Seasonal variations of microbial abundances and virus- versus flagellate-induced mortality of picoplankton in three peri-alpine lakes

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Viruses and small heterotrophic flagellates are known to exert an important control on bacterial populations. In parallel with the study of picoplankton dynamics (abundance and distribution) in surface waters (0–50 m) of Lakes Geneva, Bourget and Annecy, we used a dilution technique during different seasonal periods in order to assess flagellate- versus virus-induced mortality of heterotrophic bacteria, picocyanobacteria and small eukaryotic phytoplankton. Although it was not always possible to detect a significant viral effect (typically in winter), viral lysis and protozoan grazing could be responsible for up to 71% of the bacterial mortality (in summer). Viral impact, considered alone, never equalled or exceeded predation for heterotrophic bacteria, but could for picocyanobacteria, typically in autumn. In addition, during summer, complex interactions between grazing- and virus-induced mortality of bacteria (e.g. synergism versus antagonism) could be highlighted (for instance with bacterial lysis susceptible to enhance picocyanobacterial growth).

The temporal variations observed for experimental viral parasitism and flagellate predation were consistent with the in situ dynamics and statistical relationships found between the targeted communities. This study thus provides new evidence on the critical role played by viruses and small flagellates in the functioning of freshwater microbial food webs and also that these mortality processes vary strongly throughout the seasons.

INTRODUCTION

In aquatic microbial ecology, the term picoplankton refers to a complex community of small size microbes (0.2–2 μm), including picophytoplankton (i.e. tiny autotrophs) and heterotrophic planktonic groups (i.e. mainly bacteria but also archaea and small eukaryotes). The picophytoplankton, composed of picocyanobacteria (e.g. Synechococcus like and Prochlorococcus) and picoeukaryotes, may contribute substantially to both total phytoplankton biomass and production in aquatic ecosystems (Campbell and Vaulot, 1993; Li, 1994; Bustillos-Guzman et al., 1995; Binder et al., 1996; Buck et al., 1996; Campbell et al., 1998; Callieri and Stokner, 2002), especially in oligotrophic waters where they can account for up to 90% of the total photosynthetic biomass and carbon production (Li et al., 1983; Campbell et al., 1994; Jardillier et al., personal communication). It is now recognized that the bacterio-plankton play a substantial role comparable to that of the primary producers in terms of element cycling in...
the water column (Williams, 1981; Kirchman et al., 1982). The roles of heterotrophic bacteria as decomposers but also as producers of biomass have been well established since the emergence of the "microbial loop" concept (Azam et al., 1983; Fenchel, 2008).

Heterotrophic nanoflagellates have been recognized as the main consumers of suspended or attached bacteria (Fenchel, 1986; Sanders et al., 1992; Boenigk and Arndt, 2002) and of picophytoplankton (Campbell and Carpenter, 1986; Hagström et al., 1988; Sherr and Sherr, 2002) in a variety of aquatic systems. During the last two decades, a significant body of results has revealed that viruses may also control bacterial and phytoplankton abundance through high lytic activities in both marine and limnetic environments. There are still only a few data published about the dynamics of viral communities and their potential hosts in aquatic ecosystems, especially in freshwaters (Bettarel et al., 2003; Goddard et al., 2005; Pradeep Ram et al., 2005; Duhamel et al., 2006; Personnic et al., 2009). Available data have generally revealed a tight coupling between viruses and bacteria, suggesting a close relationship between these two communities (Bratbak et al., 1990; Maranger and Bird, 1995; Weinbauer and Peduzzi, 1995; Sime-Ngando et al., 2003; Weinbauer, 2004), while such a relationship between viruses and phytoplankton is rarer or detectable only when bloom situations occur (Jacquet et al., 2002; Brussaard, 2004). Viral lysis may be responsible for 10–60% of daily bacterial production removal (reviewed in Weinbauer, 2004; Jacquet et al., 2005), while such a percentage of virally induced mortality can reach 100% in the context of microalgal blooms (Jacquet et al., 2002; Brussaard, 2004; Nagasaki, 2008). Whatever the importance of these viruses in killing bacterial hosts, a process which may vary considerably with time (Pradeep Ram et al., 2005), space (Seymour et al., 2006) and host presence and activity (Peduzzi and Schiemer, 2004), viruses have also been shown to play a key role in modifying, sustaining and structuring bacterial diversity (Weinbauer and Rassoulzadegan, 2004). Finally, it is important to note that the viral activity is highly dependent on life strategies (i.e. mainly lytic versus lysogenic). However, the different viral life cycles and the real impact of viruses on bacteria and phytoplankton remain complex to study especially when one knows that they are likely to vary dramatically within short time and spatial scales (Thomas et al., submitted for publication).

Recently, Pernthaler (Pernthaler, 2005) and Miki and Jacquet (Miki and Jacquet, 2008) reminded that the interplay between viruses and flagellates in the control of aquatic prokaryotes is still poorly understood, with, on one hand, evidence that viral infection and lysis may be favoured by intense protozoan grazing (Sime-Ngando and Pradeep-Ram, 2005; Jacquet et al., 2007; Weinbauer et al., 2007; Pradeep-Ram and Sime-Ngando, 2008) while, on the other hand, protists can be the targets for viral attack (Garza and Suttle, 1995; Massana et al., 2007) or potential feeders on viruses (Gonzales and Suttle, 1993; Bettarel et al., 2005).

