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PRIMARY RESEARCH PAPER

Seasonal and spatial variability of virio-, bacterio-, and picophytoplanktonic abundances in three peri-alpine lakes

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Abstract Flow cytometry (FCM) was used to assess microbial community abundances and patterns in three natural, large and deep peri-alpine hydrosystems, i.e., lakes Annecy (oligotrophic), Bourget, and Geneva (mesotrophic). Picocyanobacteria, small eukaryotic autotrophs, heterotrophic prokaryotes, and viruses were studied in the 0–50 m surface layers to highlight the impact of both physical and chemical parameters as well as possible biotic interactions on the functioning of microbial communities. Some specificities were recorded according to the trophic status of each ecosystem such as the higher number of viruses and heterotrophic bacteria in mesotrophic environments (i.e., Lakes Geneva and Bourget) or the higher abundance of picocyanobacteria in the oligotrophic Lake Annecy. However, both seasonal (temperature) and spatial (depth) variations were comparatively more important than the trophic status in driving the microbial communities' abundances in these three lakes, as revealed by principal component analysis (PCA). A strong viral termination of the heterotrophic bacterial blooms could be observed in autumn for each lake, in parallel to the mixing of the upper lit layers. As virus to bacteria ratio (VBR) was indeed very high at this period with values varying between 87 and 114, such important relationships between viruses and bacteria were likely. The magnitudes of seasonal variations in VBR, with the highest values ever reported so far, were largely greater than the magnitude of theoretical variations due to the trophic status, suggesting also a strong seasonality in virioplankton production associated to prokaryotic dynamics. FCM analyses allowed discriminating several viral groups. Virus-Like Particles group 1 (VLP1) and group 2 (VLP2) were always observed and significantly correlated to bacteria for the former and chlorophyll *a* and picocyanobacteria for the latter, suggesting that most of VLP1 and VLP2 could be bacteriophages and cyanophages, respectively. On the basis of these results, new ways of investigation emerge concerning the study of relationships between specific picoplanktonic groups; and overall these results provide new evidence of the necessity to consider further viruses for a better understanding of lake plankton ecology.

Keywords Viruses · Bacteria · Flow cytometry · Lake · Trophic status

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Introduction

During the past two decades, the emergence of epifluorescence microscopy (EFM) and flow cytometry (FCM), combined with the development of highly efficient nucleic acid dyes, have considerably increased our knowledge of micro-organisms by providing relatively quick, easy, and accurate methods for determining their abundances (Courties et al., 1994; Marie et al., 2000; Rose et al., 2004). These techniques have also played an essential role in the detection and quantification of free viruses in the water column (Xenopoulos & Bird, 1997; Noble & Fuhrman, 1998; Marie et al., 1999; Brussaard, 2004) and in sediments (Danovaro et al., 2001; Duhamel & Jacquet, 2006).

Viruses have now been recognized as an active component of the planktonic and microbial food webs in both freshwater and marine systems (Wommack & Colwell, 2000; Weinbauer, 2004; Suttle, 2007; Breitbart et al., 2007). The high abundance of viruses (i.e., 10^6–10^9 particles ml^{-1}) and their main functional roles (as agents of host organisms mortality and diversification through genetic transfer or as drivers of the enrichment of the dissolved organic matter (DOM) pool, Brussaard et al., 2008) have led to the development of the concept of the viral loop (Bratbak et al., 1992), only 10 years after that of the microbial loop proposed by Azam et al. (1983). The latter which describes the flow of dissolved organic carbon (DOM) from the prokaryotes (i.e., the heterotrophic bacteria) and the small eukaryotes to the grazing food chain has highlighted the importance of pico- and nanoplanktonic communities. Heterotrophic bacteria take up DOM, which originates predominantly from primary producers (eukaryotic phytoplankton and picocyanobacteria), and small grazers such as flagellates and ciliates consume heterotrophic bacteria, which are in turn consumed by larger grazers (i.e., the metazooplankton) so that the organic matter comes back to the grazing food chain.

In addition to the physical and chemical influence of the environment, viral infection depends mainly on the abundance of the viruses and their hosts (Murray & Jackson, 1992; Clasen et al., 2008). Consequently, prokaryotic viruses (i.e., viruses infecting photo- and heterotrophic bacteria and archaea) are likely to dominate the viral community (Paul & Jiang, 2001; Hambly & Suttle, 2005; Suttle 2005, 2007). As viruses are supposed to kill the most abundant members of the prokaryotic and/or eukaryotic community (Wommack & Colwell, 2000), they also probably have a considerable impact on bacterial species diversity (Weinbauer & Rassoulzadegan, 2004). Hence, viral lysis of bacterioplankton results not only in the removal of bacterioplankton and changes in its diversity, but also in the release of lysis products which may be an important mechanism by which organic matter is lost from the phagotrophic food web as DOM (Bratbak et al., 1990; Wilhelm and Suttle, 1999; Miki & Jacquet, 2008).

Although the need to understand the spatial distribution of viruses in relation to the distribution of bacteria and phytoplankton was pointed out in the early 1990s (Cochlan et al., 1993), still only a few studies have focused on the simultaneous variation of viruses, bacteria, and small phytoplankton abundances so far (Bratbak et al., 1990; Rodriguez et al., 2000; Jacquet et al., 2002; Vrede et al., 2003; Anesio et al., 2004; Larsen et al., 2004; Goddard et al., 2005). In this study, for a year and a half we studied the distribution of viral, bacterial, and small phytoplankton communities in the surface waters (0–50 m depth) of the three largest French and/or occidental natural lakes, i.e., Lakes Annecy, Bourget, and Geneva. Our main objective was to investigate and understand the distribution of these microorganisms (especially viruses) and the existing relationships between these communities and their biological, chemical, and physical environments. With this goal in mind, bivariate and multivariate data analyses were used to analyze the relationships and interdependency among the variables and their relative weights.

Methods

Study sites

Lake Geneva is the largest western European lake. It is situated between France and Switzerland at an altitude of 372 m, with an area of 582 km^2, a maximum width and length of 13 and 72 km, respectively, and a volume of 86×10^9 m^3. The quality of the lake water has been monitored uninterruptedly since 1960. Before 1960, Lake Geneva

had an oligotrophic status, but it became eutrophic in the 1970–1980s. Following restoration programs, it has been reported to be mesotrophic in recent years. Lake Bourget is also located in eastern France (Savoie). It is the largest natural French lake situated at an altitude of 232 m, with an area of 44.2 km^2, maximum width and length of 3.5 and 18 km, respectively, and a volume of 3.6×10^9 m^3. This lake suffered from eutrophication until the mid 1980s, before an important policy program helped to restore this ecosystem. It can be viewed as having a similar status as Lake Geneva. Lake Annecy is also located in the eastern part of France (Haute-Savoie). It is the second largest lake located entirely within France, with an area of 28 km^2, a width of 3.2 km, a length of 14.6 km, and a volume of 1.2×10^9 m^3, for an altitude of 447 m. It has been reported to be oligotrophic since the late 1960s.

Sampling strategy

Samples were collected monthly twice from July 2002 to December 2003 for all three lakes. For Lake Geneva, the sampling station, which is the reference station known as SHL2 (46°27′12N; 6°35′39E) is located over the greatest depth of the largest basin of the lake (309 m). Eight samples were taken per date at depths of 2.5, 7.5, 10, 15, 20, 25, 30, and 50 m. Samples in Lake Annecy were taken at 3, 10, 15, 20, 25, 30, and 45 m in the center of the largest basin (45°52′36N; 6°09′87E), where the maximum depth is about 65 m. For Lake Bourget, samples were also carried out at the reference station, known as B (45°44′81N; 5°51′36E), located above the deepest point of the lake (i.e., 147 m) at the depths of 2, 6, 10, 15, 20, 30, and 50 m. We chose to sample this 0–50 m layer as fully representative of the autotrophic activity.

