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Estimates of protozoan- and viral-mediated mortality of bacterioplankton in Lake Bourget (France)

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SUMMARY

1. We performed three, 1-week *in situ* experiments in March-April (expt 1), May (expt 2) and August (expt 3) 2003 in order to assess protozoan and virus-induced mortality of heterotrophic bacteria in a French lake. Viral and bacterial abundances were obtained using flow cytometry (FCM) while protozoa were counted using epifluorescence microscopy (EFM).
2. A dilution approach, applied to pretreated grazer-free samples, allowed us to estimate that viral lysis could be responsible for 60% (expt 1), 35% (expt 2) and 52% (expt 3) of daily heterotrophic bacterial mortality. Flagellate (both mixotrophic and heterotrophic) grazing in untreated samples, was responsible for 56% (expt 1), 63% (expt 2) and 18% (expt 3) of daily heterotrophic bacteria removal.
3. These results therefore suggest that both viral lysis and flagellate grazing had a strong impact on bacterial mortality, and this impact varied seasonally.
4. From parallel transmission electron microscopy (TEM) analysis, we found that the burst size (i.e. the number of viruses potentially released per lysed cell) ranged from nine to 25 (expt 1), 10 to 35 (expt 2) and eight to 25 (expt 3). The percentage of infected heterotrophic bacteria was 5.7% (expt 1), 3.4% (expt 2) and 5.7% (expt 3) so that the calculated percentage of bacterial mortality induced by viruses was 6.3% (expt 1), 3.7% (expt 2) and 6.3% (expt 3).
5. It is clear that the dilution-FCM and TEM methods yielded different estimates of viral impact, although both methods revealed an increased impact of viruses during summer.

Keywords: bacteria, lake, mortality, protists, viruses

Introduction

Over the past 15 years, it has been realised that viruses are an important component of aquatic microbial food webs. They have been shown to be important controlling agents in planktonic community composition, diversity and succession, playing a key role in cell mortality and nutrient cycles (Bergh *et al.*, 1989; Suttle, 1994; Maranger & Bird, 1995; Fuhrman, 1999; Wommack & Colwell, 2000; Weinbauer & Rassoulzadegan, 2004). Of additional ecological significance, the ability of aquatic viruses to transfer genetic material has been demonstrated (Chiura, 1997; Clokie *et al.*, 2003). A large majority of aquatic viral ecological studies have been carried out in seawater (see Wommack & Colwell, 2000; Sime-Ngando *et al.*, 2003; Weinbauer, 2004). Some freshwater systems have also been investigated, although less often, and these include rivers (Mathias, Kirschner & Velmirov, 1995; Farnell-Jackson & Ward, 2003), Antarctic lakes (Kepner, Wharton & Suttle,

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1998; Laybourn-Parry, Höfer & Sommaruga, 2001), oligotrophic lakes (Klut & Stockner, 1990; Tapper & Hicks, 1998; Hoffer & Sommaruga, 2001; Bettarel *et al*., 2003b, 2004; Vrede, Stensdotter & Lindström, 2003), mesotrophic lakes (Hennes & Simon, 1995; Maranger & Bird, 1995; Leff *et al*., 1999; Wilhelm & Smith, 2000), and eutrophic lakes (Sommaruga *et al*., 1995; Weinbauer & Höfle, 1998; Fischer & Velimirov, 2002; Bettarel *et al*., 2003a; Weinbauer *et al*., 2003; Bettarel *et al*., 2004), as well as lake sediments (Maranger & Bird, 1996). In France, the three largest natural freshwater ecosystems (Lakes Annecy, Bourget and Geneva) have not yet been investigated from this perspective and only the oligotrophic Lake Pavin and the eutrophic Lake Aydat and Sep Reservoir have received recent interest in France (Bettarel *et al*., 2003a,b, 2004; Pradeep Ram *et al*., in press).

We used a variation of the dilution technique to assess the virus-induced mortality of heterotrophic bacteria in Lake Bourget. The dilution approach initially introduced by Landry & Hassett (1982), and refined by Landry, Kirshtein & Constantinou (1995), has been used routinely as a field technique to quantify grazing of phytoplankton by microzooplankton and to estimate phytoplankton growth rate (Campbell & Carpenter, 1986; Weisse & Scheffel-Möser, 1990; Landry, Monger & Selph, 1993; Calbet & Landry, 2004). Briefly, the technique consists of incubations of water samples after dilution at different levels of the original sample to reduce the abundance of algal and/or bacterial predators and thus to render less likely contact and interactions between predator and prey species. The net growth rate of the prey can therefore be expected to be highest in the most diluted fractions. Overall, the method allows an estimate of the grazing impact of zooplankton. To the best of our knowledge, this technique has been used to study the impact of viruses on microorganisms on very few occasions, for both heterotrophic and autotrophic groups. Wilhelm, Brigden & Suttle (2002) studied the 'rebound' in virus numbers following dilution in virus free water. Evans *et al*. (2003) estimated virus-induced mortality in a coastal and marine phytoplankter.

Our objective was to assess the impact of viruses on the heterotrophic bacterial communities at different periods of the year in Lake Bourget and then to compare these findings with those obtained in various other European lakes. Parallel grazing experiments were also performed in order to estimate the potential impact of protozoan predators on the bacterial community and to permit a comparison of bacterial mortality induced by viral lysis and protozoan predation. The grazing data were also compared with those from various other European freshwater ecosystems.

Methods

Study site

Lake Bourget (45°44'N, 05°51'W, 231 m altitude), on the western edge of the Alps, is the largest natural lake in France. It is an elongated and north–south orientated lake (length, 18 km; width, 3.5 km; area, 44×10^6 m^2; volume, 3.5×10^9 m^3; maximum depth, 145 m; mean depth, 80 m; residence time, 10 years) and is warm and meromictic. The catchment is about 560 km^2, with maximum and average altitudes of 1845 and 700 m, respectively. More details (including a map of the lake) are available in Jacquet *et al*. (in press).

Assessment of in situ microbial community dynamics

In situ dynamics of the microbial community (i.e. viruses, heterotrophic bacteria and picocyanobacteria) were assessed using flow cytometry (FCM) on samples of water from the reference station, known as 'B', which is located in the middle and deepest part of the northern basin. This station is more than 1.5 km from each bank and more than 5 and 10 km from the Sierroz and Leysse rivers (the two main freshwater inputs in the lake), respectively. Cell or particle concentrations for the different assemblages were measured at seven different depths between 0 and 50 m (2, 6, 10, 15, 20, 30 and 50 m) and sampled on average every 2 weeks between March and September 2003. In addition, water temperature, transparency, and nitrate and phosphate concentrations were assessed on the same sampling occasions.

Environmental variables

A conductivity-temperature-depth measuring device (CTD SBE 19 Seacat profiler, Seabird, SBE, Bellevue, WA, U.S.A.) was used to obtain vertical profiles (from the surface to the bottom) of water temperature, and to make sure that in each of the three experiments we sampled in the mixed surface layer and never below the epilimnion (see below). Nutrient

concentrations (total phosphorus, P-PO_4, N-NO_3, N-NH_4 and Si-SiO_2) were measured at the Institut National de la Recherche Agronomique (INRA) Hydrobiological Station chemistry laboratory (details available at http://www.thonon-inra-chimie.net/pages/public/analyses.asp). Water transparency was measured using a Secchi disk, before and after the experiments, and during routine surveys of the lake, and all measurements were performed by the same person. The underwater light intensity was measured using a LI-1400 current meter and data logger combined with a spherical quantum sensor LI-193SA (LI-COR, Lincoln, NE, U.S.A.).

Experimental set-up for estimating the impact of viruses on bacteria

Three similar experiments were conducted from 31 March to 4 April (expt 1), 19–23 May (expt 2) and 18–22 August (expt 3), which correspond to distinct periods in terms of the microbial planktonic dynamics and diversity in surface waters of the lake (Comte *et al*., in press; Jacquet *et al*., in press). For each period, an integrated >60-L sample was taken in the 0–10 m surface layer of the second reference station (called A) located in the middle and deepest part (100 m) of the southern basin of the lake, located at a distance >1 km from each bank and 6 km from B. The decision to use water of station A, rather than B, was mainly on the grounds of convenience, as the former was nearer than B, where the incubations were carried out, but also because we demonstrated in a previous study that communities and dynamics were similar between stations A and B (Comte *et al*., in press).

