

Detection of Subgroups From Flow Cytometry Measurements of Heterotrophic Bacterioplankton by Image Analysis

Stefan Andreatta,* Manfred M. Wallinger, Thomas Posch, and Roland Psenner

Institute of Zoology and Limnology, University of Innsbruck, Innsbruck, Austria

Received 9 October 2000; Revision Received 8 March 2001; Accepted 18 April 2001

Background: Flow cytometry is an invaluable tool for the analysis of large series of samples in aquatic microbial ecology. However, analysis of the resulting data is often inefficient or does not reflect the complexity of natural communities. Because bacterioplankton assemblages frequently fall into several clusters with respect to their cellular properties, these subgroups seem to be a promising level of abstraction. Image analysis was used to detect clusters from flow cytometry data. The method was tested on a bacterial community under heavy protozoan grazing pressure.

Methods: A bivariate histogram of flow cytometry data was transformed into a gray-scale image for image analysis. After low-pass filtration, regional maxima were delimited by a watershed algorithm. The resulting areas were then used as gates on the original measurements.

Results: Three clusters could be detected from the bacterial assemblage. Protozoan grazing had a strong impact on the bacterial community, which could be analyzed in detail at the level of individual subgroups.

Conclusions: Investigation at the level of bacterial subgroups allowed a more detailed analysis than whole-community statistics and delivered essential and ecologically meaningful information. Image analysis proved to be an adequate tool to detect the subgroups without a priori knowledge. Cytometry 44:218–225, 2001.

© 2001 Wiley-Liss, Inc.

Key terms: bacteria; aquatic microbial ecology; flow cytometry; image analysis; data analysis; cluster; subgroups; community structure

In recent years flow cytometry has been established as an efficient tool for the analysis of aquatic bacteria from natural samples. Compared with epifluorescence microscopy, it increases tremendously the number of bacteria and samples that can be analyzed and a wide range of fluorescent stains is used to target different cellular properties (1). Flow cytometry thus offers unparalleled opportunities to the microbial ecologist (2). In particular, it is an ideal tool to process large series of samples to monitor the bacterial community with high frequency in space or time.

However, analysis of flow cytometric results still largely depends on visual interpretation of histograms or basic statistical values that are generally calculated for manually selected populations of cells that often comprise the entire bacterial community. At present, there seems to be a wide gap between the possibilities provided by current instruments and staining protocols, on the one hand, and the poor tools that aid analysis and interpretation of the results, on the other. This makes the use of flow cytometry particularly difficult for scientists with less experience in this special field. Further, it complicates the communication of flow cytometric results to the scientific community.

Adequate data analysis is especially important when cells are not analyzed for the clear presence or absence of properties but for quantitative interpretation of measurements. Natural bacterial samples are complex because they usually comprise a large number of bacterial cells belonging to different species in different physiologic states. When analyzed for certain cellular properties, they can fall into several close or overlapping subgroups. Advances in the analysis of flow cytometry data therefore are particularly important for further progress in aquatic microbial ecology.

In general, any method of data analysis is assessed by four criteria. (a) The method should be reproducible and independent of user interaction. (b) Relevant information must be preserved and clearly displayed. (c) The results should be efficiently visualized and concisely presented.

Grant sponsor: Austrian Science Fund; Grant numbers: P-12718-Bio and P-14387-Bio; Grant sponsor: Austrian National Bank; Grant number: 6513; Grant sponsor: Austrian Academic Exchange Service; Grant number: 2000/22.

*Correspondence to: Stefan Andreatta, Institute of Zoology and Limnology, University of Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria.

E-mail: stefan.andreatta@uibk.ac.at

(d) The resulting data should allow for comparison with other data and statistical testing. Thus, the benefit of any method of data analysis for flow cytometry will depend on its potential to reduce the data to a manageable set of values that still adequately characterize the population under investigation.

If two conventional analytical techniques are judged by these standards, their drawbacks become obvious. Uni- or bivariate histograms provide very detailed information, but they are not efficient in presenting large series of measurements and do not allow for easy comparison with other data or statistical testing. Conversely, basic descriptive statistics of the entire bacterial community provide concise and easily comparable data. Nevertheless, statistical values such as mean or standard deviation only adequately represent unimodal distributions. The use of such central indices entails the loss of important information whenever measurements show a two- or multimodal distribution.

Natural bacterial communities usually are not distributed unimodally. On the contrary, in most cases they exhibit different clusters with respect to fluorescence or scatter signals. Such bacterial subgroups have been described by several researchers (3,4), particularly with respect to DNA content (5). Groups with high DNA content or large size have been reported to comprise the active fraction of the bacterioplankton (6,7). It is a promising approach to distinguish these subgroups and calculate statistics for each of them individually (8). Thus, the bacterial assemblage under investigation can be described in greater detail and the calculated values can be readily visualized and compared with other data.

It has to be stressed that we used the term *subgroup* in a purely operational way to describe the clusters of individual measurements visible from flow cytometric analysis of cellular properties. Specifically, we do not imply that these subgroups are linked to taxonomic entities.

Several methods have been applied thus far to extract clusters of similar cells from the total set of measurements. Manual gating is used widely to define clusters of events in all applications of flow cytometry. However, bacterial subgroups are frequently located very close to each other and do not show clear boundaries. According to our experience, in such cases interactive delimitation of the individual clusters is highly operator dependent and difficult to reproduce.