Physical, chemical, and chlorophyll *a* measurements

Concentrations in milligram per liter of dissolved orthophosphates ($P–PO_4$), nitrates ($N–NO_3$), ammonium ($N–NH_4$), silicates (SiO_2), and total organic carbon (TOC) were measured by the chemical laboratory of the Thonon hydrobiological station according to French normalized (AFNOR) protocols (http://thononin8.win3.hebergement.com/pages/public/index.html). The temperature (°C), dissolved oxygen concentration (mg l^{-1}), and fluorescence-based chlorophyll *a* (chl*a*) concentration (mg m^{-3}) were measured using a submersible multiparametric probe CTP 90 SST 24610 for Lakes Annecy and Geneva, and using a CTD SBE 19 Seacat profiler Seabird in Lake Bourget. Note that the two CTDs are inter-calibrated every year. The water column transparency was measured using a white 25-cm diameter Secchi disk.

FCM analysis

Autotrophic small eukaryotes, picocyanobacteria, heterotrophic bacteria, and viruses were counted using a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air cooled laser providing 15 mW at 488 nm. Note that such an analysis was performed only a few hours (<4 h) the same day after the sampling and samples were kept at <6°C in a cool box filled with ice packs during transfer and finally stored in a 4°C refrigerator in the laboratory. Viruses were fixed with glutaraldehyde (0.5% final concentration, grade I, Merck) for 30 min in the dark, then diluted in 0.02 µm filtered TE buffer (0.1 mM Tris–HCL and 1 mM EDTA, pH 8), and incubated with SYBR Green I (at a final 5×10^{-5} dilution of the commercial stock solution; Molecular Probes), for 5 min at ambient temperature, followed by 10 min at 75°C, and then another 5 min at room temperature, prior to FCM analysis (based on Brussaard, 2004 and modified by Jacquet unpubl.). Heterotrophic bacterial counts were performed on samples that had also been fixed with glutaraldehyde (0.5% final concentration) for 30 min, but the samples were then diluted in 0.02 µm filtered 50-m water sample, and incubated with SYBR Green I (10^{-4} dilution of the commercial stock solution) for 15 min. For photosynthetic cells (i.e., the picocyanobacteria and small eukaryotes) neither fixative nor fluorochrome were used since it is well known that fixation can induce considerable cell loss (Vaulot et al., 1989; Jacquet et al., 1998). Analysis was thus made on fresh samples in which we added a suspension of 1-µm beads (molecular probes). Flow cytometer Listmode files obtained were then transferred and analyzed on a PC using the custom-designed software CYTOWIN (Vaulot, 1989). More details about the FCM analysis and data treatment can be obtained elsewhere (Marie et al., 1999, 2000).

Statistical analysis

The normal distribution of the data was checked using the Kolmogorov–Smirnov test. Because not all the variables followed a normal distribution, we applied log transformation to meet the requirements for parametric statistics. Comparisons between the lakes for the different variables were done using a *t*-test, with a confidence level of 95% ($\alpha = 0.05$). PCA was performed on the whole dataset for each lake. The cluster analysis plot was carried out to describe the association of samples, and the principal component variable loading plot was computed. PCA analysis allowed us to identify seasonal and spatial modes of variability for each lake, then considering consistent periods, we could compute partial correlation coefficients. The PCA was used as an exploratory technique for each lake. Moreover, taking the pooled datasets from all three lakes together, bivariate analyses were used to describe global relationships between some variables, especially between bacteria and chlorophyll *a* or viruses. We used a linear regression analysis to make comparisons with relationships previously reported in the literature. All statistical analyses were performed using Statistica 6.0 software (Statsoft Inc., Tulsa, USA).

Results

Physical and chemical environments

Water column stratification occurred from July to September, with a thermocline extending between 10 and 20 m for Lake Annecy, and between 10 and 25 m for the other two lakes (Fig. 1A–C). Lower oxygenation concentrations were observed in the deeper water, but there was no marked deoxygenation in the upper 50 m (Fig. 1D–F). Higher concentrations of TOC were measured in Lake Bourget than in Lakes Annecy and Geneva with an annual mean value of 1.96 mg l^{-1} (SD ± 0.27), 1.80 mg l^{-1} (SD ± 0.32) and 1.23 mg l^{-1} (SD ± 0.34), respectively, for each lake (Fig. 1J–L). High values of TOC were also measured in Lake Annecy in December that we assumed to be of allocthonous origin. The mean and maximum depths of transparency were 7 and 10 m, 6.7 and 15 m, and 6 and 14 m for Lakes Annecy, Bourget, and Geneva, respectively (data not shown).

The annual cycle of $P–PO_4$ in Lake Annecy was different from that recorded in the other two lakes (Fig. 2A–C). *t*-Tests displayed that the mean $P–PO_4$ concentration measured in Lake Annecy was significantly lower than the values measured in Lakes Bourget and Geneva ($P < 0.001$). The $P–PO_4$ concentration in Lake Annecy was always below 0.005 mg l^{-1} in the upper 50 m, except between September and December, when concentrations could reach levels of up to 0.025 mg l^{-1} (annual mean ± SD: 0.002 ± 0.001). The seasonal patterns of $P–PO_4$ concentration were quite similar in Lakes Bourget and Geneva, with undetectable concentration of $P–PO_4$ between 0 and 45 m from May to December. However, $P–PO_4$ increased markedly during the destratification period, from December to April, reaching 0.025 mg l^{-1} in February and March in these two lakes (annual mean ± SD: 0.01 ± 0.001 and 0.01 ± 0.008 for Lakes Bourget and Geneva, respectively). In contrast with $P–PO_4$, there was no obvious seasonal cycle in Lake Annecy for the $N–NO_3$ concentrations which ranged from 0.1 to 0.4 mg l^{-1}. The highest values (i.e., 0.4 mg l^{-1}) were measured at depths between 20 and 50 m from October to December 2002, and from July to December 2003. The overall pattern of $N–NO_3$ levels in Lakes Bourget and Geneva over an annual cycle was similar to that of $P–PO_4$. For these two lakes, $N–NO_3$ concentrations ranged from 0.1 to 0.6 mg l^{-1} (Fig. 2D–F). A *t*-test also revealed that $N–NO_3$ concentrations were significantly lower in Lake Annecy ($P < 0.001$) than in Lakes Bourget and Geneva. Similarly, for $N–NH_4$ concentrations, Lake Annecy also displayed significantly lower concentrations than the other two lakes (Fig. 2G–I). $N–NH_4$ was generally below 0.01 mg l^{-1} in the top 50 m of all three lakes. Two $N–NH_4$ peaks were recorded in 2003 for both Lakes Bourget and Geneva in spring at around 10–15 m depth (probably in relation to zooplankton activity, not shown). For Lake Bourget, a deeper (>50 m) water peak was also observed in December 2003. SiO_2 concentrations ranged from 0.5 to 5, 0.5 to 3.5, and 0.5 to 1.5 mg l^{-1} in Lakes Annecy, Bourget, and Geneva, respectively (Fig. 2J–L). This nutrient increased gradually with depth, and for all three lakes SiO_2 patterns followed the seasonal water stratification (i.e., a decrease occurred throughout the stratification process).