In this study, simultaneous monitoring and experiments were carried out in order to assess the impact of both viruses and flagellated predators on heterotrophic bacterial and picocyanobacterial mortalities in lakes Annecy, Bourget and Geneva. In situ experiments used the modified dilution technique of Evans et al. (Evans et al., 2003) on 14 occasions corresponding to different seasons of the year. Microcosms were used and incubated in situ for 48 h, consisting of a series of dilutions of <11 μm raw water. Such an approach was initially introduced by Landry and Hassett (Landry and Hassett, 1982) and refined by Landry et al. (Landry et al., 1995) in order to quantify grazing of phytoplankton by microzooplankton. Evans et al. (Evans et al., 2003), then Jacquet et al. (Jacquet et al., 2005) and Tijdens et al. (Tijdens et al., 2008) proposed some modifications of this approach in order to estimate viral lysis in small eukaryotes (i.e. Micromonas), cyanobacteria or heterotrophic bacteria, in either coastal marine or lake waters but only for restricted periods of the year. Our aim is to extensively apply the dilution method during different seasons of the year, for comparative assessment of virus-induced and protozoan grazing mortality of various microbial communities (i.e. heterotrophic prokaryotes, picocyanobacteria and small eukaryotes) in three different freshwater lake ecosystems.

METHOD

Study site

Lake Geneva is located on the border between France and Switzerland. It is the largest natural western European lake situated at an altitude of 372 m, with an area of 582 km², a maximum width, length and depth of 13 km, 72 km and 310 m respectively, and a volume of app. 86 × 10⁹ m³. Lake Bourget is the largest natural French lake, located in the Eastern part of France, at an altitude of 232 m, with an area of 44.2 km², a maximum width, length and depth of 3.5 km, 18 km and 147 m respectively, and a volume of 3.6 × 10⁹ m³. Lake Annecy, also located in the eastern part of France, is the second largest French lake with an area of 28 km², a width of 3.2 km, a length of 14.6 km, a maximum depth of 65 m and a volume of 1.2 × 10⁹ m³, at an altitude of
447 m. All these lakes are of glacial origin and situated in a same eco-region. Following restoration programs, Lakes Geneva and Bourget have been reported to be mesotrophic in recent years with total phosphorus concentrations varying between 20 and 25 μg P L⁻¹ (Jacquet et al., 2008; Lazzarotto et al., 2008). In contrast, Lake Annecy has been reported to be oligotrophic since the late 1960s with total phosphorus concentrations lower than 8 μg P L⁻¹ (Gerdeaux et al. 2008). More details on the study sites can be found in Anneville et al. (Anneville et al., 2002), Domaizon et al. (Domaizon et al., 2003) and Jacquet et al. (Jacquet et al., 2005).

Dynamics of microbial communities and environmental parameters

Microbial counts were performed on samples taken at the reference station, located in the middle and deepest part of each lake, every 2 weeks between January 2005 and November 2006, and at seven different depths between 0 and 50 m. Water temperature and transparency, and the concentrations of nitrogen and phosphorus forms were obtained at the same sampling occasions. Water temperature was obtained with a conductivity–temperature–depth measuring device (CTD SBE 19 Seacat profiler, SEABIRD). Water transparency was measured using a Secchi disk. Nutrient concentrations (P-PO₄, N-NO₃, N-NH₄ and Si-SiO₂) were analysed the same day or the day after sampling according to standardized protocols (AFNOR).

Flow cytometry

Viruses, heterotrophic bacteria, picocyanobacteria and small eukaryotic phytoplankton were counted using a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm and its original filter set-up. Heterotrophic bacterial and viral counts were obtained on fixed samples using glutaraldehyde (0.5% final concentration) for 30 min, then diluted in either 0.02 μm filtered fresh water (for the bacteria) or 0.02 μm filtered TE buffer (0.1 mM Tris–HCl and 1 mM EDTA, pH 8) for the viruses. Heterotrophic bacteria were then incubated with SYBR Green I (at a final 10⁻⁴ dilution of the commercial stock solution, Molecular probes) for 15 min. Viruses were incubated with SYBR Green I (5 × 10⁻⁵ final dilution), for 5 min at ambient temperature, followed by 10 min at 75°C and again 5 min at room temperature, prior to flow cytometry (FCM) analysis (modified from Brussaard, 2004). For photosynthetic cells (i.e. the picocyanobacteria and small eukaryotes), neither fixative nor fluorochrome were used. Analysis was made on fresh samples in which we added a suspension of 1-μm beads (Molecular probes). FCM listmode files were then analysed on a PC using CYTOWIN (Vaulot, 1989).

Epifluorescence (EFM) and inverted light microscopy

Glutaraldehyde (1% final concentration) was used to fix flagellates. Samples (25–30 mL) were filtered (pressure <100 mm Hg) on polycarbonate membranes (diameter: 25 mm, pore size: 0.8 μm), then stained with primulin (modified from Caron, 1983) and stored for less than 1 week at −20°C before analysis. Slides were examined under UV light to count the heterotrophic nanoflagellates using epifluorescence (EFM). Ciliates were preserved with mercuric bichloride (2.5%) and counted according to Sime-Ngando et al. (Sime-Ngando et al., 1991). The sedimentation of samples (100 mL) was done during 48 h in an Uthermol column, and the observations were made with an inverted light microscope (500 ×).

