution rate.

The chemostat dialysis experiment showed a clear chemical effect of the flagellate grazers on the bacterial morphologies (Fig. 11). The *Flectobacillus* cell length in the treatments where bacteria were exposed to flagellate excretory products (Fig. 11 C, D) continuously increased and reached a median cell length of > 10 µm after about one week of continuous cultivation. The proportion of resistant morphotypes (defined as cells > 7 µm) was not as high as in the treatment with direct grazing impact (Fig. 11B) but still exceeded 50 % of the population. The fact that treatments with different prey bacteria inside the dialysis bags, *Flectobacillus* GC5 and *P. putida* MM1, produced similar results, makes it less probable that "infochemicals" were released by the bacteria itself. Therefore, this experiment can be seen as the first strong evidence that a grazing-resistant morphology in bacteria might be induced by excretory products of bacterivores.

It has long been speculated that grazer-mediated chemicals might trigger phenotypic responses in bacteria and the development of grazing-resistance (**19**, **38**) similar to that known from other predator-prey interactions in planktonic systems (**27**, **53**). However, attempts to demonstrate inducible phenotypic shifts in bacteria, e.g., by adding grazer conditioned water to bacterial cultures, have failed until now (Jürgens, unpublished results). One reason might be that it depends on the right balance between bacterial growth and grazing losses in such experimental systems to allow the development of resistant bacteria. The chemostat results further indicate that sufficiently long exposure to a grazer-mediated environment is required for the shift towards the development of filaments.

This fact is in line with other published examples showing that it may take several days of protist predation impact until filamentous bacteria develop, e.g. in chemostat experiments (**15**) and in freshwater plankton (**39**). The batch dialysis experiments did not produce a higher proportion of filamentous *Flectobacillus* despite presumably considerable DOC excretion by flagellates (Fig. 9).

The faster shift towards filament dominance in batch experiments containing grazers is mainly due to the rapid elimination of non-resistant morphotypes, not necessarily due to strong growth of the filaments. It remains to be seen whether really a predator-released

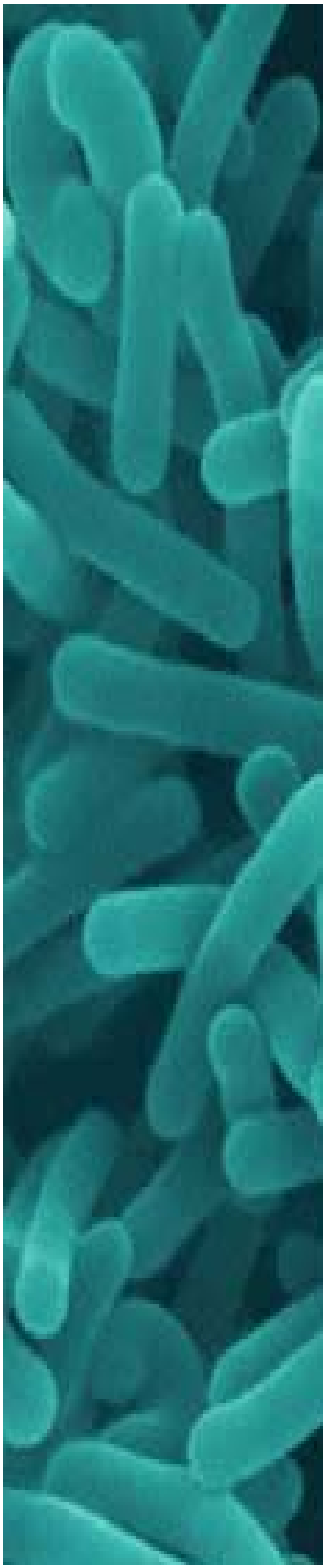
"infochemical" is involved or whether the flagellate excretions change the bacterial growth medium in a way which favours filamentous morphotypes. Unless the active chemical compound has been determined it will not be possible to answer this question.

Underlying mechanisms of bacterial filament formation

The most detailed laboratory studies on grazing-mediated changes in bacterial populations with the capacity to develop resistant morphologies (filaments, aggregates) has been performed by Hahn and co-workers (**15**, **14**, **17**). From their experimental results with several phenotypic plastic bacterial taxa which are able to develop filaments, the authors presented an intriguingly simple theory on the underlying mechanism as to how flagellate grazers shift the bacterial populations towards the dominance of resistant morphologies. Similarly as in our study, the authors observed the shift towards filamentous cells in three bacterial strains, *Comamonas acidovorans* and two *Flectobacillus* species, in predation experiments with bacterivorous flagellates. However, an increase in mean cell size was also observed in the absence of grazers but when the growth rate was increased, either in logarithmic growth in batch culture or at increased dilution rates in chemostat cultures. This was particularly pronounced for the *Flectobacillus* strains grown in continuous cultivation, much less so for *C. acidovorans* (**17**). Therefore the suggested underlying mechanism of filament formation was a growth-rate controlled increase in cell length. In chemostat predation experiments this size increase was achieved by flagellate grazing because grazers reduce overall bacterial biomass, eliminate non-resistant competitors and excrete dissolved substrates which might result in an increase in the specific growth rate of ungrazed bacteria.

The pattern of filament formation observed in our experiments with *Flectobacillus* GC-5 resembles in several aspects the phenomenon observed by Hahn *et al.* (**17**). In all these strains a high morphological variability occurred, with cell length in the range of < 1 µm to > 10 µm and thus with co-occurring resistant and vulnerable size classes even under strong grazing pressure. We also observed some dependence of cell size on growth conditions, e.g., no elongated cell forms under starvation conditions. However, we could not observe a strong increase in the proportion of filamentous cells, such as reported for *Flectobacillus* sp. strain MWH38, in any of the treatments without predators. If filament formation were controlled by growth rate, this should have been visible particularly in the batch experiments where nearly maximum growth rates were achieved. In our case, a simple negative selection mechanism of filamentous cells would be sufficient to explain the observed shift in a polymorphic *Flectobacillus* population as long as there are substrates available that allow further growth of

resistant morphotypes. Our chemostat dialysis experiments imply, however, that this selection process becomes enhanced due to a chemically stimulated transformation of cells into filamentous morphotypes. It remains to be elucidated how widespread this mechanism is and whether it also plays a role under natural conditions, e.g. during short-lasting blooms of filamentous bacteria in planktonic systems (**39**). Situations, in which protist grazing is the only selective predation pressure are probably only temporary events and vulnerability towards metazoan filter feeders (**29**), bacteriophages (**56**) and predatory bacteria (**43**) have also to be considered and imply multiple trade-offs in natural bacteria (**2**).



Chapter III

Impact of productivity on the interactions between freshwater planktonic bacteria and bacterivorous flagellates

Impact of productivity on the interactions between freshwater planktonic bacteria and bacterivorous flagellates

Abstract

To examine how system productivity influences predator-prey interactions between planktonic bacteria and bacterivores we used carbon-limited chemostats with differing substrate input to simulate a productivity gradient. We analysed population dynamics, diversity and functional attributes of the mixed bacterial assemblage and bacterivorous nanoflagellates, and assessed

the relative importance of edible and inedible bacteria. In accordance with existing models on predator-prey interactions, relative importance of grazing-resistant prey increased with increasing productivity. Additionally, experiments revealed that the type of resistance mechanism changes along the productivity gradient

Introduction

The effects of limiting factors on ecological systems and their potential influence on structure and composition of the communities are one of the most debated topics in modern ecology. The understanding of processes related to biological communities is extremely difficult, because of their extraordinary complexity. Early studies from Brooks and Dodson (**61**), and Cody (**62**), demonstrated a direct relation between community processes and the effect of one single factor, considering predation and competition as the most effective. Following this way, models considering the relative effects of several factors were developed and used to identify factors that may determine the relative role of each process (**63**, **71**).

Different models predict that productivity of the system influences the relative importance of predation and competition in determining community patterns (**72**; **64**): when production is low, competition is the most important factor influencing community patterns, but at high productivity predation is predicted to be the main structuring factor. In this case, organisms able to avoid or reduce predatory mortality are assumed to dominate those communities.

This sort of trade-off between being successful in competition for resources and avoiding predation was proposed in several articles (**73**, **74**, and **75**). The limitation of these studies was their reduced and artificial community composition: prey populations were often limited to two species, sharing a common resource and predator. The development of a theoretical model (**64**) where the relative importance of predation and competition for resources was tested through a gradient of productivity predict the existence of an intermediate level where both prey types, the more competitive and the more resistant to predation were coexisting in a rather instable equilibrium. This theory was confirmed experimentally by direct observations from Bohannan and Lenski (**60**) on two *E. coli* populations exposed to predation by the bacteriophage T2 at different substrate concentrations in continuous culture system: vulnerable bacteria were dominant in absence of phages and inedible dominate populations under predation pressure, but the advantage obtained

from ability in competition was higher at the lower substrate concentration while, the advantages due to invulnerability were higher at the high substrate concentration.

Our aim was to study the relative importance of competition and predation for complex populations of planktonic bacteria. In line with the studies by Leibold (**64**) and Bohannan and Lenski (**76**) we wanted to use an experimental productivity gradient to examine predator-prey interactions and the impact on prey abundance and community structure. Predation pressure was exerted by bacterivorous nanoflagellates, organisms recognized as the main and most common predators for aquatic bacteria (**77**, **78**). Competition was regulated designing the systems with a gradient of substrate input and thus productivity. Resource competition between microorganisms has been demonstrated in several laboratory studies (reviewed in **74**), and the impact of productivity on competition was demonstrated on laboratory bacterial communities using different quantities of substrate supply by Bohannan and Lenski (**76**, **60**). For our study it was important to assume a trade-off between competitive ability and vulnerability towards predators: bacteria more resistant to predation are assumed to be less competitive for resource utilisation (**73**, **79**, **74**, **75**, and **59**).

Laboratory communities of microorganisms have been proposed as ideal model systems in several studies on interactions prey-predator (**80**, **76**, and **81**). Environmental variables as light, temperature, dilution rates and productivity of the system can be easily manipulated and the fluxes of nutrients are always under control.

For our experiments we used microbial communities composed of bacteria (prey), and different hetero- and mixotrophic flagellates (predators). These communities have several advantages as ecological model communities. They are composed of organisms having short generation times and which can be easily maintained in large populations. Many of them can be stored (-80°C) for further analysis also after the end of the experiment. Microbes can be grown under simple and defined conditions, close to the assumption of many theoretical models. Finally, bacterial and flagellates diversity is so huge that it allows us to study communities with very different species composition, good for the evaluation of their response not only at the different environmental conditions we settled in the system, but also at the changes on their diversity due to predation impact.

The aim of our two experiments was to test the predictions described above (relative impact of predations and productivity on prey communities' abundances, activities and diversity) on different bacterial assemblages, with different variables, always using natural populations of prey and predators.

By using bacterial inocula from different seasons (winter, summer) we obtained different bacterial assemblages for the two experiments. Predators were varied from a single flagellate species to a mixed natural flagellate community.

The two series of chemostats were designed trying to examine microbial populations with a composition close to the *in situ* populations in the lake in early winter or in mid summer. The two experimental conditions were adapted to these moments: the gradient of the substrate supply was larger and reached much higher concentrations in the summer one, and also temperature was raised for the summer experiment.

Analysis of the population dynamics of bacteria (prey) and flagellates (predator) and assessment of the relative importance of edible and inedible bacteria was performed to evaluate the possibility of feed-back mechanisms to compensate for grazing losses. Our predictions were compared with the experimental data to test our hypothesis on bacterial and predators dynamics, to evaluate the dependence of their biomasses on productivity of the system and, finally, to examine the degree of bacterial grazing resistance in dependence of predation and substrate supply.

Methods

Isolation of bacterial communities

Several plankton samples were taken from a mesotrophic lake (Schöhsee, North Germany) at two different periods of the year: winter (November 2002) and summer (June 2003). The samples were filtered through 0.8 μm polycarbonate filters (Millipore) to eliminate bacterial predators, and stored at experimental conditions of light and temperature. Immediately after filtration, samples were enriched with 5 mg L^{-1} of yeast extract (BactoYE).

After 5 and 10 days of incubation (13 ± 1°C for the winter experiment and 16 ± 1 °C for the summer one), the pre-cultures where checked by light and epifluorescence microscopy for protistan growth. Predator-free flasks were pooled and, at day 10, inoculated into the chemostats filled with culture media. Just 6 hours before the inoculum in the chemostats, a eukaryotic antibiotic (Cycloheximide, 40 mg L^{-1}) was added to the flasks, in order to kill small protists which potentially passed the filters. The inhibitor was eliminated by dilution with the inoculum in the chemostats vessels.

Flagellates isolation

The heterotrophic nanoflagellate *Spumella* sp. isolated from Lake Shöhsee and used in previous studies (**82**; **66**), was selected as predator in the winter experiment.

For the summer experiment we used as predators a natural community of flagellates (from visual analysis at the microscope mainly composed by the genera *Spumella*, *Ochromonas*, *Bodo*, *Paraphysomonas*), to develop experimental conditions closer to the lake environment. Several summer plankton samples from Schöhsee were filtered through 10 µm to eliminate ciliates and other larger organisms, then incubated for 15 days at experimental conditions, without any addition of organic substrate but periodically fed with heat-killed bacteria (*Pseudomonas putida*, strain MM1). After 15 days flagellate flasks were checked for growth of other organisms by light and epifluorescence microscopy and suitable flasks pooled before inoculation of the experiment.

Continuous cultures design

Both winter and summer experiments were performed in one-stage chemostats. Basic inorganic growth medium (WC, **12**) was supplemented with different amounts of yeast extract, in all cases maintaining carbon limitation of bacterial growth in the absence of predators. Concentrations were at 0.25-0.50-1.00-2.00-4.00-8.00 mg YE L^{-1} for the winter experiment, and at 0.10-1.00-4.00-8.00-16.00-32.00 mg YE L^{-1} for the summer one. Two replicate chemostats (A, B) were run for each substrate concentration.

The twelve cylindrical reactors (750 ml volume) for every chemostat experiment where continuously aerated by fine bubbling with sterile air from the bottom and filled with 550 ml of enriched medium, pumped from a 20 L reservoir, by peristaltic pumps to achieve a dilution rate of D = 1.0 d^{-1}. Chemostats systems were assembled in a climate chamber (13 ± 1°C for the winter experiment and 16 ± 1 °C for the summer one) in the dark.

Bacteria from pre-cultures were inoculated (1ml, $6x10^6$ cells in winter and $5x10^6$ in summer, respectively) in every reactor. After 3 days of growth, predators were inoculated in one of the replicate chemostats (B) of each substrate concentration. For the winter experiment the inoculum consisted of 5ml of *Spumella* sp. culture with a density of 25 x 10^4 cells ml^{-1}, for the summer one in 5 ml of mixed culture of flagellates as mentioned above (density 31 x 10^4 cells ml^{-1}). Chemostat experiments were run for 35 (summer) and 62 (winter) days, respectively.