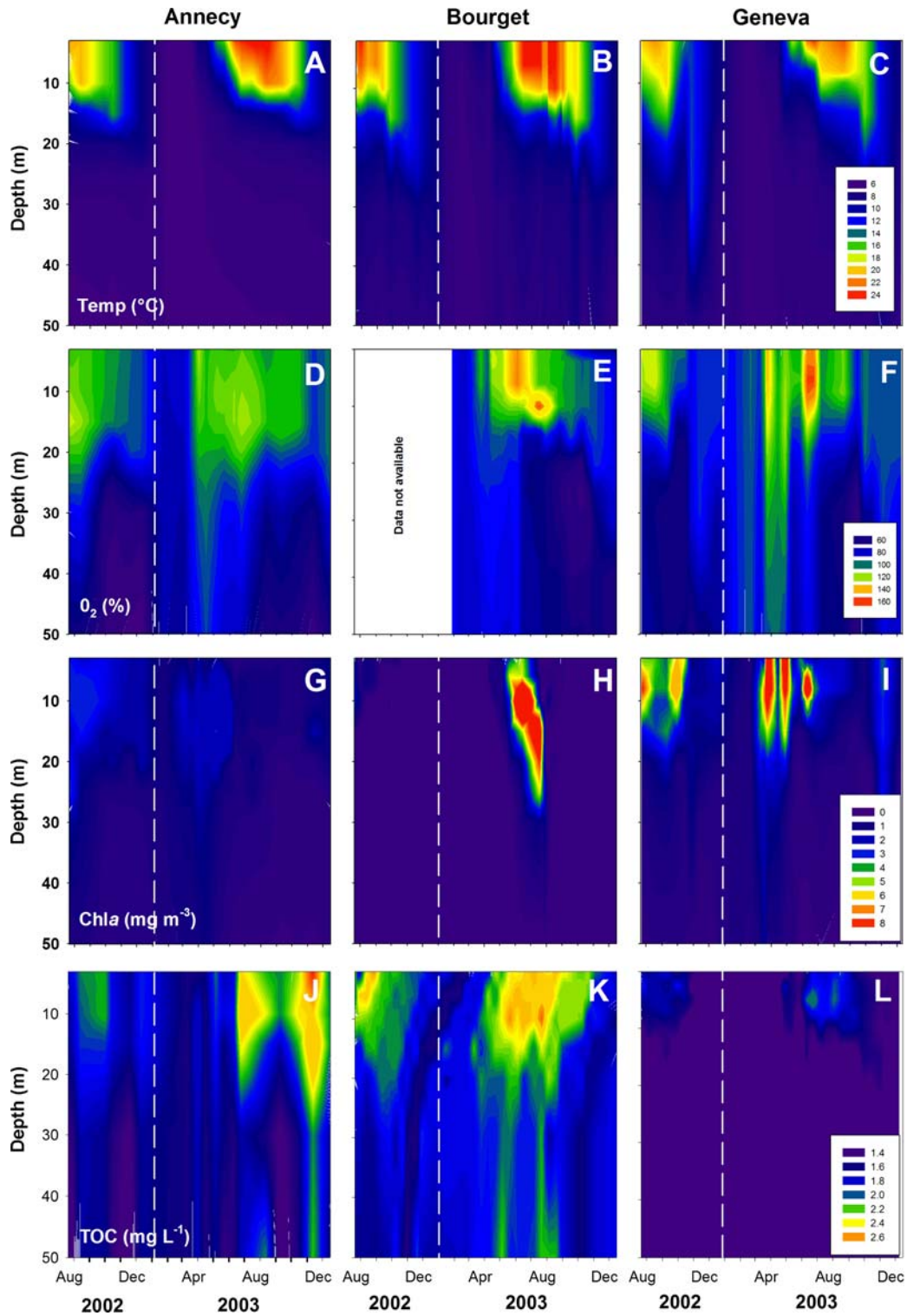

Fig. 1 Time series obtained for water temperature (**A**, **B**, **C**), oxygen saturation (**D**, **E**, **F**), chlorophyll *a* concentration (**G**, **H**, **I**) and total organic carbon (**J**, **K**, **L**) between August 2002 and December 2003 in the surface waters (0–50 m) of Lakes Annecy, Bourget, and Geneva. Data not shown were in fact not available due to O_2 probe problems

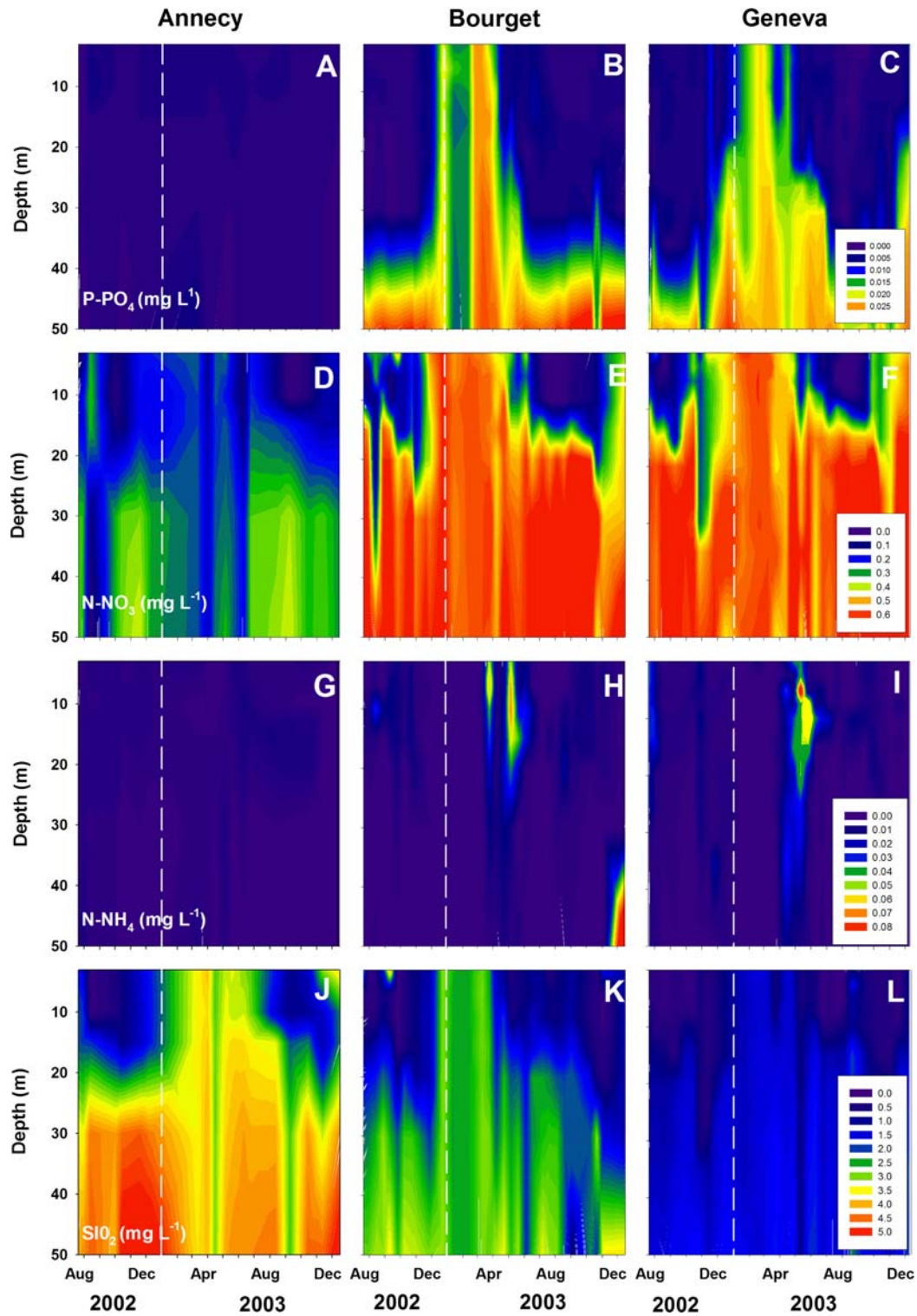

Fig. 2 Time series obtained for data sets for $P–PO_4$ (**A**, **B**, **C**), $N–NO_3$ (**D**, **E**, **F**), $N–NH_4$ (**G**, **H**, **I**) and SiO_2 (**J**, **K**, **L**) concentrations between August 2002 and December 2003 in the surface waters (0–50 m) of Lakes Annecy, Bourget, and Geneva

Distribution and development of the various microbial communities

Chl*a* concentrations ranged from 0 to 8 μg l^{-1} (Fig. 1G–I), and the maximum for each lake was always obtained during the stratified period and in the top 10–15 m. For Lake Annecy, concentrations were lower than in two other ecosystems ($P < 0.001$), with values varying between 2 and 3 μg l^{-1} in the 0–25 m surface layer during August–September 2002, and from March to June 2003. At the same depths, the Chl*a* concentration reached 3–8 μg l^{-1} for Lake Bourget (from June to August), and for Lake Geneva (but over a longer period, from August to October 2002 and from March to July 2003).

