

Genetic changes in the bacterial community structure associated with protistan grazers

E. J. van Hannen¹, M. Veninga², J. Bloem², H. J. Gons¹ and H. J. Laanbroek¹

With 4 figures and 1 table

Abstract: Besides controlling bacterial production, bacterivorous protists are thought to govern bacterial community structure. Experimental work has shown that both grazers and bacteria developed strategies that may influence the bacterial community structure. However, evidence from the natural environment is still lacking. Advances in molecular techniques now allow the profiling of natural bacterial assemblages. We used denaturing gradient gel electrophoresis (DGGE) to profile a bacterial community in a continuous flow system that was fed with living and detrital cyanobacterial cells. Although attempts were made to remove eukaryotic grazers, heterotrophic nanoflagellates were growing in the second stages of the system. Analysis of DGGE patterns by nonmetric multidimensional scaling showed a large change in the bacterial community structure coinciding with the peak in protist numbers. Our results show that DGGE analysis can be used to facilitate studies on the effect of protistan grazing on natural bacterial communities.

Introduction

Although the importance of heterotrophic nanoflagellates as regulators of bacterial production is widely recognized (FENCHEL 1982, AZAM et al. 1983) little is known about the ability of protist grazers to influence bacterial community structure and species composition. JÜRGENS & GÜDE (1994) postulated that bacterivory should be one of the major forces shaping the genetic and phenotypic composition of bacterial communities.

Experimental work has shown that heterotrophic nanoflagellates graze at higher rates on motile bacteria than on non-motile bacteria (MONGER & LANDRY 1992, GONZÁLEZ et al. 1993), have a preference for larger, dividing

¹ **Authors' addresses:** Netherlands Institute of Ecology, Centre for Limnology, Rijksstraatweg 6, Nieuwersluis, P.O. Box 1299, NL-3600 BG Maarssen, The Netherlands. E-mail: vanhannen@cl.nioo.knaw.nl

² DLO-Research Institute for Agrobiological and Soil Fertility, NL-6700 AA Wageningen, The Netherlands.

cells (GONZÁLEZ et al. 1990 b, SHERR et al. 1992, ŠIMEK & CHRAZNOWSKI 1992) and digest different bacterial species at different rates (GONZÁLEZ et al. 1990 a). Bacteria on the other hand may escape grazing by increasing their growth rate or elongating their cells and become inedible (PERNTHALER et al. 1997, ŠIMEK et al. 1997). Thus, both predator and prey developed feeding or defense strategies that may induce shifts in bacterial morphotypes to either minute or large, inedible cells forms. Indeed, inedible, so-called grazing resistant bacteria were frequently observed in experimental systems where high grazing occurred (GÜDE 1979, BIANCHI 1989, SHIKANO et al. 1990). Both morphotypes can be maintained in natural bacterioplankton communities (GÜDE 1989, SHERR et al. 1992, JÜRGENS & STOLPE 1995, SOMMARUGA & PSENNER 1995) suggesting that these predator-prey interactions stimulate bacterial phenotypic plasticity or govern microbial community structure by specifically removing genotypes from the bacterial population. Recently, PERNTHALER et al. (1996) demonstrated that protistan grazing could increase the biomass of very small and large cells in a natural freshwater bacterioplankton community. It has only been shown in laboratory experiments that these morphological changes were partly due to genetic shifts in the bacterial community (ŠIMEK et al. 1997, PERNTHALER et al. 1997, HAHN & HÖFLE 1998). However, field evidence is still lacking, mainly because of the high complexity of natural microbial communities. Advances in molecular techniques and especially the application of denaturant gradient gel electrophoresis (DGGE) (FISHER & LERMAN 1979) to problems in microbial ecology (MUYZER et al. 1993) now allows the characterisation of complex microbial communities without the need of cultivation.

We used DGGE of the 16S rRNA gene to profile the bacterial community composition in a continuous flow system that was grazed by protists. The experiment was originally designed to follow the growth of a natural consortium of bacteria on detritus derived from selected phytoplankton species. For this purpose, we used a two-stage continuous flow system with the capability of producing fresh detritus by killing phototrophs with UV-C light (VAN HANNEN & GONS 1997). However, attempts to remove eukaryotic organisms from a lake water inoculum by filtration (1 µm) were unsuccessful and heterotrophic nanoflagellates were growing in the second stages. Although the experiment lacked a control since exclusion of protistan grazers from the flow system failed, we applied DGGE in order to assess its potential in describing how protistan grazing may govern bacterial community structure.