Cluster analysis is a statistical tool to distinguish groups of similar events from multidimensional sets of data (4,8). However, for the size of data sets usually encountered in flow cytometry, it is impossible to analyze all possible subdivisions or calculate the complete distance matrix on desktop computers. Partitional clustering techniques such as k-means clustering are faster and were used, for example, to group phytoplankton cells (8). However, they are less accurate and require the number of clusters to be determined beforehand. To overcome this dilemma, a combination of k-means clustering and subsequent nearest-neighborhood clustering has been applied for the analysis of blood cells (9).

Supervised clustering methods such as artificial neural networks can be superior to unsupervised techniques and have been used successfully for the identification of phytoplankton (10,11) and bacterial groups (12). However, they require a training set with a priori knowledge about the actual group membership of the cells. Therefore, these methods are not adequate for the analysis of natural samples of unknown composition.

Another method for subgroup detection attempts to fit mathematical models to the flow cytometry data. This has been demonstrated for the detection of phytoplankton groups from bi- and trivariate data sets (13). This method also requires a predefinition of the number of subpopulations to be fitted.

We propose a new approach that employs image analysis to detect subgroups from flow cytometry data. Histograms are basically matrices of natural numbers that describe the number of events that fall into each single channel or combination of channels. Gray-scale images are just another special case of matrices. Digital image analysis provides a wide range of algorithms that can be applied to those matrices. In the present study, we tested whether image analysis can be used to extract subgroups from two-dimensional histograms. We also explored whether the analysis at the level of subgroups does, in fact, yield additional information and delivers ecologically meaningful data.

The new method was tested on data from a laboratory experiment studying the interactions between bacteria and protists. Flagellates and ciliates are the most important consumers of heterotrophic bacterioplankton (14). Selective protistan grazing has a strong impact on the abundance, size distribution (15,16), taxonomic composition (17,18), and activity (19,20) of a bacterial community. Laboratory experiments allow investigation of these mechanisms under more controlled conditions than in ecosystem studies (21,22). The experiment was particularly promising for a test of a subgroup detection method, as distinct changes in the bacterial community could be expected.

MATERIALS AND METHODS

Experimental Setup

The samples for this study were taken during a laboratory experiment in May 1999. The principal setup of the system has been described in detail (16). The unicellular algae *Cryptomonas* sp. was grown on an inorganic medium in a laboratory, two-stage, flow-through system with an associated mixed bacterial assemblage depending on the extracellular organic carbon released by the algae (16,22). The taxonomic composition of the bacterial community in this and prior experiments was investigated by fluorescent in situ hybridization. A large part of the bacteria was affiliated to *Caulobacter* sp., *Aquabacterium* sp., or an uncultivated line of a freshwater *Actinobacterium* sp. (17,23). Bacteria and algae were pumped continuously through parallel second-stage vessels at a dilution rate of 0.27 d^{-1} . To these bottles different protozoan grazers were added. The impact of these grazers on the

bacterial community was followed for 17 days. For this study we used only samples that were taken from one of the second-stage vessels that were inoculated with the scuticociliate *Cyclidium glaucoma* on day 1 and then with the mixotrophic flagellate *Ochromonas* sp. on day 8.

Protozoan and Bacterial Samples

Samples were taken daily for 17 days, fixed with Lugol's solution (0.5 % final concentration) and formaldehyde (3 %), and unstained with sodium thiosulfate (0.5 %). Protist samples were stained at a final concentration of 2 µg/ml of 4', 6'-diamidino-2-phenylindole (DAPI) for 7 min and filtered on black polycarbonate filters (1.0-µm pore size). They were then enumerated by microscopic counting on an epifluorescence microscope (24).

Flow Cytometry

Bacterial samples were analyzed by flow cytometry. For each sample 250 µl was stained with DAPI at a final concentration of 1 µmol for 15 min. In addition, 25 µl of 1-µm TransFluoSpheres yellow fluorescing microspheres (Molecular Probes, Eugene, OR) in suspension was added as the fluorescence standard and for absolute cell counts. The stock concentration of beads was determined by a Coulter Counter (Beckman Coulter, Fullerton, CA). The final concentration in the sample was $3.8 \times 10^5 \text{ ml}^{-1}$.

Flow cytometric analysis was performed on a MoFlo cell sorter (Cytomation, Fort Collins, CO) equipped with two water-cooled argon-ion lasers. DAPI blue fluorescence and yellow signals from microspheres were excited by 50 mW of ultraviolet light (350–365 nm). The orthogonal signal was reflected by a neutral 95/5 beamsplitter and split by a 525-nm long-pass dichroic. DAPI blue fluorescence passed through a 450/65 bandpass filter. The yellow signal from the beads was detected after being passed through a 570/40 bandpass filter. An excitation of 50 mW at 514 nm was used at the second observation point. The signal was split by a 560-nm short-pass dichroic to detect red autofluorescence and green side scatter (SSC, orthogonal scatter) through 670/40 and 514/10 bandpass filters, respectively. SSC signal was also filtered through a neutral density filter with an optical density of 1.3.

Photomultiplier tubes were obtained from Hamamatsu Photonics (model R-1477; Hamamatsu City, Japan). The detector voltage was 550 V for blue and yellow fluorescences, 500 V for SSC, and 750 V for red autofluorescence. Amplification was logarithmic for all signals. Measurement of an event was triggered by a logical combination (OR) of logarithmically amplified blue or yellow fluorescence to measure DAPI-stained bacteria and yellow fluorescing beads. This was made possible by a special multiple trigger board (Cytomation). Between 2.0×10^3 and 1.0×10^5 bacteria were analyzed per sample.