The water sample was first filtered through a series of 200- and 20-μm mesh filters (NYCOM, Buisine, France) and then through a 2-μm filter (Nuclepore, Whatman, San Diego, CA, U.S.A.) twice, which theoretically should eliminate all the bacterivores (i.e. nanoflagellates and ciliates). However, as some flagellates may not have been entirely removed by filtration, phagotrophic protists were counted in less than the 2 μm fraction using a Nikon TE 200 epifluorescence microscopy (EFM). A fraction of this 2-μm filtered water was subjected to tangential ultra-filtration using a mini-Ultrasette with a 100 kDa cut-off membrane (Vivaflow, Vivasciences, Hannover, Germany) in order to eliminate all organisms (including viruses). The purity of the water was checked using both EFM and FCM. The two fractions were mixed in order to obtain percentages of the initial 2-μm filtered whole water of ca 20, 40, 70 and 100%. Dilution reduced the background of free viruses by adding virus-free water, thus reducing the amount of contact between viruses and bacteria. A control bottle containing none of the original 2-μm filtered water was also prepared for each experiment, in order to confirm the absence of background contamination. For each level of dilution, duplicates were prepared in acid-washed, water rinsed and autoclaved 250 mL polycarbonate bottles (Nalgene, Bioblock, Illkirch, France). Immediately after preparation, the bottles were attached to the side of a bridge in the eastern part of the lake and then incubated at a depth of 1 m, for 5 days. Population dynamics were monitored during the whole week but the time of incubation for assessing the viral impact was limited to the first 24 h, assuming that there were no nutrient limitation and/or confinement effects during the first day of incubation. All bottles were sampled early each morning, and samples were subsequently prepared for FCM analysis.

Flow cytometry sample analysis

We used a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) equipped with an air-cooled laser providing 15 mW at 488 nm with the standard filter set-up. One millilitre samples were analysed without adding any fixative or dye to analyse the picocyanobacterial community dynamics and also to check for the absence/presence of eukaryotic autotrophic organism in the <2 μm treated samples. Fluorescent microbeads (Molecular Probes Inc., Eugene, OR, U.S.A.) of 1 μm in diameter were added to each sample as an internal standard. Another 1 mL sample was fixed with 0.25% glutaraldehyde (final concentration, Sigma, Lyon, France) and used for bacterial and viral counts via FCM. Briefly, for heterotrophic bacteria, samples were diluted with 0.2-μm filtered water from the lake while for viruses, samples were diluted with 0.02-μm filtered TE (Tris-EDTA, pH = 8) buffer, and then heated for 10 min at 75 °C. Samples were stained with SYBR Green I (1/10 000 final dilution of stock supplied by manufacturer) for 15 min in the dark. We found this protocol was the most accurate and the results will be published elsewhere (V. Dorigo, S. Personnic & S. Jacquet, in revision; some details can

also be found in Marie *et al.*, 1999). Cytofiles were analysed using Cytowin (Vaulot, 1989).

Analysis using transmission electron microscopy

Samples were also taken in the <2 μm filtered sample at time zero and fixed in formalin (5% final concentration) for the analysis of infected cells by transmission electron microscopy (TEM). For analysis, the samples were kept at 4 °C for about 3 weeks. In the formalin-fixed samples, the bacteria contained in 8 mL subsamples were harvested by ultracentrifugation onto 400 mesh NI electron microscope grids with carbon-coated Formvar film, by using a Centrikon TST 41.14 Swing-Out-Rotor (Kontron Instruments, Buckinghamshire, U.K.) run at 70 000 × g for 20 min at 4 °C (Weinbauer & Peduzzi, 1994; Sime-Ngando *et al.*, 1996). Each grid was then stained for 30 s with uranyl acetate (2% wt/wt) and examined using a JEOL 1200EX TEM (JEOL, Peabody, MA, U.S.A.) operated at 80 kV at a magnification of ×40 000. A cell was considered to be infected when the phages inside could clearly be recognised on the basis of shape and size. At least 600 bacterial cells were inspected per sample for a target number range of infected cells of 20–25 per sample. The minimum number of phages found in an infected cell was five. To estimate the virus-induced bacterial mortality (VIBM), the frequency of visibly infected cells (FVIC, as a percentage) was first related to the frequency of infected cells (FIC) as follows: $FIC = 9.524 \times FVIC - 3.256$ (Weinbauer, Winter & Höfle, 2002). The FIC was then converted to the VIBM according to Binder (1999): $VIBM = (FIC + 0.6\ FIC^2)/(1 - 1.2\ FIC)$. In a steady-state system, the bacterial mortality because of viral lysis matches the bacterial production which is removed by lysis (Suttle, 1994). Thus, multiplying the lysed bacterial production by the burst size (i.e. the number of viruses produced per cell) yields the viral production (Weinbauer & Höfle, 1998).

Assessment of flagellate grazing

In addition to the dilution experiments, grazing measurements were made both in the <2 μm fraction and in the original untreated water sample, in order to assess the potential impact of bacterivores which could have passed in the <2 μm fraction, and the total impact of bacterivores in the original water sample. Briefly, experiments were conducted in duplicate in 250 mL glass containers in which 0.5-μm tracer particles (Fluoresbrite Plain Microspheres, Polysciences Inc., Warrington, PA, U.S.A.) were added (0.5 μm represented a particle size close to the mean length of heterotrophic bacteria in Lake Bourget, Comte *et al.*, in press). After a few minutes to allow the plankton to acclimatise to the glass bottles, the beads were injected (microspheres final concentration = 5–20% of bacterial concentration in the lake). Bead concentration was estimated by EFM after filtration onto 0.2 μm pore size polycarbonate black filters (Millipore, St Quentin, France).

Based on preliminary measurements of predation kinetics in prealpine lakes (I. Domaizon, S. Viboud & D. Fontvieille, 2003; Domaizon, unpublished data), we chose an incubation time of 15 min. For the three experimental series, we conducted the grazing measurements *in situ*, in two experimental bottles in which we took and analysed samples at $t = 0$ min and 15 min. Ice cold glutaraldehyde was added to each sample (2% final concentration) for minimising the egestion of particles (Sanders *et al.*, 1989), and the microbeads ingested by flagellates were counted after filtering each sample (30 mL) onto a 0.8 μm polycarbonate black membranes (Nucleopore, Bioblock) and staining by Primulin, according to the protocol described by Caron (1983); 200 μg primulin per millilitre of distilled water with 0.1 mol L^{-1} Tris-HCl. Flagellates and microbeads were observed at a 1250 × magnification under UV and blue light. Briefly, UV light allowed the display of microbeads and all flagellates which were primulin-stained. In the same microscopic field, pigmented flagellates were distinguished by switching on blue light (500 nm) to observe the autofluorescence of chlorophyll *a*. Taxa which had plastids and ingested microbeads were considered as mixotrophs. In order to compare grazing by flagellates with daily viral lysis (expressed as the percentage of bacterial standing stock removed daily), we converted flagellate grazing to a number of bacteria potentially consumed by flagellates over 24 h, which allowed us to evaluate the percentage of bacterial standing stock consumed daily by flagellates.

The clearance rate (nL flagellate^{-1} h^{-1}) was calculated for each taxon by dividing the number of beads ingested per hour by the concentration of beads in the bottle. The ingestion rate of each taxon (bacteria flagellate^{-1} h^{-1}) was calculated by multiplying the corresponding clearance rate by the concentration of

heterotrophic bacteria. The grazing impact (bacteria h^{-1} L^{-1}) of a taxon was estimated by multiplying its ingestion rate by its concentration.