Cell abundances, sorting, and biovolumes

Samples (2 ml) for the enumeration of bacteria and flagellates were taken every 24 or 48 hours and fixed with 0.2 µm-filtered formaldehyde (final concentration 2%). Staining with 4',6-diamidino-2-phenylindole (DAPI, **40**) and filtration onto 0.2 µm pore-size polycarbonate filters was required for cell counting by epifluorescence microscopy.

Several bacterial morphotypes and their biovolumes were quantified from DAPI stained samples, using an automated image analysis system (SIS GmbH, Münster, Germany) and a detection and analysis procedure similar as described in Massana *et al.*, (**31**) or, for large morphotypes (e.g., filaments) directly measuring cells length with the help of an ocular grid.

Each bacterial sample from the chemostats was analyzed for the phenotypic features under examination, covering cell size, morphology (including chains and aggregates formation) and motility. Morphotypes such as aggregates (cluster of more than 10 bacterial cells), filamentous forms (single cells or cells chains longer than 7 or 10 µm respectively for winter and summer experiment) were defined as inedible for grazers; freely suspended bacterial cells shorter than 10 (7 in winter) µm of length and aggregates composed of less than 10 of these cells, were defined as edible for predators. This classification was allowed after the visual analysis of the different predators strategies (in the mixed cultures some large flagellates of the genus *Paraphysomonas* were present; they were able to predate even bacterial aggregates of 8-9 small cells) and a comparison with former studies on size-controlled predation on bacteria (see **83**; **84**).

Bacterial motility: Bacterial swimming behaviour was documented microscopically by means of a standard video camera, VCR and tracking software MedeaLab 3.1 (1994-97, Medea AV GmbH, Erlangen, Germany) as described by Matz and Jürgens (**35**). Bacterial cells faster than 60 µm s^{-1} were assumed as inedible for grazers (**85**; **66**).

Data analysis

We employed regression analysis to evaluate the relative importance of bacterial and flagellate abundances, biovolumes and their distribution through functional prey classes. Data that were not normally distributed were log transformed before the analysis. Rm ANOVAs and Tukey's post hoc tests were used to test for significant differences between time series of bacterial abundance data from the different treatments during the period day 5-END for both experiments (END at day 35 for summer and day 62 for winter). Additionally, student *t*-tests (Bonferroni corrected) were performed to test for differences between means of the same periods in bacterial and flagellates biovolumes between treatments and between different bacterial morphologies (considering as unlike classes of prey all the inedibles and all the edibles). All statistical tests were performed using JMP, Version 5.0.1 (SAS Institute Inc.).

Results

Bacterial and grazers abundances

During the first 5 days bacterial populations in both experiments were adapting to the chemostats conditions and a constantly increased in abundances reaching the equilibrium number at day 4-5. For our evaluations we considered only the period between day 5 and the end of the experiment.

Dynamics of winter and summer bacterial populations, in the absence of predators, through the gradient of substrate supply showed (Figure 1B and 1A) a constant relation between bacterial abundance and amount of substrate supplied. In the summer experiment (Figure 1A) the larger range in the nutrient gradient, corresponded to bacterial concentrations (Table 2) between 24 x 10^6 cells ml^{-1} in the richest treatment and about 4 x 10^6 in the poorest treatment on average. In the winter experiment (Figure 1B), the richest treatment reached 18 x 10^6 cell ml^{-1} in average and the poorest one stayed around 6 x 10^6.

The presence of bacterial predators, a mixed community of flagellates for the summer experiment and the single species *Spumella* sp. for the winter one, was drastically changing the abundances, biovolumes and general patterns of the bacterial communities. Grazing effect was in general reducing bacterial abundance through the whole substrate gradient (Figure 1C, 1D), but impact of predation was higher on vessels where the substrate supply was high. In those vessels the reduction in bacterial abundances was much higher than in the poorest ones.

Flagellates abundances (Figures 1E, 1F) were correlated both with the richness of substrate and with bacterial fluctuations during the experiment. In fact, a classic Lotka-Volterra type of predator-prey population dynamics can be recognized in every B vessel, both during summer and winter experiment (compare figures 1C-1E and figures 1D-1F). Flagellates abundances were also constantly increasing with the substrate supply (Figure 1E, 1F).

A more detailed analysis must take in consideration also the biovolumes of every population. In this study we decided to show them as mean of the period 5-END, in order to make the differences between treatments more comprehensible.

Biovolume means of the bacterial assemblages of the summer experiment during period 5-END (Figure 2A), in absence of predation, goes from 3.6 x 10^6 $\mu m^3 ml^{-1}$ for the poorest YE reactor, supplied with just 0.10 mg YE L^{-1}, to 15.2 x 10^6 $\mu m^3 ml^{-1}$, mean of the same period for the richest reactor, supplied with 32.00 mg YE L^{-1}. The higher bacterial biomass was strictly correlated to the

SUMMER -FLAG (x 10^6cells ml^{-1})						
YE conc.	0.10	1.00	4.00	8.00	16.00	32.00
Mean	3.62	3.64	5.04	7.77	12.38	15.17
St. dev	*± 1.91*	*± 1.62*	*± 2.08*	*± 2.17*	*± 3.49*	*± 2.56*
min	1.61	1.39	1.85	4.74	7.53	11.98
max	6.00	6.04	9.85	13.21	22.38	22.52
SUMMER +FLAG (x 10^6cells ml^{-1})						
YE conc.	0.10	1.00	4.00	8.00	16.00	32.00
Mean	9.66	1.39	1.23	2.31	4.25	6.22
St. dev	*± 1.35*	*± 1.01*	*± 1.12*	*± 1.94*	*± 3.25*	*± 3.76*
min	0.16	0.39	0.34	0.40	1.48	2.63
max	1.03	1.73	1.68	2.82	5.99	9.41
WINTER -FLAG (x 10^6cells ml^{-1})						
YE conc.	0.25	0.50	1.00	2.00	4.00	8.00
Mean	2.99	4.01	5.09	5.72	7.30	10.54
St. dev	*± 0.71*	*± 1.19*	*± 1.53*	*± 1.38*	*± 1.51*	*± 2.49*
min	1.43	1.85	2.31	3.48	4.75	5.46
max	4.30	6.72	8.25	9.16	9.94	17.26
WINTER +FLAG (x 10^6cells ml^{-1})						
YE conc.	0.25	0.50	1.00	2.00	4.00	8.00
Mean	2.05	1.37	2.12	1.75	2.04	3.02
St. dev	*± 0.59*	*± 0.56*	*± 0.60*	*± 0.58*	*± 0.61*	*± 0.88*
min	1.17	0.32	0.97	0.72	1.28	1.46
max	3.47	2.75	3.33	3.22	3.71	4.86

Table 1: Means, standard deviations, minimum and maximum values reached by bacterial abundances from all the vessels, during the experimental period. It was between days 5-END where END was at day 35 for the summer experiment and day 62 for the winter experiment.

substrate supply how is clearly exposed in Table 1 and confirmed by statistics on differences between abundances in Table 2.

The same dynamics were characterizing also the winter experiment, except that the smaller range of the substrate gradient was reducing the differences in total bacterial abundance (Figure 1B)

and thus also in biovolume means: from 3.0 x 10^6 $\mu m^3 ml^{-1}$, mean of the period 5-END days for the poorest, supplied with 0.25 mg YE L^{-1}, to 10.5 x 10^6 $\mu m^3 ml^{-1}$, mean of the same period for the richest reactor, supplied with 8.00 mg YE L^{-1} (Figure 2B).

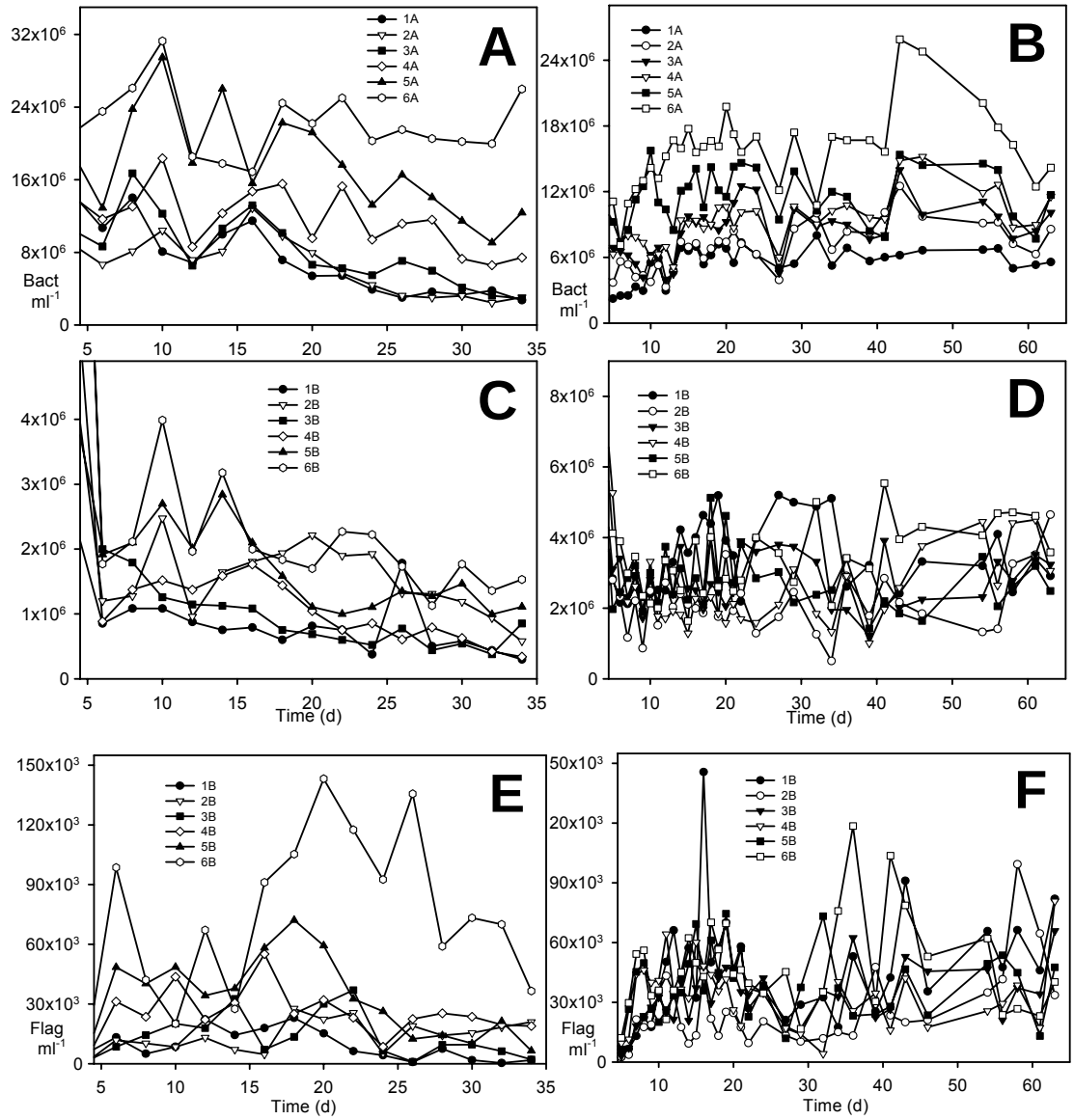


Figure 1: Populations dynamics in chemostats supplied with media enriched with different amount of YE, following the gradient from 1 to 6 (as explained in the text). Left graphs (A, C, E) refer to the summer experiment, right graphs (B, D, F) to the winter. Bacterial development in predators-free treatments (graph A and B) and under grazing pressure (graphs C and D). Bacterial abundances dynamics (Graphs A, B, C and D) and flagellate abundances dynamics (graphs E and F) are measured as number of cells ml^{-1}. Experimental period was between days 5-END where END was at day 35 for the summer experiment and day 62 for the winter experiment. Time series from treatments 1B, 3B, 5B and 6B (summer) and 1A, 2B, 5B and 6B (winter) aren't normally distributed. They were log-transformed before statistical and then tested, as well as the normally distributed time series, for statistical significance by one-way ANOVAs.

Presence of predators dramatically reduced the bacterial biomass: mean bacterial biovolume (Figure 2A, summer exp.) during period 5-END days was 0.4 x 10^6 $\mu m^3 ml^{-1}$ in the poorest reactor (0.10 mg YE L^{-1}) and of 1.7 x 10^6 $\mu m^3 ml^{-1}$ in the richest one (32.00 mg YE L^{-1}).

Mean bacterial biovolume (Figure 2B) in the winter experiment during the period 5-END days in the poorest reactor (0.25 mg YE L^{-1}) was 2.1 x 10^6 $\mu m^3 ml^{-1}$, while in the richest one (8.00 mg YE L^{-1}) during the same period was 3.0 x 10^6 $\mu m^3 ml^{-1}$.

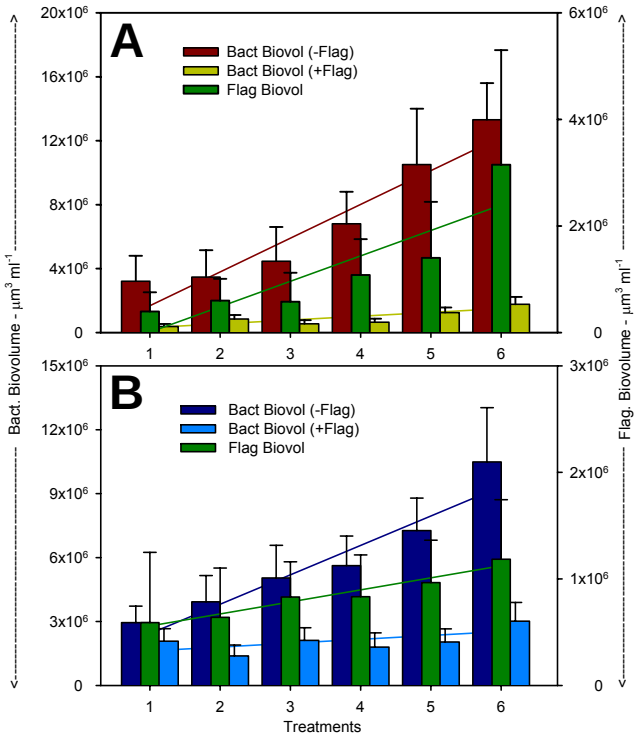


Figure 2: Means (+ st. dev.) of the period 5-END days of bacterial and flagellate biovolumes (in $\mu m^3 ml^{-1}$) in chemostats supplied with media enriched with different amount of YE, following the gradient from 1 to 6 (as explained in the text). Graph A is referring to the summer experiment, graph B to the winter one. Means were statistically compared with one-way ANOVAs (Tukey's corrected).

Flagellate abundances and biovolume (Figures 2A and 2B) were also indirectly influenced by substrate availability for bacteria and mean biovolume of the mixed flagellate populations in the summer experiment during the period 5-END days was increasing from 3.9 x 10^5 $\mu m^3 ml^{-1}$ in the poorest treatment to 3.1 x 10^6 $\mu m^3 ml^{-1}$ in the richest one (32.00 mg YE L^{-1}). In the winter experiment mean biovolumes of *Spumella* sp. were between 5.9 x 10^5 $\mu m^3 ml^{-1}$ in the poorest treatment and 1.2 x 10^6 in the richest one (8.00 mg YE L^{-1}) $\mu m^3 ml^{-1}$.