Data Analysis

Manual gates were applied to distinguish the bacterial community from the electronic background caused by the beads, protozoan and algal cells, and electronic background noise. These discriminations were clearly visible and could be done without ambiguity. Identical gates

were applied to all 17 samples. From these gated files 2,000 events were randomly selected and merged into a new listfile to represent the entire series of measurements in one artificial sample. A bivariate histogram was calculated for SSC versus DAPI fluorescence (256×256 channels). For image analysis, that bivariate histogram was transformed into a 256×256 pixel image where gray values represented the number of events within each combination of channels.

For detection of the bacterial subgroups from the gray-scale image, an automated routine was employed. The image was first smoothed by a low-pass filtration, with an exponential kernel of size 7×7 . A constant value of 1 was subtracted to remove small isolated areas within the background. Regional maxima were subsequently detected as the centers of the subgroups, and the borders between those groups were calculated by a watershed algorithm. The resulting zones of influence were saved as binary images and used to gate the listfiles and separate the bacterial subgroups.

To concentrate on the dominating subgroups of the bacterial community, only the inner 90% of the bacterial community were used for all evaluations (hereafter, *total bacterial community*). The corresponding area was calculated from the same manually gated and merged file that was used for the detection of subgroups and applied to all individual samples. The mean of DAPI fluorescence and SSC was calculated for each subgroup and the total community. To minimize between sample variation, the mean values were normalized by dividing by the mean of yellow fluorescence or SSC of the standard beads that were analyzed together with each sample. Absolute abundance of bacteria also was calculated with the known concentration of beads. Principal component analysis was performed for the 17 samples by using mean DAPI fluorescence, mean SSC, and relative abundance (percentage of total bacterioplankton) for each subgroup. Absolute abundance was added for the total bacterial community only to avoid redundancy. All variables were standardized by dividing by their means.

Software

Manual gating and calculation of basic descriptive statistics for bacterial groups was done with Summit 2.0 (Cytomation). Image analysis was implemented in Visilog 5.1 (Norpix, Montreal, QC, Canada). Principal component analysis was calculated with Statistica 99 (StatSoft, Tulsa, OK).

To transform flow cytometry standard (FCS) files to gray-scale images and gate the listfiles with binary images, the program FCS-Flounder was written. It was used with FCS files generated by Summit on a Windows NT platform. However, the tool follows the FCS 2.0 standard (25,26) and was written with the platform-independent, free, Python programming language (CNRI, Reston, VA). Therefore, it should be functional with standard FCS files on different platforms. The program is available from the first author.

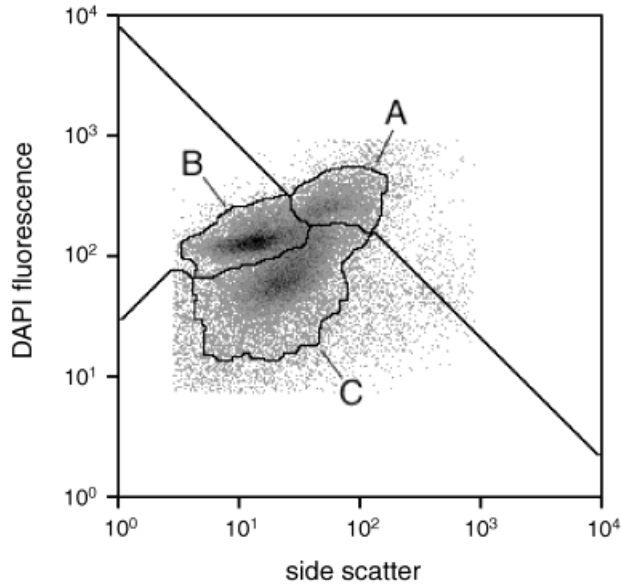


Fig. 1. Bivariate histogram composed of 2,000 events from each of the 17 samples. Borders between subgroups A, B, and C are superimposed, as detected by image analysis and the outer limits of the area containing 90% of the events.

RESULTS

Detection of Subgroups

From the bivariate histogram of SSC versus DAPI fluorescence, which was merged from 17 individual samples, three distinct subgroups could be distinguished by image analysis. Figure 1 shows an overlay of the merged histogram, with the borders of subgroups and the area comprising 90% of the events in the histogram. Group A shows high SSC and DAPI fluorescence. Group B shows medium DAPI and the lowest SSC signal among the three groups. Group C is characterized by medium SSC and low DAPI fluorescence. Care has to be taken, particularly for mixed communities, when translating fluorescence or scatter signals quantitatively to cellular properties. However, a positive relation between DAPI fluorescence and DNA content (2) and between SSC and cell size can be safely assumed (7). Thus, group A represents large cells with high DNA content in comparison with groups B and C.

The subgroup areas were then applied to each sample. In general, a good fit between the groups' borders and the individual histograms was found with visual inspection, but group A was not visible on all days. Three of the 17 samples are depicted in Figure 2. Those samples represent different phases of bacterioplankton development before and after the onset of grazing by *Cyclidium glaucoma* and *Ocbromonas* sp. Its impact is clearly displayed by the bacterial size versus DNA distribution.