Materials and methods

Experimental setup

Modified Kluver flasks were used as culture vessels (VAN LIERE et al. 1975). The system consisted of one 2 l vessel (first stage) and two 1 l vessels (second stages). Both

second stages were connected in parallel to the first stage, see VAN HANNEN & GONS 1997 for details. Both second stages received medium including biomass of the non-axenic cyanobacterium *Oscillatoria limnetica* from the first stage. One second stage (D-stage; for "Detritus") received dead *O. limnetica* cells, killed on-line by a UV-C lamp (Philips TUV 6W). The UV-C lamp was placed in front of a small quartz tube (inner diameter: 2 mm, outer diameter: 4 mm, length 350 mm). The other second stage (L-stage; for "Living cells") received living *O. limnetica* cells. *O. limnetica* (Centre for Limnology, strain MR1) was grown under light limiting conditions in the first stage ($D = 0.36 \text{ d}^{-1}$). The culture medium used was a modified Guillard medium (VAN HANNEN & GONS 1997). The culture was grown at constant temperature (20°C), pH (8.0 ± 0.2) and light (circular Philips fluorescent lamp TLE 32W/33). Samples to determine chlorophyll-a (Chl-a) concentration and total suspended solids (TSS) were taken every 24 h. The Chl-a and TSS measurements have been described previously (VAN HANNEN & GONS 1997). After 30 days the total suspended solids and Chl-a concentration reached a steady state. Both second stages were then completely filled with filtered ($1 \mu\text{m}$ polycarbonate membrane filter, Porectics, Livermore, CA, USA) lake water (sampled: 9 October 1995, Lake Loosdrecht, The Netherlands) and connected to the first stage. The lake water was filtered in order to remove eukaryotic microorganisms, but it turned out that small protists were not removed completely. Lake Loosdrecht water was chosen because *O. limnetica* is the dominant cyanobacterium in this shallow lake (VAN TONGEREN et al. 1992). Both second stages had a dilution rate of 0.30 d^{-1} . Every day a sample of 50 ml was withdrawn from both second stages. Twenty ml were used to measure Chl-a content and another 20 ml for total suspended solids. One ml was fixed with formaldehyde to a final concentration of 0.5 % for prokaryotic and eukaryotic cell enumeration and stored at -80°C . Another 1 ml volume for PCR and DGGE was stored at -80°C .

Cell enumeration and bacterial biomass

Samples were diluted depending on cell numbers and filtered onto prestained, black polycarbonate membrane filters (Poretics Corporation) and stained with DAPI (PORTER & FEIG 1980). For bacterial counts, $0.2 \mu\text{m}$ filters were used. Eukaryotic microorganisms were counted on $1.0 \mu\text{m}$ filters. Samples were enumerated using an Axiophot microscope (Carl Zeiss Jena GmbH, Jena, Germany) and a magnification of $\times 1,250$. Bacterial numbers were determined by counting 20 microscope fields. Eukaryotic cell numbers were determined in 50 microscope fields. Some samples were counted 10 times to estimate counting error. The errors were $<10\%$.

Cell volumes of bacterial cells were estimated by confocal laser scanning microscopy (Leica Laser Technik GmbH, Heidelberg, Germany) and automatic image analysis (BLOEM et al. 1995). Cells were divided into two classes, i.e. edible bacteria (length $<3 \mu\text{m}$) and inedible bacteria (length $>3 \mu\text{m}$) based on the results of PERNTHALER et al. (1996) and ŠIMEK et al. (1997).