Picocyanobacteria (with two groups referred to as Picocyano1 & 2 for Lake Annecy), several pigmented eukaryotes, heterotrophic bacteria, and at least two virus populations for each lake (VLP1 & 2) were identified using FCM from either Chl*a* or PE fluorescence versus side or forward angle light scatter, as described by many authors before us (Marie et al., 1999, 2000; Li & Dickie, 2001; Castberg et al., 2001; Chen et al., 2001; Jacquet et al., 2002; Larsen et al., 2004; Goddard et al., 2005; Duhamel et al., 2006; Gobler et al., 2008). Typical FCM signatures are reported in Fig. 3.

Picocyanobacterial (PC) abundance ranged from 0 to 3×10^5 cell ml^{-1}, the highest concentrations being always observed in the top 20 m of the water column (Fig. 4A–C). For Lakes Bourget and Geneva, the highest values (3×10^5 and 7×10^4 cell ml^{-1}, respectively) were recorded during the stratification period, i.e., between May and October, in the top 15 m. For the other months and depths investigated, the mean abundance was 3×10^3 cells ml^{-1}. The comparison between the three lakes for this community revealed that Lake Annecy had significantly higher PC abundances than Lakes Bourget and Geneva, by a factor of 1.6 and 8.6, respectively ($P = 0.04$ and $P = 0.001$). Moreover, FCM analysis allowed us to clearly identify two distinct populations of picocyanobacteria (i.e., Picocyano1 and 2) in Lake Annecy (Fig. 3A). The first peak was mainly dominated by Picoyano1, with maximum concentrations of 1.6×10^5 cell ml^{-1} in the top 15 m in May 2003, while the second peak could be attributed to the second group, with 1.5×10^5 cell ml^{-1} in May to September 2003, and located deeper down, between 15 to 25 m. It is noteworthy that in February, March, and April 2003, the highest densities were observed at depths greater than 50 m.

Pigmented pico- and nanoeukaryotes, considered here as a single group, displayed concentrations ranging over three orders of magnitude depending on depth and season (i.e., between 7×10^1 and 4.8×10^4 cells ml^{-1}). The highest concentrations were recorded between 0 and 25 m from March to June 2003 in Lake Annecy, between 0 and 15 m from April to September in Lake Bourget, and to a lesser extent between 0 and 15 m from March to July 2003 in Lake Geneva (Fig. 4D–F). The mean abundance of these small, pigmented eukaryotes was significantly lower in Lake Geneva than in the other two lakes ($P < 0.01$).

In Lake Annecy, the highest concentrations of heterotrophic bacteria (3 to 4×10^6 cells ml^{-1}) were recorded in the top 25 m from July to December in 2002 and in 2003. In the two mesotrophic lakes, large increases in heterotrophic bacteria abundance were observed in spring, summer, and early autumn (Fig. 4G–I). In Lake Bourget, the heterotrophic bacterial distribution displayed four main periods of high bacterial densities with concentrations exceeding 5×10^6 cells ml^{-1}, in the top 15–20 m (August 2002, March–April 2003, May–July 2003 and August–October 2003). The same kind of pattern was observed in Lake Geneva where the highest abundances $>5 \times 10^6$ cells ml^{-1} were also found in the top 15–20 m (August–September 2002, March 2003, April–May 2003, July–October 2003). On average, the highest bacterial concentrations were observed in Lake Bourget (2.7×10^6 cells ml^{-1}), followed by Lake Geneva (2.6×10^6 cells ml^{-1}), whereas Lake Annecy (2.2×10^6 cells ml^{-1}) had significantly lower heterotrophic bacterial abundances ($P < 0.01$).

FCM allowed us to distinguish up to four viral groups but only two throughout the year. These two groups (Figs. 4J–L, 5) were referred to as VLP1 and VLP2 (virus-like particles, groups 1 and 2). The concentration of the most abundant group, VLP1, ranged from 2×10^7 to 2×10^8 particles ml^{-1}. The concentration of VLP2 was always lower, and ranged from 2×10^6 to 2×10^7 particles ml^{-1}. Like those of bacteria, the abundances of VLP1 in Lake Annecy were significantly lower than in the two other lakes ($P < 0.01$). In Lake Annecy, viral densities ranged

