Permanent Presence of Grazing-Resistant Bacteria in a Hypertrophic Lake

RUBEN SOMMARUGA* AND ROLAND PSENNER

Institute of Zoology and Limnology, University of Innsbruck, Innsbruck 6020, Austria

Received 31 March 1995/Accepted 20 June 1995

The size structure of planktonic bacteria from a hypertrophic lake was investigated at 5- to 15-day intervals by means of a semiautomatic image analysis system during 1 year. Characteristic of this bacterial assemblage was the permanent presence of large filamentous bacteria and small cocci with cell sizes of $<0.01 \mu\text{m}^3$. These filamentous bacteria, sometimes longer than $200 \mu\text{m}$ and with cell volumes of up to $276 \mu\text{m}^3$, are larger than nanoflagellates ($<20 \mu\text{m}$) and, even, metazoans living in the lake. Although they account for only 4 to 16% of bacterial abundance, their contribution to total bacterial biovolume was between 45 and 86%. An analysis of the food web structure indicates that this particular bacterial size structure may be the consequence of a strong bacterivory pressure by nanoflagellates and the absence of other larger bacterivores. The persistence of bacterial forms resistant to grazing has important consequences for the carbon flow within the microbial food web.

Pelagic bacteria from marine and freshwater systems constitute an important carbon pool available to protists and metazoans (2, 23). Typical bacterial assemblages living in the plankton of marine and freshwater systems have cell sizes of between 0.01 and $0.1 \mu\text{m}^3$. However, complex morphological forms with larger cell volumes have been observed during short periods of intense protistan grazing (8, 10, 22). Temporal and spatial shifts in bacterial cell size may reflect changes in species composition within the community as well as cell plasticity (9). These shifts may be the consequence of different biological or environmental factors (8). Since the recognition of the microbial loop, sufficient information has accumulated to show the important role of phagotrophic protists as consumers of picoplankton biomass (1, 18). However, less attention has been paid to the role of protists in structuring size and species composition of planktonic bacteria (9). An early attempt was made by Güde (7) to study the effect of predatory protists on the size structure of the bacterial assemblage using chemostat experiments. He observed drastic changes in taxonomic composition as well as in morphological structure of bacteria under strong grazing pressure by flagellates. The increased abundance of filamentous, spiral, and aggregated forms was interpreted as a replacement of small and free-living bacteria by forms resistant to grazing. Phenotypic plasticity of bacteria in response to predators has been described too. Shikano et al. (19) observed that the parental bacterial population, with cells $1.5 \mu\text{m}$ long, persisted after the introduction of a predatory ciliate, a *Cyclidium* sp., but a filamentous form of up to $20 \mu\text{m}$ long appeared. A hypothetical model for the expected bacterial size structure under bacterivorous pressure by protists and cladocerans was introduced by Güde (8). This model establishes that when grazing pressure is mainly exerted by flagellates, small and large (e.g., filamentous) forms are dominant. However, these large grazing-resistant forms disappear when cladocerans, e.g., *Daphnia* spp., are present, and in this case small cells are expected to dominate.

Field evidence showing the occurrence of grazing-resistant bacteria and the conditions that favor their growth are scarce (9). Here, we present results on the particular size structure of the bacterial assemblage from a hypertrophic lake studied by means of image analysis. Moreover, we explore which factors may explain the permanent existence of grazing-resistant bacteria in the plankton of this system.

Sampling site. Lake Rodó is a small (area, 1.3 ha) man-made lake situated in the south of Uruguay ($34^{\circ}55'\text{S}$, $56^{\circ}10'\text{W}$). The lake is typical for continuous dominance by filamentous cyanobacteria, mainly *Planktothrix agardhii*, because it is shallow (maximum depth, 1.1 m), nutrient enriched (mean total phosphorus content, $430 \mu\text{g liter}^{-1}$), poorly flushed, and well mixed. Accordingly, phytoplankton biomass estimated as chlorophyll *a* is very high (mean at 0 m , $223 \mu\text{g liter}^{-1}$). The surface mean temperature is 19°C (maximum, 28°C), the lake water is alkaline, and oxygen concentration oscillates between 50 and 170% saturation. The euphotic zone is restricted to the first 50 cm of the water column, and transparency measured with a Secchi disk changed from 15 cm in summer to 30 cm in winter. Other characteristics of the lake are described elsewhere (24).