DOM release in the second stages

The specific lysis rate of *O. limnetica* in the second stages was used as an estimation of the DOM release. This specific lysis rate is based on the disappearance of Chl-a in the

second stages and has been described by VAN HANNEN & GONS (1997). The specific lysis rate is defined by:

$$[\text{Chl } a]_t = \frac{D \cdot [\text{Chl } a]_1}{(D + L)} \cdot (1 - e^{-(D+L) \cdot t}) \quad (1)$$

where $[\text{Chl } a]_t$ is the Chl-a concentration at time t , $[\text{Chl } a]_1$ is the Chl-a concentration in the first stage, D is the dilution rate of the second stage, and L is the specific lysis rate.

DNA extraction, PCR and DGGE

Samples of 0.5 ml were heated in a microwave oven for 3 min at maximum power to release DNA from the cells. Five μl of each microwave-treated sample were subjected to PCR and DGGE. Amplification of bacterial DNA was performed using universal bacterial-specific primers against the V3 region of the 16S rRNA gene (MUYZER et al. 1993) in 30 cycles of PCR. DGGE was performed on the Bio-Rad Protean II system as described previously (MUYZER et al. 1993). Briefly, similarly-sized PCR-products were separated on a 1.5-mm thick, vertical gel containing 8 % (w/v) polyacrylamide (37.5:1 acrylamide: bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30 % at the top of the gel to 55 % at the bottom. Here, 100 % denaturant is defined as 7M urea and 40 % v/v formamide. Electrophoresis was performed in a buffer containing 40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.6 ($0.5 \times \text{TAE}$) and 75 V were applied to the submerged gel for 16 h. Nucleic acids were visualized by staining for 1 h in $0.5 \times \text{TAE}$ buffer containing 0.5 mg/l ethidium bromide, followed by destaining for 5 min in demineralized water and photographing the gel using a CCD camera (The Imager, Appligene, Illkirch, France). Digitized images were inverted using the software Photostyler (Aldus Corporation, Seattle, USA). Contrast and gray balance of the entire image were adjusted to reduce background. To facilitate comparison of the samples, the DGGE banding patterns were converted to a binary matrix. The presence or absence of a nucleic acid band at the same height in each lane as judged by eye was marked with 1 or 0, respectively. Gel images were enlarged two times to facilitate band detection. Since bacteria could have multiple copies of their ribosomal RNA gene, care should be taken with the interpretation of the number of species present in a DGGE pattern. We, therefore, refer to a band as a sequence type rather than a species.

Data analysis

The binary data generated from the DGGE patterns were used to calculate a distance matrix after NEI & LI (1979) according to the equation:

$$D_i = 1 - \frac{2 \times N_{AB}}{(N_A + N_B)} \quad (2)$$

where D_i is the distance between the patterns detected in lanes A and B, N_{AB} is the number of bands shared by both lane A and lane B, N_A is the number of bands in lane

A and N_B is the number of bands in lane B. This distance matrix was then used in a nonmetric multidimensional scaling analysis (NMDS). This analysis constructs a 'map' showing the relationships between a number of observations given only a table of distances between them. The data is presented in a Euclidean plane such that highly similar measurements are plotted close together. Such a graphical representation is much easier to interpret than the original table of distances. The dimensions (axes) in the map have no special significance and can be rotated or mirrored without influencing the relative distances between the points. As a measure of the goodness of fit of the reproduced distances to the observed distances, the stress value is used. When stress values are <0.1 , the NMDS plot is considered to be an acceptable representation of the original data. Interpretation of the NMDS plot can be achieved by explaining single dimensions or by finding structures or patterns in the multidimensional space (KRUSKAL & WISH 1978, BORG & LINGOES 1987).

Applied to DGGE data, the NMDS map shows every banding pattern, – the community structure at a particular point in time – as one dated point and by connecting consecutive points, relative changes in the community structure can be visualised and interpreted. NMDS has proved to be useful as an analysis tool for genetic structures (LESSA 1990). NMDS was performed using the statistical software package Statistica (Statsoft Inc. Tulsa, Oklahoma, USA).