Bacterial populations' composition

As was already explained before, we identified two different functional groups of bacteria with respect to flagellate: filaments, aggregates and fast swimming bacteria were operationally defined as "inedible" for flagellates; the remaining, freely suspended cells, were defined as "edible". During the period 5-END edible bacteria were predominant in all experiments in absence of predators: in

the summer chemostat (mean of days 5-END, Figures 3A, 4A) their proportion was between 72 to 90% of the total bacterial biovolume and was also statistically correlated to the availability of substrate, decreasing from the poorest reactor to the richer ones (comparison of mean biovolumes of assemblages from different reactors were always $p < 0.01$ for all treatments). Inverse trend was thus followed by the inedible cells, always a minority in respect of edibles, but constantly increasing ($p < 0.01$ between treatments) with substrate: from 10% in the poorest reactor to 28% in the richest.

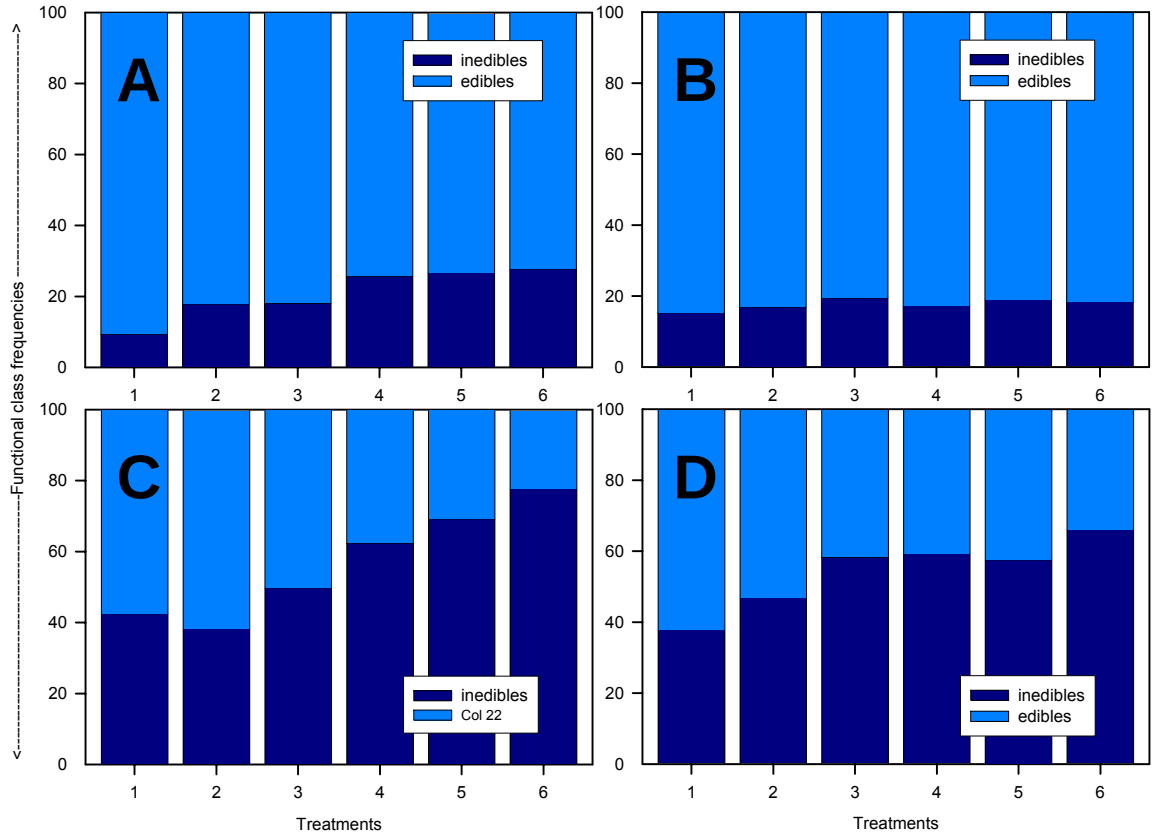


Figure 3: Development of frequency distribution of different bacterial functional groups (relative abundance) of DAPI-stained cells from chemostats bacterial communities during growth in the presence (C, D) and in the absence (A, B) of bacterivorous flagellates. Graphs A and C show summer expoeriment, graphs B and D show winter experiment.

In the winter reactors (mean of days 5-END, Figures 3B and 4B), without grazing pressure by the flagellates, the proportion of edible bacteria was about 80% through the whole substrate gradient, thus also the proportion of inedible bacterial forms was quite constant, between 16 and 19%.

Very different was the composition of the communities which developed under grazing pressure by flagellates: in summer chemostats (Figures 3C and 5A) the proportion of small and medium-sized, edible, bacteria was reduced to about 22-30% of total bacterial biovolume due to flagellates grazing in the 3 richer vessels, but in the 3 poorer ones the proportion of edible cells was still higher than 50% (61% in vessel 2B).

Summer	1A	2A	3A	4A	5A	6A
1A	x					
2A	—	x				
3A	—	—	x			
4A	●●●	●●●	—	x		
5A	●●●	●●●	●●●	●●●	x	
6A	●●●	●●●	●●●	●●●	●	x
	●●●*	●●●	●●●*	●●●	●●●*	●●●*
	1B	2B	3B	4B	5B	6B

Summer	1B	2B	3B	4B	5B	6B
1B	x					
2B	—	x				
3B	—	—	x			
4B	—	—	—	x		
5B	—	—	—	—	x	
6B	—	—	—	—	—	x
	●●●*	●●●	●●●*	●●●	●●●*	●●●*
	1A	2A	3A	4A	5A	6A

Winter	1A	2A	3A	4A	5A	6A
1A	x					
2A	●*	x				
3A	●●●*	●●	x			
4A	●●●*	●●●	—	x		
5A	●●●*	●●●	●●●	●●	x	
6A	●●●*	●●●	●●●	●●●	●●●	x
	●●●*	●●●*	●●●	●●●	●●●*	●●●*
	1B	2B	3B	4B	5B	6B

Winter	1B	2B	3B	4B	5B	6B
1B	x					
2B	●●	x				
3B	—	—	x			
4B	—	—	—	x		
5B	—	●*	—	—	x	
6B	—	●*	—	—	—	x
	●●●*	●●●*	●●●	●●●	●●●*	●●●*
	1A	2A	3A	4A	5A	6A

— = P > 0.05	● = P < 0.05	●● = P < 0.01	●●● = P < 0.001

Table 2: Statistics analysis of bacterial abundances and fluctuations during the period 5-END for the summer and the winter experiment. Treatments without predators (A series, vessels 1-6) and treatments under grazing pressure (B series, vessels 1-6) are fully compared by one way ANOVAs and their means tested by Tukey's Post Hoc. The same analysis was performed also between treatments A-B at the same substrate concentration (1 to 6). Results of comparison from log-transformed data because originally not normally distributed are indicated with *. Degree of significance, expressed in ●, is evaluated for p>0.05 (-), 0.05>p>0.01 (●), 0.01>p>0.001 (●●) and p>0.001 (●●●).

The relative importance of inedible bacteria increased, compared to correlated reactors without flagellates, to 33% (in the poorest) and 50% (in the richest) following the substrate gradient (the treatments didn't showed statistically significant differences and trends in increasing the relative importance of inedible cells between the three poorer treatments but the differences became significant comparing this three treatments with each one of the richer ones, with $p < 0.01$).

Also in the winter chemostat (Figures 3D and 5B) the impact of grazers was mainly affecting the relative proportion of edible bacteria, reducing it to 62-34% of the total biovolume and, at the same time increased the proportion of inedible forms to 38-66%, correlated to the substrate gradient.

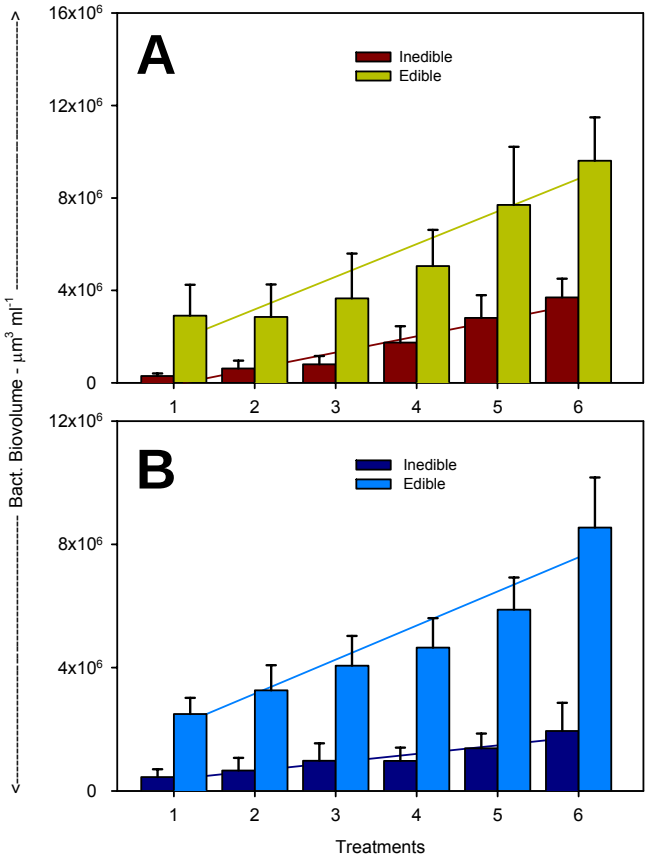


Figure 4: Means (+ st. dev.) of the period 5-END days of functional bacterial group populations' biovolumes (in $\mu m^3 ml^{-1}$) in chemostats (see text) in absence of predators. Graph A is referring to the summer experiment, graph B to the winter one. Biovolume means of edible bacteria (grey bars) and inedible bacteria (black bars) were statistically compared using one-way ANOVAs (Tukey's corrected).

In both experiments the impact of grazing was mainly affecting the group of the "edible" bacteria. Comparison of reactors from the same treatment, with and without flagellates, generally showed an average reduction in biovolume of edible bacteria by around 80% (in winter) and 90% (in summer).

In general, in the winter experiment, the presence of predators was not affecting the growth of inedible bacteria, and in some cases (Figure 5B) seemed to positively stimulate it. Is interesting to compare winter and summer treatments where substrate supply was similar: reactors winter-3 and summer-2 (1.0 mg YE L^{-1}), winter-5 and summer-3 (4.0 mg YE L^{-1}) and winter-6 and summer-4 (8.0 mg YE L^{-1}). Winter bacterial communities always appeared more productive, both comparing abundances in absence of predators: means in winter-3 was 5.1 x 10^6 cells ml^{-1}, in summer-2 just 3.6 x 10^6 cells ml^{-1}, winter-5 had about 7.3 x 10^6 cells ml^{-1}, while summer-3 only 5.0 x 10^6 cells ml^{-1}, and for winter-6 the mean was of 10.5 x 10^6 cells ml^{-1}, for summer-4 7.8 x 10^6 cells ml^{-1}. Predation was affecting the two bacterial communities with different results: in winter, bacteria under grazing pressure were limited to abundances of 2 - 3 x 10^6 cells ml^{-1}, independent of substrate concentration (Table 1).

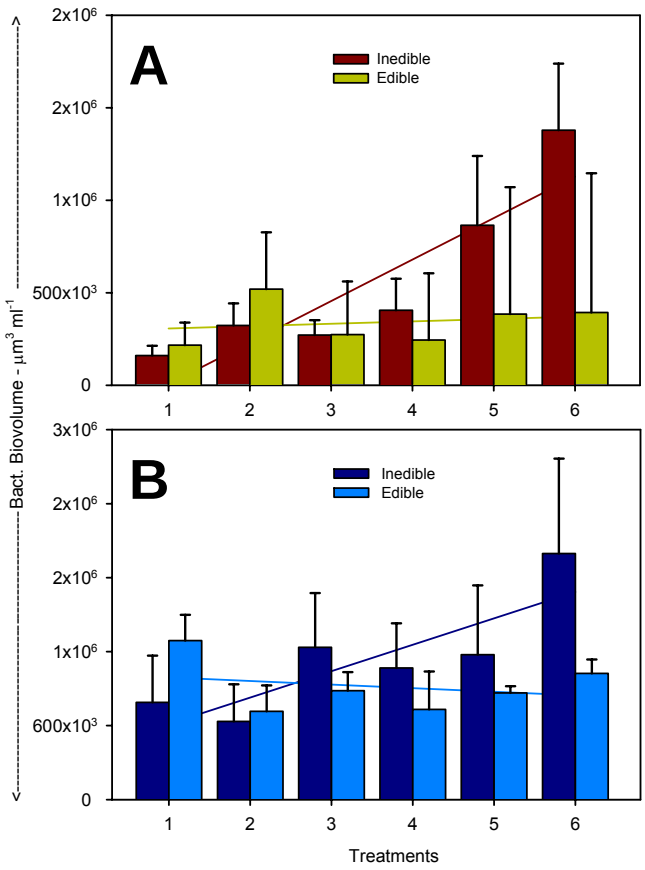


Figure 5: Means (+ st. dev.) of the period 5-END days of functional bacterial group biovolumes (in $\mu m^3 ml^{-1}$) in chemostats (see text) under high grazing pressure by flagellates. Graph A is referring to the summer experiment, graph B to the winter one. Biovolume means of edible bacteria (grey bars) and inedible bacteria (black bars) were statistically compared using one-way ANOVAs (Tukey's corrected).

In summer the reduction in bacterial abundances was also evident but still, the substrate had a little control on bacterial densities: in summer-2 the mean of bacterial cells under grazing pressure was

about 1.4 x 10^6 cells ml^{-1}, in summer-4 was about 2.3 x 10^6 cells ml^{-1}, thus increasing, slightly, with the substrate availability. The *Spumella* sp. population in the winter experiment achieved slighter high biovolumes then the mixed flagellate community in summer (*Spumella* biovolumes means were at 0.83, 0.86 and 1.18 x $10^6 \mu m^3 ml^{-1}$ in the vessels compared above, while the mixed communities of flagellates at 0.60, 0.58 and 1.08 x $10^6 \mu m^3 ml^{-1}$). Proportion of inedible forms was similar for all the six compared vessels in absence of predators 18-19% of the total bacterial biovolume, except for treatment summer-4 where it reached 25.7%. Presence of predators increased the fraction of inedible cells to 58, 57 and 66% on the winter population (and here as well the proportion of resistant cells, as well as the abundance of the population, was constant through the gradient). In summer that percentage was of 38, 50 and 62 respectively, thus constantly increasing with substrate supply in the treatment.