Field samples and analysis. Twenty-nine samplings were made at the deepest part of the lake at intervals of 5 to 15 days. The first 50 cm of the water column was sampled with a 5-liter Schindler-Patalas sampler. Bacterial samples were fixed with formalin (final concentration, 2% [vol/vol]) and stained with the fluorochrome 4',6-diamidino-2-phenylindole (DAPI; final concentration, 0.2% [wt/vol]) according to the method of Porter and Feig (13). Bacteria were counted on black membrane filters (pore size, $0.2 \mu\text{m}$; Poretics) at $\times 800$ and $\times 1,250$ magnification in a Nikon epifluorescence microscope with a BP 365, FT 395, and LP 397 filter set. The sizes of the bacteria were determined with an image analysis system with an interference filter (450 to 490 nm) connected to a high-sensitivity camera, Hamamatsu C2400-08. The image analysis software LUCIA (Laboratory Imaging, Prague, Czech Republic) was used in combination with a real-time image processor (MATROX; MVP-AT, Dorval, Quebec, Canada). Digitized images were resolved with 512×512 pixels, using 8 bits of memory. Between 600 and 1,000 cells were analyzed. Background subtraction was used to minimize variability associated with non-

* Corresponding author. Mailing address: University of Innsbruck, Institute of Zoology and Limnology, Technikerstr. 25, 6020 Innsbruck, Austria. Phone: (512) 507-6121. Fax: (512) 507-2930. Electronic mail address: Ruben.Sommaruga@uibk.ac.at.

FIG. 1. Fluorescence micrographs of a DAPI-stained sample from the plankton of Lake Rodó. (A) Shown are a filamentous bacterium ca. 75 μm long and a heterotrophic flagellate (arrow) of ca. 6 μm . Bar, 15 μm . (B) Overview showing FB and small bacteria. Bright objects crossing the field are filaments of the cyanobacterium *Planktothrix agardhii*. Bar, 30 μm .

uniformity in illumination and electronic noise. Filtering of the digital image was done by using a nonlinear filter known as General Filtration (25). Cell volumes were computed with the formula $V = (w^2 \times \pi/4) \times (l - w) + (\pi \times w^3/6)$, where V is volume (in cubic micrometers), w is width (in micrometers), and l is length (in micrometers). Accuracy of the algorithms used was confirmed with independently calibrated microspheres of known size (Molecular Probes). Other characteristics of the image analysis system are described elsewhere (14).

Filamentous bacteria (FB), defined here as cells $>10 \mu\text{m}$ in length, were found on all occasions. The largest bacterium observed was 210 μm long. This is 105 times the size of the smallest phagotrophic flagellates and also larger than most ciliates and the most abundant bacterivorous rotifer, *Anuraeopsis fissa*, found in this lake (Fig. 1). Other morphologically complex forms of budding-type bacteria were also present. Filamentous forms presented different lengths and also widths, but most (65%) FB were 50 to 210 μm long and ca. 0.6 μm wide. They were gram-negative, unbranched, and multicellular (individual cells, 5 to 6 μm by 0.6 μm) and had characteristic empty spaces (necridia), typical for bacteria of the *Herpetosiphon* type (15). This genus belongs to the category of the so-called green nonsulfur bacteria and represents a primitive form, as indicated by the unusual cell wall structure (15) and by the results of the 16S rRNA studies (4). FB were never attached to particles or forming flocs, but very often we observed small bacteria attached to them. While the contribution of FB to total bacterial abundance (1.5×10^6 to 2.0×10^7 cells ml^{-1}) was only 4.5 to 16%, they contributed 45 to 86% to the total biovolume (Fig. 2). No clear seasonal pattern of FB abundance was observed. Some FB, 2 μm wide and of uncertain taxonomic affiliation, even reached single-cell volumes of 276 μm^3 . Cell volume ranged 5 orders of magnitude, from that of very small cocci ($<0.01 \mu\text{m}^3$) to that of these enormous bacteria (Fig. 3). In most cases, the cell volume distribution showed a clear discontinuity at values ca. $1.0 \mu\text{m}^3$. Bacterial cells with volumes between 0.01 and $0.1 \mu\text{m}^3$ were the most representative.