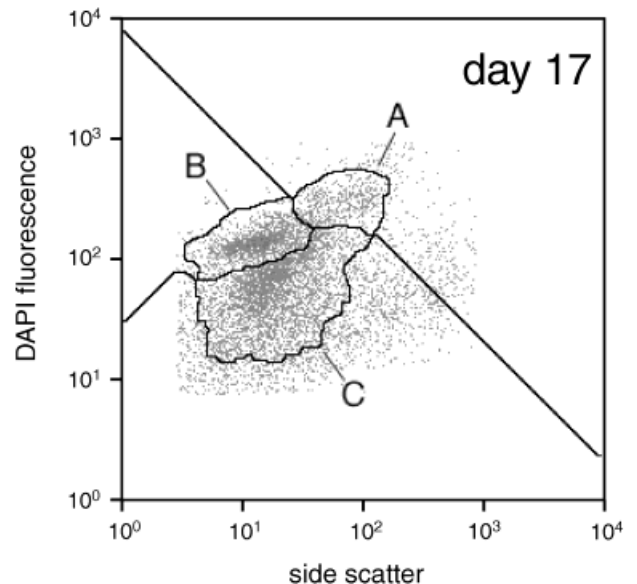
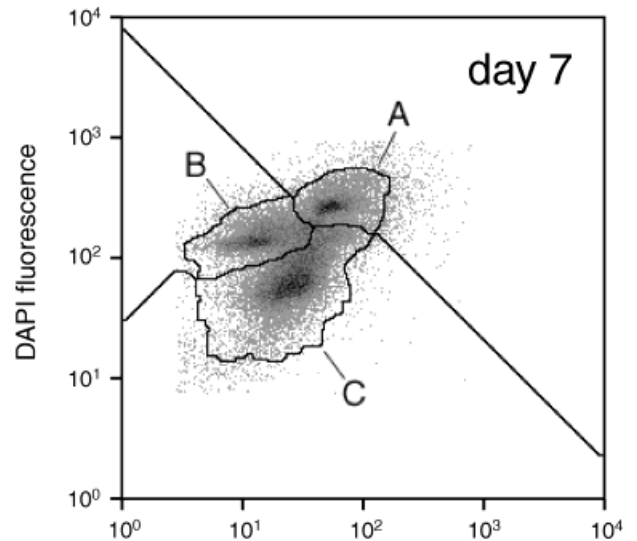
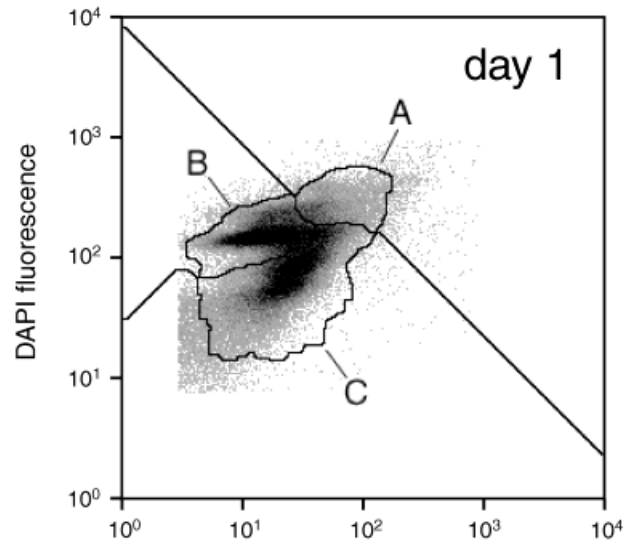


Fig. 2. Borders between subgroups and 90% limits superimposed on samples from 3 days during the experiment. Day 1: Before the onset of protozoan grazing. Day 7: Heavy grazing pressure by *Cyclidium glaucoma*. Day 17: At the end of the experiment; strong impact of *Ocbromonas* sp. DAPI, 4', 6'-diamidino-2-phenylindole.