Missing data

Because of FCM problems, no data were available for either the picocyano- or the heterotrophic bacterial communities for the last experiment after day 3. No virus data were available for the last experiment for the same reason.

Results

Environmental conditions

Between the end of March and the end of August, the water temperature just under the surface of Lake Bourget increased from 7.5 °C (expt 1) to 16.3 °C (expt 2) and then to 24.8 °C (expt 3). Stratification began in spring, and lasted at least until early autumn (Fig. 1a). No significant differences were recorded between the temperatures at a depth of 1 m at the two sampling stations (both A and B) and the place where the bottles

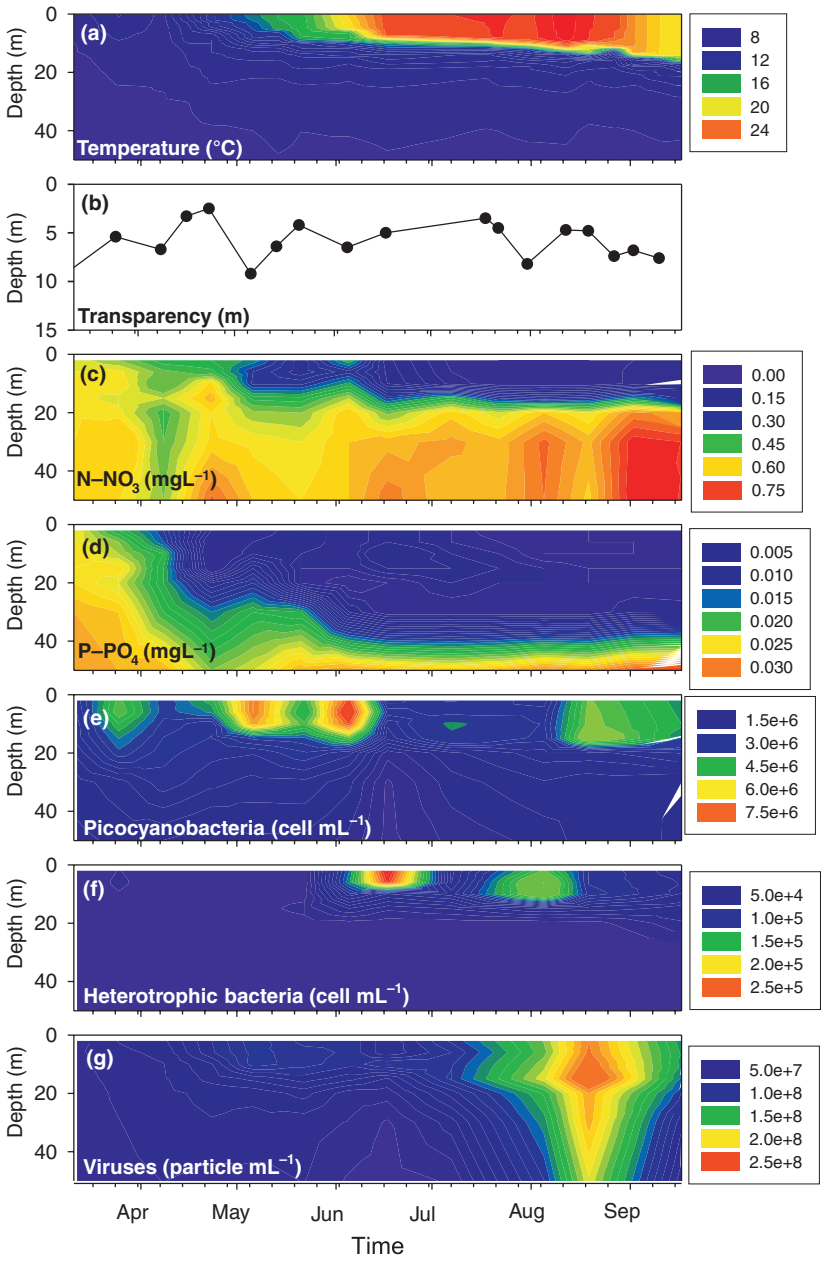

Fig. 1 Data sets for water temperature (a), water transparency (b), N-NO_3 (c), P-PO_4 (d), picocyanobacteria abundance (e), heterotrophic bacteria abundance (f) and viruses abundance (g) over 0–50 m between March and September 2003.

were incubated near the shore (not shown). The water transparency changed from 5.4 to 6.7 m between 25 March and 8 April, from 6.4 to 4.2 m between 14 and 21 May, and was equal to 4.8 m on 19 August (Fig. 1b). Nutrient concentrations ($P\text{-}PO_4$, total P, $N\text{-}NO_3$, $N\text{-}NH_4$, and SiO_2) measured at the beginning of each experiment are summarised in Table 1. Overall, both total phosphorus and phosphate concentrations decreased from the first to the third experiment in near surface waters, whereas the ratio of nitrates to phosphates increased dramatically, suggesting a definite increase in P limitation over the year (see also Fig. 1c,d). Concentrations of the other elements remained relatively high (Table 1).

Development of the microbial community

In situ *dynamics in the middle of the lake (station B).* From March to September, different patterns could be observed for the microbial community analysed by FCM, with a succession over time of the different groups. The highest concentrations were always recorded in the top 10–15 m layer, first for the picocyanobacteria, then the heterotrophic bacteria, and finally the viruses. Counts of 2.8×10^5 cell mL^{-1} for the picocyanobacteria (Fig. 1e) and 8.3×10^6 cell mL^{-1} for the heterotrophic bacteria (Fig. 1f) were obtained in early and late June, respectively, and of 2.6×10^8 part mL^{-1} for the viral community (Fig. 1g) in mid-August. No significant correlation was observed between the heterotrophic bacteria and the picocyanobacteria or between the picocyanobacteria and the viruses on the basis of FCM counts. However, there was a relatively tight linear regression between heterotrophic bacteria and viruses ($r = 0.4$, $n = 70$, $P < 0.01$). Viral development either lagged behind that of its potential hosts or was recorded virtually simultaneously but at a different depth (especially in summer). This could probably account for the lack of a really close and clear positive correlation between viruses and their bacterial hosts (with a $r > 0.4$).

Table 1 Nutrient concentrations measured at the beginning of the three experiments. Concentrations are given in μg L^{-1}.

	Expt 1 (31 March)	Expt 2 (19 May)	Expt 3 (18 August)
Total P	24	19	10
$P\text{-}PO_4$	7	5	1
$N\text{-}NO_3$	140	240	100
$N\text{-}NH_4$	13	54	46
$Si\text{-}SiO_2$	820	640	1003
$N\text{-}NO_3/P\text{-}PO_4$	20	48	100

Temporal changes in viral and bacterial abundances in the experiments. The lowest abundance of heterotrophic bacteria was found during the first experiment, with an average of 6.2×10^6 cells mL^{-1} over the incubation period (Fig. 2a). These values increased as the year progressed to 6.9×10^6 cells mL^{-1} for expt 2 and to 1.2×10^7 cells mL^{-1} for expt 3. Bacterial dynamics were very different in each experiment, with a regular increase in cell number during expt 1, a bell shape in expt 2, and a very considerable increase in expt 3. The net growth rate estimated during the first 24 h of each experiment was 0.23 day^{-1} (expt 1), 0.72 day^{-1} (expt 2) and 1.15 day^{-1} (expt 3). Note that the initial concentration of heterotrophic bacteria was rather similar in all three experiments, ranging from 3.2 to 4.3×10^6 cell mL^{-1}.

The initial concentration of the viruses (Fig. 2b) and the value of VBR (i.e. the ratio between viruses and heterotrophic bacteria; Fig. 2c), was different in expt 1 (with 6.2×10^7 mL^{-1} and a VBR of 19) and expt 2 (8.9×10^7 mL^{-1} and a VBR of 27). Mean concentration of viruses, and the VBR, throughout the incubation period was about 6.7×10^7 mL^{-1} and 12 (expt 1) and 8.6×10^7 mL^{-1} and 14 (expt 2), respectively. However, the concentration of viruses varied markedly, with an opposite pattern in the two experiments (Fig. 2b). In contrast, the pattern of VBR was relatively similar between the two experiments and a clear decrease was recorded along the 5 days of incubation (Fig. 2c).