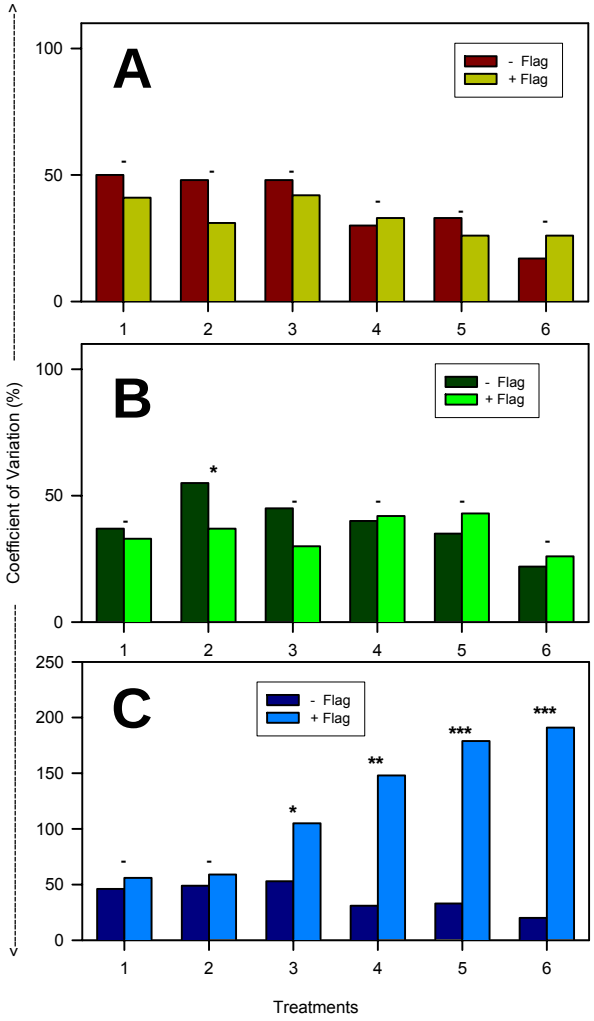


Figure 6: Coefficient of variation (CV) for bacteria biovolume during the period 5-END days for the summer experiment. Graph A: total bacterial biovolume; graph B: inedible bacterial biovolume; graph C: edible bacterial biovolume. Treatments with and without flagellates were statistically compared using student *t*-tests (Bonferroni corrected): - =p >0.05; * = 0.05>p>0.01; ** = 0.01>p>0.001; *** = p<0.001.

Stability of prey communities was evaluated by measuring, for the summer experiment, the coefficient of variation (CV) for each bacterial community during the period 5-END day (Figure 6).

Fluctuations of undefined predation-free prey bacterial populations (Figure 6A, black bars) appeared quite stable through the gradient of substrate supply with a slight trend in reduction that become significant just comparing treatment 1 or 2 or 3 against treatments 4 or 5 (always $p < 0.05$) or, better, treatment 6 ($p < 0.05$). Comparing CVs of inedible bacteria (Figure 6B, grey bars) we found significant difference only in two treatments and also no trend through the gradient was visible. Finally, the comparison between treatments predation-free and treatments subjected to predation didn't show any significant difference between the coefficients of variation of the 6 couples of treatments (Figure 6A).

Despite this evidence, the detailed analysis of the coefficients of variation for the two functional classes of prey (edible and inedible) showed a radically different impact of substrate availability and predations on the two classes of prey. Inedible bacteria didn't show any significant difference (with the only exception of treatment 2) both, between couple of treatments at the same level of substrate supply but in presence or absence of predators, and through the substrate gradient, from the poorest to the richest treatment. Edible bacteria (Figure 6C) showed completely different trends: in absence of predators, coefficients of variation decreased following the same trends of the total (Figure 6A) that were, in fact, largely composed by edible cells. In presence of predators there was a large and highly significant decrease in stability (i.e., increase in coefficient of variation) increasing with substrate supply ($p < 0.05$ in treat. 3, $p < 0.01$ in treat. 4 and $p<0.001$ in treatments 5 and 6).

Discussion and conclusions

The relative importance of population and community regulation by resources (bottom-up control) and predators (top-down control) is still a hardly discussed topic in experimental and theoretical ecology. Many improvement resulted from the modelling and experimental studies by Abrams (**86**), Leibold (**64**), Bohannan-Lenski (**76**; **60**), Gasol and Duarte (**87**), Horner-Devine et al. (**88**), tried to answer the question that Power (**71**) formulated as essential for further studies in this field: "What factors may modulate resource limitation and predation in a system, determining when and where predators or resources will dominate in regulating populations?"

Heterogeneity in prey edibility was recognized as a determinant factor in the study of the relative importance of predation. Theoretically, only a proper estimation of prey heterogeneity allows to correctly evaluating the balance between top-down and bottom-up control (**63**; **64**; **86**).

From a proper evaluation of variations in heterogeneity it was possible to determine that productivity can determine the relative importance of competition and predation in determining community structure.

This pattern has been observed in natural systems (**89**; **90**), and then reproduced in laboratory in continuous culture systems by Bohannan and Lenski (**59**) using a very simple prey-predator community composed by one bacterial strain as prey and a bacteriophage as predator at two levels of productivity.

With our study we confirm the results of Bohannan and Lenski in more complex communities of prey, composed by a mixture of bacterial strains which do not only have to cope with predation but also with competition for substrate. To have a more detailed view of the relative effects of predation and competition we developed continuous culture systems, very similar to the ones used by Bohannan and Lenski, but increasing the levels of substrate supplied, from two to six, and enlarging the gap in substrate concentrations from the poorest to the richest treatment.

Bacterial abundances and biovolumes, in our predation-free treatments, were significantly correlated to the substrate supplied (Table 1 and 2, Figures 1A, 1B, 2) for both our experiments, except for very low substrate concentrations treatments where the differences in bacterial abundances and biovolumes between the couple of treatments, despite graphically following a visual trend related to the substrate supplied, were statistically non-significant (Table 2).

In absence of predators, production was clearly determined by the substrate supplied, confirming the observations from several articles recently summarized and improved by Horner-Devine *et al.* (**88**): bacterial abundance increased with increasing primary productivity. Despite differences in bacterial abundances and biovolumes between treatments were, due to the shorter range of the substrate gradient, smaller in the winter experiment, the comparison of relative bacterial productions for treatments supplied with the same substrate concentrations showed that winter bacterial populations were better adapted to the experimental conditions and thus more productive than the summer ones.

Direct relation between bacterial abundance and primary productivity was reported also from other aquatic systems (**91**). They suggest that, despite the presence of bacterivorous predators, top–down effects are in general less important than bottom–up effects in determining the overall abundance of bacteria in aquatic systems. In this case, results from our study (Table 1) suggest that this can be true for treatments poor in substrate supply, where competition for resources is fundamental, but is much less important, in treatments with high substrate supply. In fact (Figures 1C, 1D, 2) we found that the impact of predation was generally the main limiting factor for the prey community.

In all treatments of the winter experiment, prey populations, significantly different in abundances in absence of grazers, were reduced to non-significantly different stressed and small populations by the impact of predation. The situation was a bit less dramatic for the summer experiment were, probably because of the higher substrate concentration for the treatments 4, 5 and 6, that prey populations were not just predation-controlled, but statistically the results were the same than for the winter experiment.

Bacterial abundances for each YE level (Table 1) were controlled, in presence of predators, mainly by grazing pressure then by substrate availability. Bacterial biovolume was significantly higher just in the richest reactor; while through the other five steps of the gradient was impossible to define a clear positive (or negative) effect of nutrients availability on bacterial biovolumes.

Likewise also flagellates abundance (Figure 1E) was not influenced by different substrate availabilities for bacteria while, fluctuations in flagellates abundances were, like for the summer experiment (Figure 1F), strictly correlated to bacterial abundance (availability of prey), and controlling it as main limiting factor.

More, in Figure 2, is possible to see an unambiguous geometrical increase in flagellates' abundance with a relative increase in productivity of the system. Part of the higher amount of substrate supplied in those treatments was not just transformed in bacterial biomass, but, through grazing activity, transferred to the higher trophic level.

In several studies it obviously appeared that bacterial resistance to predation imposes a cost in terms of reduced competitiveness for resources (reviewed in **20**). Inedible bacteria always appeared as less competitive in substrate uptake, and more, their complex and usually large size required a high amount of nutrients for being maintained: in the poorest treatments the chances, for a size-resistant strain to be able to develop and positively compete with other edible (and more active) strains were limited by the low substrate supply.

Our results are consistent with those predictions, as well as Bohannan and Lenski (**59**) study, but using gradients of substrate concentrations and mixed model communities of prey we can go in detail in the observations of trends in community composition and, more evaluate the relative success strategy at different productivity levels (Figures 3, 4 and 5).

At lower substrate input we observed that the population density of inedible bacteria, poor competitors for resources, declined, even though it was less vulnerable to predation. Average substrate supply treatments presented inedible bacteria and edible bacteria shown quite similar trends, middle of the extreme concentrations trends.

At high productivity, we observed that the population density of the group of edible bacteria relatively declined, even though it was the better competitor for resources. In extreme (lower end and upper end of the gradient of nutrients concentration) treatments the decline of the inferior

competitor was due to a combination of resource and apparent competition as explained by Bohannan *et al.* (**92**).

Analysing bacterial abundances for each substrate level (Figures 3, 4 and 5) is clear that some bacterial strains could find a refuge against grazers at lower end of the gradient and others at the upper end, while at average substrate concentration bacterial biomass seems to be highly controlled by grazing pressure. Very small fast bacteria (not shown in the graphs) at lower substrate concentrations and, more important in percentiles and in biovolume, filaments and aggregates (Figure 2) at the opposite side of the gradient found very good conditions for survival in these extreme environments were high grazing pressure was coupled ones, at a very low nutrients availability, that was making impossible the development of big grazing-resistant cells, and in the second case, at very high substrate concentration giving to strains able to resist to predation almost unlimited resources for their strategy (Figure 3).

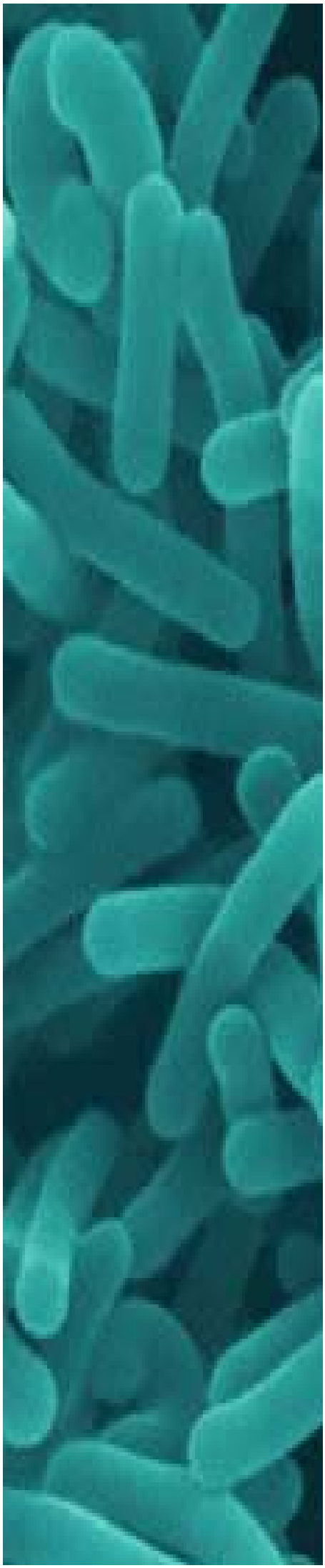
The completely different impact of predation and competition on the two functional prey groups is even more understandable when expressed in terms of variability of the two groups (Figure 6). To properly evaluate it we compared the coefficients of variation of whole bacterial populations, edible and inedible groups for every treatment during the summer experiment. Bohannan and Lenski (**59**) showed that in their experiments population stability was unaffected by enrichment in the control chemostats. Our observations on these communities are consistent with their predictions and more, with a detailed analysis of the functional groups we can describe different impact on them.

In fact, we couldn't find any significant impact of predation on the whole populations in terms of an increase of variability (increase of the percentile of the coefficient of variation, Figure 6A) and also observing the impact of predation on inedible bacteria (Figure 6B) was not showing significant results.

What is highly significant is the impact of predation on the inedible bacteria and its trend through the gradient of nutrient concentration. At lower productivity (treatments 1 and 2), where the available substrate is poor and the competition for resources is higher the impact of predation is reduced.

Not enough carbon available for the development of long filaments and, more, too few substrate available to allow inedible bacteria to compete for it. Then (treatment 3, 4 and 5) the impact of predation is constantly increasing with the increase in substrate supply, and thus is increasing the risk of extinction for the edible bacteria. At the end of the gradient (treatment 6) the productivity of the system is reaching the maximum and the predation impact as well. Here, for edible bacteria, coefficient of variation and thus risk of extinction is highest as well as edible bacteria abundance fluctuations.

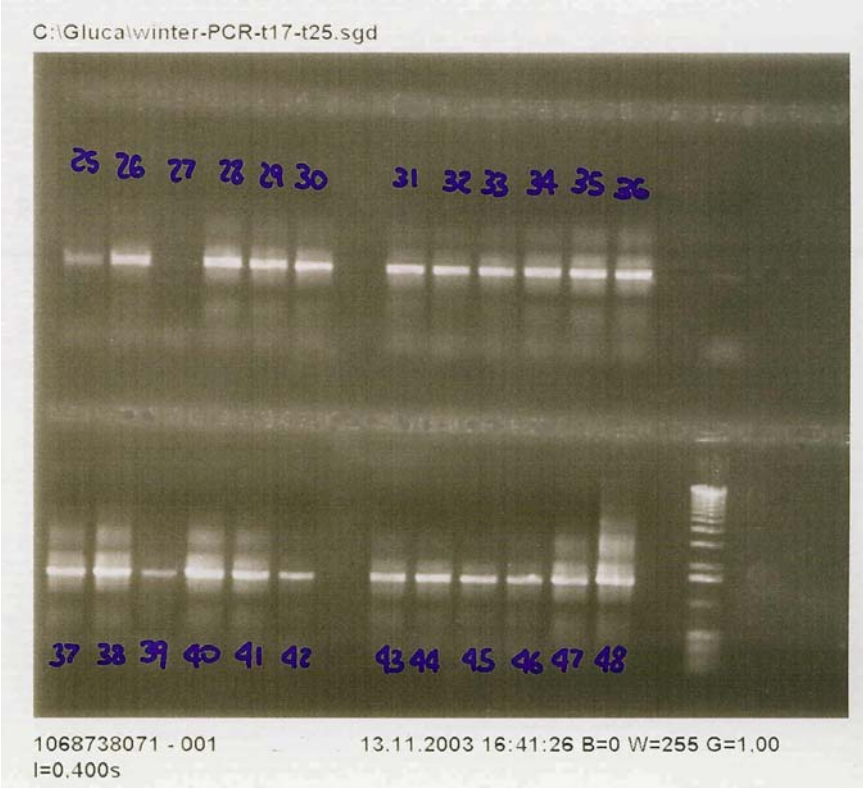
We can finally argue that flagellates, strongly reducing the presence of edible bacterial populations, were indirectly helping the inedible populations reducing the number of competitors for the same resources.



Chapter IV

Impact of flagellates predation and productivity on phenotypic and genotypic composition of freshwater bacterial communities

Impact of flagellates predation and productivity on phenotypic and genotypic composition of freshwater bacterial communities



Abstract

Carbon-limited chemostats, enriched with different substrate input, were performed in order to examine the influence of system productivity on predator-prey interactions in classical artificial communities of planktonic bacteria and bacterivores.