To discern which factors may explain the continuous presence of large FB in this lake, we considered the grazing impact of heterotrophic nanoflagellates and the presence of other potential bacterivores. Bacterivory experiments made in parallel, using fluorescently labeled bacteria as prey (24), showed that between 4 and 26% of the bacterial standing stock was removed per hour by a very abundant heterotrophic nanoflagellate assemblage (mean, $1.2 \times 10^4 \text{ ml}^{-1}$; maximum, $3.0 \times$

10^4 ml^{-1} in summer). The uptake rates (up to 130 bacteria flagellate $^{-1} \text{ h}^{-1}$) were also very high and were similar to those observed with cultures (3). Accordingly, bacterial turnover times between 5 and 42 h (leucine incorporation) were extremely fast (24). Further indirect evidence of the high grazing pressure comes from the recurrent observation of the low percentage of bacteria with sizes of around 0.5 to $1 \mu\text{m}^3$ (Fig. 3). Several studies of experiments using fluorescently labeled bacteria as surrogates have shown that bacteria with cell volumes in this range are the most efficiently taken up by flagellates (11, 21). Jürgens and Güde (9), using microspheres of different diameters, also showed that for two flagellate species the maximum efficiency was observed with $1\text{-}\mu\text{m}$ ($0.52\text{-}\mu\text{m}^3$) spheres. Thus, we conclude that the strong bacterivory pressure observed in this lake may partially explain the presence of large grazing-resistant bacteria.

However, an increase in cell size, while protecting bacteria from protistan grazing, may have negative consequences because of increased vulnerability to larger bacterivores like *Daphnia* species (8). In our case, the crustacean zooplankton was subjected to harsh conditions, because of the dominance of inedible filamentous cyanobacteria and the presence of planktivorous fish (24). Therefore, cladocerans were absent from the plankton during most of the year, and *Daphnia parvula*, the only daphnid species present, reached a maximum abundance of 3 organisms liter^{-1} in spring. At present, we do not know the fate of the large FB, but changes in abundance of *Herpetosiphon* spp. due to fragmentation are a possibility.

Güde (8) suggested that small-sized bacteria can also be

FIG. 2. Relative contribution of FB to the total abundance of bacteria and biovolume during the study period.

FIG. 3. Frequency distribution of bacterial cell volumes measured by image analysis. The number of cells measured varied between 500 and 1,000. Measurements were divided into 15 size classes.

considered grazing-resistant forms. Although flagellates are able to ingest small viruses (5) and incorporate dissolved macromolecules (17), the encounter rates predicted by direct interception models suggest a refuge for small-sized bacteria (20). A close look at the volume distribution in Fig. 3 shows also the permanent presence of DAPI-stained particles with sizes of between 0.001 and 0.01 μm^3 . These small particles, although not necessarily all bacteria (e.g., presence of viruses), may also find a refuge from flagellate grazing (20).

Free-living bacteria of ca. 20 μm have been observed during short periods in the plankton of other aquatic systems (9, 10, 22). Moreover, *Herpetosiphon* spp. can grow more than 1,200 μm long, have been found in polluted parts of Lake Constance, Germany (6), and are typical for zones rich in decaying organic matter (15). Our results show for the first time the permanent presence of free-living large bacteria inhabiting the plankton of a lake comprising the bulk of biomass. Furthermore, they support the hypothetical model of bacterial size structure distribution introduced by Güde (8).

Size is one of the most important properties of living organisms, and therefore, many biological processes depend on it. The singular size structure of this bacterial assemblage may have important consequences for the functioning and carbon flow within the microbial food web. As an example, the extremely fast bacterial turnover times measured in the lake may be a necessary consequence of the high grazing pressure on smaller bacteria. Also, the characteristic predator-prey size relationships found in other food webs (12) differed greatly in this lake. We suggest that high nutrient concentrations may be a prerequisite for maintaining the large biomass of FB, although this point needs further investigation. It may be also possible that FB have a very slow turnover time.

The smallest eukaryotic-prokaryotic predator-prey system known, i.e., flagellate-bacteria, may also be the system with the longest period of coevolution. The development of filamentous

forms seems to be a winning strategy under high protist grazing pressures. While it is too early to generalize, the permanent presence of large FB may be common in shallow hypertrophic lakes and organic polluted systems with a low abundance of larger bacterivores.

We thank D. Conde, D. Fabian, and M. L. Arin for assistance in the field; M. Krössbacher for assistance with the image analysis; and R. Pöder and U. Peintner for helping to identify *Herpetosiphon* spp. K. Jürgens provided useful comments to an early version of the manuscript, and J. Wieser improved the English.

This work was supported by the North-South Dialogue Programme, Ministry of Foreign Affairs, Austria.