Experimental set-up for estimating picoplanktonic mortality

We performed 14 dilution experiments from autumn 2005 to summer 2006, following the experimental design proposed by Evans et al. (Evans et al., 2003). These 2 day long experiments were conducted at distinct periods of the year (early autumn, winter, spring and summer). For each experiment, an integrated >20 L sample was taken in the 0–10 m surface layer of the reference station of each lake using a pump and a long flexible pipe controlled from the surface on the boat (via an electric cable and a 12-V battery). In the laboratory, experimental samples were pre-filtered through 20 μm mesh filters, and then filtered twice through 11 μm mesh filters (NYCOM, Buisine, France) to eliminate metazooplankton, and ciliates. This size fractionation used for grazers (<11 μm) was chosen according to previous studies in these lakes (Domaizon et al., 2003; Comte et al., 2006; Duhamel et al., 2006) where it was shown that the majority of the heterotrophic flagellates were in the 5–11 μm size range and abundant during the year (Domaizon et al., 2003; Comte et al., 2006). Thus, this “<11 μm” size fraction contained only bacterioplankton, viruses, heterotrophic flagellates and small phytoplankton. Half of this filtrate (<11 μm) was kept as such while the second half was used to constitute two types of diluents (<0.2 μm filtrate and 30-KDa filtrate). To do this, the <11 μm fraction (around 10 L) was filtered through 2 μm (Nuclepore, Whatman) and 0.2 μm filters (Nuclepore, Whatman) to eliminate all grazers and bacterioplankton, respectively. Finally, the <0.2 μm water
was split into two sub-samples, one of these was subjected to tangential ultra-filtration using a mini-Ultrasound with a 30 kDa cut-off membrane (Vivaflow, Vivasciences) in order to eliminate all organisms and obtain virus-free water. Figure 1 shows the quality of the dilutions obtained for all experiments combined.

Thus, three different size fractions were constituted: $<$11 $\mu$m, $<$0.2 $\mu$m and $<$30 kDa. The efficiency of each filtration step, i.e. the theoretical absence or presence of the different microbial communities, was tested in each related fraction, both at the beginning and at the end of each experiment (data not shown). The $<$11 $\mu$m water was diluted with either the $<$0.2 or with the $<$30 kDa diluent in order to obtain dilution percentages of the initial (11 $\mu$m filtered) water of ca. 20, 40 and 70%. Two extra bottles containing 100% of each diluent were also added to the experimental design to confirm and follow the absence for contamination (data not shown). Each treatment was conducted in duplicate. Raw and diluted samples were prepared in 1 L polycarbonate bottles (Nalgene, Bioblock) acid-washed, rinsed three times with MilliQ water and autoclaved. Once filled, all bottles were incubated at a depth of 1 m in Lake Geneva for convenience since this lake is in front of the laboratory. Samples were taken in the field at T0, T24 and T48 hours for subsequent analysis using FCM and at T0 and T48 hours for EFM.

Statistical analyses of data from *in situ* dynamics

Data from the *in situ* dynamics survey were subjected to one-way analysis of variance (the absence of replicates for variables preventing the use of two-way ANOVA) to test for effects of time or depth (SigmaStat 3.1). In addition, a matrix of data was produced for non-linear redundancy analysis based on polynomial regression (Makarenkov and Legendre, 2002). The Freeware (available at http://www.bio.umontreal.ca/casgrain/en/lab/plrdacca.html) allowed us to build data tables with explanatory and response variables which in turn enabled identification of combinations of variables that account for the largest amounts of the total variance observed and how the different variables match when combined. Response variables (heterotrophic bacteria and picocyanobacteria) were related to physical (temperature), chemical (P-PO4, N-NH4, N-NO3 and SiO2) and biological (heterotrophic flagellates, ciliates and different groups of viruses separated from FCM) explanatory variables. This analysis was conducted on the integrated epilimnion data of the three lakes between January 2005 and October 2006. The program produced the output required to draw biplot diagrams.

Analysis of data from the dilution experiments

Net growth rate of prokaryotes is considered to be a product of the instantaneous growth and the mortality known mostly from grazing and viral lysis. It is assumed that the net growth rate remains constant with respect to the level of dilution, whereas mortality is considered to be density-dependant (Evans *et al.*, 2003). In each experiment, the net growth rate was calculated between T0 and T24 hours and between T0 and T48 hours. Then, it was displayed graphically as a function of the dilution. The slope between the dilution and the net growth rate gives the percentage of mortality due to grazing (diluent 0.2 $\mu$m) or grazing plus lysis (diluent 30 kDa) following the equation of Suzuki *et al.* (Suzuki *et al.*, 2002): \% of mortality=\((1-\exp(\text{Slope}))\times 100.