Biolog Eco Plates tested the utilisation of several carbon substrates by different bacterial communities was tested and the results shows that bacterial communities well adapted against

predation were usually worst in competition for resources and that the ability in substrate uptake was generally related to the poorness of the treatment of origin.
With a detailed analysis on the morphology and functionality of bacterial cells it was possible to recognize different mechanisms of resistance along the productivity gradient.
Performing T-RFLP analysis of bacterial populations we noticed that predator-mediated increases in phenotypic diversity were not necessarily reflected in changes of the genetic diversity of our bacterial populations. Relative importance of single OMUs and OTUs was measured and their presence or absence evaluated as possible consequence of competition and predation pressure.

Introduction

The extreme complexity of biological communities makes them almost impossible to be observed and studied in all their aspects without approaching at first fundamental questions on very simplified model communities.

This, particularly successful approach, is called "community module" approach, and has been used primarily by theoretical ecologists trying to clarify general processes, direct and indirect relations between a reduced number of subjects (usually two), and then trying to transpose their results to complex ecological communities (**86**; **72**; **153**; **154**). Not only theoretical, but also experimental ecologists largely used the "community module" approach. In the last ten years, several studies implemented our knowledge in general ecology studying simplified model communities (**156**; **155**; **59**; **88**).

Many of those studies used microbial model communities that are ideal for studies in ecology and evolution (**157**): they are composed of microbes mostly growing in laboratory conditions, having short generation times and are easy to be maintained in large populations and thus, allow the study of communities on both evolutionary and ecological scales. Moreover, ecological and genetic variables can be easily manipulated in laboratory model communities and the possibility to store most of them for indefinite time in a non-evolving state makes microbes the best subject for such kind of studies (**60**).

Due to a lack in appropriate techniques, theoretical studies developed innovative ecological models (e.g. **151**) many years before experimental microbial ecologists could study microbial model communities for testing the proposed theories in laboratory and in field conditions (**76**; **81**). The developing of modern techniques of genetic analysis of microbial diversity gave the chance to use

microbial communities in a proper way, for testing of models and theories formerly proposed. A review of the studies made by Bohannan and Lenski (**60**) summarized direct and indirect relations between prey and predators in very simple model communities, performed in chemostats, using bacteria (*E. coli*) as prey and phages as predators (e.g. **76**; **59**). They studied the relative importance of predation and competition for resources in these model communities, allowing two levels of bacterial productivity, and distinguishing edible and inedible prey.

Laboratory systems utilizing mixo- or heterotrophic flagellates as predators and bacteria as prey gave us a large and well defined view of the importance of phenotypic plasticity for bacterial strains under grazing pressure (**20**).

In the present study, performing chemostat experiments with mixed bacterial communities, we increased the complexity of the Bohannan and Lenski experiments in terms of biological complexity of the model communities and range of productivities with six substrate levels of the system.

In order to estimate the importance of phenotypic plasticity we assessed bacterial diversity at the genetic and at the morphological level. However, predation in our systems was just one of the factors affecting our prey communities. A second key factor for the composition of the communities was competition for substrates which occurred between bacterial strains and, more important for our study, between functional bacterial groups, either investing in defences against grazing or in increasing competitive ability.

Brooks and Dodson (**61**) and, later, Cody (**62**), studied and tested effects of single factors on community processes, considering predation and competition as the most effective ones. Theory predicts that productivity of the system influences the relative impact of predation and competition in determining community patterns (**64**): at low production levels, competition should be the main factor influencing communities' structure, whereas at high production levels, predation is expected as the most important limiting factor. In both cases organisms which are better in adapting to the main selection factor are supposed to become dominant in the community.

This study tries to elucidate the relative impacts of competition and protist predation on the abundance, genotypic and phenotypic composition of several bacterial communities, exposed to a gradient in substrate supply and predation by bacterivorous flagellates. For this, traditional techniques for measurements of bacterial abundance and biomass were combined with techniques to analyse the physiology (substrate uptake), genetic diversity (DNA-fingerprints), and morphological diversity of the bacterial assemblages.

Methods and definitions

Bacterial communities' isolation

Several plankton samples were taken from mesotrophic Lake Schöhsee (North Germany) in June 2003, filtered through 0.8 µm filters to eliminate bacterial predators, and stored at experimental conditions of light and temperature (outlined below). Directly after filtration, samples were supplemented with 5 mg L^{-1} of yeast extract (BactoYE) to enhance bacterial growth and a eukaryotic inhibitor (Cycloheximide, 40 mg L^{-1}, and exposition time 6 hours) in order to eliminate small protists which potentially had passed the filter. After 5 and 10 days incubation time, pre-cultures where checked by light and epifluorescence microscopy for protistan growth. Predator-free flasks were pooled and, at day 10, inoculated into the continuous systems for the experiments.

Flagellates isolation

During the same period several plankton samples from Lake Schöhsee were filtered through 10 µm filter to eliminate larger protozoans and metazoans, then grew for 15 days at experimental conditions, without any addition of organic substrate but periodically fed with heat-killed bacteria (*Pseudomonas putida*, strain MM1). After 15 days flagellate flasks were checked by light and epifluorescence microscopy and appropriate flasks were pooled before using them for inoculation of the experiment.

Continuous cultures design

The experiment was performed in one-stage continuous culture systems, carbon limited and 35 days running. Altogether twelve chemostat systems were run in parallel (6 substrate concentrations, each in two parallels A and B). Basic inorganic growth medium (WC, Guillard and Lorenzen, **12**) was supplemented with different amounts of Bacto-Yeast Extract (YE). Chosen YE concentrations were at 0.10-1.00-4.00-8.00-16.00-32.00 mg YE L^{-1}. The twelve cylindrical chemostat reactors were continuously aerated by fine bubbling with sterile air from the bottom and filled with 550 ml of enriched medium, pumped from a 20 L reservoir by peristaltic pumps to achieve a dilution rate of D = 1.0 d^{-1}. Chemostats systems were assembled in a climate chamber (16 ± 1 °C) with a night-day period of 12 h.

Bacteria from pre-cultures were inoculated (1ml, $5x10^6$ cells) in every reactor. After 3 days of bacterial growth, 5ml (density 3.1 $x10^5$ cells ml^{-1}) from the flagellate pre-culture were inoculated in every B reactor, one for each YE concentration.

Bacterial and protists abundances and biovolumes

Bacterial and flagellate cell numbers were daily determined from formalin (2 % final concentration) fixed samples from chemostats treatments stained with 4',6-diamidino-2-phenylindole (DAPI, **40**), filtered onto 0.2 µm polycarbonate filters and counted by epifluorescence microscopy. At least 400 bacteria and 100 flagellates were counted per sample. Bacterial and protists cell size measurements were done from DAPI-stained samples and to obtain biovolumes by using an automated image analysis system (SIS GmbH, Münster, Germany). Area and perimeter of 300-500 cells were measured, which were used to calculate cell dimensions (volume, length, width) according to the algorithms given in Massana *et al.* (31).

Biolog community profiling

The substrate utilisation patterns of the different chemostat bacterial communities were assessed after 15 and 32 days from the sole carbon source utilization in Biolog ECO-microtiter plates which contain a triple set of 31 (plus water as control) pre-dried carbon sources enriched with tetrazolium violet redox dye that turns purple upon bacterial substrate utilization (**158**).

Sub-samples of 150 µl from each reactor were distributed in each Biolog plate's well and incubated for 44 h at 30°C. Thus, bacterial concentrations in the inocula were different, similar as in the chemostat reactors. This device allows a double analysis of bacterial substrate utilisation in wells: a community substrate utilisation, influenced by abundance in the treatment, and the substrate utilisation per cell, where the community values were divided by the number of bacteria in the wells, obtained from the DAPI preparation at time 44 h. The utilization of each carbon source was indicated by the optical density (OD), measured spectrophotometrically at 595 nm (OD_{595}) in each well and recorded with a microtiter-plate reader and associated software (Benchmark; Bio-Rad Laboratories, California). Absorbance values from each micro-well at incubation-time 22, 33 and 44 h, were compared after normalization by the average colour development (AWCD) as described by Garland and Mills (**158**):

$$\mathrm{AWCD} = [\ \sum (\ C - R\ )\ ] / n$$

where C is the colour production within each well (measurement of the, OD), R is the OD value of the no-substrate control well of each series, and n is the number of substrates (n = 31). Each blank-

corrected well was then divided by the plate AWCD: (*C* - *R*)/AWCD (**158**; **159**). To normalize the analysis, a standard AWCD of 0.5 was adopted (**160**; **161**).

Nucleic Acid Extraction

For DNA extraction 50–80 ml of chemostat samples were filtered on 25-mm Durapore filters (Millipore) and stored in 1 ml lysis buffer at -20°C at day 3, 8, 16 and 32. Nucleic acid extraction was performed directly from the filters using the Mobio Bacterial Soil Kit. DNA integrity was checked by agarose gel electrophoresis, and DNA yield was quantified by a Hoechst dye fluorescence assay (**68**). Nucleic acid extracts were stored at -80°C until analysis.

T-RFLP analysis

The primers used for PCR were the bacteria-specific primer 27F-FAM 5'-AGA GTT TGA TCC TGG CTC AG-3' and the universal primer 1492R 5'-GGT TAC CTT GTT ACG ACT T-3' (**28**). 27F-FAM was 5' end-labelled with phosphoramidite fluorochrome 5-carboxyfluorescein (5' 6-FAM) by Eurogentec (Searing, Belgium).

Each 50-µL PCR consisted of 0.3 µM of primers, 1.5 mM $MgCl_2$, 200 µM of each dNTP, Tris-HCl, KCl and 1.5 U of *Taq* polymerase. In the cocktail an amount of 2-5 µl of DNA extracts was usually diluted. Samples were amplified by use of an initial denaturation step at 94°C (3 min), followed by 30 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), and an extension at 72°C (1 min). Cycling was completed by a final extension at 72°C (7 min). The FAM-labelled PCR products were purified with Qiaquick PCR columns (Qiagen). Purified PCR products were digested by the restriction enzyme *Cfo* I at 37°C for 4 h (for bacterial PCR product) or by the enzyme *Msp* I in the same conditions (for eukaryotic PCR products).

For T-RFLP analysis, 2 µL restriction digest was denatured in the presence of 2.5 µL deionised formamide at 90°C for 2 min. Terminal restriction fragments (T-RFs) were resolved by electrophoresis at 3000 V for 5.5 h in a 6% polyacrylamide gel with an ABI PRISM 3100 automated sequencer. The sizes of T-RFs (which were defined as different operational taxonomic units, OTUs) were determined with the software GeneScan 3.1 at 1-bp resolution by using the size standard Rox 1000 or Rox 2000 (Applied Biosystems). The intensity of each T-RF was measured using the peak area measured by the software and were manually checked to correct size and shape. The relative frequency of the individual T-RFs among the total bacterial community was calculated from the relative peak areas using all peaks with >1% of the total peak area.

Richness in OTUs, and thus biodiversity of the community, was measured as the number of relevant peaks, as described above, independently by their relative quantitative importance. For a real diversity index, the Index of Shannon (*H*) was calculated for every sample by using the peaks as OTUs and the peak areas as relative abundance:

$$H = -\sum_{i=1}^{S} p_i \ln p_i$$

where *S* is total number of species in the community (richness), p_i the proportion of *S* made up of the *i*th species.

Phenotypic classification

Samples of bacteria and flagellates were daily taken and preserved with formaldehyde for a final concentration of 2%.

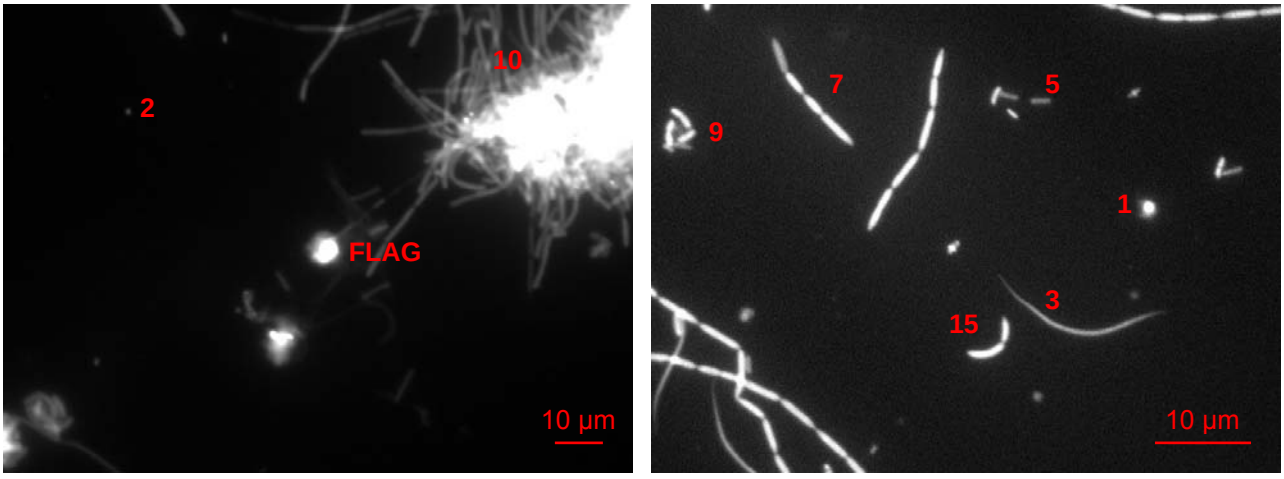


Figure 1: Microphotographs of DAPI-stained bacterial cells, representing several different OMUs, Samples were taken at day 16 of the chemostat run from vessel 5B. Numbers refer to single characteristic OMUs, described in Table 1. FLAG indicate a eukaryotic cell (probably *Ochromonas* sp.)

Cell numbers were counted microscopically after staining with DAPI (**40**, Figure 1). 16 distinct bacterial operational morphotypes or associations (OMUs), such as filaments and aggregates, were recognized and thus quantified during the experimental period (Table 1). Richness in OMUs and thus morphological diversity of the community was measured as the number of OMUs seen in the sample, independently by their relative abundance.

OMU	Shape	Description	Size max	Resistance against grazing	References
1		Large cocci	> 4µm	NO	---
2		Small cocci	0.5 - 4µm	NO	---
3		Shaped long bacilla	7 – 12µm	YES	---
4		Large bacilla	4 – 7µm	NO	Posch et al. (2001)
5		Small bacilla	0.5 - 4µm	NO	---
6		Long filaments	> 10µm	YES	Pernthaler et al. (1997)
7		Chains of bacilla	> 10 µm	YES	Matz et al. (2002)
8		Small cocci aggreg.	< 10 cells	NO	---
9		Small bacilla aggr.	< 10 cells	NO	---
10		Filaments aggreg.	> 10 cells	YES	Van Hannen et al. (1999)
11		Large cocci aggreg.	> 10 cells	YES	Hahn and Höfle (1999)
12		Large bacilla aggr.	> 10 cells	YES	---
13		Small cocci aggreg.	< 10 cells	NO	---
14		Very small bacteria	< 0.5µm	partly	Bohannan and Lenski (1999)
15		C-shaped bacilla	3 – 7µm	NO	---
16		Chains of filaments	> 10 µm	YES	Matz et al. (2002)

Table 1. OMUs (Operational Morphological Units) description and classification. Max size of the bacterial cell or the bacterial aggregation of cells and its attitude against predation is reported as well as references to former studies on grazing resistance for such morphologies of bacteria.