Bacterial growth and mortalities in dilution experiments. The FCM analysis revealed clearly that the ultrafiltrate used to dilute the natural sample was devoid of any particles, including viruses. Therefore, there was a clear gradient in the concentration of viruses over the series of dilutions (Fig. 3). In the three dilution experiments conducted on March-April (expt 1), May (expt 2) and August (expt 3), significant relationships were found between the level of dilution and the apparent growth rate of the heterotrophic bacterial community (Fig. 4). The regression analysis of dilution plots allowed us to estimate both the specific growth and mortality rates in the incubation bottles as being 0.48 and −0.29 day^{-1} for expt 1, 1.04

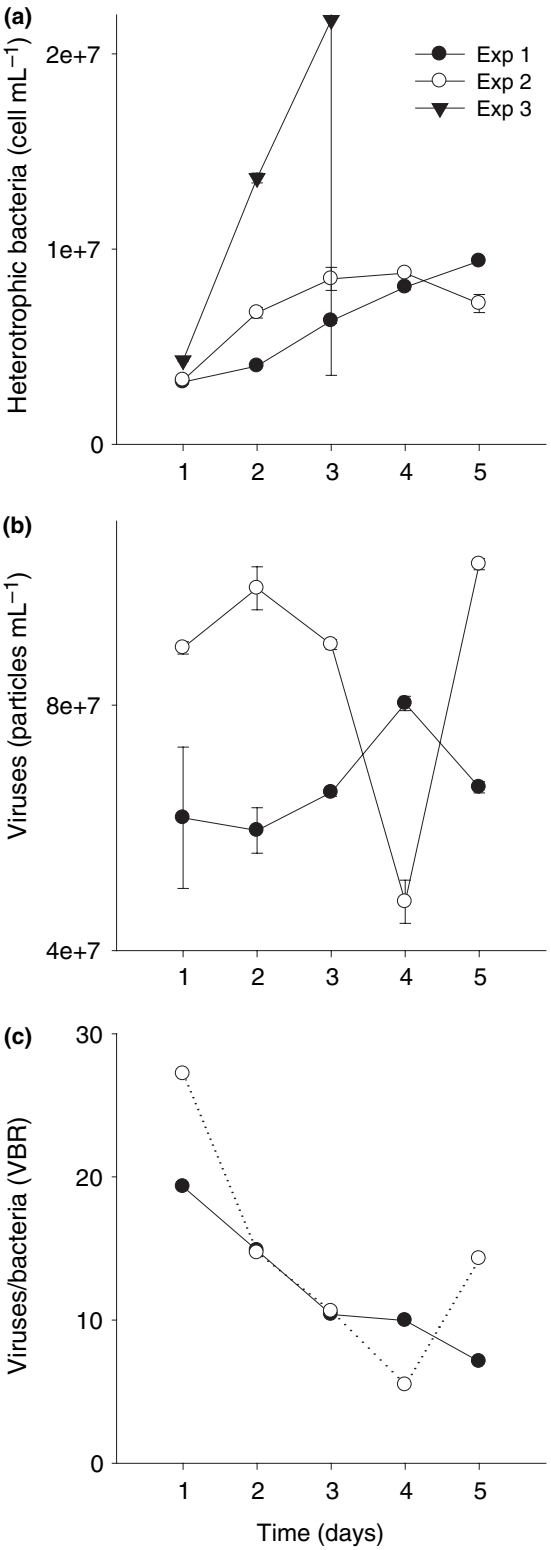

Fig. 2 Time series obtained over 3–5 days for the heterotrophic bacterial (a) and viral (b) communities and of the VBR (viruses-to-bacteria ratio) (c) in the control (undiluted) bottle of the different dilution experiments.

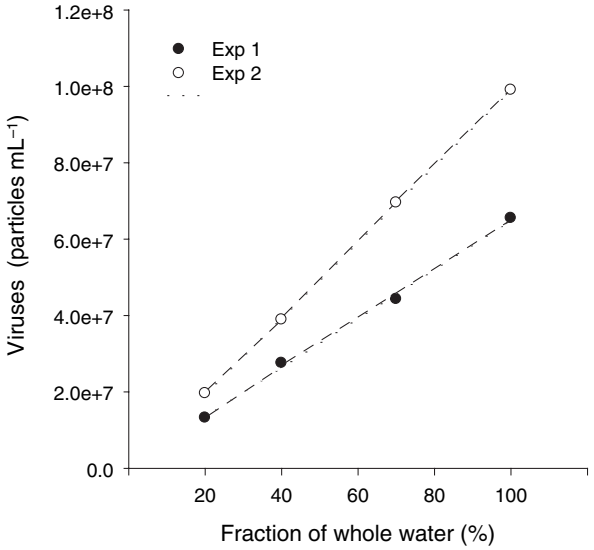

Fig. 3 Virus concentration against the fraction of whole water for expt 1 and 2 dilution series.

and −0.36 day^{-1} for expt 2, and 2.2 and −1.14 day^{-1} for expt 3, respectively. We verified that these values were in agreement with the net growth rate mentioned above (Table 2). Thus, the impact of viruses

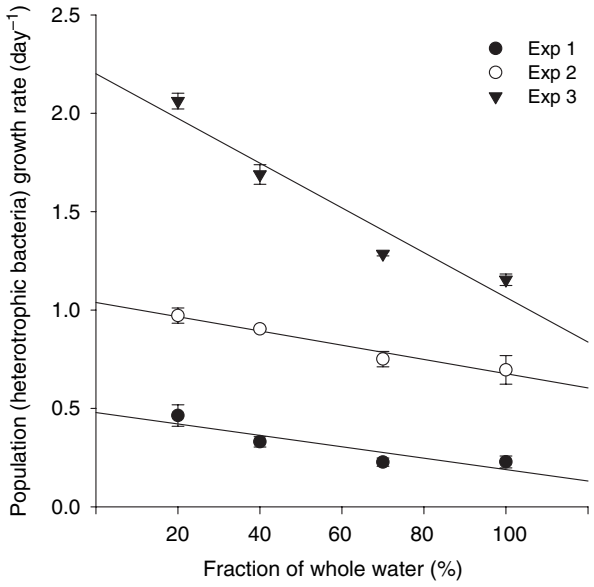

Fig. 4 Dilution plots and analysis when virus- and grazer-free water was used to dilute the <2 μm filtered sample for each experiment. The equations of the different plots are as follows for the heterotrophic bacteria: expt 1: $y = 0.4791–0.2889 \times$ ($r^2 = 0.93$); expt 2: $y = 1.0385–0.3617 \times$ ($r^2 = 0.98$) and expt 3: $y = 2.2012–1.1366 \times$ ($r^2 = 0.99$).

Table 2 Comparison between the specific gross growth rate of heterotrophic bacteria given by the regression lines in Fig. 4 and the specific gross growth rate calculated from the net growth rate observed in Fig. 2 and the mortality coefficient from the regression lines in Fig. 4

	Net growth rate (day^{-1})	Viral-mediated mortality rate (day^{-1})	Calculated gross growth rate (day^{-1})	Estimated gross growth rate (day^{-1})
Expt 1	0.23	0.29	0.52	0.48
Expt 2	0.72	0.36	1.08	1.04
Expt 3	1.15	1.14	2.29	2.2

was calculated to be equivalent to daily bacterial removal rates of 60.3, 34.6 and 51.8%, respectively.