The significance of all correlation coefficients was statistically tested by linear regression (Table I) to know...
### Table I: Percentage of heterotrophic bacterial and picocyanobacterial removal due to flagellate grazing with or without viral lysis calculated on a daily base scale (see Results) obtained at different periods of the year (Autumn, Winter, beginning (A) and end of Spring (B), Summer) for Lakes Geneva, Bourget and Annecy

<table>
<thead>
<tr>
<th>Season</th>
<th>Lake</th>
<th>Host</th>
<th>Diluent</th>
<th>Instantaneous growth rate</th>
<th>Regression equation</th>
<th>Linear fit</th>
<th>Viruses+Grazer mediated mortality (% day⁻¹)</th>
<th>Grazer mediated mortality (% day⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T0–T24</td>
<td>T0–T48</td>
<td></td>
<td>R²</td>
<td>P-value</td>
<td>P limit</td>
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<td>Autumn</td>
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<td>0.2 µm</td>
<td>0.14</td>
<td>y = -1.8608x + 2.1738</td>
<td>0.9716</td>
<td>0.014</td>
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<td></td>
<td></td>
<td>Cyanobacteria</td>
<td>30 kDa</td>
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<td>0.014</td>
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<td>-0.09</td>
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<td>0.2 µm</td>
<td>-0.23</td>
<td>y = -0.464x + 0.2281</td>
<td>0.8353</td>
<td>0.087</td>
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<td>30 kDa</td>
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<td>0.8934</td>
<td>0.05</td>
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<tr>
<td></td>
<td>Annecy</td>
<td>Heterotrophic</td>
<td>0.2 µm</td>
<td>0.04</td>
<td>y = 0.1432x + 0.013</td>
<td>0.7755</td>
<td>0.115</td>
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<td>Cyanobacteria</td>
<td>30 kDa</td>
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<td>0.9063</td>
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<td>Winter</td>
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<td>0.01</td>
<td>y = -0.0012x + 0.1016</td>
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<td>Spring A</td>
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<td>0.01</td>
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<td>30 kDa</td>
<td>-0.05</td>
<td>y = -0.0006x + 0.2519</td>
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Continued
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<th>Season</th>
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<th>Host</th>
<th>Diluent</th>
<th>T0–T24</th>
<th>T0–T48</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>$P$ value</th>
<th>$P$ limit</th>
<th>Viruses + Grazer mediated mortality (% day $^{-1}$)</th>
<th>Grazer mediated mortality (% day $^{-1}$)</th>
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<tr>
<td>Spring B</td>
<td>Geneva</td>
<td>Heterotrophic bacteria</td>
<td>0.2 μm</td>
<td>-0.02</td>
<td>-0.48</td>
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<td>36.8*</td>
<td>37.3*</td>
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<td></td>
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<td>0.24</td>
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<td>$y = 0.1011x + 0.3928$</td>
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<td>10.66*</td>
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<td>Heterotrophic bacteria</td>
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<td>$y = 0.2888x + 0.1496$</td>
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<td></td>
<td>Cyanobacteria</td>
<td>30 kDa</td>
<td>0.13</td>
<td>0.43</td>
<td>$y = 0.2911x + 0.196$</td>
<td>0.52976</td>
<td>0.27</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>0.2 μm</td>
<td>0.08</td>
<td>-0.01</td>
<td>$y = -0.179x + 0.069$</td>
<td>0.4355</td>
<td>0.34</td>
<td>NS</td>
<td>-0.02</td>
<td>8.19</td>
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<td></td>
<td></td>
<td></td>
<td>30 kDa</td>
<td>-0.06</td>
<td>-0.09</td>
<td>$y = 0.0004x - 0.1019$</td>
<td>0.0000</td>
<td>0.99</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring B</td>
<td>Geneva</td>
<td>Heterotrophic bacteria</td>
<td>0.2 μm</td>
<td>0.46</td>
<td>-0.08</td>
<td>$y = -1.4474x + 1.8281$</td>
<td>0.88612</td>
<td>0.058</td>
<td>&lt;0.01</td>
<td>71.0*</td>
<td>76.5*</td>
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<tr>
<td></td>
<td></td>
<td>Cyanobacteria</td>
<td>0.2 μm</td>
<td>0.29</td>
<td>0.44</td>
<td>$y = 0.3035x + 0.0659$</td>
<td>0.54041</td>
<td>0.26</td>
<td>NS</td>
<td>-31.54*</td>
<td>-35.47</td>
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<td></td>
<td></td>
<td></td>
<td>30 kDa</td>
<td>0.35</td>
<td>0.14</td>
<td>$y = 0.2742x + 0.0251$</td>
<td>0.84596</td>
<td>0.06</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
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<tr>
<td>Bourget</td>
<td>Heterotrophic bacteria</td>
<td>0.2 μm</td>
<td>0.28</td>
<td>0.06</td>
<td>$y = -0.7663x + 0.9596$</td>
<td>0.87025</td>
<td>0.037</td>
<td>&lt;0.05</td>
<td>25.10*</td>
<td>56.83*</td>
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<td>Cyanobacteria</td>
<td>0.2 μm</td>
<td>0.11</td>
<td>0.47</td>
<td>$y = 0.2461x - 0.1805$</td>
<td>0.70049</td>
<td>0.16</td>
<td>NS</td>
<td>-22.55*</td>
<td>-25.05</td>
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<td>30 kDa</td>
<td>0.07</td>
<td>0.45</td>
<td>$y = 0.1808x - 0.1164$</td>
<td>0.9756</td>
<td>0.024</td>
<td>&lt;0.05</td>
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<tr>
<td>Annecy</td>
<td>Heterotrophic bacteria</td>
<td>0.2 μm</td>
<td>0.23</td>
<td>0.39</td>
<td>$y = -0.6943x + 0.819$</td>
<td>0.54961</td>
<td>0.30</td>
<td>NS</td>
<td>32.97</td>
<td>46.74</td>
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<tr>
<td></td>
<td>Cyanobacteria</td>
<td>30 kDa</td>
<td>0.34</td>
<td>0.22</td>
<td>$y = 0.3376x + 0.5175$</td>
<td>0.29444</td>
<td>0.35</td>
<td>NS</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.2 μm</td>
<td>0.36</td>
<td>0.90</td>
<td>$y = 0.1514x + 1.331$</td>
<td>0.2812</td>
<td>0.4</td>
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<td>-49.34*</td>
<td>-23.38</td>
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<td>0.97827</td>
<td>0.002</td>
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</table>