Data analysis

One way ANOVAs and Tukey's post hoc tests were used to test for significant differences between time series of bacterial abundance data from the different treatments during the period 5-34. Basic student *t*-tests (Bonferroni corrected) were performed to test for differences at selected time points

in Shannon Indexes, OTU and OMU richness between treatments at the same substrate concentration but with and without predators. Bonferroni corrected *t*-tests were also performed to test for differences between substrate utilisation on Biolog Eco-Plates wells after 22, 33 and 44 hours from the inoculum of bacteria. All tests were performed using JMP, Version 5.0.1 (SAS Institute Inc.).

Results

Bacterial and protists abundances and biovolumes

These variables were already presented and discussed in detail in chapter III. Here are summarised only the major results as important background data for the following results on biodiversity and survival strategies.

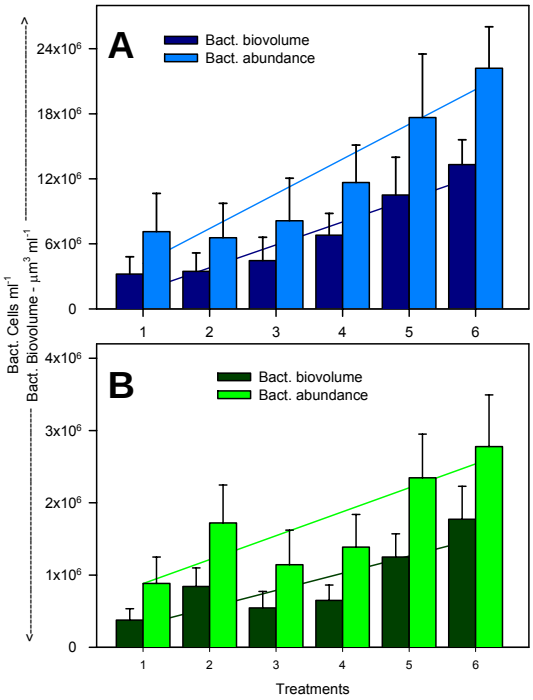


Figure 2: Average values of bacterial biovolume and abundances (± st. dev.) and relative regression lines, for the period day 5 – day 34, for the six different substrate concentrations (1-6, see text). A: Treatments without predators (A series); B: treatments with predators (B series). Biovolume (dark coloured bars) and abundance (light coloured bars) means of couples of treatments with the same amount of substrate supply were statistically compared using student *t*-tests (Bonferroni corrected, results are discussed in the text).

Due to the considerable range in the substrate gradient, bacterial communities developed with significantly different abundances and biovolumes: from 3.2 ± 1.5 x 10^6 $\mu m^3 ml^{-1}$, mean of the

period 5-34 (days) for the poorest YE reactor, supplied with just 0.10 mg YE L^{-1}, to 13.3 ± 2.3 x 10^6 $\mu m^3 ml^{-1}$, mean of the same period for the richest reactor, supplied with 32.00 mg YE L^{-1} (Figure 2A). In the presence of predators, bacterial biovolume during the period 5-34 days ranged from 0.4 ± 0.2 x 10^6 $\mu m^3 ml^{-1}$ in the poorest reactor (0.10 mg YE L^{-1}) to of 1.8 ± 0.5 x 10^6 $\mu m^3 ml^{-1}$ in the richest one (32.00 mg YE L^{-1}, Figure 2B).

Flagellates abundances was measured during the period 5-34 days. The average concentration increased with increasing substrate concentration and was between 8.2 ± 7.6 x 10^3 cells ml^{-1} in the poorest and 66.0 ± 45.1 x 10^3 cells ml^{-1} in the richest chemostat system.

Biolog community profiling

Bacterial substrate utilisation was measured at day 32, with 35 different carbon substrates belonging to five main groups: polymers, carbohydrates, carboxylic acids, amino acids and amines. Substrate utilisation activity per cell, measured after 44 hours (Figure 3) was different for the different carbon sources.

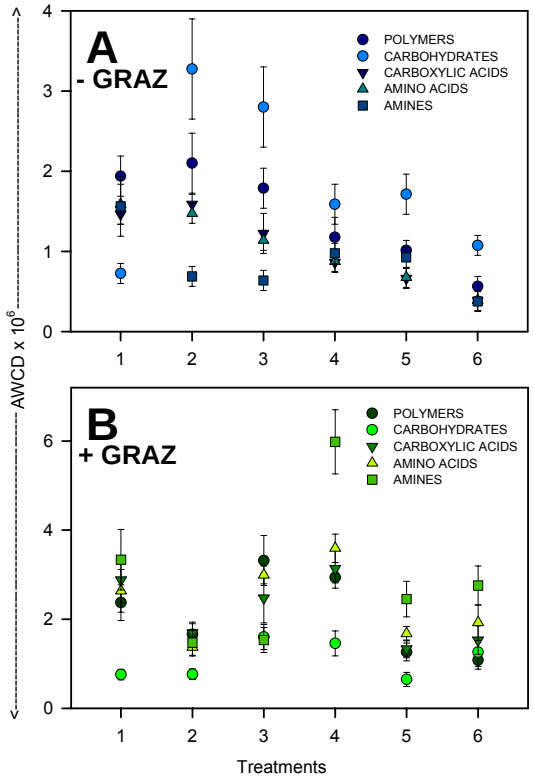


Figure 3: Biolog plates substrates utilisation determined by average well colour development (AWCD, Garland and Mills 1991) on 5 groups of substrates separately considered, measured per cell as explained in the text, by means (± st. dev.) of AWCD at day 32 and after 44 h of growing in the well. Every value is the mean of three replicates. Graph A refer to treatments (1-6) predators free, graph B to treatments with predators.

When plotting the cumulative values from different substrates utilisation activities per cell for each treatment, it became obvious that bacteria from predators-free treatments showed a trend to higher total substrate utilisation activities when inocula were derived from vessels poor in substrate (except for treatment 1A, Figure 4).

Carbohydrates were the major substrate used (Figure 4A). Bacteria from grazing treatments did not show any trend in ability of utilisation through the gradient and different substrate groups were used similarly (Figure 4B).

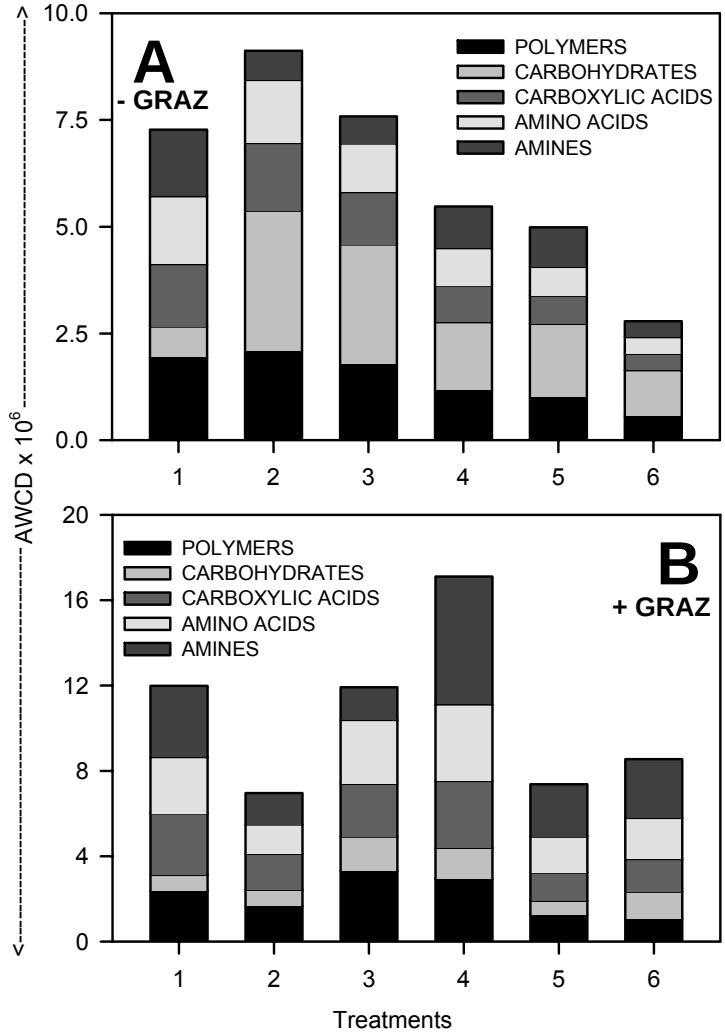


Figure 4: Substrate utilisation pattern in Biolog plates measured in AWCD per cell at day 32 and after 44 h of bacterial growth in the wells. Bars are representing the computation of the utilisation mean of different substrates. Graph A: Bacterial inocula from treatments without predators; graph B: bacteria from treatments with predators. Numbers 1 - 6 refers to the different substrate levels of the chemostat systems.

Substrate utilisation dynamics for single substrates, measured at time 22, 33 and 44 hours after the inoculum of bacterial cells in plates' wells, showed diverse activities for different bacterial populations: bacterial cells from treatments free from predation pressure appeared more active but

not for all substrates: five different developments, one for each group of substrates, are shown as examples in Figure 5. The higher activities with the predator-free inocula seemed to be correlated with the cell volume of the bacteria subjected to grazing pressure, which is about 1.5 times the volume of a bacterium from the same vessel at lower substrate levels, and about 3 times at higher substrate concentrations. Activity per unity of biovolume (μm^3), was thus always higher for bacteria growing free from predation.

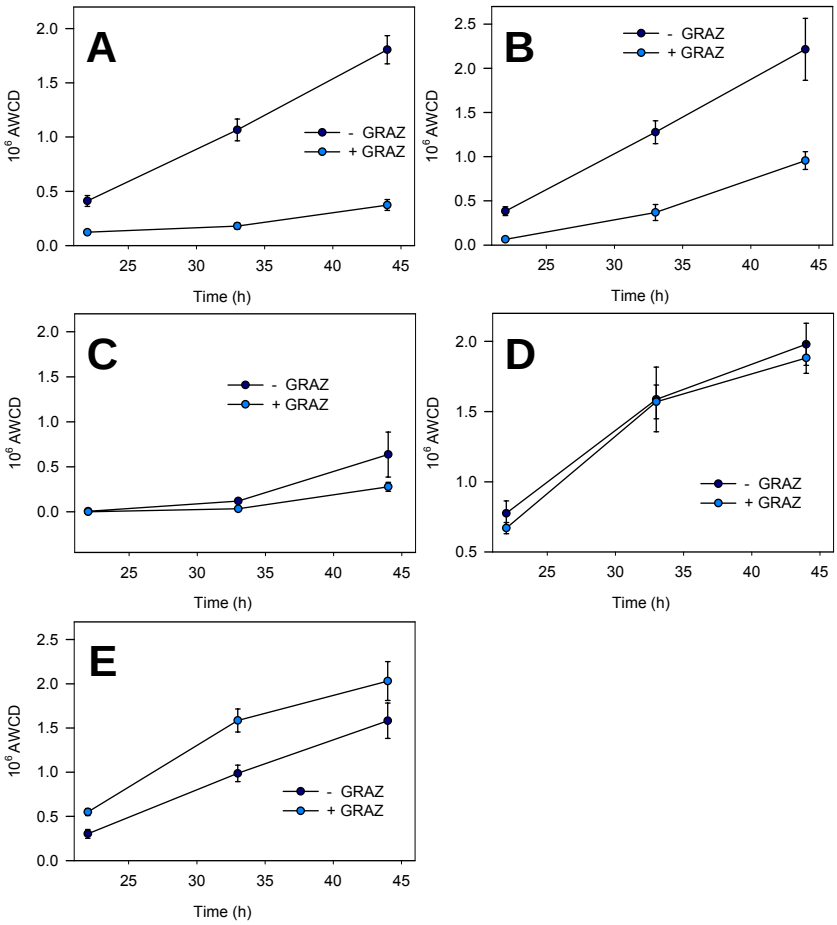


Figure 5: Temporal dynamics of Biolog substrate utilisation patterns with bacterial inocula from treatments 4 (enriched with 8 mg YE ml^{-1}), measured in AWCD per unity of biovolume (μm^3). Shown is the development of 5 substrates belonging from the 5 main groups: A: Glycogen (polymer) B: α-D-Lactose (carbohydrate), C: Glucose 1-phosphate (carboxylic acid), D: L-aspargine (amino acid), E: putrescine (amine). All lines represent trends of uptakes at time 22, 33 and 44 hours after the inoculum in wells, at day 32. Values are expressed as means (± st. dev.) of three replicates.

Genotypic classification:

From the first T-RFLP analysis (Figure 7) on bacterial diversity at day 3, at the beginning of the experimental phase, the richness in OTUs (Figure 6A) was rather similar in all 12 vessels, in total between 7 and 11 OTUs per vessel. At time 3 (day) the differences in OTU abundances between grazing-free treatments and treatments with predators were never significant ($p > 0.05$). However,

after 32 days of chemostat run, significant differences between the treatments were observed with respect to OTU richness (Figure 6B). Treatments without grazers resulted in a higher reduction of richness at lower substrate levels: e.g. in the vessel 1A, the poorest, only 3 OTUs remained from the 8 which were present at day 3. OTU richness increased with substrate supply and, after 32 days, 13 OTUs were found were found at the highest substrate level. Treatments B (Figure 6B, green bars) were under grazing pressure; at day 32 this resulted in a significantly different bacterial richness (treatments 6: $p < 0.001$, 5: $p < 0.01$, 4: $p < 0.05$) compared to vessels from group A, at high substrate supplied concentrations.