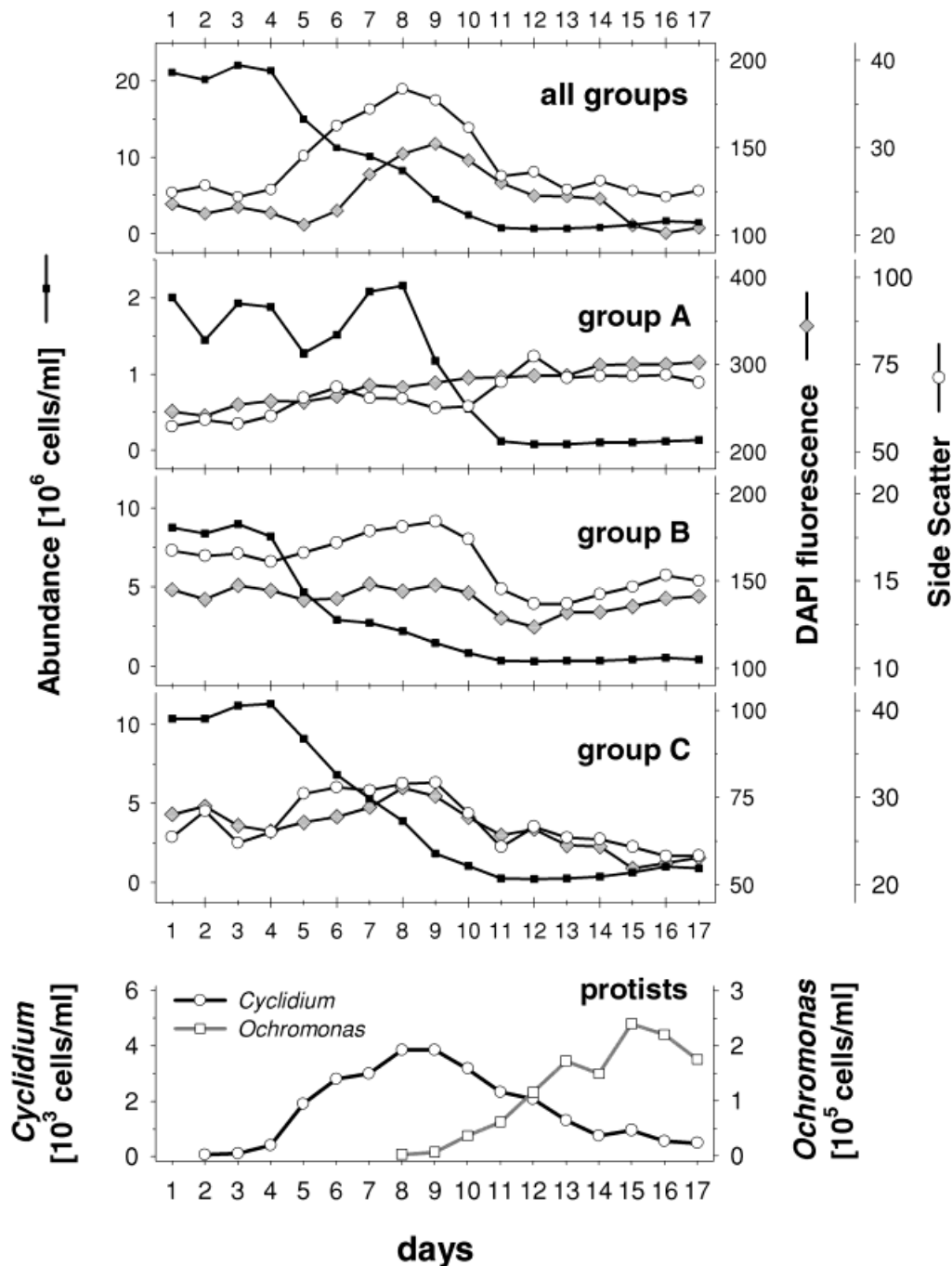


FIG. 3. Line plots showing the abundance, mean 4', 6'-diamidino-2-phenylindole (DAPI) fluorescence, and mean side scatter of total bacterioplankton the three subgroups throughout the experiment. Protist abundances are depicted in the lowest graph. Note the different scales of the individual graphs; scales for DAPI fluorescence and side scatter always span a twofold increase.

Total Community and Subgroup Statistics

To analyze the effects of protozoan grazing quantitatively, mean DAPI fluorescence, mean SSC, and abundance were calculated for the total bacterial community and the three subgroups detected by image analysis (Fig. 3). With regard to total bacterioplankton abundance, three phases may be distinguished. During the first 4 days, the situation was quite constant. After day

4, when the number of *Cyclidium glaucoma* started to increase, bacterial abundance dropped off and kept decreasing until day 11. After the onset of *Ochromonas* sp., growth abundance became stable, although approximately one order of magnitude below its initial values. DAPI fluorescence and SSC of the total community started to rise after days 4 and 5 and decreased again after days 8 and 9, respectively.

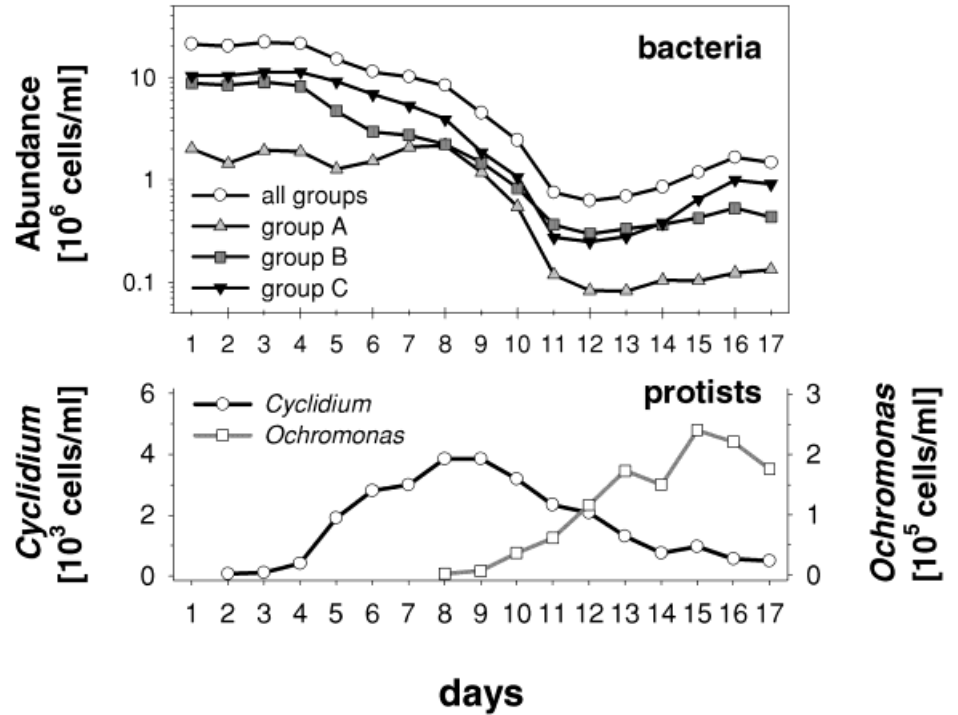


FIG. 4. Line plots showing abundances of the entire bacterial community and the three subgroups on a logarithmic scale compared with protist abundance.