*symbolizes significant mortality percentage values ($P < 0.1$ or $P < 0.05$). Equations describe linear regressions of apparent growth of heterotrophic bacteria and picocyanobacteria. Instantaneous growth rates of heterotrophic bacteria and picocyanobacteria were calculated in standard enclosure (100%, no dilution) after 24 or 48 h of experiments.
whether the slope value could be used as such. As the incubation time should be consistent with the generation time of the cell host to obtain an accurate estimate of virus-induced cell mortality, we reported the percentage of heterotrophic bacterial and picocyanobacterial mortality per day (Table I) due to grazers alone or to both grazers and viruses, calculated with bacterial net growth rate between T0 and T24 hours (in summer) and between T0 and T48 hours (in autumn, winter and spring). Based on the estimated instantaneous growth rate of heterotrophic bacteria, incubation time of 24 or 48 h was chosen to obtain accurate estimates of picoplankton mortality in autumn, winter and spring compared with summer (see Discussion).

RESULTS

Physico-chemical characteristics

Patterns recorded for temperature were almost similar in the three lakes. Water temperature changes were typical of temperate lakes, with stratification starting in spring and finishing in autumn (Fig. 2A–C). During winter, the temperature was homogeneous throughout the 0–50 m water column. Seasonal thermal stratification was more pronounced in the top 10 m layer with temperature ranging from 7.3 to 25°C between May and November, and from 4.2 to 11.8°C between December and April. The stratification was a little bit more pronounced with deeper warmer waters in 2005 and the highest abundances spread lasted July to September 2006 (Fig. 3D–E) in surface waters. In Lake Annecy (Fig. 3F), the bacterial seasonal pattern was different and did not show a strong peak in spring. In Lake Bourget, mean values of heterotrophic bacterial abundance were always higher in autumn (3.3 × 10^6 cells mL^-1), winter (1.6 × 10^6 cells mL^-1) and spring (3.2 × 10^6 cells mL^-1) than in the two other lakes. In summer, mean values were higher in Lake Geneva (3.1 × 10^6 cells mL^-1). In Lake Annecy, heterotrophic bacterial abundance ranged between 3.1 × 10^5 and 5.1 × 10^6 cells mL^-1. Maximal abundances recorded in Lakes Annecy, Bourget and Geneva were at 5.1, 10.8 and 9.9 × 10^6 cells mL^-1, respectively.

Heterotrophic flagellates were more abundant in Lake Bourget than in Lake Geneva with annual mean abundances of 1.1 × 10^3 and 7.2 × 10^3 cells mL^-1, respectively. This community reached its highest values from April to September between 0 and 20 m in Lake Bourget (Fig. 3H). In Lake Geneva, the heterotrophic flagellates were more abundant between 0 and 15 m in 2005 and the highest abundances spread lasted July to September 2006 (Fig. 3G). No data were available for Lake Annecy.

When considering the ciliate community, a similar pattern could be observed for the two mesotrophic lakes where the highest abundances (i.e. 1.2 × 10^7 cells mL^-1) were observed in the top 20 m between April and October 2005 while they did not really increase during 2006 (Fig. 3I and J). No data were available for Lake Annecy.

Viruses displayed very comparable patterns in the two mesotrophic Lakes. There were two main periods of high densities in summer and autumn (Fig. 3K and L). In Lakes Geneva and Bourget, virus abundances ranged from 1.1 × 10^7 to 1.9 × 10^8 part mL^-1 and from 1.1 × 10^7 to 1.2 × 10^8 part mL^-1, respectively. Maximal abundances were recorded in the top 20 m in summer. In Lake Annecy, there was no real peak of abundance in summer, but high density was always observed between 15 and 25 m in September and October.

Between two and four groups of viruses could be distinguished using FCM as previously reported (Castberg et al., 2001; Chen et al., 2001; Goddard et al., 2005). In this study, we especially identified two groups of viruses. The first group, i.e. the one with the lowest green fluorescence (data not shown) is referred to as VLP1 (virus-like particles, Group 1). It is assumed that this group,
which represented $>90\%$ of the total viral community, is composed mainly of bacteriophages (Marie et al., 1999; Li and Dickie, 2001; Jacquet et al., 2002; Larsen et al., 2004; Duhamel et al., 2006). In Lakes Geneva, Bourget and Annecy, the VLP1 mean abundances in the top 10 m were $6.9 \times 10^7$, $7.4 \times 10^7$ and $4.4 \times 10^7$ part mL$^{-1}$, respectively (Fig. 4A–C). The second group, labelled VLP2 and characterized with a higher green fluorescence signature, represented $<7\%$ of the total viral community, and may be typical of
cyanophages (Castberg et al., 2001; Sandaa and Larsen, 2006; Personnic et al., 2009). These VLP2 reached annual mean abundances in the upper 10 m of 5.7, 4.7 and $2.8 \times 10^4$ part mL$^{-1}$ in Lakes Geneva, Bourget and Annecy, respectively (Fig. 4G–I). Two other groups, labelled VLP3 and VLP4 and representing less than 3% of total viruses, were identified and followed during the 2 years. However, these two groups, which are thought to be eukaryotic viruses (Duhamel et al., 2006, Personnic et al., 2009), were strongly affected by
the filtration step of the dilution technique and thus, their dynamics were not considered in the context of this study.