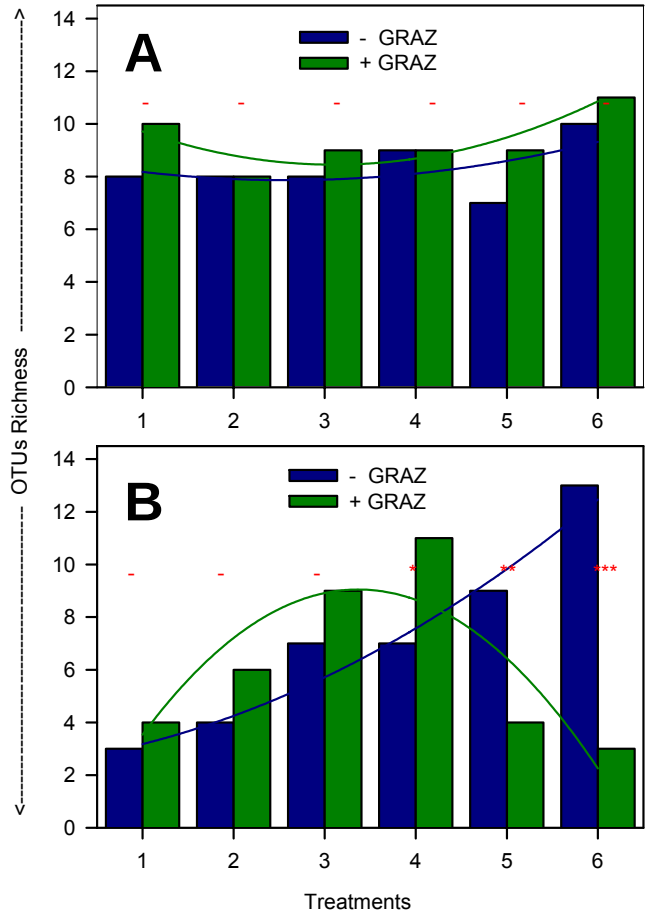
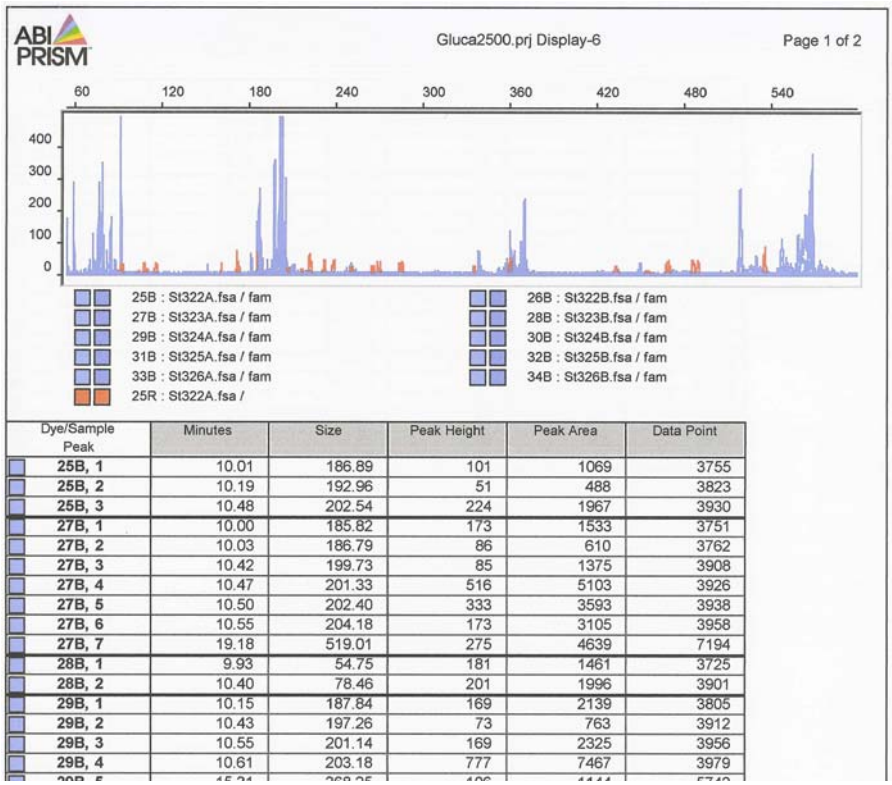


Figure 6: Bacterial community diversity measured as richness in OTUs (Operational Taxonomic Units) for 6 substrate levels with and without grazers at day 3 (A) and at day 32 (B). Quadratic regression ($y = y_0 + ax + bx^2$) of the values emphasize trends through the gradient of substrate supply. Significance of the difference between values from couples of vessels enriched with the same amount of substrate is expressed as: (-) $p>0.05$, (*) $0.05>p>0.01$, (**) $0.01>p>0.001$, (***) $p<0.001$ (student *t*-test, Bonferroni corrected).

In treatments with lower substrate supply (1B-4B) presence of grazers resulted in an increase of bacterial diversity compared to predators-free vessels (1A-4A). Conversely, in the richest vessels (5B-6B) the presence of predators resulted in a strong reduction of bacterial diversity, where just 4 and 3 OTUs survived from the 9 and 11 present at day 3. Trends of richness through the gradient at the end of experiment (day 32) showed a constant increase from the lower to the richest vessel in

absence of predators. The presence of predators resulted in a hump-shaped curve, strongly impacting the bacterial diversity of the richest treatments (5B-6B), much less the poorer ones. An increase in bacterial richness is obvious only from treatments 1B to 4B.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
25B, 1	10.01	186.89	101	1069	3755
25B, 2	10.19	192.96	51	488	3823
25B, 3	10.48	202.54	224	1967	3930
27B, 1	10.00	185.82	173	1533	3751
27B, 2	10.03	186.79	86	610	3762
27B, 3	10.42	199.73	85	1375	3908
27B, 4	10.47	201.33	516	5103	3926
27B, 5	10.50	202.40	333	3593	3938
27B, 6	10.55	204.18	173	3105	3958
27B, 7	19.18	519.01	275	4639	7194
28B, 1	9.93	54.75	181	1461	3725
28B, 2	10.40	78.46	201	1996	3901
29B, 1	10.15	187.84	169	2139	3805
29B, 2	10.43	197.26	73	763	3912
29B, 3	10.55	201.14	169	2325	3956
29B, 4	10.61	203.18	777	7467	3979

Figure 7: Example of electropherogram and corresponding table with T-RFLP obtained fragments length and correlate peaks dimensions produced by software Genescan. Peaks in red refer to the size standard, peaks in blue to T-RF.

Shannon index (*H*) of bacterial diversity (Figure 8A) confirmed the communities' composition and biodiversity measured as number of OTUs. Values for *H* were for all treatments with and without predators, at day 3 in the range 1.51 - 2.04 without any significant difference. At day 32, *H* (Figure 8B) was 0.94 for treatments without predators for the vessel 1A, and then rose through the gradient up to treatment 6A where it reached a value of 2.05, according with bacterial biodiversity measured as OTUs richness. *H* values followed the trends of richness also for treatments with predators. The effect of a weighted evaluation of different OTUs resulted in smoothened trends, but without significant changes in their dynamics.

From the analysis of the distribution of single OTUs (Table 2) it is possible to better understand the diversity of bacterial communities through the gradient, with or without predators, and their evolution during the 30 days of experiment.

24 different OTUs, corresponding to fragment peaks of diverse length, were recognized trough the treatments series. Some of them just occasionally appeared in some treatments: the peaks 197, 218,

234 and 365 bp appeared just in one treatment (2A, 6B, 1A and 5B respectively) at day 3, and then these OTUs rapidly disappeared. Other OTUs appeared during the experiment, in a single treatment, for example 339 bp and 449 bp in treatments 5B and 4B.

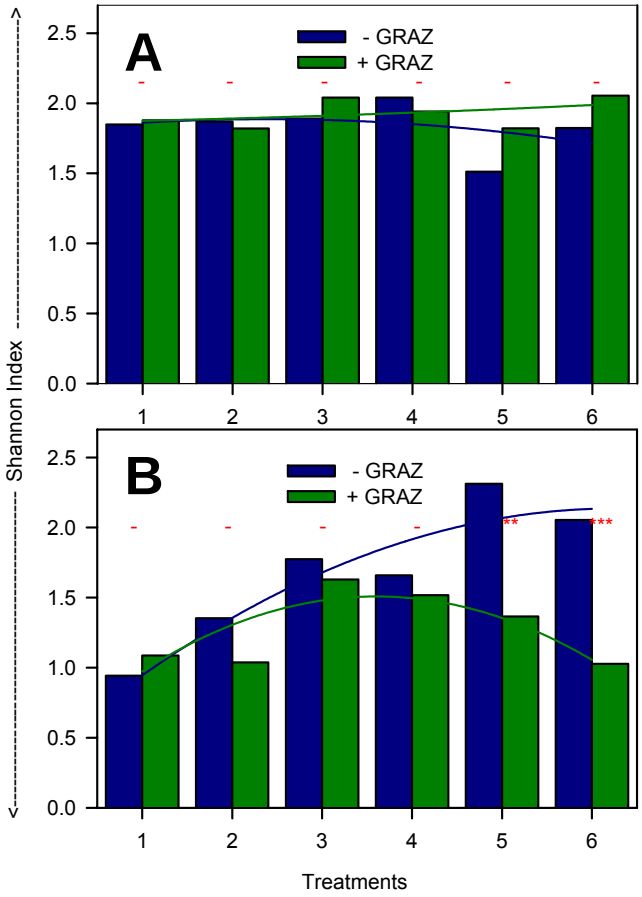


Figure 8: Shannon Index of bacterial diversity, measured on OTUs, in the 6 couples of vessels (with grazers and without) through the substrate gradient at day 3 (graph A) and at day 32 (graph B). Quadratic regression ($y = y_0 + ax + bx^2$) of the values emphasize trends through the gradient of substrate supply. Significance of the difference between values from couples of vessels enriched with the same amount of substrate is expressed as: (-) p>0.05, (*) 0.05>p>0.01, (**) 0.01>p>0.001, (***) p<0.001 (student *t*-test, Bonferroni corrected).

The OTU 203 bp was probably the most common in the chemostat. It was present in every treatment with important populations except in the poorest vessels. OTU 201 bp is also very well adapted, but seems to suffer the presence of predators, reducing its presence in vessels under grazing pressure.

Phenotypic classification

Bacterial morphologic community composition was different in the treatments depending both on the presence of predators and the amount of substrate supplied (Table 3).

peak / treat	TABLE 2 - Peak Area Percentage												
	1A	2A	3A	4A	5A	6A	1B	2B	3B	4B	5B	6B	
185	-	4.0	-	-	-	-	-	4.0	-	-	8.3	-	
187	6.7	14.0	20.7	16.9	-	-	11.4	3.8	-	3.4	4.2	-	
189	14.5	-	-	-	-	6.4	7.4	19.3	-	8.8	3.5	17.0	
191	9.9	-	8.8	-	-	-	7.2	18.1	4.7	-	-	4.3	
195	-	18.3	-	-	-	-	-	-	-	-	-	-	
197	-	6.8	-	-	-	-	-	-	-	-	-	-	
199	-	-	3.0	7.9	-	-	-	-	-	4.6	-	-	
201	-	17.1	16.8	8.8	26.0	1.8	-	10.4	14.0	3.8	21.5	3.7	
203	20.2	29.3	-	30.9	5.6	10.4	23.0	10.4	-	17.5	30.9	14.8	
205	31.9	5.4	30.1	4.5	-	-	-	31.0	9.8	30.2	-	-	D
207	-	5.2	5.4	8.5	45.3	34.9	34.3	-	27.7	16.5	-	39.6	A
211	-	-	-	-	7.9	3.0	2.1	-	11.2	-	-	6.5	Y
218	-	-	-	-	-	-	-	-	-	-	-	2.6	
234	4.8	-	-	-	-	-	-	-	-	-	-	-	3
339	-	-	-	-	-	-	-	-	-	-	-	-	
348	-	-	-	-	-	-	-	-	-	-	-	1.6	
358	-	-	-	-	3.5	3.3	1.8	-	-	-	-	2.7	
365	-	-	-	-	-	-	-	-	-	-	1.3	-	
368	3.5	-	-	4.1	8.3	5.4	1.8	3.1	-	4.8	-	5.0	
370	-	-	-	3.7	3.6	2.2	-	-	3.7	10.4	3.6	2.2	
373	-	-	-	-	-	-	4.5	-	11.7	-	-	-	
449	-	-	-	-	-	-	-	-	-	-	-	-	
519	8.5	-	9.9	14.6	-	5.5	6.4	-	7.9	-	5.9	-	
526	-	-	5.4	-	-	27.2	-	-	9.3	-	20.8	-	
185	-	-	7.7	-	-	-	-	-	2.9	-	-	-	
187	24.0	-	3.1	11.6	6.8	3.8	-	-	-	5.7	-	-	
189	-	-	-	-	3.0	7.9	-	-	-	-	23.0	15.6	
191	-	-	-	-	-	-	-	6.6	-	-	-	-	
195	-	-	-	-	-	-	-	-	-	6.6	-	-	
197	-	-	-	-	-	-	-	-	-	-	-	-	
199	-	-	6.9	4.1	-	5.2	-	-	22.9	11.5	-	-	
201	-	17.7	25.6	12.6	15.7	14.3	-	41.0	17.1	21.1	-	-	
203	16.0	20.6	18.0	40.5	26.9	18.6	27.3	22.3	7.4	-	23.2	44.5	
205	60.0	30.5	15.6	-	-	6.3	38.7	13.9	4.7	1.4	-	-	D
207	-	-	-	-	14.0	5.6	-	-	-	-	-	-	A
211	-	-	-	-	-	2.5	-	-	-	-	-	-	Y
218	-	-	-	-	-	-	-	-	-	-	-	-	
234	-	-	-	-	-	-	-	-	-	-	-	-	32
339	-	-	-	-	-	-	-	-	-	-	21.6	-	
348	-	-	-	-	-	-	-	-	-	-	-	-	
358	-	-	-	-	6.6	10.4	-	-	-	-	-	-	
365	-	-	-	-	-	-	-	-	-	-	-	-	
368	-	-	-	6.2	6.1	7.0	21.7	-	-	2.7	-	-	
370	-	-	-	5.5	16.1	4.3	-	-	1.8	3.1	32.2	-	
373	-	-	-	-	-	-	-	-	2.3	30.8	-	-	
449	-	-	-	-	-	-	-	-	-	5.0	-	-	
519	-	31.3	23.2	19.4	4.7	8.1	12.4	6.6	15.2	9.7	-	-	
526	-	-	-	-	-	6.2	-	9.5	25.8	2.3	-	39.9	

Table 2. OTUs distribution and relative importance. Percentage of the relative area of different peaks (identified with bp length) is measured on T-RFLP data from samples at day 3 and day 32 for vessels 1A-6A (in absence of predators) and 1B-6B (in presence of predators).

At the beginning of the experiment (day 3) the composition in OMUs was rather similar in all treatments. After 32 days, adaptations to environmental conditions provided very different bacterial communities. Few OMUs demonstrated to be very well adapted to all chemostat conditions: OMU 2 and OMU 5 (small cocci and small bacteria) were present in every treatment at day 3 and they survived almost everywhere at day 32 (the only exception is the absence of OMU 5 in treatments 5B and 6B).