TEM analysis

From the TEM analysis, we estimated that the burst size ranged from nine to 25 (expt 1), 10 to 35 (expt 2) and eight to 25 (expt 3). The percentage of infected heterotrophic bacteria was 5.7% (expt 1), 3.4% (expt 2) and 5.7% (expt 3), and the VIBM was 6.3% (expt 1), 3.7% (expt 2) and 6.3% (expt 3). These values of the percentage daily loss of bacterial production because of viral lysis, revealed by TEM, were obviously different from the estimates based on dilution-FCM. Electron micrographs (Fig. 5) allowed us to observe that the viruses of Lake Bourget, both inside and outside bacterial cells, have a different size and appearance (tailed or not). Most of the particles were <70 nm in diameter. This viral diversity is not surprising when we consider that the bacterial diversity in Lake Bourget is probably very high and changes with the seasons (Comte *et al.*, in press; V. Dorigo, J.F. Humbert, D. Fontvieille, B. Leberre & S. Jacquet, unpublished data), not to mention the genetic diversity of the viruses themselves (Dorigo, Jacquet & Humbert, 2004; S. Jacquet, unpublished data).

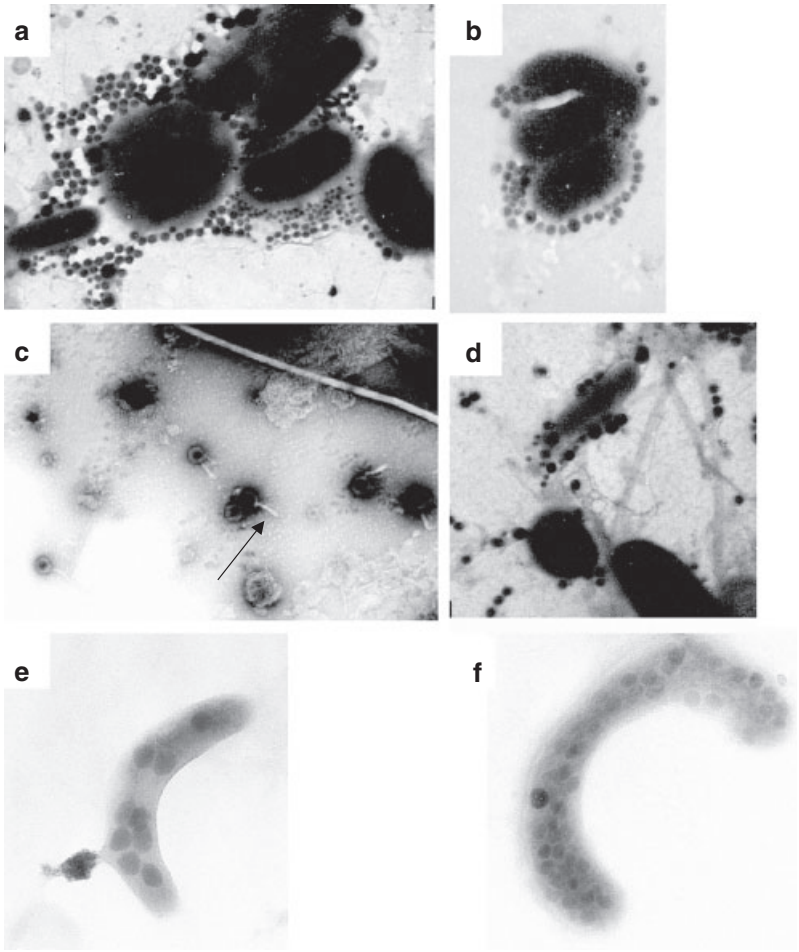

Fig. 5 Transmission electronic microphotographs of free viral particles and bacteria infected with viruses from Lake Bourget. (a and b) Clusters of viruses of similar size and shape surrounding rod-shaped bacteria. (c) Tailed phages (arrowed). (d) Viruses of different sizes surrounding bacteria. (e and f) Phages evenly distributed inside curved-rod bacteria.

Protozoan grazing of bacteria and the assessment of viral lysis versus predation pressure

Some small flagellates slipped throughout the smallest filtration cut-offs, because of their amoeba-like or oval–shaped. The initial abundance of heterotrophic and mixotrophic flagellates in untreated water was 3×10^3 cell mL^{-1} (expt 1), 2.9×10^3 cell mL^{-1} (expt 2), 1.7×10^3 cell mL^{-1} (expt 3) and 1.2×10^4 cell mL^{-1} (expt 1) 1.1×10^4 cell mL^{-1} (expt 2), 3.4×10^4 cell mL^{-1} (expt 3), respectively. The 'contamination' rate in the <2-μm filtered water varied largely according to taxon. The potentially mixotrophic flagellates (mainly some *Cryptomonas* and *Dinobryon* spp.) were usually mainly eliminated by filtration, as only 0.6% (expt 1), 6% (expt 2) and 10% (expt 3) of their initial abundances were observed in the <2 μm fraction. Flagellates which slipped through the filtering process consisted mainly of small heterotrophic flagellates, and their abundance was reduced to 18% (expt 1), 11% (expt 2) and 35% (expt 3) of the initial concentration found in the untreated water. Among these small heterotrophic flagellates we observed picoflagellates (such as unidentified cells; 2–3 μm), chrysomonads (such as *Spumella*, 3–4 μm) and cryptomonad forms (such as *Katablepharis*, 7 μm).

Ingestion rates varied largely according to taxon, and ranged from 1 to 45 bacteria $flagellate^{-1}$ h^{-1}. *Per capita* ingestion rate of bacteria by heterotrophic flagellates was 2.6×10^3 mL^{-1} h^{-1} (expt 1), 2.1×10^3 mL^{-1} h^{-1} (expt 2), and 1.7×10^4 mL^{-1} h^{-1} (expt 3). In the same water sample (<2 μm fraction), mixotrophic flagellates grazed 5×10^2 bacteria mL^{-1} h^{-1} (expt 1), 4.3×10^3 mL^{-1} h^{-1} (expt 2), and 2.1×10^3 mL^{-1} h^{-1} (expt 3). In the <2-μm filtered water, the fluorescent microbeads technique estimated that flagellate grazing could remove 1.9% (expt 1), 4.8% (expt 2) and 7.8% (expt 3) of heterotrophic bacteria in the first 24 h of incubation. The potential impact of grazing in experimental bottles thus appeared rather low compared with estimates of the mortality induced by viruses made using the dilution method.

In order to compare the impact of viral lysis to total flagellate grazing, we also assessed the grazing pressure in the untreated water samples. It appeared that flagellate grazing (both mixotrophs and heterotrophs) could account for 56.5% (expt 1) 63.2% (expt 2) and 18.2% (expt 3) of the daily removal of heterotrophic bacteria. Thus, flagellate grazing was at least equal to the impact of viruses in expts 1 and 2, but lower in expt 3 (August). Indeed, if we accept the estimates of viral impact from the dilution method, the ratio of mortality because of flagellates and viruses was 0.95 (expt 1), 1.8 (expt 2) and 0.35 (expt 3). The impact of viral lysis estimated using the percentage of visibly infected cells led to quite different results, with higher bacterivory to viral lysis ratios, i.e. 9 (expt 1), 17 (expt 2) and 3 (expt 3). All results revealed an increased impact of viruses during summer.

Note that our estimates of grazing mortality considered not only heterotrophic nanoflagellates but all bacterivorous flagellates. The grazing impact of mixotrophs sometimes exceeded that of the heterotrophs, particularly during the Spring (exps 1 and 2), because of the relatively high ingestion rates of *Dinobryon* and large *Cryptomonas* (six to 16.5 bacteria $flagellate^{-1}$ h^{-1} and three to 45.5 bacteria $flagellate^{-1}$ h^{-1}, respectively). Using the microbeads method, the percentage of bacteria removed daily because of grazing by mixotrophic and heterotrophic flagellates was estimated to be 37.3% (expt 1) 46.7% (expt 2) 3.5% (expt 3) and 19.2% (expt 1) 16.5% (expt 2) 14.7% (expt 3), respectively.