Results

O. limnetica lysis rates in the second stages

At steady state growth of *O. limnetica* in the first stage, the mean concentrations of Chl-a and TSS were 1,435 $\mu\text{g/l}$ and 64.35 mg/l , respectively. At the same time, both second stages, filled with filtered ($<1\mu\text{m}$) lake water simultaneously received effluent from the first stage at a dilution rate of 0.30 d^{-1} . After 5 days, both second stages reached a steady state for Chl-a and TSS (Chl-a and TSS of L-stage: 1,136 $\mu\text{g/l}$ and 50 mg/l , respectively and D-stage: 882 $\mu\text{g/l}$ and 42 mg/l , respectively). From these steady state values specific lysis rates were calculated according to Eq. (1). Lysis rates in the L-stage and D-stage were 0.066 and 0.180 d^{-1} , respectively.

Bacterial biomass and protozoan numbers

Mean cell length of edible bacteria in both second stages was $0.7\mu\text{m}$ with a mean cell width of $0.6\mu\text{m}$. Mean cell length of inedible cells of both second stages were $7\mu\text{m}$ with a mean cell width of $4\mu\text{m}$. During the course of the experiment no changes were found in the mean cell volume of both edible and inedible bacteria so all measurements together of a continuous culture were used to calculate a mean cell volume for that stage (Table 1). These mean values were then used to calculate the bacterial biomass by assuming a carbon

Table 1. Mean cell length, width and volumes of edible ($<3\ \mu\text{m}$) and inedible ($>3\ \mu\text{m}$) bacteria in the second stages of the continuous flow system. n = the number of bacteria analyzed by confocal laser scanning microscopy. The range of the measurements is given in parenthesis.

	Length (μm)	Width (μm)	Volume (μm^3)	n
L-Stage				
edible	0.7 ± 0.2 (0.4–2.8)	0.6 ± 0.2 (0.2–1.3)	0.16 ± 0.15 (0.01–1.09)	584
inedible	6.6 ± 3.1 (3.1–18.1)	0.5 ± 0.1 (0.3–0.7)	1.03 ± 0.60 (0.20–3.01)	43
D-Stage				
edible	0.7 ± 0.3 (0.4–2.8)	0.6 ± 0.2 (0.2–1.4)	0.17 ± 0.16 (0.01–2.19)	1446
inedible	7.4 ± 3.7 (0.4–20.6)	0.4 ± 0.1 (0.3–0.6)	1.05 ± 0.59 (0.21–3.30)	88

content of $200\ \text{fg}/\mu\text{m}^3$ (BRATBAK & DUNDAS 1984, SCAVIA & LAIRD 1987, BLOEM et al. 1988, BLOEM et al. 1995).

D-stage. Immediately after connecting the first stage to the second stage, bacterial biomass associated with edible bacteria began to rise steadily and reached a maximum biomass at day 7 (Fig. 1). After day 7, edible bacterial biomass decreased till a much lower level was reached after 20 days. As soon as the numbers of protists, mainly consisting of heterotrophic nanoflagellates with cell sizes of $2\text{--}5\ \mu\text{m}$, began to rise, bacterial biomass associated with inedible bacteria increased till a maximum biomass on day 14. After this day, inedible bacterial biomass declined till the end of the experiment. During the experiment, edible bacterial numbers ranged from 0.8 to $8 \cdot 10^7/\text{ml}$, while inedible bacterial numbers were between 0.1 and $1 \cdot 10^7/\text{ml}$. Protists reached highest numbers on day 12.

L-stage. During the first 16 days after the start of the second stages bacterial biomass associated with edible bacteria was rather constant (Fig. 1). The edible biomass decreased slightly during the next 11 days till the end of the experiment. Bacterial biomass associated with inedible bacteria increased gradually but declined also after day 16. Edible bacterial numbers ranged from: 0.5 to $2 \cdot 10^7/\text{ml}$, while inedible bacterial numbers were between 0.01 and $0.2 \cdot 10^7/\text{ml}$. Protists reached highest numbers on day 12. Bacterial numbers of the first stage were in general 2–15 times lower than bacterial numbers in the D-stage and 1–4 times lower than the L-stage.