With the goal of interpreting VLP1 variations in parallel with the bacterial dynamics, we followed the virus (VLP1) to bacteria ratio (VBR), from January 2005 to September 2006 (Fig. 4D–F). The lowest VBR was recorded in spring for the three lakes. In Lakes Geneva and Bourget, the first increase of viral abundance over the year was observed in May, whereas bacteria bloomed at the beginning of April. Minimal/maximal values of the VBR were 8.6/37.6 for Lake Geneva, 5.9/69.1 for Lake Bourget and 6.8/114.2 for Lake Annecy. Wide variations for the VBR were recorded in Lake Annecy, followed by Lake Bourget, while VBR appeared relatively stable in Lake Geneva (Fig. 4D–F). We also examined the VCR (i.e. the ratio of VLP2 on picocyanobacteria, assuming that VLP2 were indeed related to picocyanobacteria following Personnic et al., 2009). In Lake Annecy, the VCR was relatively constant.
with only small variations. In contrast, a strong peak of VCR was observed between November and May in both Lakes Bourget and Geneva.

Relationships between the different variables

The results of the one-way ANOVA showed that each parameter was significantly affected by time or depth. Typically, we found a significant effect ($P < 0.05$) of both depth and season for each of the tested parameters (i.e., physical, chemical and biological variables). Considering the organization of variables in the epilimnion, polynomial RDA allowed us to identify two canonical axes explaining together 87.8, 89.1 and 75.2% of heterotrophic bacterial and picocyanobacterial variations in Lake Geneva, Bourget and Annecy, respectively (Fig. 5). In RDA biplots, the angle between response variables (heterotrophic bacteria and picocyanobacteria) and the explanatory variables (VLP1, VLP2, heterotrophic flagellates, ciliates, N-NH$_4$, N-NO$_3$, P-PO$_4$ and SiO$_2$, temperature for Lakes Geneva, Bourget and without the protozoa for Lake Annecy) reflected their correlations (Makarenkov and Legendre, 2002). As a rule for the three lakes, we noted that the variable VLP1 was associated with heterotrophic bacteria in the three lakes suggesting a clear host-parasite link. Moreover, in RDA biplot, projecting a sampling date at right angles on a species axis (cyanobacteria and heterotrophic bacteria) approximates the value recorded at this date along the species axis (Makarenkov and Legendre, 2002). Thus, it appears that the highest viral and heterotrophic bacterial abundances were tightly linked especially during the stratification period (from May to October). Concerning grazers (data not available for Lake Annecy), we observed an association between: (i) picocyanobacteria and ciliate dynamics and (ii) flagellates and heterotrophic bacteria dynamics in Lake Geneva, while, in Lake Bourget, ciliates and flagellate dynamics were linked equally to both picocyanobacteria and heterotrophic bacteria. While temperature had a central position between the heterotrophic bacteria and picocyanobacteria axis in Lake Geneva and Lake Annecy, this energetic parameter had a different position for the biplot obtained for Lake Bourget for which temperature was associated to heterotrophic bacteria but not to picocyanobacteria.

It appears that the biplot obtained for Lake Bourget is slightly different from the two others. In the mesotrophic lake Bourget, not only N-NH$_4$ and N-NO$_3$ were explanatory variables which seemed to influence the increase of heterotrophic bacterial abundances, but
all the variables were grouped and, the dynamics of heterotrophic bacteria were correlated with several explanatory variables (temperature, dissolved nitrogen and VLP1). Phosphorous concentrations were also associated with high abundances of bacteria and picocyanobacteria. For the two other lakes, the position of the phosphorus vector, in opposition to picocyanobacteria axis, highlighted the phosphorus depletion in summer. It seems that, in Lake Bourget, this summer phosphorus decrease was not so important in the explanation of picoplankton abundance variance, and high picocyanobacteria densities could be concomitant with high phosphorus concentrations, while in the other lakes, they appeared during the summer phosphorus depletion. Finally, the V2 group was clearly associated with phosphorus both in Lake Geneva and Annecy but not in Lake Bourget.

### DISCUSSION

#### Methodological considerations

When using the dilution method, it is of critical importance to verify the accuracy of each dilution in the various enclosures. As summarized in Fig. 1, we obtained a fairly good dilution of viral abundance in agreement with the theory (Landry and Hassett, 1982; Evans et al., 2003). However, we observed that the four VLP groups were not similarly affected by the filtration step. When considering the fraction of whole water in the $<0.2\ \mu m$ treatments, we noticed that VLP1 reductions were about 2 to 10% and between 7 and 22% for VLP2. It was not possible from our results to test whether such percentages could dramatically affect virus-host interactions. This aspect was clearly a main cause for the lack of reliable results for the assessment of viral impacts on small eukaryotic phytoplankton, since the filtration on 0.2 $\mu m$ eliminated between 80 and 90% of the VLP (not shown), which are strongly suspected to be eukaryotic viruses (Duhamel et al., 2006). The huge loss of this viral group seems to be a clear limitation to the use of the dilution approach for the assessment of viral impact on small eukaryotic phytoplankters.