Discussion

As a novel work on the field of aquatic viral ecology, this is the first study dealing with natural aquatic viruses of Lake Bourget, both in terms of dynamics at different periods of the year and of their potential functional role as mortality agents of bacteria. To date, there are few investigations of the seasonal fluctuations in the abundance of these particles in large natural freshwater ecosystems (Hennes & Simon, 1995; Tapper & Hicks, 1998; Hoffer & Sommaruga, 2001; Fischer & Velimirov, 2002; Bettarel *et al*., 2003b, 2004; Vrede *et al*., 2003). Also, this study is rare in that both predation by protozoa and parasitism by viruses were investigated simultaneously (Simek *et al*., 2001; Fuhrman & Noble, 1995; Guixa-Boixereu *et al*., 1996; Guixa-Boixereu, Lysnes & Pedros-Alio, 1999; Almeida, Cunha & Alcantara, 2001; Bettarel *et al*., 2003a, 2004) using a combination of methods and at different periods of the year. Although some limitations and/or drawbacks were either unavoidable, or subsequently identified, our results provide a first estimation of what are the potential impacts of both the protozoan and viral communities on the bacterial

community of this large lake. It also shows how these different impacts may vary over time (Hennes & Simon, 1995; Fischer & Velimirov, 2002; Vrede *et al*., 2003).

Environment and microbial dynamics

Increasing concentrations of the different groups were found from March to September. It is suspected that temperature, which increased in the surface waters of Lake Bourget, could be an important factor in controlling viral abundance, in that temperature controls bacterial growth rates and has a significant positive effect on bacterial production (White *et al*., 1991; Jiang & Paul, 1994). The VBR increased gradually from winter to summer, and we did indeed find faster bacterial growth and greater biomass in summer. The first peak of heterotrophic bacteria in June was not followed by a marked increase in viral abundance. The second peak of heterotrophic bacteria in August was clearly followed by an increase in the viral abundance and VBR reached 134. It was more difficult to explain why there was a relatively high concentration of viruses throughout the water column (0–50 m) in August, that was not related to levels of the other groups mentioned above. This pattern could not be related to any analytical problems, which suggests that it could be linked to other planktonic groups, such as heterotrophic pico- or nanoplankters (e.g. Vaulot, Romari & Not, 2002), or to the adsorption of viruses to small sedimenting particles. It has previously been shown that this last possibility may make a contribution to high sediment abundances (Danovaro *et al*., 2001).

From the dilution experiment, we found that viruses could play a key role in controlling bacterial biomass and might exceed the impact of grazing flagellates. The different bacterial peaks could be related to genetically different groups, which are probably susceptible to different forms of pathogens and predators. Such a scenario was supported by the study of Comte *et al*. (in press), where we showed from fluorescent *in situ* hybridization (FISH) and microscopic analyses that there was indeed a clear seasonal variation in both the bacterial and the protozoan assemblages. The impact of lysis was clearly lower in May, contrasting with the predation (see below). It is possible that this feature could be related to a decrease in bacterial diversity occurring during the spring clear-water phase, as has been reported elsewhere, and which is mainly because of zooplankton grazing (Höfle, Haas & Dominik, 1999; Yannarell *et al*., 2003). However, low diversity is likely to cause higher specific host–virus encounters and, therefore, a higher lysis rate (Thingstad & Lignell, 1997).

For the picocyanobacterial community, lower values for cell concentrations were found in May than earlier in the year or thereafter. Such a finding could also be related to the clear water phase, and a major expansion in the population of cladocerans that can graze very efficiently these small phtyoplankters (Jürgens, 1994; Degans *et al*., 2002; Domaizon *et al*., 2003; Comte *et al*., in press). The bloom of heterotrophic bacteria in June occurred after that of the picocyanobacteria, suggesting that the heterotrophic bacteria could use material resulting from the decline of the picocyanobacteria. Nutrients did not seem to play a critical role compared with that of the temperature at the end of winter-early spring, as the lowest cell concentrations were recorded at that time. In contrast, low cell concentrations during the summer could suggest that nutrients may play a key role in terms of growth limitation.

As previously reported when using FCM for counting viruses, it is reasonable to assume that most of the viruses were bacteriophages (Marie *et al*., 1999; Larsen *et al*., 2004). The correlation we found between heterotrophic bacteria and viruses agreed with this assumption. Moreover, the numbers we recorded are comparable to the bacteriophage abundance generally reported for both marine and freshwater ecosystems (e.g. Wommack & Colwell, 2000; Sime-Ngando *et al*., 2003). The number of viruses increased considerably either at the same time or shortly after the bacterial bloom in August. These coinciding peaks of bacteria and viruses support the idea that bacteriophages cause bacterial lysis and thus lead to changes in bacterial abundance that reduce bacterial production (Wilhelm & Suttle, 1999). Assuming that the majority of viruses were bacteriophages, we used the FCM numbers to calculate the VBR ratios. The numbers ranged from 9 to 134 (mean = 30, $n = 84$). Such ratios fall generally between 3 and 10 but can be high for nutrient-rich, productive areas (Wommack & Colwell, 2000; Almeida *et al*., 2001; Vrede *et al*., 2003). VBR values <10 are thought to indicate low levels of viral-mediated bacterial mortality, whereas a ratio >10 corresponds to conditions that favour bacterial lysis

(Wilcox & Fuhrman, 1994). The ratios observed in our material may thus indicate that bacteriophages accounted for an important part of the bacterial mortality. This was supported by the dilution experiments.

According to TEM observations, most viruses were between 50 and 70 nm in size, agreeing with previous studies in freshwaters and suggesting that these viruses were indeed mainly bacteriophages. For example, Hennes & Simon (1995) reported a predominance of 30–60 nm viruses within the bacterioplankton. We did not observe the large viruses that can be relatively common in freshwaters (Sommaruga *et al*., 1995; Kepner *et al*., 1998; Pina *et al*., 1998). The production of bacteriophages (as well as the frequencies of cells containing the mature phage, the burst size, the phage head sizes and the distribution patterns of phages inside cell) is strongly influenced by the structure of the bacterial community, i.e. by the relative abundances of the various morphotypes (Weinbauer & Peduzzi, 1994). These differences may also be because of various physiological differences that may or may not be associated with the morphotypes. We showed in a previous study that the bacterial structure changed considerably during the same period of the year as that covered by this study (Comte *et al*., in press). Lysogeny and host diversity may explain the differences in the FIC observed during the experiments. Our results confirm previous studies showing that the impact of phages may vary strongly on both spatial and temporal scales in response to host diversity and/or changes in host physiology (Weinbauer & Peduzzi, 1994).

Methodological questions

Sampling strategy. We decided always to sample the epilimnion of the lake but at different times in order to compare temporarily the same part of the water column. This was logistically feasible, but of course means that we assessed only a small portion of the water column, as Lake Bourget reaches a depth of 145 m. It is thus inevitable that we obtained only a partial picture, and that further investigations would be necessary to find out whether bacterial grazers and lysis varies with depth, as has been suggested elsewhere (Weinbauer & Höfle, 1998; Hoffer & Sommaruga, 2001; Bettarel *et al*., 2004). In addition, we sampled in the middle of the lake. It is also possible that lysis and grazing varies horizontally, and that there could be major variation with proximity to the shore. At least, an effort should be made to obtain high frequency sampling (several times a day) to have a better picture of phage-bacteria dynamics (Winter, Herndl & Weinbauer, 2004).

The filtration process and contamination problems. We could not avoid contamination by small heterotrophic flagellates (undetermined uniflagellates, small chrysomonads and cryptomonads) in the <2 μm final sample. In some situations, significant numbers of grazers may even pass through a 0.8 μm filter although we did not find such contamination in preliminary tests (unpublished data). Therefore we used the <2 μm filter as the final step; as further filtration would have been very time consuming. The contamination rate varied depending on the period, and especially according to taxon. Among the potential mixotrophs, some *Cryptomonas* also slipped through filters. The estimated grazing rate in the <2 μm fraction allowed us to conclude that the presence of grazers induced relatively little mortality, compared with the virus-mediated mortality, for the first 24 h incubation. In addition, it is possible that these small flagellates could also have reduced the concentrations of viruses by unselected grazing (Gonzales & Suttle, 1993) so that the impact of the viruses we found may in fact be under-estimated. Also, it has been reported recently that grazing may enhance viral activity so that the reduction/elimination of the predators could lead to under-estimations of viral activity (Simek *et al*., 2001; Weinbauer *et al*., 2003). At the very least, the filtration process could be responsible for cell destruction, with nutrient enrichment contributing in some way to the bacterial growth but probably different according to the period. Although the filtration process has inherent problems, our results suggest that fractionation of the microbial community by filtration may nevertheless be useful in studying community interactions, as long as sufficient care is taken and an assessment of possible bias (contamination) is made.