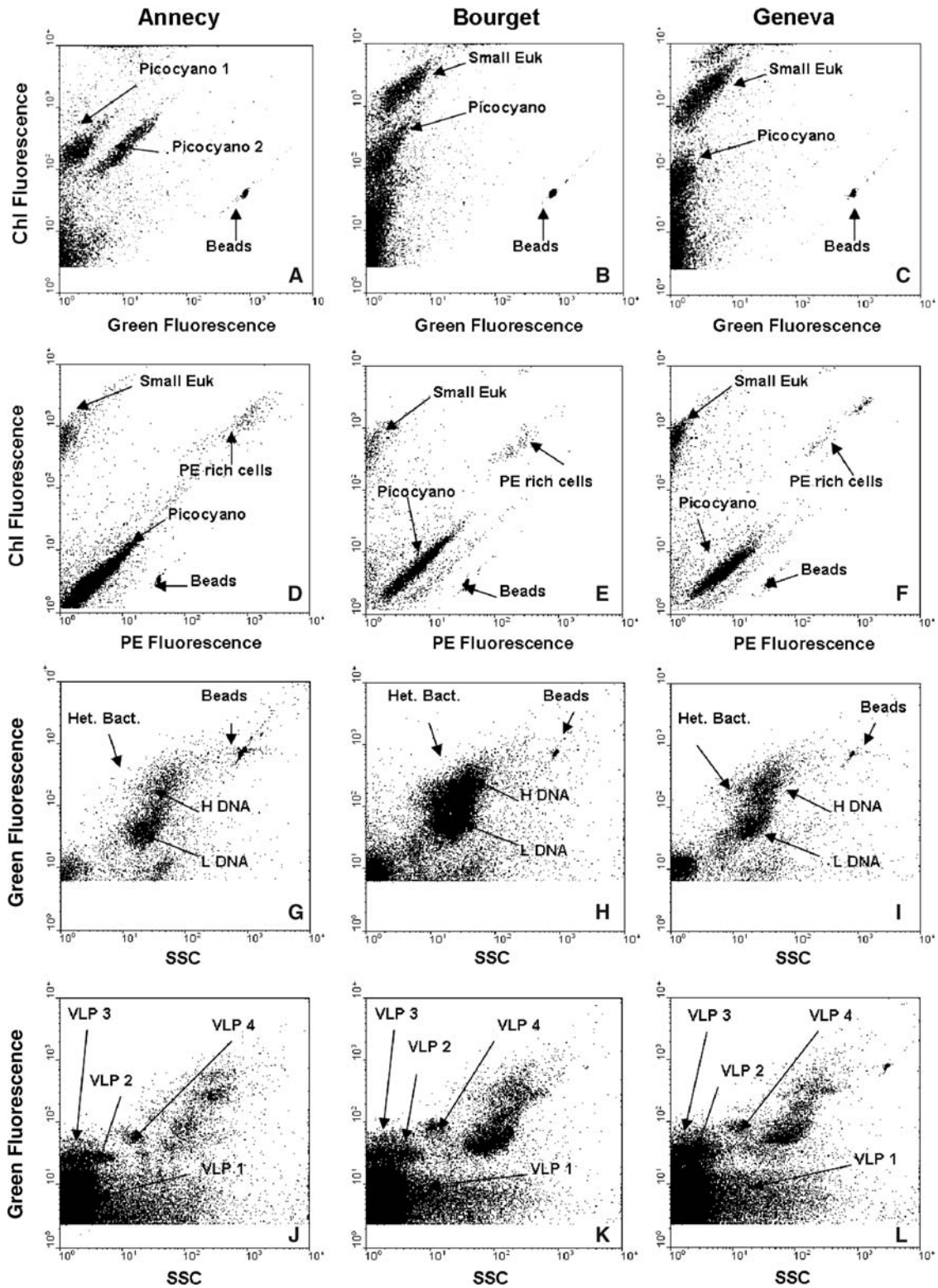

Fig. 3 Typical FCM signatures of the picocyanobacterial (**A**, **B**, **C**), small eukaryotic (**D**, **E**, **F**), bacterial (**G**, **H**, **I**) and viral communities (**J**, **K**, **L**) in the surface waters of Lakes Annecy, Bourget, and Geneva

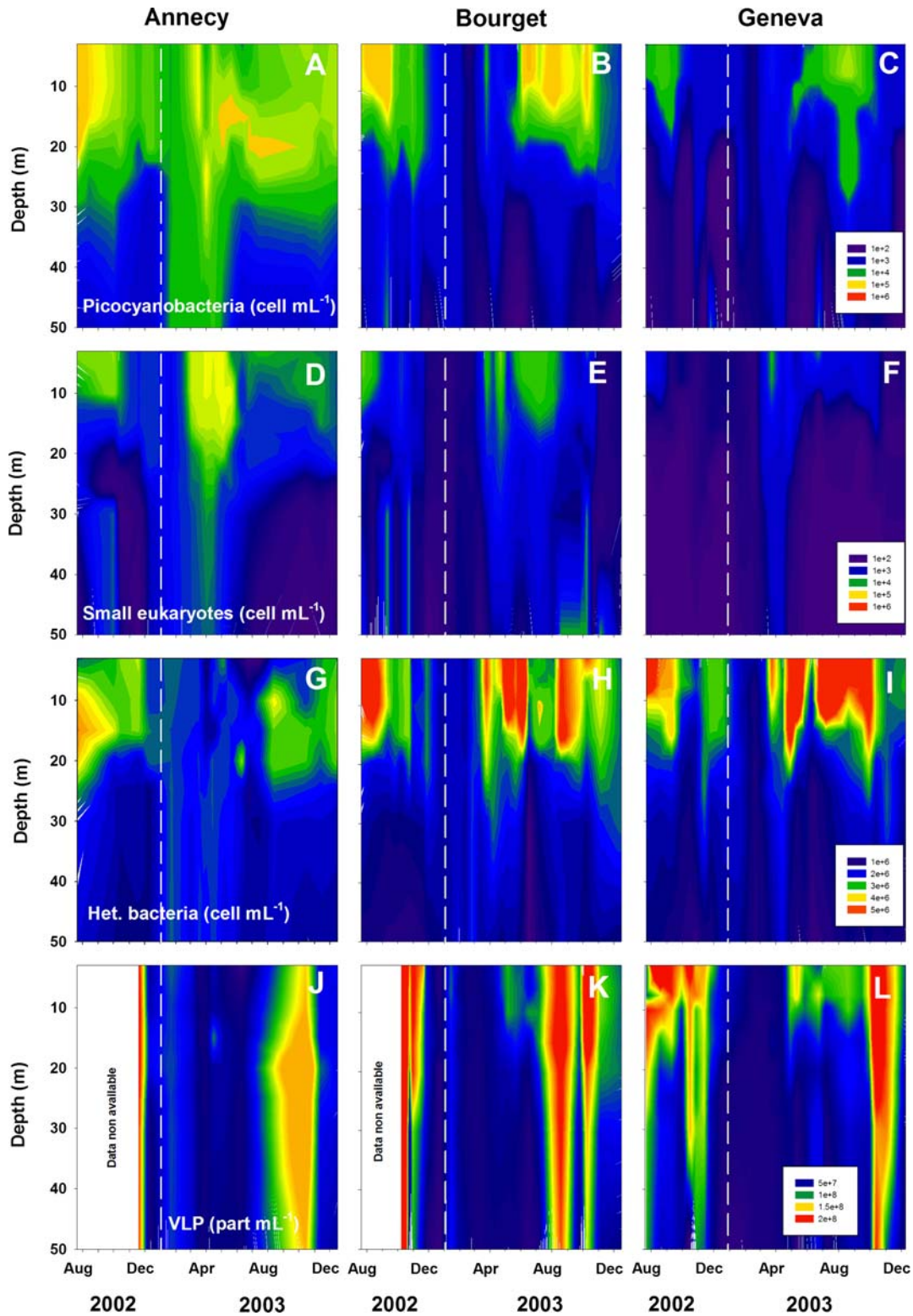

Fig. 4 Development of picocyanobacteria (**A**, **B**, **C**), small eukaryotes (**D**, **E**, **F**), heterotrophic bacteria (**G**, **H**, **I**) and virus-like particles (**J**, **K**, **L**) between August 2002 and December 2003 in the surface waters (0–50 m) of Lakes Annecy, Bourget, and Geneva