Bacterial genetic community structure

Both second stages showed a markedly changing bacterial DGGE pattern during the course of the experiment (Fig. 2). In the D-stage, 43 different bacterial

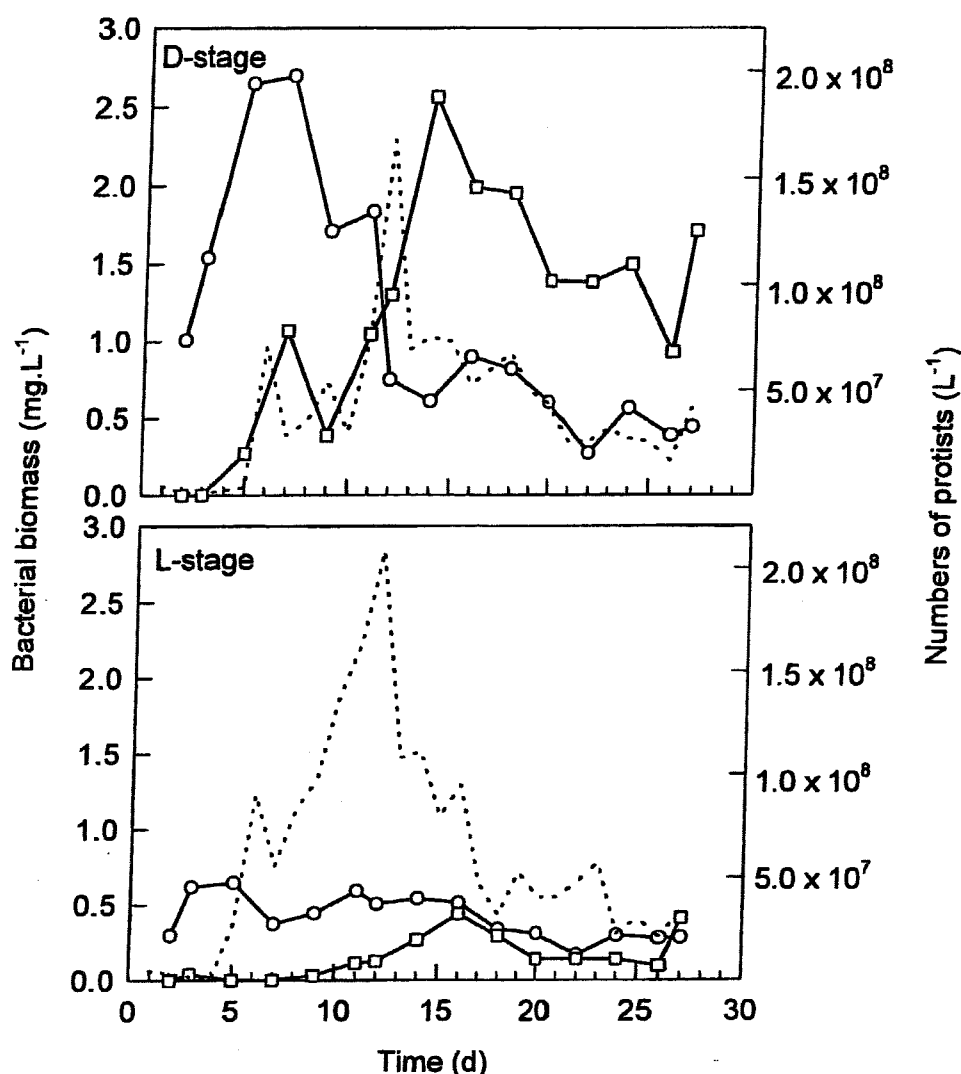


Fig. 1. Biomass of edible and inedible bacteria and numbers of protist grazers in the second stages of the continuous culture system during the course of the experiment. Circles: edible bacteria (length $<3\ \mu\text{m}$), squares: inedible bacteria (length $>3\ \mu\text{m}$), dashed line: numbers of protist grazers.

sequence types could be detected while in the L-stage only 20 different bacterial sequence types were counted.