For more detailed analysis, the same statistics as those for the total bacterial community were calculated for the three subgroups (Fig. 3). Groups B and C showed a similar development over time. Abundance was constant until day 4 and then decreased to a lower level, where it stabilized from day 11 on. SSC and DAPI increased with considerable fluctuations until day 10 and then dropped sharply. At this point, the situation changed: both signals increased again after day 12 in group B but kept decreasing until the end of the series in group C. Group A, representing the largest bacteria, exhibited a completely different pattern, with high, albeit fluctuating, abundance until day 8, which dropped by more than one order of magnitude within 3 days and then stabilized at approximately 1×10^5 cells/ml. In contrast, DAPI and SSC signals increased steadily during the first 14 days and stayed constant for the last 4 days. For further comparison, abundances of the three groups and the total community were plotted together (Fig. 4), showing that the groups B and C were affected by *Cyclidium glaucoma* and *Ochromonas* sp. grazing, whereas group A was affected by *Ochromonas* sp. Thus, the increase in mean cell size and DNA content was due to the preferential grazing of *Cyclidium glaucoma* on smaller, low-DNA bacteria.

Principal Component Analysis

Principal component analysis (Fig. 5) for the 17 samples summarized and indicated the main trends that could be shown by the development of individual parameters. Factor loadings (Fig. 5B) showed a high coupling between DAPI and SSC signals for each subgroup, with groups B and C closely related and contrasting with group A. Relative abundances were different between groups and dis-

tributed independently from DAPI and SSC signals. Factor scores (Fig. 5A) showed the main phases of the bacterial community during this experiment. Days 4–5 were grouped closely together, strongly influenced by total abundance. Samples from days 6–10 formed a more heterogeneous cluster, influenced by high DAPI and SSC signals of groups B and C and high relative abundance of large cells (group A). The days after the onset of *Ochromonas* sp. grazing (11 through 17) were clearly set apart and formed two distinct groups (11 to 14 and 15 to 17) characterized by high DAPI and SSC signals in group A and a relative high proportion of group B cells.

DISCUSSION Protozoan Grazing

The addition of two protists had a very strong impact on the bacterial community in this experiment. Data from the parallel control stage also confirmed that the extreme changes were caused by protist grazing (27). The effects also were consistent with those of previous experiments (16) and literature data (14,15). The size-specific effects of protozoan grazing agreed with those of investigations using microscopy and image cytometry of bacterial cells (16). Differences in feeding behavior between the two grazers were obvious. *Cyclidium glaucoma*, which was added at day 1 of the experiment, grazed predominantly on smaller bacteria, which also showed relatively low DNA content (groups B and C). In contrast, *Ochromonas* sp. preferred larger cells with high DNA content from group A. Further, the impact from *Ochromonas* sp. could be detected sooner after inoculation than the impact from *Cyclidium glaucoma*. That result might be related to the