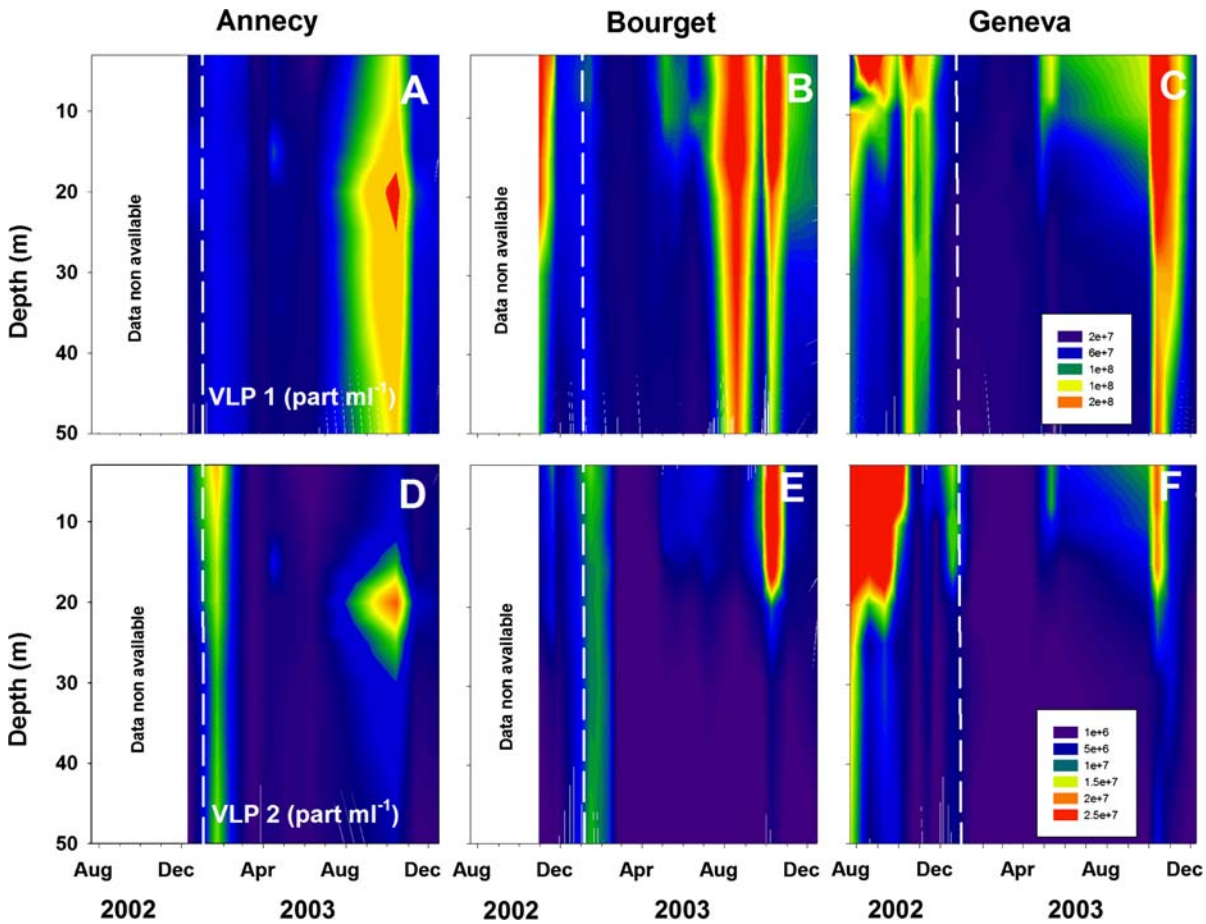

Fig. 5 Development of virus-like particles VLP 1 (**A**, **B**, **C**) and VLP 2 (**D**, **E**, **F**) between August 2002 and December 2003 in the surface waters (0–50 m) of Lakes Annecy, Bourget, and Geneva

from 2×10^7 to 8×10^7 particles ml^{-1}, the highest densities being recorded between August and October 2003. In Lake Bourget, VLP1 abundance reached its three highest values in October 2002, August 2003, and October 2003, respectively, with concentrations exceeding 2×10^8 particles ml^{-1} between 0 and 50 m, whereas the highest abundances of VLP2 (2×10^7 particles ml^{-1}) occurred only in October (2002 and 2003) between 0 and 20 m. Similarly, in Lake Geneva, the two highest abundances of VLP1 were recorded in early autumn (October 2002 and 2003), with concentrations exceeding 2×10^8 particles ml^{-1} in the top 50 m. VLP2 viruses reached their maximum densities in late summer and early autumn (August–October 2002 and October 2003), with 2×10^7 particles ml^{-1} observed between 0 and 20 m.

Relationships between variables

The VBR, an indicator of the relationship between viral and bacterial communities, always indicated the numerical predominance of viruses over bacteria. For each of the lakes, the mean values calculated for each month ranged from 20 to 87 (mean: 36), 15 to 99 (mean: 51), and 16 to 114 (mean: 40), respectively (Table 1). Although the highest ratios were found in the two mesotrophic lakes (Bourget and Geneva), statistical analysis did not reveal any significant difference between the lakes. In contrast, clear seasonal changes in the VBR were recorded. The highest ratios were found in early autumn, and significant differences between VBR values were measured in spring, summer, autumn, and winter ($P < 0.05$). During summer and especially during the

Table 1 Mean values (out of 309 samples) in 2003 of virus–bacteria ratios per month and per depths for the three lakes

2003	Lake Annecy		Lake Bourget		Lake Geneva	
	VBR	SD	VBR	SD	VBR	SD
Months						
January	35.6	1.2	45.2	11	22.8	7.5
February	32.8	6	25.3	9	27.9	3
March	23.8	6.4	15	5	16.1	5.3
April	26.5	16.4	20.7	4	19.9	6.5
May	20.8	9.8	21.7	4	22.5	7.1
June	19.3	4.1	27.8	15	25.7	11
July	24.4	24	35.8	7	20.8	8.7
August	25.4	12.3	74.6	39	31.8	7.1
September	30	7.9	27.7	3	25.3	7.7
October	87.3	23.6	98.7	19	113.7	36.5
November	32.2	8.2	34.9	1	73.2	2.7
December	28.3	8.1	37.4	4	29.3	3.2
Depths						
<3 m	29.5	16.3	29.5	15.2	29	23.4
6–7.5 m	27.6	15.3			28.4	23.4
10 m	30.6	22.1	31.7	22	27.5	21.7
15 m	29.6	18.9	32.1	21.5	28.7	22.5
20 m	33.4	23	31.8	21.3	33	27.6
25 m			35.7	24.6	35.1	29.8
30 m	36.4	30	40.3	33.2	34	24
50 m	36.5	28.2	40.3	27.6	43.4	44.7

VBR Virus to bacteria ratio, *SD* standard deviation

autumn, the VBR clearly increased. The highest VBR was always observed in October in all three lakes, with high values (>80) recorded both in 2002 and 2003. Considering the dataset as a whole (all three lakes together), we noted an inverse relationship between the VBR and bacterial abundance ($P < 0.01$, $n = 423$, $r = -0.25$), with the highest VBR values being recorded at times of relatively low bacterial abundance. This has been reported in many studies for both marine and freshwater ecosystems (Jiang & Paul, 1995; Maranger & Bird, 1995). In addition, highest VBRs were recorded at depths greater than 20–30 m for the three lakes (Table 1). Indeed, between 0 and 20 m, VBR annual mean values were about 29.3, 31.2, and 30.1 in Lakes Geneva, Bourget and Annecy, respectively. These values reached 36.3, 37, and 35.4 between 20 and 50 m.

We used linear regression to describe the relationships between bacteria and the other parameters (Table 2). Based on the analysis of all the data (for the three lakes), we found a significant linear relationship between bacteria and chlorophyll *a* concentrations (Eq. 1, Table 2). We did not find any significant correlation between total viral abundance (VLP) or VLP1 and chlorophyll *a* concentrations. In contrast, VLP2 was observed to be significantly linked to Chl*a* and PC concentrations (Eqs. 6 and 7, Table 2). Significant relationships between VLP2 and Chl*a* or PC could be confirmed separately for Lakes Bourget and Geneva, but not for Lake Annecy. The abundance of the free viruses was observed to increase with increasing heterotrophic bacterial concentrations (Eqs. 2 and 3, Table 2), and quite similar relationships were obtained for VLP and VLP1. Very similar equations were obtained (Eqs. 2 and 3, Table 2) independently, for each lake (slopes from 0.565 to 0.596 and intercept from 9.361 to 9.876), suggesting that this relationship remains constant from one lake to another.

Using PCA, we investigated the dataset obtained for each lake, the three eigenvalues (Table 3) accounted for the explanation of 79.6, 76.7, and 69.1% of variance of the data for lakes Geneva, Bourget, and Annecy, respectively. The first mode of variability for all three lakes was "depth," which accounted for 50.9, 51.4, and 33.4% of the overall variability for Geneva, Bourget, and Annecy, respectively. The first axis was able to discriminate between the "hypolimnion" and the other depths. This analysis exhibited a similar association of the parameters on the axis 1 for Lakes Bourget and Geneva. This axis opposed, on one hand, "P–PO_4" concentrations and, on the other hand, "temperature-TOC-Picocyanobacteria" which were associated with viruses and bacteria. We noticed the marked impact of the structuring summer stratification characterized by high productivity and the progressive depletion of P–PO_4 concentrations in the epilimnion, whereas the hypolimnic zone remained rich in dissolved nutrients. Interestingly, a different projection was obtained for Lake Annecy. Although the axis reflected the structuring effect related to depth, the projection of the variables was different from that obtained for Lakes Geneva and Bourget. Indeed, P–PO_4 concentrations were associated with high abundances of viruses, heterotrophic bacteria, and picocyanobacteria, highlighting the importance of this element as a limiting factor in the productivity in this oligotrophic lake.