After the start of the second stages, a rapid increase in detectable sequence types in the D-stage was seen, reaching a peak of 35 sequence types at day 8 (Fig. 3). From day 8, the number of sequence types decreased sharply to 18 sequence types at day 12, coinciding with a sharp increase in protist numbers. From here on, the number rose again to 23 sequence types, but after day 19 decreased steadily to 8 at the end of the experiment. The number of detectable sequence types in the L-stage showed a similar pattern with maximum of 14 sequence types on day 10. The number of sequence types decreased till 11 on day 12, coinciding with the peak in protist numbers. Then, the number stayed

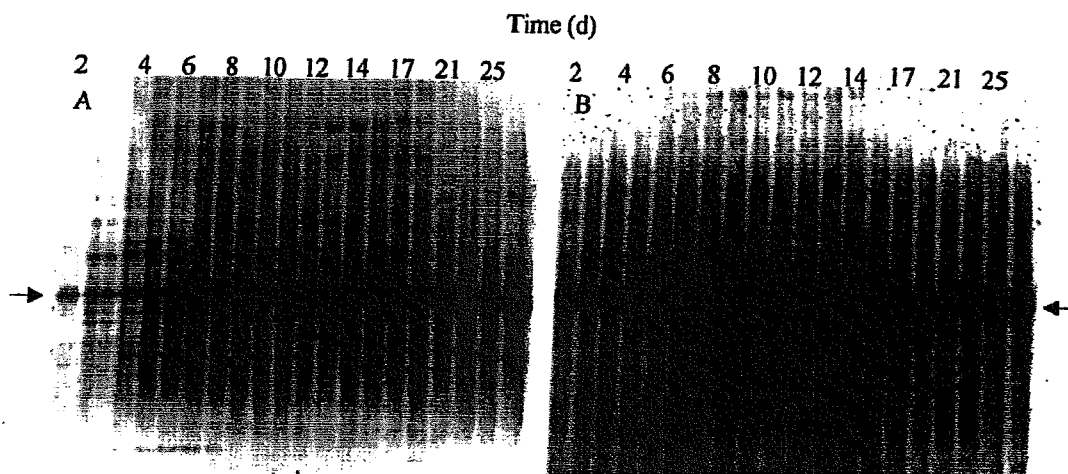


Fig. 2. Denaturant gradient gel electrophoresis patterns of the second stages of the continuous culture system. A = bacterial pattern of the Detritus-stage, B = bacterial pattern of the Living cells-stage. Denaturing gradient: 30–55 %. The arrows indicate the band belonging to *Oscillatoria limnetica*. Band positions between gels cannot be compared due to small gradient differences.

constant until day 21 after which the number decreased till 8 at the end of the experiment.

Nonmetric multidimensional scaling analysis of the D-stage bacterial DGGE showed an initial large change in the pattern from day 3 till 4 and a gradual change in the next 7 days (Fig. 4). A second large change in the DGGE pattern was seen from day 11 to 12 coinciding with the sharp increase in protist numbers. From day 12 on, changes in the pattern were gradual again. The DGGE patterns of the L-stage changed gradually from day 3 till day 6. The major change in the pattern occurred from day 6 to 7. Hereafter, changes were gradual again till the end of the experiment.

Discussion

Molecular techniques have been used increasingly to reveal patterns in the microbial world (NOLD & ZWART 1998). By directly sampling the natural microbial diversity, problems of non-culturability are circumvented but the molecular techniques can introduce PCR-based errors (VAN HANNEN et al. 1998 and references herein). DGGE presents complex microbial communities as banding patterns which show the dominant bacterial species (MUYZER et al. 1993). These banding patterns can be analysed by clustering techniques to show differences in microbial communities (VAN HANNEN et al. 1998) or by ordination techniques to reveal patterns in the changing microbial community.

We used DGGE to show a large change in the bacterial community that correlated with a peak in the abundance of heterotrophic nanoflagellates. We