OTU / treat	TABLE 3 - Single OTU percentage on total bacterial biovolumes												
	1A	2A	3A	4A	5A	6A	1B	2B	3B	4B	5B	6B	
1	-	25.2	21.8	26.8	17.8	12.2	-	12.0	16.1	20.4	21.6	15.8	
2	38.6	26.6	20.3	14.5	14.0	23.8	40.1	20.3	17.2	15.8	19.3	10.7	
3	-	-	-	-	-	-	-	-	2.1	-	-	-	
4	9.7	14.4	25.9	24.6	23.8	29.3	6.6	6.5	31.3	22.1	24.7	16.9	
5	22.8	21.6	18.3	16.8	23.1	25.9	29.6	27.6	15.1	10.8	13.5	15.2	
6	-	2.2	-	2.2	3.5	1.8	1.3	0.9	3.1	2.9	3.7	1.1	D
7	1.4	-	-	1.7	1.0	0.3	-	0.9	-	-	2.7	2.0	A
8	-		5.1	-	-	-	-	-	6.8	-	-	-	Y
9	4.8	2.9	5.6	7.3	2.1	0.6	2.6	-	3.1	7.9	4.7	11.0	
10	-	-	-	-	-	-	-	-	-	-	-	-	3
11	-	-	-	-	6.3	2.1	-	-	-	14.2	2.0	13.2	
12	-	-	-	-	-	0.9	-	1.8	-	-	1.0	4.5	
13	-	7.2	3.0	6.1	8.4	-	-	20.7	5.2	5.8	-	-	
14	22.8	-	-	-	-	-	19.7	9.2	-	-	-	-	
15	-	-	-	-	-	3.0	-	-	-	-	6.8	9.6	
16	-	-	-	-	-	-	-	-	-	-	-	-	
1	1.6	5.6	17.5	34.0	36.4	13.0	-	17.3	3.6	-	5.7	6.4	
2	35.5	39.3	36.7	29.9	7.0	21.7	30.0	27.4	44.6	15.5	5.2	4.7	
3	-	-	-	-	-	-	-	-	-	-	-	11.8	
4	3.2	2.2	26.5	8.8	18.7	17.5	-	-	-	-	-	-	
5	41.1	46.3	18.1	16.3	25.1	37.5	18.3	30.2	24.7	6.2	-	-	
6	0.8	-	-	-	0.5	0.5	1.7	4.0	7.8	2.5	3.8	9.8	D
7	-	-	-	-	-	0.4	-	1.2	1.8	-	9.4	13.5	A
8	-	1.9	1.2	8.8	-		6.4	3.6	-	1.9	-	4.7	Y
9	4.0	-	-	-	-	1.8	-	1.2	-	3.1	-	5.1	
10	-	-	-	-	-	-	-	-	6.0	8.7	49.1	9.4	32
11	-	-	-	-	-	1.1	14.9	6.9	8.4	42.9	-	11.4	
12	-	-	-	-	5.3	-	-	2.0	-	19.3	17.0	8.4	
13	2.4	-	-	2.0	-	0.7	5.5	-	-	-	-	-	
14	11.3	4.8	-	-	-	-	23.2	6.0	3.0	-	-	-	
15	-	-	-	-	7.0	6.0	-	-	-	-	8.0	8.4	
16	-	-	-	-	-	-	-	-	-	-	1.9	6.4	

Table 3. OMUs distribution and relative importance. Percentage on total community biovolume of cells belonging from every single morphological unit is measured at day 3 and day 32 for vessels 1A-6A (in absence of predators) and 1B-6B (in presence of predators). OMSs number refer to the different morphologies described in Table 1.

Other OMUs (e.g. OMU 4, large bacilla) were well adapted to substrate availability, but also completely vulnerable to grazing. Moreover, there were OMUs (e.g. OMU 6, OMU 7, OMU 16,

filaments and chains) that, to be present required the presence of grazers, but also high substrate availability. Finally, few OMUs (e.g. OMU 3, shaped long bacteria) happened just in one or two vessels. Small, free living bacteria were dominating in absence of predators and in vessels poor in substrate supply. Free living bacteria, and aggregates of small cocci and rods dominated communities in vessels rich in substrate in absence of predators.

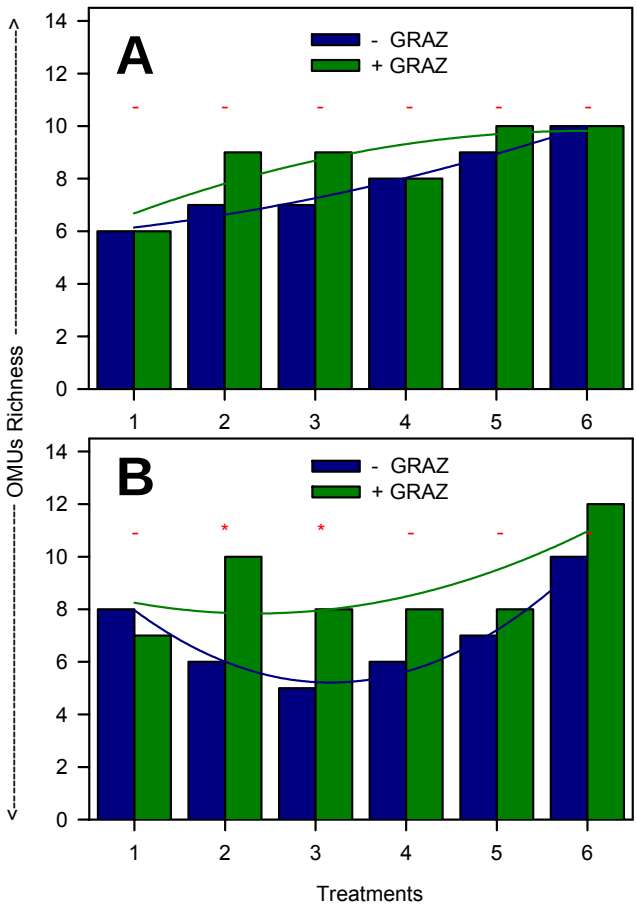


Figure 9: Bacterial phenotypic diversity measured as richness in OMUs (Operational Morphological Units, described in Table 1) richness in the 6 couples of vessels (with grazers and without) through the substrate gradient at day 3 (graph A) and at day 32 (graph B). Quadratic regression ($y = y_0 + ax + bx^2$) of the values emphasize trends through the gradient of substrate supply. Significance of the difference between values from couples of vessels enriched with the same amount of substrate is expressed as: (-) p>0.05, (*) 0.05>p>0.01, (**) 0.01>p>0.001, (***) p<0.001 (student *t*-test, Bonferroni corrected).

The presence of grazers strongly reduced the importance of those "medium-size" free living cells. In presence of predators, aggregates, very small cells and long filaments and chain of cells (but only where substrate supplied was higher) dominated the communities. A quantitative analysis of the biodiversity measured from bacterial phenotypes at day 3 (Figure 9A), showed slight differences between different populations: 6 OMUs in the poorer vessels (1A, 1B), 10 OMUs in the richer ones (6A, 5B, 6B) and any significant difference between treatments with and without predators. OMU richness at day 32 (Figure 9B) was generally comparable to the richness at the beginning of the

experiment. Treatments where bacteria were growing under grazing pressure showed higher number of morphological units, but just in two cases (treatments 2 and 3) this difference became significant ($p < 0.05$). Numbers of OMUs in vessel 5B and 6B, at day 32, were 8 and 12, respectively, despite the low OTU values (4 and 3) measured in the same treatments at the same day.

Discussion

Due to the design of the experiment in the continuous culture systems the selective pressure of relative competition between bacterial strains was determined by the productivity of the system which was given by the different amounts of substrate supplied (substrate availability per cell).

On the other hand, grazing pressure increased with productivity (more substrate → higher productivity → more prey available → more flagellates). Predator grazing activity was resulting in a reduction of bacterial abundances throughout the whole substrate gradient; thereby limiting the effects of competition on bacterial production due to substrate availability. Therefore competition pressure should have been higher in vessels with very low productivity and was gradually reduced with an increase of substrate supply.

Confirming the conclusions of Horner-Devine *et al.* (**88**), the bacterial concentrations in the absence of predators correlated to the substrate concentration supplied. In the presence of predators, the effect of grazing was resulting in a strong reduction of bacterial abundances throughout the whole substrate gradient, limiting the differences in bacterial production due to substrate supplied but channelling the increased bacterial production into predator biomass (see Chapter III).

The impact of competition and, in the B treatments, predation, drastically changed the original equilibrium of the vessel community composition.

Biolog Eco-plates analysis demonstrated that grazing had a strong impact not only on bacterial community composition but also on single cell, and thus community, physiological activities (Figure 3). Except for treatments 1 treatments grazing-free clearly showed a reduction in bacterial cell uptake activities with the increase of available substrate, and thus, reduction in competition. Grazing changed this trend: in treatments where bacteria where subjected to grazing pressure the ability in substrate uptake seemed to be a secondary limiting factor, and this impact was not showing any trend along the gradient of nutrients supplied (Figure 4).

The ability in utilising different substrates was tested with bacteria growing in the presence of predators and bacteria growing predator-free. Considering the utilisation of substrate per cell, the generally larger bacteria in vessels under predation were utilising higher amount of substrates. However, measuring the ability and speed in utilising substrates per unit of biovolume (μm^3), then just for few substrates similar trends were recorded whereas bacteria growing free from predation seemed to be more competitive in substrate utilisation. Just in few cases bacteria growing under grazing pressure demonstrate to be more efficient in uptake, usually on amines (Figure 5).

T-RFLP fingerprints at the end of the experiment (day 32) revealed several deviations in bacterial compositions and diversity compared to the beginning of the experiment. After 32 days, in treatment series A, without predators (Figure 6B, blue bars), the high competition for resources resulted in a higher selection at lower substrate levels and, then, constantly following the gradient, from the lowest to the richest reactor (Treatment 6A). Throughout the A vessels, in absence of predators, competition was thus controlling the development of the bacterial community, and this impact seemed to be very high at lower substrate levels which had reduction in bacterial diversity of about 65%.

In the B treatment series, the impact of grazers changed the bacterial communities (Figure 6, green bars) compared to the A vessels. At low substrate supply (1B-4B), and thus in conditions for bacteria close to starvation and potentially very high substrate competition, the presence of flagellates resulted in a relative increase of bacterial diversity compared to the vessels predators free (1A-4A). This reduction in the impact of competition may be due to grazing effects. The selective predation and the additional source of nutrients due to grazer excretions mitigated the strong control of competition for resources (**67**).

In the richest vessels (5B-6B) the presence of predators resulted in a strong reduction of bacterial diversity. In this case the additional source of carbon represented by the excretion of the grazers was not necessary because the vessels were already supplied with very high amount of nutrients. In these vessels predation by flagellates was clearly reducing the richness of the bacterial communities.

When competition was the only limiting factor, bacterial abundances and bacterial diversity revealed a constant relation. In contrast, during grazing this relation disappeared: at day 32, vessels 5B and 6B were much more productive than 3B or 4B, but poorer in bacterial diversity.

Analysing single OTU distributions, it is possible to define some OTUs as potentially grazing resistant (189, 370, 373 bp) and also, usually requiring a high amount of available substrate. Other OTUs, seemed to be adapted against grazing pressure as well, but their distribution suggest us that a high phenotypic plasticity might be characteristic in this group (e.g. 526 bp, dominant at day

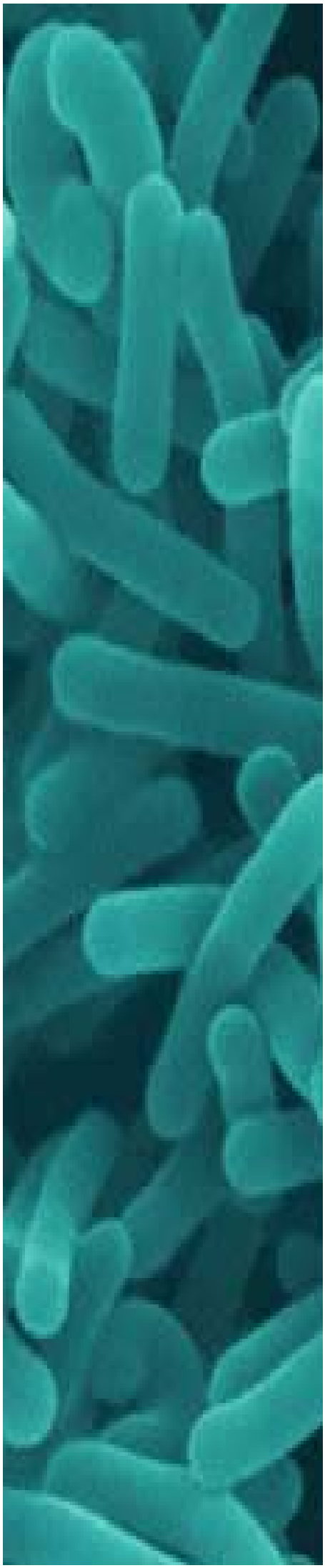
32 in vessel 6B were OMUs richness is highest). OTUs 201 and 205 bp were well adapted to compete for resources but they seemed vulnerable to grazing and their relative presence in vessels under predation pressure was strongly reduced.

These patterns were confirmed by the OMU richness and distribution data: in the richer vessels OMU richness was much higher than OTU richness (Figure 9). The high grazing pressure in such rich environments may select grazing resistant strains which are phenotypically plastic, able to develop different strategies to survive in the presence of grazers. At the same time, the survival of flagellates was enabled by the presence of edible, or partly edible, OMUs.

Conclusions

With this study on biodiversity in a system where predation and competition impacts were simultaneously affecting bacterial communities it was possible to clarify several aspects about distribution and richness of our artificially designed prey communities. Bacteria (prey) biodiversity is stimulated by a combination of factors which control the communities, and is limited when the control by one of this factor becomes too strong.

Morphological plasticity is an efficient refuge against predation (**19**) but at the same time it requires sufficient amount of substrate for the formation of long cells or colonial structures. These results are consistent with the ecological theory which predicts that the relative importance of competition and predation will change with the productivity of the environment (**63**). They are also in line with previous experimental proofs from a laboratory system studying bacteria–bacteriophage interactions (**60**), and they are also consistent with the observations of an increasing abundance of morphologically resistant bacteria in more productive aquatic environments.



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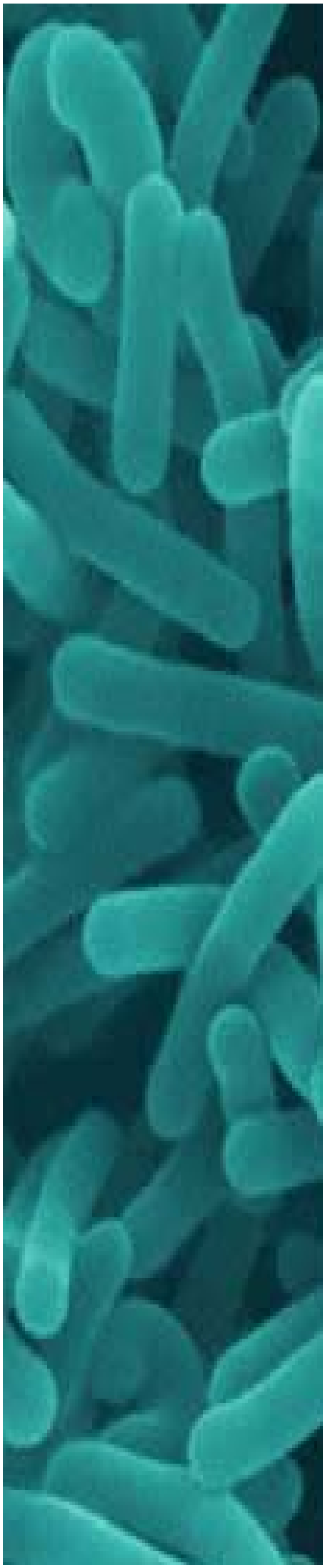

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