The dilution experiment. The impact of viruses on bacterial mortality has been studied using different approaches: by direct observation of infected cells by using TEM, the analysis of viral decay rates by using the cellular poison potassium cyanide, radiotracer

incorporation into virioplankton biomass and by the utilisation of fluorescently labelled viruses as tracers. Only a couple of studies have used the dilution method. For instance, Evans *et al.* (2003) used it to quantify simultaneously both grazing and viral-induced mortality in coastal waters of the Norwegian Sea, and they estimated the viral-induced mortality of the picophytoplankter *Micromonas* spp. standing stock to be 9–25% per day. It was found that, on a particular day, viral lysis caused the loss of 50% of *Micromonas* spp. production. The reverse approach, consisting of increasing the concentration of active viruses in water, has been used more often but again usually applied to marine bacterio- or phytoplankton (Proctor & Fuhrman, 1992; Hewson *et al.*, 2001; Eissler & Quinones, 2003). As a recent example, Noble, Middelboe & Fuhrman (1999) were able to increase bacterial mortality attributed to viral infection and stimulate the growth of subpopulations of non-infected heterotrophic bacterioplankton. In virioplankton-enriched seawater incubations lasting 24 h, a 25–40% decline in bacterioplankton abundance has generally been observed (Wommack & Colwell, 2000; Sime-Ngando *et al.*, 2003). A very interesting perspective will be to compare these two methods (dilution versus enrichment) to assess viral-induced bacterial mortality and diversity changes.

We can also point out that there may be a fundamental difference in using Landry & Hassett (1982) approach to estimate zooplankton grazing rate, and using the same approach to estimate mortality resulting from viral lysis. In the Landry & Hassett (1982) approach, it is assumed that the loss of bacteria is the result of collision between a predator and a cell. However, in the viral dilution experiment, cell lysis may initially be the result of infections that occurred before the experiment was begun. Depending on the length of the lytic cycle and the time of infection, lysis resulting from infections that occurred before the experiment could be maintained for a considerable period of time. The length of the lytic cycle is usually relatively tightly tied to the growth rate of the bacteria. In the case of the studies presented here, we chose to focus on the first 24 h of incubation. This is in the same time-frame as the length of the lytic cycle. Consequently, we should recall that many of the infection events that occurred during the incubation might not have resulted in cell lysis during the period that the experiment was monitored.

Lysis of bacteria: estimates by dilution-FCM versus TEM. The two methods led to different conclusions, although both indicated a potentially important role for viruses as causes of bacterial mortality. This agrees with the recent work of Fischer & Velimirov (2002), who studied viral control of bacterial production in a eutrophic oxbow lake of the River Danube. Using several approaches (viral decay method, estimation of the frequency of infected bacterial cells), they also observed large variations between the methods. The problem is that we do not know which method is the most accurate. With TEM, assumptions are necessary to obtain an estimate of virus-mediated bacterial mortality, so that errors or bias are possible (Binder, 1999). It is also possible that some infected cells could be lost by disruption during ultracentrifugation, and that they could have been inappropriately scored as non-infective because of the darkening of cells by electrons. In addition, samples analysed by TEM were kept for a few days before analysis. This makes it tempting to suggest that these steps between sampling and analysis could account for the large differences between the results of the two methods. An effort will have to be made in the future to reduce or eliminate this delay or at least to make it similar for the different methods. Finally, the TEM/FIC method does not take into account diel cycles in viral infection of bacterioplankton and the estimate is thus dependent on the time of day when the samples are taken. This problem has been recently highlighted by Winter *et al.* (2004).

Grazing. The benefits and advantages of the fluorescent microbeads technique have been discussed extensively in the literature (Vaqué, Gasol & Marrassé, 1994; Bratvold, Srienc F. & Taub, 2000; Cleven & Weisse, 2001; Sherr & Sherr, 2002). Although there are possible problems inherent to the method, we used the tracer approach as a 'near standard' method during this study. Its main shortcomings are the possible selective grazing behaviour against microspheres as an artificial prey, the possible quick egestion of beads by some taxa, and the size selectivity of bacterivorous flagellates. Although previous work has shown that protozoa can discriminate strongly against fluorescent microspheres (Pace & Bailiff, 1987; Sanders *et al.*, 1989), Boenigk *et al.* (2002) recently demonstrated the absence of any selectivity between inert or living particles in interception-feeding

bacterivorous nanoflagellates. Moreover, beads resist digestion and are easy to see in vacuoles of bacterivores and make it possible to measure taxon-specific grazing rates. Therefore, fluorescent microspheres appeared be a reasonable approach for comparing relative changes in ingestion rate and measuring the relative predation impact of different bacterivores (Carrias, Amblard & Bourdier, 1996; Thouvenot *et al.*, 1999). Recently, Bettarel *et al.* (2003a) used the fluorescent microbeads method to compare the potential impact of heterotrophic nanoflagellates grazing with viral lysis on heterotrophic bacteria.

We attempted to optimise the technique as far as possible, taking into account the predation kinetics for the main flagellate taxa and the bacterial size structure in Lake Bourget. Also, we have to keep in mind that the technique used to correct for the grazer 'contaminants' involved a short-term incubation looking at ingestion. It is not impossible that this contrast between the 15 min experiment and its extrapolation to 24 h to compare with the viral impact may be a source of potential errors or bias.

Impact of viruses versus protozoa

Results from the dilution experiments suggested that the daily removal of bacteria by viral lysis ranged from 35 to 60%, depending on the season. As we have already said, these values are in the range reported for some aquatic ecosystems (Wommack & Colwell, 2000; see Table 3). The impact of lysis in Lake Bourget seemed to be higher than in the oligotrophic Lake Pavin (6–16%) but definitely lower than in the eutrophic ecosystems such as Lake Plussee (up to 97%) or the Sep reservoir (up to 60%). The same can be said concerning our burst size values and those reported in the literature. It seems, therefore, that our data support the general proposal that viral production increases with eutrophication in freshwater ecosystems (Weinbauer *et al.*, 2003).

Concerning grazing impact, both filtration and ingestion rates measured for flagellates were in the range of values reported in other freshwater systems (Table 4). However the predation rates measured for heterotrophic flagellates were higher in Lake Bourget than those measured in the oligotrophic Lake Annecy or the oligomesotrophic Lake Pavin (Carrias *et al.*, 1996; Bettarel *et al.*, 2003a; Domaizon *et al.*, 2003), because of the higher abundance of flagellates. The bacterial mortality induced by mixotroph grazing was also high, but remained lower than that reported for Lake Annecy. Finally, we considered only grazing by flagellates but we may speculate upon the relative importance of ciliates. In fact, parallel investigations

Table 3 Virioplankton abundance, VBR (virus:bacteria ratio), BS (the burst size or the number of virus particles released upon host cell lysis), FVIC (the fraction of visibly infected cells), FIC (the estimation of the total fraction of infected cells) and VIBM (the virus-induced bacterial mortality) in some European lakes