REFERENCES

- Berninger, U. G., D. A. Caron, R. W. Sanders, and B. J. Finlay. 1991. Heterotrophic flagellates of planktonic communities, their characteristics and methods of study, p. 39–56. In D. J. Patterson and J. Larsen (ed.), *The biology of free-living heterotrophic flagellates*. Systematic Association, Clarendon Press, Oxford.
- Cho, B. B., and F. Azam. 1988. Major role of bacteria in biogeochemical fluxes in the ocean's interior. *Nature (London)* **332**:441–443.
- Fenchel, T. 1982. Ecology of heterotrophic microflagellates. II. Bioenergetics and growth. *Mar. Ecol. Prog. Ser.* **8**:225–231.
- Gibson, J., W. Ludwig, E. Stackebrandt, and C. R. Woese. 1985. The phylogeny of the green photosynthetic bacteria: absence of a close relationship between *Chlorobium* and *Chloroflexus*. *Syst. Appl. Microbiol.* **6**:152–156.
- González, J. M., and C. A. Suttle. 1993. Grazing by marine nanoflagellates on viruses and virus-sized particles: ingestion and digestion. *Mar. Ecol. Prog. Ser.* **94**:1–10.
- Gräf, W., and G. Perschmann. 1970. Über eine neue Spezies von *Vitreoscilla* (*Vitreoscilla proteolytica*) in Bodensee. *Arch. Hyg. Bacteriol.* **154**:128–137.
- Güde, H. 1979. Grazing by protozoa as selection factor for activated sludge bacteria. *Microb. Ecol.* **5**:225–237.
- Güde, H. 1989. The role of grazing on bacteria in plankton succession, p. 337–364. In U. Sommer (ed.), *Plankton ecology: succession in plankton communities*. Springer-Verlag, New York.
- Jürgens, K., and H. Güde. 1994. The potential importance of grazing-resistant bacteria in planktonic systems. *Mar. Ecol. Prog. Ser.* **112**:169–188.
- Jürgens, K., and G. Stolpe. 1995. Seasonal dynamics of crustacean zooplankton, heterotrophic nanoflagellates and bacteria in a shallow, eutrophic lake. *Freshwater Biol.* **33**:27–38.
- Monger, B. C., and M. R. Landry. 1992. Size-selective grazing by heterotrophic nanoflagellates: an analysis using live-stained bacteria and dual-beam flow cytometry. *Arch. Hydrobiol. Beih. Ergeb. Limnol.* **37**:173–185.
- Pimm, S. L. 1982. Food webs. Chapman and Hall Publishers, London.
- Porter, K. G., and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**:943–948.
- Psenner, R. 1993. Determination of size and morphology of aquatic bacteria by automated image analysis, p. 339–345. In P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. Cole (ed.), *Handbook of methods in aquatic microbial ecology*. Lewis Publishers, Boca Raton, Fla.
- Reichenbach, H. 1992. The genus *Herpetosiphon*, p. 3785–3805. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes*. Springer-Verlag, New York.
- Sherr, B. F., and E. B. Sherr. 1984. Role of heterotrophic protozoa in carbon and energy flow in aquatic ecosystems, p. 412–423. In M. J. Klug and C. A. Reddy (ed.), *Current perspectives in microbial ecology*. American Society for Microbiology, Washington, D.C.
- Sherr, E. B. 1988. Direct use of high molecular weight polysaccharide by heterotrophic flagellates. *Nature (London)* **335**:1225–1227.
- Sherr, E. B., and B. F. Sherr. 1987. High rates of consumption of bacteria by pelagic ciliates. *Nature (London)* **335**:1225–1227.
- Shikano, S., L. S. Luckinbill, and Y. Kurihara. 1990. Changes of traits in a bacterial population associated with protozoan grazing. *Microb. Ecol.* **20**:75–84.
- Shimeta, J. 1993. Diffusional encounter of submicrometer particles and small cells by suspension feeders. *Limnol. Oceanogr.* **38**:456–465.
- Šimek, K., and T. H. Chrzanowski. 1992. Direct and indirect evidence of size-selective grazing on pelagic bacteria by freshwater nanoflagellates. *Appl. Environ. Microbiol.* **58**:3715–3720.
- Sime-Ngando, T., G. Bourdier, C. Amblard, and B. Pinel-Aloul. 1991. Short-term variations in specific biovolumes of different bacterial forms in aquatic ecosystems. *Microb. Ecol.* **21**:211–226.
- Simon, M., B. B. Cho, and F. Azam. 1992. Significance of bacterial biomass in lakes and the ocean: comparison to phytoplankton biomass and biogeochemical implications. *Mar. Ecol. Prog. Ser.* **86**:103–110.
- Sommaruga, R. 1995. Microbial and classical food webs: a visit to a hypertrophic lake. *FEMS Microbiol. Ecol.* **17**:257–270.
- Zamperoni, P. 1989. Methoden der digitalen Bildsignalverarbeitung. Friedrich Vieweg & Sohn Verlag, Braunschweig, Germany.