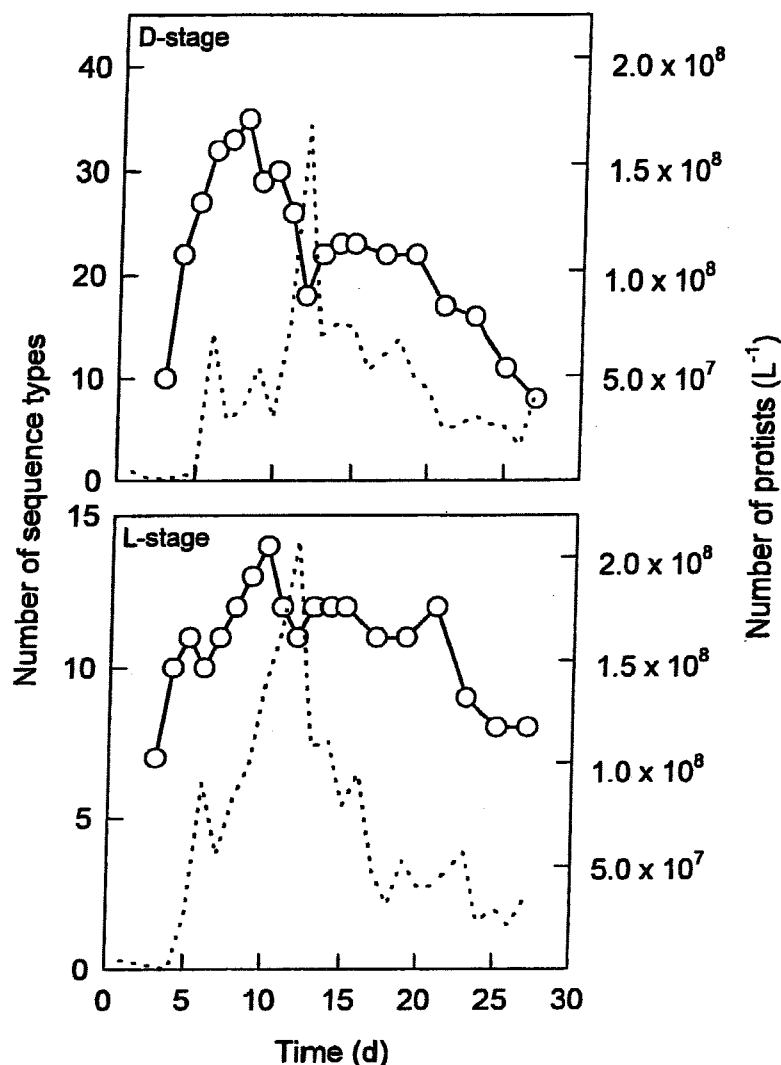


Fig. 3. Number of detected sequence types (circles) and numbers of protist grazers (dashed line) during the course of the experiment.

fed a natural assemblage of bacteria with detritus (high DOM) and living cells (low DOM) in a continuous culture system to follow the growth of heterotrophic bacteria. At the start of the experiment all biomass was allocated to small, edible bacteria (mean cell length: 0.7 μm) and during the first days their biomass increased especially in the stage that was fed with detritus (Fig. 1). Although attempts were made to remove small protists, populations of heterotrophic nanoflagellates developed in both second stages and with the increase in protist abundance more biomass was allocated to larger bacteria (mean cell length: 7 μm). These large bacteria were found to be inedible for heterotrophic nanoflagellates (PERNTHALER et al. 1996, ŠIMEK et al. 1997) and this induction of grazing resistant morphotypes during high grazing pressure in experimental systems has been reported many times (for review see JÜRGENS & GÜDE 1994).