Lake and location	Status	Abundance ($\times 10^6$ Part mL^{-1})	VBR	BS	FVIC or *FIC* (%)	VIBM (%)	Reference
Gossenköllesee, Austria	O	<0.02 to <5; 1–10	0.1–11; 4–31	4–45	0.9–2.3	5–28	Pina *et al.* (1998); Hoffer & Sommaruga (2001)
Rédo, central Pyrenees	O	3 to 30	9–43				Pina *et al.* (1998)
Constance, Germany	M	10 to 40		21–121	0–1.8; *1–17*	0.11–18.4	Hennes & Simon (1995)
Plussee, Germany	E	0.3 to >200	19–35	19–87	0.5–3.4	7.7–97.3	Bergh *et al.* (1989); Demuth, Neve & Witzel (1993); Weinbauer & Höfle (1998)
Kalandsvannet, Norway			50		*2–16*	2–24 h^{-1}	Heldal & Bratbak (1991)
Pavin, France	O–M	10 to 54	4–13	13–54	0.5–3.1	2–74	Bettarel *et al.* (2003a,b, 2004)
Aydat, France	E	25 to 99	4–14	16–60	0.4–2.8	1–38	Bettarel *et al.* (2003a,b, 2004)
Sep Reservoir, France	O–M	8 to 130	2–12	8–140	0.5–3.7	5–60	Pradeep Ram *et al.* (in press)
Alte Donau, Austria	E	17 to 117	4–39		2.3–9	55–63	Fischer & Velimirov (2002)
Rimov reservoir, Czech Republic	M–E	8 to 47; 13 to 69	4–40	19–40	1.7–4; 1.1–5.2	15–37 day^{-1}; 18–66 day^{-1}	Simek *et al.* (2001); Weinbauer *et al.* (2003)
Gäddtjärn	O	22 to 23.5	3–12	6–18	*9–41 (23)*		Vrede *et al.* (2003)
Fisklösen (Sweden)	O	29 to 31	5–24	6–21	*10–43 (25)*		
Bourget, France	M	47 to 100	5–27	11–49	1.2–1.9; *8–14.5*	9.3–19.2	Jacquet *et al.* (this study)

O, Oligotrophic; M, Mesotrophic; E, Eutrophic; nd, Not determined.

Table 4 Ingestion rates, *per capita* grazing impact of heterotrophic and mixotrophic flagellates and flagellates grazing-induced bacterial removal in lakes differing in their trophic status

Ingestion rates (bacteria individual^{-1} h^{-1}) (large variation according to taxon)	Per capita grazing rates (bacteria L^{-1} h^{-1})	% loss because of grazing	Method	Site	Status	Reference
Heterotrophic flagellates						
1–45	Max: 36×10^6	BSS: 14.7–19.2	FMS	Lake Bourget, France	M	Jacquet *et al.* (this study)
1.8–72.3	Max: 30.7×10^6	BP : 0.5–115	FMS	Lake Pavin, France	O–M	Bettarel *et al.* (2003a,b)
0–15.6	Max: 6.1×10^6	BSS: 0.2–11.7; BP: 0.1–62.3	FMS	Lake Annecy, France	O	Domaizon *et al.* (2003)
0–31		BP: 0.5–48.3	FMS	Lake Constance, Germany	M–E	Cleven & Weisse (2001)
21–53		BSS: 8–28; (<20 μm bacterivores)	FLB	Reservoir Rimov, Czech Republic	E	Simek *et al.* (1997); Simek & Kojeka (1999)
0–3.3	Max: 1.5×10^6		FMS	Reservoir Sep, France	O–M	Thouvenot *et al.* (1999)
2.2–26.5		BP: 0.3–20	FLB	Alte Dauno, Austria	E	Wieltschnig *et al.* (1999)
		BP: 2.9–108		Plussee, Germany	E	Weinbauer & Höfle (1998)
10–37			FLB	Lake Erie, U.S.A.	M	Hwang & Heath (1997)
1.6–92.4	Max: 18.9×10^6		FMS	Lake Pavin, France	O–M	Carrias *et al.* (1996)
2–53		BSS: Max: 20%; BP: Max: 77%	FMS	Lake Oglethorpe, U.S.A.	E	Sanders *et al.* (1989)
Mixotrophic flagellates						
3.7–86	Max: 63×10^6	BSS: 3.5–46.7	FMS	Lake Bourget, France	M	Jacquet *et al.* (this study)
0–54.8	Max: 68×10^6	BSS: 6–71.5	FMS	Lake Annecy, France	O	Domaizon *et al.* (2003)
0–38.3			FLB	Artificial pond, England	E	Hitchman & Jones (2000)
0–137.6	Max: 2.6×10^6		FMS	Reservoir Sep, France	O–M	Thouvenot *et al.* (1999)
1.3–87	Max: 4.5×10^6		FMS	Lake Pavin, France	O–M	Carrias *et al.* (1996)
2–53			FMS	Lake Oglethorpe, U.S.A.	E	Sanders *et al.* (1989)

O, Oligotrophic; M, Mesotrophic; E, Eutrophic; BSS, bacterial standing stock; BP, bacterial production; FLB, fluorescent labelled bacteria; FMS, fluorescent microspheres.

allowed us to estimate that, for all three experiments, predation pressure by the ciliates on bacteria was very low (with a maximum grazing impact of 1.2% of bacterial mortality).

Few studies have investigated the comparative importance of viral lysis and protozoan grazing simultaneously, especially in freshwater ecosystems. All but three of the available studies have reported lower mean viral lysis than protist grazing. Fischer & Velimirov (2002) have reported less grazing predation on bacteria than viral lysis, the latter being responsible for 55.7 to 62.7% of bacterial production removal, and thus was 11-fold greater than grazing by heterotrophic nanoflagellates. Bettarel *et al.* (2004) also reported that viral lysis could prevail over protistan grazing in the anoxic hypolimnion zone of the eutrophic Lake Aydat at various times of year. Similar observations were reported by Weinbauer & Höfle (1998) and predicted by Pedros-Alio, Calderon-Paz & Gasol (2000). We were in a similar situation, with viral lysis exceeding predation in our last experiment, i.e. in summer, but still lower than that reported by Fischer & Velimirov (2002) (by a factor of two).

When we considered the *per capita* grazing impact of heterotrophs and mixotrophs, viral control exceeded flagellate grazing only during the third experiment but, if we considered only heterotrophs grazing (as did Fischer & Velimirov, 2002) the virus-mediated mortality (measured by the dilution method) exceeded HNF grazing for all three experiments. In their study, Simek *et al.* (2001) reported that protist grazing and viral lysis were responsible for 50 and 25%, respectively, of bacterioplankton mortality. As stated above, one of their experiments was grazer-free, and this showed that in such a situation, there was a lower percentage of cell mortality because of viruses (i.e. 17%) suggesting an apparent synergism between grazing and virus-induced mortality. Simek *et al.* (2001) speculated that their fractionation (i.e. <0.8 μm) reduced bacterial diversity and that there

may be a reciprocal relationship between the diversity of a bacterial community and virus-induced mortality. Alternatively, infection rates may shift with changes in individual cells. Cell-specific production and activity are stimulated by grazing because of the release of organic and inorganic nutrients. Higher growth rates might be associated with enhanced receptor formation on the cell surface, which may result in a greater chance of phage attachment and thus higher infection frequencies.

Seasonally induced changes act as important structuring forces for the Lake Bourget bacterial assemblage. Viruses and protozoa are important controllers of bacterial biomass. As with previous studies, our study shows that parasitism by viruses may have a broadly similar impact as protozoan bacterivory on bacterial loss (Weinbauer & Höfle, 1998; Fischer & Velimirov, 2002; Bettarel *et al*., 2004). Many avenues of research are now possible. Among them, the relative importance of phytoplankton (including picocyanobacteria) compared with bacterioplankton as a significant host reservoir for viruses in fresh water needs to be further investigated. Heterotrophic protists may be indirectly related to viruses via the bacterial compartment, but no correlation between the two communities has ever been found hitherto. In the near future, we will also focus on other large prealpine lakes such as Lakes Annecy and Geneva. Indeed, potential ecosystem differences in micrograzer activity, viral lysis and, ultimately, trophic structure and function still constitute a major uncertainty for biogeochemical models that seek to predict the role of the microbial community role in carbon cycling on the basis of bacterial parameters alone. There are still insufficient data, especially in freshwaters, on seasonal and depth-related variability of virus-mediated bacterial mortality as well as relationships related to the trophic status of a system. Lastly, it will also be necessary to initiate studies to compare the different methodologies used in order to assess virus-mediated bacterial mortality.

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