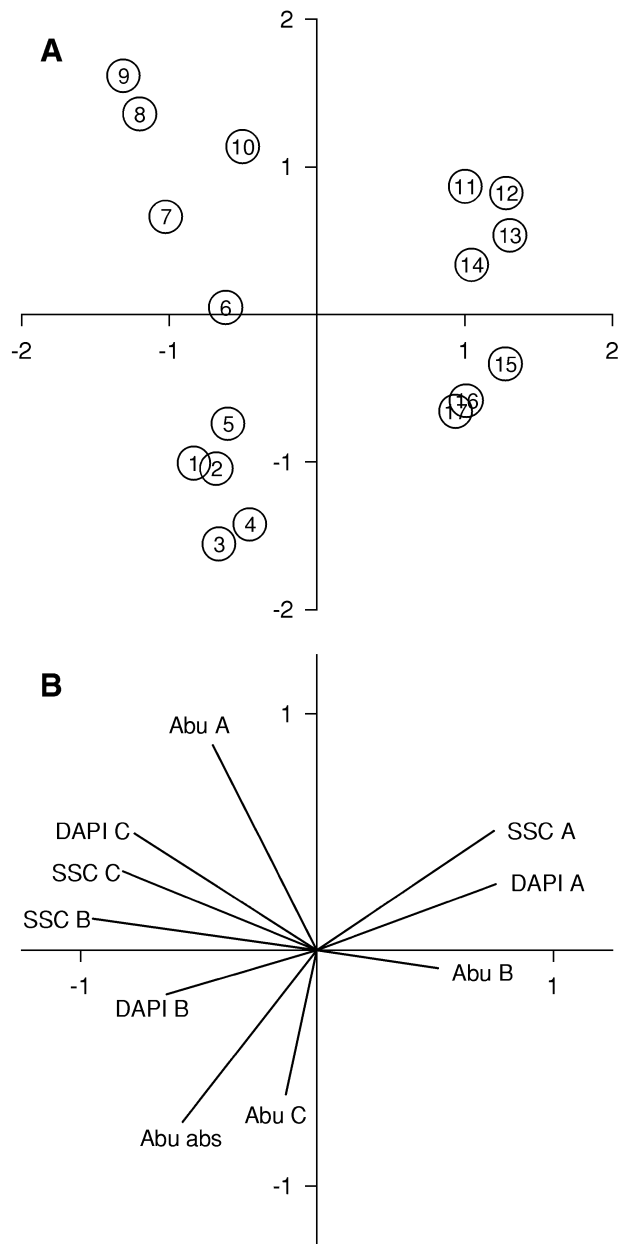


FIG. 5. Principal component analysis of the 17 daily samples based on absolute abundance of the entire bacterial community (Abu abs), relative abundance (Abu A-C), 4', 6'-diamidino-2-phenylindole fluorescence (DAPI A-C), and side scatter (SSC A-C) of subgroups A-C. **A:** Factor scores of the 17 samples. **B:** Factor loadings of parameters.

much higher growth rates of that flagellate. In addition, the potential for mixotrophy of *Ochromonas* sp. might subsist better at decreasing bacterial abundances.

Detection of Subgroups in Bacterioplankton

The analysis of bacterioplankton at the subgroup level conveyed significantly more information than the conventional total community approach. Trends that were visible from total community statistics (e.g., increasing average SSC) could be attributed to specific changes within certain

compartments (decreasing number of small cells) in this study. Apparently, bacterial subgroups as defined in this study act as ecological units, at least under certain circumstances.

Data from individual subgroups could be presented conveniently in line plots (Figs. 3 and 4). The presentation of bivariate histograms therefore was limited to a small number of typical cases for the purpose of illustration. Further, the data on subgroups provided a good data basis for further statistical analysis. Principal component analysis reduced the complexity of the many parameters and enabled a summary interpretation of the entire data set.

The present analyzed samples were derived from a laboratory experiment. However, the flow cytometric characteristics of this mixed bacterial community were similar to those of natural samples from oligo- and mesotrophic lakes (unpublished data). Thus, the present findings also can be used for field studies. Further, the method for detecting subgroups of events from bivariate histograms obviously can be applied with any other flow cytometry data where similar requirements exist.

Image analysis proved to be an adequate tool to detect bacterial subgroups in an objective and reproducible way. A priori knowledge such as the number of clusters to be detected is not required. However, the automatic detection of subgroups can be combined with manual gating if such a combined approach is favorable. Computing easily can be carried out on common personal computers. During the development of image processing routines, the use of a sophisticated interactive image analysis package has proved beneficial. However, for the repeated transformation of files, a custom tool had to be written. Because of the various programs involved, application is laborious and not very user-friendly at the moment. Moreover, the computations currently cannot be performed online during analysis at the flow cytometer. Thus, easy sorting on detected subgroups is not possible at this stage. However, specific physical isolation of individual subgroups would be an important tool for the further characterization of bacterial groups by auxiliary investigations. Therefore, the entire process ideally should be integrated into a standard flow cytometry software system to enable online analysis and sorting.

It is an advantage that detection of subgroups can be successful even when only two parameters (SSC and DAPI) are used. However, for more general applications, extending the method to higher-dimensional input data would be desirable. Because there is no principal obstacle to applying the same algorithms to multivariate data, this would be primarily a matter of programming and testing the appropriate routines. Nevertheless, cluster analysis (8,9) currently might be advantageous for highly multivariate data, whereas model fitting (13) might be preferred for the detection of a known number of overlapping populations.

In this study, borders between subgroups were detected from a merged file representing the entire series of samples and subsequently applied to individual samples. Thus, analysis was based on static subgroup areas. Such an approach may not be reasonable or possible for other

series of data. In particular, when subgroups show larger shifts in signal intensity between samples, a dynamic detection of clusters will be necessary. However, when the detection is based on individual samples, small differences can cause abrupt changes in the detection of subgroup borders between adjacent samples. We successfully tested a combination of the static and dynamic approaches for such situations (data not shown). Subgroup calculations based on a smaller number of samples (e.g., five), analogous to a "running average", allowed us to create dynamic cluster limits and avoid erratic changes between samples. However, such an approach usually will return subgroups that can be found only in some samples, which can cause severe logical problems for interpretation and statistical analyses in particular because the properties of individual groups can be determined only for a part of the series. If subgroups divide or merge to form other groups, they also cannot be seen to just disappear and thus are not adequately represented by missing values.

CONCLUSION

The analysis of bacterioplankton at the subgroup level beyond question provides significant and ecologically meaningful information that is otherwise lost by conventional analysis. Therefore, we expect sophisticated data analysis to become a substantial part of investigations into aquatic microbiology as modern instrumentation allows for analysis of increasing numbers of samples. To this end, the application of image analysis for the detection of bacterial subgroups is a promising tool. However, more efforts are necessary to streamline its application in terms of software tools. Further, its field of application could be extended by dynamic subgroup detection and the adaptation to multidimensional data.

ACKNOWLEDGMENTS

We thank Karel Šimek, Jaroslav Vrba, Jan Jezbera (Czech Academy of Sciences, Budejovice) Jakob Pernthaler, Annelie Hentschke (Max Planck Institute of Marine Microbiology, Bremen Germany), and Bettina Sonntag (University of Innsbruck, Austria) for their cooperation during the laboratory experiment.