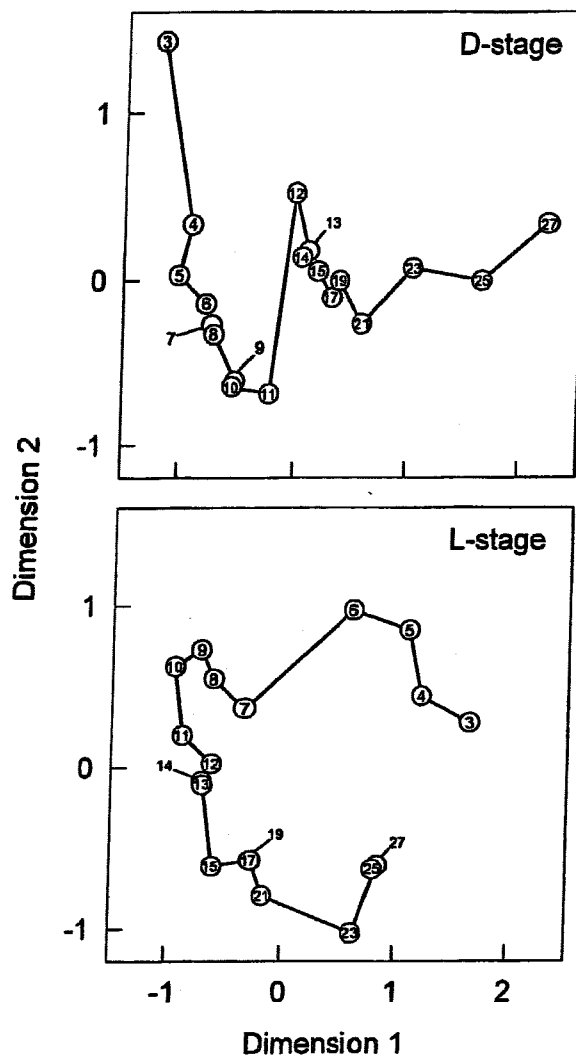


Fig. 4. Nonmetric multidimensional scaling maps of the bacterial DGGE patterns. Both the analyses were done using 2 dimensions. Numbers indicate time in days from the start of the second stages. Detritus-stage stress value: 0.085. Living cells-stage stress value: 0.095.

Although only the D-stage supported a high bacterial biomass, most likely due to the higher DOM input, both stages showed similar protist densities. The extra DOM release due to a higher lysis rate of *O. limnetica* in the D-stage may explain these observations. This DOM release clearly stimulated bacterial growth yet most of the biomass was allocated to large bacteria as soon as the protists appeared. Since these large bacteria are considered to be grazing resistant, the protists in the D-stage could not exploit these bacteria as a food source.

DGGE analysis showed that in both stages the bacterial richness – the number of sequence bands – decreased during the peak in protist numbers (Fig. 3) and NMDS analysis of the DGGE patterns reveal a large shift in the

bacterial community structure of the D-stage coinciding with this peak in protist abundance (Fig. 4). Although large bacteria were also present in the L-stage, the pronounced succession from edible to inedible bacteria was not as clear as in the D-stage. Here, molecular analysis detected only a small decrease in bacterial richness during the peak in protists and the NMDS analysis showed no large change in the bacterial community structure. HADAS *et al.* (1990) also found only an increase in heterotrophic nanoflagellates numbers and not in bacterial biomass after the addition of bacterial substrate. Apparently, bacterial growth is induced but protists keep bacterial biomass low and the detection limit of the DGGE method hampers the visualization of changes in the bacterial community structure. This detection limit is clearly shown in Fig. 3, where in the beginning of the experiment only a few sequence types were detected. With the increase in biomass more sequence types came visible. Since the continuous culture system was closed to microorganisms from outside we can only conclude that these newly appearing sequence types belonged to bacteria already present in the original lake water but were not detected because their numbers were too low. We amplified bacterial DNA directly from 5 μ l of culture medium. DNA isolation from a large sample volume prior to PCR could easily increase DGGE sensitivity.

The correlation between protist abundance, decrease in bacterial richness and changes in the genetic composition of the bacterial community suggest that grazers were controlling the bacterial community structure by effectively removing specific bacterial species from the population. However, from our data we cannot conclude that grazing was the solely cause of these changes since the experiment lacks a control, i.e. a stage without grazers. These changes in the bacterial community structure were likely the effect of many factors such as the introduction of organic compounds, the selectivity of the culture method and grazing by protists. Based on numerous studies, JÜRGENS & GÜDE (1994) synthesized a general succession pattern for experimental systems where bacterial assemblage were fed with organic compounds. In an initial phase, single-celled and free-living bacteria are dominating the community structure. Then, bacterivorous nanoflagellates develop high growth rates and consume the majority of the initial free bacterial assemblage. In the last phase there is a second increase in bacterial biomass which mainly consists of inedible morphotypes. Our results closely resemble these patterns (Fig. 1) and the molecular analysis showed changes in the bacterial community structure that correlated with the appearance of the protistan grazers.

Our results show that DGGE can be used to study changes in the bacterial community structure. Many studies applied DGGE to study natural assemblages of bacteria (MUYZER *et al.* 1993, FERRIS *et al.* 1996, TESKE *et al.* 1996) and this technique could facilitate the study of the supposed protistan control of natural bacterial community.

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