Another important methodological point is to test whether the dilution experiments yield interpretable results through the form of significant correlations between prey growth rate estimates and the dilution factor (Dolan et al., 2000; Worden and Binder, 2003; Agis et al., 2007). As shown in Table I, only 22 out of 56 (i.e. $\sim 1/3$) values could be considered as significant and be used to estimate virus- versus flagellate impact on either the bacteria or the picocyanobacteria.

Finally, the “incubation time” used in dilution experiments seems to be a critical aspect to take into account. Indeed, the incubation time of 24 h generally proposed for dilution experiments may be too short regarding some generation times at certain periods of the year,
typically in winter (Table I). Based on the estimated instantaneous growth rate of heterotrophic bacteria (Table I) which in autumn, winter and spring never exceeded 0.14 day$^{-1}$, while in summer they reached 0.59, 0.28 and 0.34 day$^{-1}$ in Lake Geneva, Bourget and Annecy, respectively, considering a 24-h period would have lead to inconsistent results. This is the reason why we calculated viral and grazing impacts using either an incubation time of 24 or 48 h to obtain accurate estimates of picoplankton mortality in autumn, winter and spring compared with summer. This duration is obviously a key point when applying this method (a long incubation period may result in modifications of community abundance and diversity, while a too short period could hide lytic and/or predatory effects). Abedon et al. (Abedon et al., 2003) who explored some issues of latent period evolution as a function of the density of phage-susceptible bacteria showed that both the burst size (i.e. number of viruses released from each individual host organism) and the phage generation time (i.e. time to produce new phage) are controlled by a latent period. Theory suggests that higher bacterial densities should select for a shorter phage latent period. In other words, phages also will evolve a shorter latent period when either the host density is high or the host quality is good (Wang et al., 1996; Abedon et al., 2001). In our case, in winter, with relatively low abundance of bacteria, viruses could have a long latent period, so that even 48 hours might have been too short to obtain a clear, if any, viral lysis impact.

**Predation and lyses control of picoplankton communities**

Large variations in microbial mortality according to the period were recorded as previously shown in these and other freshwater ecosystems (Bettarel et al., 2003; Domaizon et al., 2003; Comte et al., 2006). We determined that flagellate grazing was a relatively weak cause of picocyanobacterial mortality in autumn (7%), but a significant mortality source for heterotrophic bacteria in autumn (up to 42%), spring (up to 37%) and summer (up to 76%). The increase of viruses and grazer impacts on bacteria during these periods was consistent with the observations from the statistical analysis performed on the in situ survey data. The largest impact on bacteria was especially recorded in summer where a half of the biomass could be removed daily in the two mesotrophic lakes Geneva and Bourget. In comparison to the predators, viruses had only a minor mortality impact in all situations where we could detect an effect of this community on bacteria. Interestingly, approximately 10% of picocyanobacterial loss in autumn could be attributed to viral lysis alone. Such a percentage is typically in the range of data previously reported (Suttle and Chan, 1994; Suttle, 2000), even if the comparison should be considered with caution because of differences in the methods employed. In winter and early spring, i.e. at the period of relatively low productivity, no clear mortality impacts of either the viruses or the flagellates could be detected on either the bacterial or the picocyanobacterial community. Contrary to the two mesotrophic ecosystems, no clear impact was recorded all year long for the oligotrophic Lake Annecy. This suggests that the dilution method was difficult to apply to this permanent low-nutrient content ecosystem and during periods of low productivity, or that viruses and flagellates have no impact on prokaryotic mortality.

We assume that, for both bacteria and picocyanobacteria, the host specificity is relatively high, so that, absence of viral lysis detection may be due to low successful encounters between viruses and hosts as suggested by various authors (Jacquet et al., 2002; Evans et al., 2003; Colombet, 2008). In parallel to this assumption, the dilution method would not give a clear quantitative viral impact when heterotrophic bacteria and viruses are not abundant enough. Indeed, in such a situation, it may be argued that the low contact rates between bacteria and viruses or predators might explain the absence of clear results. We have considered carefully this hypothesis, and we have tried to define the minimal number of heterotrophic bacteria (MNB) or picocyanobacteria (MNC) for which the dilution technique should theoretically be used (Fig. 4). We observed, from our 14 experiments, that the dilution experiments worked as expected (satisfactory linear regression between bacterial growth rates and dilution level) when heterotrophic bacterial and picocyanobacterial abundances were at least $2.6 \times 10^6$ and $3.8 \times 10^4$ cells mL$^{-1}$, respectively. However, we also observed that, for the same probability of contact between heterotrophic bacteria and viruses or predators, and in the same lake, we could measure a significant impact of viruses in autumn, and, a non-significant impact in winter. The hypothesis involving a possible MNB and MNC was thus not appropriate.