LITERATURE CITED

- Davey HM, Kell DB. Flow cytometry and cell sorting of heterogenous microbial populations: the importance of single-cell analyses. *Microbiol Rev* 1996;60:641-696.
- Gasol JM, DelGiorgio PA. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci Marina* 2000;64:197-224.
- Button DK, Robertson BR, Juttner F. Microflora of a subalpine lake: bacterial populations, size and DNA distributions, and their dependence on phosphate. *FEMS Microbiol Ecol* 1996;21:87-101.
- Troussellier M, Courties C, Lebaron P, Servais P. Flow cytometric discrimination of bacterial populations in seawater based on SYTO 13 staining of nucleic acids. *FEMS Microbiol Ecol* 1999;29:319-330.
- Li WKW, Jellet JF, Dickie PM. DNA distributions in planktonic bacteria stained with TOTO or TO-PRO. *Limnol Oceanogr* 1995;40:1485-1495.
- Gasol JM, Zweifel UL, Peters F, Fuhrman JA, Hagstrom A. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. *Appl Environ Microbiol* 1999;65:4475-4483.
- Servais P, Courties C, Lebaron P, Troussellier M. Coupling bacterial activity measurements with cell sorting by flow cytometry. *Microb Ecol* 1999;38:180-189.
- Demers S, Junhyong K, Legendre P, Legendre L. Analyzing multivariate flow cytometric data in aquatic sciences. *Cytometry* 1992;13:291-298.
- Schut TCB, De Grooth BG, Greve J. Cluster-analysis of flow cytometric list mode data on a personal-computer. *Cytometry* 1993;14:649-659.
- Frankel DS, Olson RJ, Frankel SL, Chisholm SW. Use of a neural net computer system for analysis of flow cytometric data of phytoplankton populations. *Cytometry* 1989;10:540-550.
- Frankel DS, Frankel SL, Binder BJ, Vogt RF. Application of neural networks to flow-cytometry data-analysis and real-time cell classification. *Cytometry* 1996;23:290-302.
- Davey HM, Jones A, Shaw AD, Kell DB. Variable selection and multivariate methods for the identification of microorganisms by flow cytometry. *Cytometry* 1999;35:162-168.
- Li WKW. Bivariate and trivariate analysis in flow cytometry: phytoplankton size and fluorescence. *Limnol Oceanogr* 1990;35:1356-68.
- Güde H. The role of grazing on bacteria in plankton succession. In: Sommer U, editor. *Plankton ecology: succession in plankton communities*. Berlin: Springer-Verlag; 1989. p 337-364.
- Fenchel T. Suspension feeding in ciliated protozoa: Functional response and particle size selection. *Microb Ecol* 1980;6:1-11.
- Posch T, Šimek K, Vrba J, Pernthaler J, Nedoma J, Sattler B, Sonntag B, Psenner R. Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. *Aquat Microb Ecol* 1999;18:235-246.
- Pernthaler J, Posch T, Šimek K, Vrba J, Amann R, Psenner R. Contrasting bacterial strategies to coexist with a flagellate predator in an experimental microbial assemblage. *Appl Environ Microbiol* 1997;63:596-601.
- Šimek K, Vrba J, Pernthaler J, Posch T, Hartman P, Nedoma J, Psenner R. Morphological and compositional shifts in an experimental bacterial community influenced by protists with contrasting feeding modes. *Appl Environ Microbiol* 1997;63:587-595.
- Bird DF, Kalf J. Protozoan grazing and the size-activity structure of limnetic bacterial community. *Can J Fish Aquat Sci* 1993;50:370-380.
- del Giorgio PA, Gasol JM, Vaque D, Mura P, Agusti S, Duarte CM. Bacterioplankton community structure: protists control net production and the proportion of active bacteria in a coastal marine community. *Limnol Oceanogr* 1996;41:1169-1179.
- Güde H. Influence of phagotrophic processes on the regeneration of nutrients in two-stage continuous culture systems. *Microb Ecol* 1985;11:193-204.
- Rothhaupt KO. Stimulation of phosphorus-limited phytoplankton by bacterivorous flagellates in laboratory experiments. *Limnol Oceanogr* 1992;37:750-759.
- Pernthaler J, Posch T, Šimek K, Vrba J, Pernthaler A, Glöckner FO, Nübel U, Psenner R, Amann R. Predator-specific enrichment of *Actinobacteria* from a cosmopolitan freshwater clade in mixed continuous culture. *Appl Environ Microbiol* 2001;2145-2155.
- Sherr EB, Sherr BF. Preservation and storage of samples for enumeration of heterotrophic protists. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ, editors. *Handbook of methods in aquatic microbial ecology*. Boca Raton: Lewis Publishers; 1993. p 207-212.
- Dean PN, Bagwell CB, Lindmo T, Murphy RF, Salzman GC. Introduction to flow cytometry data file standard. *Cytometry* 1990;11:321-322.
- Dean PN, Bagwell CB, Lindmo T, Murphy RF, Salzman GC. Data file standard for flow cytometry. *Cytometry* 1990;11:323-332.
- Posch T, Jezbera J, Vrba J, Šimek K, Pernthaler J, Andreatta S, Sonntag B. Size selective feeding in *Cyclidium glaucoma* (Ciliophora, Scuticociliatida) and its effects on bacterial community structure: a study from a continuous cultivation system. *Microb Ecol*. Forthcoming.