Table 2 Slope values of the regression equations obtained for significant linear regressions ($P < 0.05$) relating heterotrophic bacterial (bact) abundances (cell ml^{-1}), chlorophyll *a* (chl*a*) concentrations ($\mu g\ l^{-1}$), total viruses (VLP), VLP1 and VLP2 abundances (particles ml^{-1}), picocyanobacterial (PC) abundances (cell ml^{-1})

Values obtained for this study were calculated from data of the 3 lakes

Regression equations	Authors	Slope (*a*)	*N*
1. Log [bact] = *a* Log [chl*a*] + *b*	Shortreed & Stockner (1986)	0.56	18
	Currie (1990)	0.33	361
	Del Giorgio & Peters (1993)	0.27	20
	Tzaras & Pick (1994)	0.24	26
	Gasol et al. (1995)	0.30	74
	Gasol et al. (1995)	0.59	18
	Del Giorgio and Scarborough (1995)	0.32	24
	Zinabu & Taylor (1997)	0.59	52
	Zinabu & Taylor (1997)	0.34	24
	Jeppesen et al. (1997)	0.29	>100
	Bouvy et al. (1998)	0.58	93
	This study	0.33	507
2. Log [VLP] = *a* Log [bact] + *b*	Maranger & Bird (1995)	0.16	27
	Guixa-Boixereu et al. (1999)	0.83	149
	Guixa-Boixereu et al. (1999)	0.92	580
	This study	0.61	423
3. Log [VLP1] = *a* Log [bact] + *b*	This study	0.57	385
4. Log [VLP1] = *a* Log [PC] + *b*	This study	0.15	486
5. Log [VLP2] = *a* Log [bact] + *b*	This study	0.87	417
6. Log [VLP2] = *a* Log [PC] + *b*	This study	0.13	376
7. Log [VLP2] = *a* Log [chl*a*] + *b*	This study	0.28	356

Table 3 Eigenvalues, total and cumulative % of variance in the factor analysis

Factor	Eigenvalues	% Of total variance	Cumulative eigenvalue	Cumulative %
Annecy				
1	3.01	33.43	3.01	33.43
2	1.88	20.89	4.89	54.32
3	1.33	14.82	6.22	69.13
Bourget				
1	5.14	51.37	5.14	51.37
2	1.42	14.16	6.55	65.53
3	1.12	11.16	7.67	76.69
Geneva				
1	5.09	50.94	5.09	50.94
2	1.75	17.54	6.85	68.48
3	1.11	11.13	7.96	79.61

The second mode of variability for all three lakes appeared on axis 2, and made it possible to pick out key periods in the variation of abundances of the microbial communities in the epilimnion. Different periods could be spotted, and corresponded to specific community structures (Fig. 6), especially in the two mesotrophic ecosystems. Indeed, for Lakes Bourget and Geneva, the PCA made it possible to clearly distinguish an 'end summer–early autumn' period during which viral abundances were highest, and occurred concomitantly with a reduction in the abundance of bacteria.

Discussion

The succession observed in the abundance of the different communities revealed the same general pattern in all three lakes. Some specific features depending on the trophic status of the lake could, however, be recorded. Even though the three systems studied are not characterized by a large difference in regards to their trophic status, the analysis of our dataset using PCA clearly highlighted the impact of reduced nutrient concentrations on the functioning of microbial communities in the more oligotrophic system. In particular, the importance of $P–PO_4$ as a productivity-limiting factor emerged clearly. In agreement with previous studies, which reported the

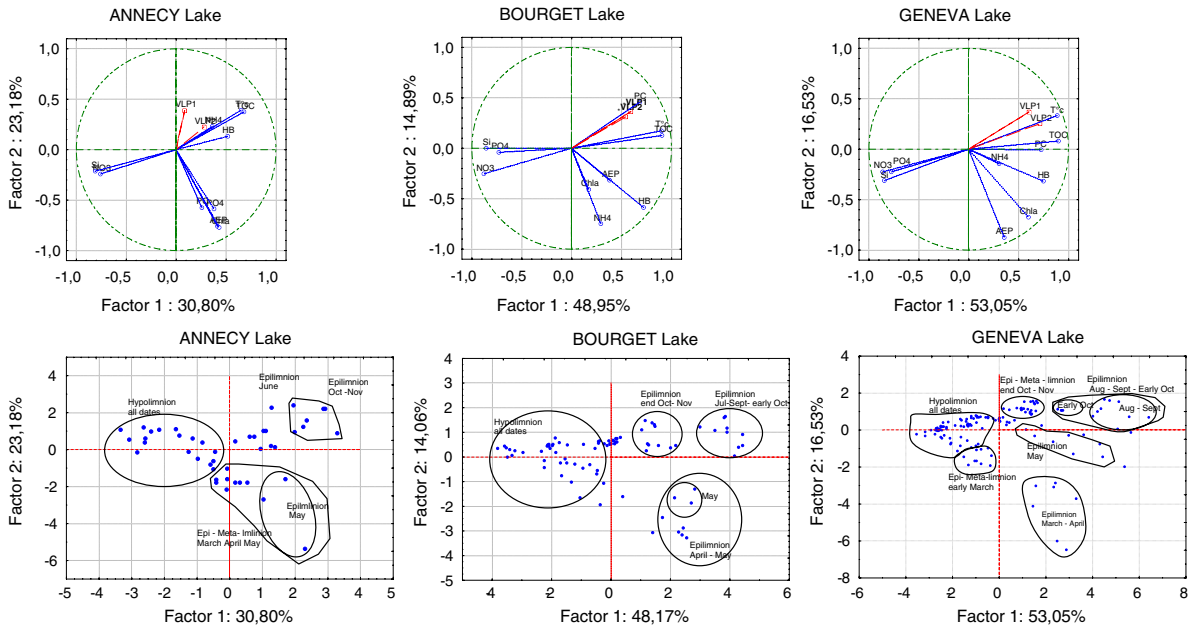

Fig. 6 Plot of principal components 1 versus 2: principal component variable loading plots are shown for each lake (**A**) displaying the projection of the variables onto 1 the factor plane 1 × 2. The second series of loading plots (**B**) represents the scores of the samples (samples characterized by the depth and date of sampling) on the plane defined by components 1 and 2. Spatial and temporal clusters are represented

increase of viruses and heterotrophic bacteria densities in productive and nutrient-rich environments (Gasol & Duarte, 2000; Wommack & Colwell, 2000), we observed higher abundances of heterotrophic bacteria and viruses in the two mesotrophic lakes. These lakes displayed almost similar distributions for chlorophyll *a*, small pigmented eukaryotes, picocyanobacteria, heterotrophic bacteria, and VLPs, in contrast to Lake Annecy where we could observe differences in the microbial and viral dynamics. In this oligotrophic lake, heterotrophic bacteria displayed a single major development peak, mainly during summer, whereas several periods of high bacterial densities were observed in the two mesotrophic lakes, in spring, summer, and autumn. Picocyanobacteria were more abundant in Lake Annecy and dominated the picophytoplankton for a rather long period, from spring to the end of autumn. It is now well known that when nutrients constitute a limiting factor, autotrophic picoplankton (APP) cells can compete against bigger phototrophic organisms, so it is not surprising that APPs were particularly abundant in this lake (Raven, 1986; Callieri, 2007). More interestingly, FCM analysis allowed us to discriminate two PC groups in Lake Annecy, in contrast to both Lakes Bourget and Geneva, in which there was only one group. We observed that picocyanobacteria peaked earlier in Lake Annecy, as soon as nutrients increased before the stratification period (spring), whereas this did not occur until the stratification period in both Lakes Bourget and Geneva. The relative importance of factors regulating PC abundance (temperature, vertical mixing, nutrients, etc.) seemed to vary according to the trophic status of the lake. We did not observe the clear bimodal pattern with a first peak in spring and a second one in autumn, as has been reported for many other aquatic systems by Weisse & Kenter (1991); Callieri & Pinolini (1995); Padisak et al. (1997) or again Callieri and Stockner (2000). Similarly to the observations of Crosbie et al. (2003) in Lake Mondsee (Austria), we could suggest that physical factors such as mixing, and especially the weak stratification in March/April, may explain the absence of the spring peak in Lakes Bourget and Geneva. A viral lysis influence could also be suspected (see below).