Hence, an explanation for the absence of a regression between dilution (of viruses and/or grazers) and the bacterial net growth rate could be that viruses and grazers had really no impact, more especially during periods of low metabolic activity, keeping in mind that what is true one day may be not the other day, regarding the dynamics of such communities. During winter (when temperature was well below 7°C), bacteria are
likely to have a slow metabolism, translating in low growth. Viruses must use bacterial metabolism to replicate and cannot maintain their abundance without healthy and active hosts. A virus in a cell characterized by a reduced metabolism is unlikely to engage the lytic mechanism, and consequently the impact on bacterial mortality should be very low or inexistent at the time scale investigated here. The statistical analysis of in situ survey data confirmed such a hypothesis by revealing temperature as a central parameter for each lake to explain the dynamics of heterotrophic bacteria. Also, several studies have suggested that physical (such as temperature) and chemical (such as the availability of nutrients) conditions may have an important influence also on viral life strategies. Typically, lysogenic infection is considered the most favourable way of bacterial infection in waters characterized by low bacterial and primary production (Jiang and Paul, 1998, Weinbauer and Suttle, 1999; Williamson et al., 2002), Bongiorni et al. (Bongiorni et al., 2005) indicated that oligotrophic conditions drive viral life strategies, towards lysogenic rather than lytic infection. The metabolic status of the host is indeed crucial for viral development, as high host growth rates can support higher viral turnover rates (Lensky, 1988; Corinaldesi et al., 2003). This feature fits well with Lake Annecy (where bacterial growth limitation has been demonstrated experimentally by Tadonlěk, unpublished data) or for the winter season for Lakes Geneva and Bourget, where viruses could have adopted a lysogenic infection as a way of life strategy. In Lake Bourget, a high proportion of lysogenic bacteria (up to 60%) can be recorded in winter (Thomas et al. submitted for publication). So, if this hypothesis held, we could expect a decrease of virus proportion in the water column during winter. During an annual succession, the VBR is likely to remain relatively high because of the necessity for the viruses to use heterotrophic bacteria for maintaining their existence in the water column. At the end of autumn, during winter and the beginning of spring, heterotrophic bacterial abundance decreased considerably. However, the VBR during these periods remained high (Fig. 3) indicating that heterotrophic bacteria decreased more than viruses. As no important lytic infection was recorded, it is expected that viral abundances would stay constant or decreased due to viral decay (Noble and Fuhrman, 2000). Surprisingly, the number of viruses remained high. A possible alternative is the existence of chronic infection where expulsion of viruses from the hosts operates through simple diffusion through the bacterial cell coat. Such a chronic infection has been reported only once at the community level in an aquatic environment (Hofer and Sommaruga, 2001). If we speculate that this chronic infection is possible, we can hypothesize that viruses in peri-alpine lakes may use lytic, lysogenic or chronic infections over the year depending on the host cell metabolism in relation to seasons and resource scarcity.

**Highlighting some complex interactions**

The dilution method initially used to estimate the mortality of bacteria due to viral lysis allowed also the identification of the complex interactions between microbial communities and the confirmation of most of the detailed analysis performed on in situ survey data. We indeed observed for all experiments that the relative impact of grazers alone was higher than the impact measured in the presence of both viruses and predators. For the picocyanobacteria, we also obtained some significant negative values suggesting that the presence of only grazers <11 μm (in spring for Lake Geneva and Bourget) and predation plus lysis (in summer for the three lakes) could favour picocyanobacterial growth. Consequently, the presence of viruses seemed to have a positive impact for bacterial growth rates, suggesting that they could favour the multiplication of non-infected bacteria. A similar result has previously been discussed (Fuhrman, 1992; Middelboe et al., 1996; Gobler et al., 1997; Middelboe, 2000; Weinbauer, 2004; Middelboe and Jorgensen, 2006) and we assume that viral lysis products such as cell content and cell wall fragments could indeed enter the organic matter pool and be exploited by bacteria. This viral shunt stimulates bacterial production and thus viruses have a positive effect on heterotrophic bacterial growth. Similarly, a positive impact could be observed for picocyanobacterial growth suggesting that the recycling of nutrients due to viral lysis may also be positive for the picophytoplankton. Thus, phages could be “beneficial” for prokaryotes (and maybe also for phytoplankton) mediated processes at the community level (Weinbauer, 2004). More than 10 years ago, Suttle (Suttle, 1996) reported lower growth rates of marine *Synechococcus* incubated and diluted in virus-free water. One explanation provided by these authors was that the dilution with virus-free water prevented nutrient recycling through viral lysis and this impacted growth rates negatively. Our results, obtained in summer during depletion of nutrients, seem to corroborate these assumptions.

In our experimental treatments, viruses and predators had a strong positive impact on picocyanobacteria in summer in all lakes. We assume that heterotrophic flagellates did not affect picocyanobacterial mortality, because heterotrophic flagellates (<11 μm) explained picocyanobacterial mortality only for two experiments...
among the 14 which were conducted. These results confirm previous studies (Pernthaler et al., 1996; Verni and Gualtieri, 1997; Callieri and Stokner, 2002; Comte et al., 2006) demonstrating that these cells are preferentially consumed by ciliates. This fact is consistent with the relationships suggested by the statistical analysis of in situ data, especially the tight coupling of picocyanobacteria and ciliate dynamics in the two mesotrophic lakes.

Flagellates can directly affect viral abundance and infectivity through direct consumption or by grazing preferentially on viral-infected cells. The interactions between viruses and grazers on bacteria are probably very complex (Miki and Jacquet, 2008) and various types of synergism effects could be involved.

In our study, we were only interested in surface waters (i.e. the upper 0–10 m layer) and selected periods of the year (i.e. a few days) separated by some weeks to months; thus, we obviously obtained a partial picture (i.e. a snapshot) of virus–predator–prey interactions. However, we could observe major changes and the complexity of viruses versus small grazers as potential regulatory factors on bacterial and picocyanobacterial dynamics in the epi-limnion of the three lakes studied.

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