The patterns for heterotrophic bacterial abundances paralleled those of the pico- and nanophytoplankton populations, suggesting that PC and EAP could provide significant resources (via DOC exudation,

cell fragments, etc.) for heterotrophic bacterial growth (Cole et al., 1988). PCA was able to distinguish these 'key' periods in the abundances of microbial communities. The partial correlations carried out for these definite periods exhibited that, depending on the period and on the trophic status of the lake, TOC was linked to the levels of picocyanobacteria, small eukaryotes, or total chlorophyll *a* concentrations suggesting changes in the origin and quality of the resources available for heterotrophic bacteria. In the oligotrophic Lake Annecy, the TOC concentration was always correlated to PC concentrations.

As regards virioplankton, lower abundances were recorded in Lake Annecy but the magnitude of seasonal variations in this lake was far greater than the inter-lake variations. A quite similar seasonal pattern was observed for the virioplankton in all three lakes, with the greatest viral abundance occurring in fall and at depth. The highest abundances of virioplankton were generally observed in the top 20 m of the water column, except during autumnal periods when the virioplankton concentration was also high and fairly uniform throughout the water column. This viral peak followed the increase in bacterial abundance at the same period as reported in other ecosystems. It was previously reported in seawater (Bergh et al., 1989), the Chesapeake Bay (Wommack et al., 1992), the Tampa Bay (Cochlan et al., 1993), the Northern Adriatic Sea (Weinbauer & Peduzzi, 1995), the backwater system of the Danube River (Mathias et al., 1995), the alkaline and hypersaline Mono lake (Brum et al., 2005), and in Norwegian coastal waters (Bratbak et al., 1996). The particularly high magnitude of these autumnal peaks could be linked to the larger initial virioplankton and bacterioplankton populations (summer) at the beginning of the autumn bloom, as suggested by Wommack & Colwell (2000). Moreover, the important concentrations of viruses found in early autumn could also be attributed to low decay rates in surface waters at that time, typically due to a lower negative effect of solar radiations. In addition, such high values, especially for VLP1, recorded throughout the water column were clearly associated with water column mixing, probably because bacteria were stimulated by both organic and inorganic nutrients.

Significant seasonal co-variations were noted for viruses and heterotrophic bacteria. Summers, and especially early autumns, were clearly characterized by high VBR. At least, higher VBR values recorded in deeper waters could also suggest a higher impact of viruses on bacteria compared to surface waters, as demonstrated elsewhere (Weinbauer & Hofle, 1998; Colombet et al., 2006). VBR values we reported were among the highest found in the literature. It is noteworthy here that our study is one of the rare where VBR values were obtained and provided with FCM, while other studies mainly used epifluorescence microscopy (EFM). As FCM allows discriminating unambiguously bacteria from viruses, it is not impossible that VBR reported by past using EFM underestimated this parameter (Marie et al., 1999). The magnitudes of seasonal variations in VBR were considerably higher than differences observed between the three lakes, suggesting seasonality in virioplankton production. Seasons in which a high VBR occurred could be expected to display greater virioplankton production and more bacterioplankton lysis. It is clear that VBR is only an indicator used to investigate possible relationships between viruses and their bacterial or phytoplankton hosts, so that it is clearly not sufficient to infer the importance of the role (lytic effects) played by the viruses. As described by many authors (Wilson & Mann, 1997; Weinbauer, 2004; Abedon, 2006), lysogenic, pseudoysogenic, and chronic cycle might be a different way of life for viruses and we recently measured that lysogeny can be high indeed (Thomas et al., unpublished). It is also noteworthy that experiments conducted in the three lakes from 2004 to 2006 clearly revealed that viruses may be an important mortality factor for both bacteria and picocyanobacteria, especially in autumn (Sime-Ngando et al., 2008; Personnic et al., unpublished)."

Based on side scatter and green DNA dye complex fluorescence, FCM data allowed the clear discrimination of two groups of viruses. The abundance of VLP1 was found to be 10- to 100-fold higher than the abundance of VLP2. Published works by different authors (Marie et al., 1999; Wommack & Colwell, 2000; Larsen et al., 2001; Culley & Welschmeyer, 2002; Payet & Suttle, 2008) indicate that most of what we called the VLP1 group is likely to consist of bacteriophages. Our data also suggest that most VLPs were phages. Using bivariate analysis, we found a power slope of 0.61 and 0.57 between heterotrophic bacteria and VLP or VLP1, respectively (considering the whole dataset). In the same way, we could suggest that VLP2 were probably linked to either

picocyanobacteria and/or small eukaryotes, as also proposed by Payet & Suttle (2008). Typically, VLP2 were observed to be significantly linked to picocyanobacteria in Lakes Bourget and Geneva ($r = 0.48$ and $r = 0.68$, respectively, $P < 0.05$), whereas this kind of relationship was only found for Lake Annecy when the distinction was made between the two PC groups ($r = 0.51$, $P < 0.05$ between VLP2 and PC1). Clearly and very interestingly, the second PC group observed in Lake Annecy did not seem to be related to VLP2. Also, we did not find any significant correlation between viral abundance (all VLPs) or VLP1 and chlorophyll *a* concentrations, suggesting that phytoplankton viruses do not contribute significantly to the total virus pool, and also that the positive effect of an increase in chlorophyll *a* on heterotrophic bacteria is not directly beneficial to virus production. However, we are aware that chlorophyll *a* represents only a crude approximation of the algal biomass and thus is probably not the best parameter to use in attempting to identify virus/parasite relationships (Gasol & Duarte, 2000). Moreover, the specificity of viruses to their hosts implies that phytoplanktonic groups must also be taken into consideration. The discrimination, using FCM analysis, between two groups of PC in the oligotrophic lake confirmed this point.

As pointed out above, we could observe up to four different viral groups using FCM. Other studies also reported such a discrimination (e.g., Castberg et al., 2001; Chen et al., 2001; Jacquet et al., 2002; Goddard et al., 2005; Duhamel et al., 2006). Unlike Larsen et al. (2004), for instance, we were not able to differentiate and observe a full seasonal cycle of other viral groups, such as VLP3 and VLP4. In this study, we only observed VLP4 during the spring period, and it was possible to correlate their abundances to the eukaryotic phytoplanktonic community (diatoms and/or chlorophytes), but not to cyanobacteria and heterotrophic bacteria (not shown). We could confirm that VLP4 was indeed associated to phytoplankton in Lake Geneva (Duhamel et al., 2006) as Goddard et al. (2005) also noticed in another freshwater ecosystem. This will deserve more attention in forthcoming years when interesting, in Lakes Annecy, Bourget and Geneva, of virus-algal interactions.

The objective of this study was to understand the variations in abundance of picophytoplankton, bacteria and viruses in the upper 50 m in three peri-alpine lakes in relation to a variety of environmental factors. We used bivariate and multivariate data analyses to obtain interdependencies among the different variables and could propose some key relationships between various parameters. Freshwater viral ecology is in need for data from natural environments, preferably covering not only standing stocks of viruses and potential hosts, but displaying also actual production rates and describing mechanisms underlying relationships between viruses and hosts. The discrimination of different viral groups by FCM opens various possible ways of investigation, especially based on the combination of methods (cell sorting, molecular analysis, PFGE as example, isolation and infection tests). This study focussed mainly on the description of the variation of microbial communities abundances in Lakes Annecy, Bourget, and Geneva but the interested reader will also find results dealing with the functional role (as mortality agents) of the prokaryotic viruses for these lakes in other studies of our group (Jacquet et al., 2005; Duhamel et al., 2006; Sime-Ngando et al., 2008; Personnic et al., unpublished).

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